Watanabe H, Kashimoto N, Kajimura J, Ishikawa M and Kamiya K	Tumor induction by monoenergetic neutrons in B6C3F1 mice	J Radiat Res	48	205-210	2007
Igaki H, Nakagawa K, Uozaki H, Akahane M, Hosoi Y, Fukayama M, Miyagawa K, Akashi M, Ohtomo K and Maekawa K	Pathological changes in the gastrointestinal tract of a heavily radiation-exposed worker at the Tokai-mura criticality accident	J Radiat Res	49	55-62	2008
Shimura T, Torres MJ, Martin MM, Rao AV, Pommier Y, Katsura M, Miyagawa K and Aladjem MI.	Bloom's syndrome helicase and Mus81 are required to induce transient double-strand DNA breaks in response to DNA replication stress	J Mol Biol	375	1152-1164	2008
Miyagawa K	Clinical relevance of the homologous recombination machinery in cancer therapy	Cancer Sci	99	187-194	2008
Enomoto A, Kido N, Ito M, Morita A, Matsumoto Y, Takamatsu N, Hosoi Y and Miyagawa K	Negative regulation of MEKK1/2 signaling by Serine-Threonine Kinase 38 (STK38)	Oncogene	in press		2007 Oct 1; [Epub ahead of print]
Sasano N, Enomoto A, Hosoi Y, Katsumura Y, Matsumoto Y, Shiraishi K, Miyagawa K, Igaki H and Nakagawa K	Free radical scavenger edaravone suppresses X-ray-induced apoptosis through p53 inhibition in MOLT-4 cells	J Radiat Res	48	495-503	2007
Suzuki T, Nishi T, Nagino T, Sasaki K, Aizawa K, Kada N, Sawaki D, Munemasa Y, Matsumura T, Muto S, Sata N, Miyagawa K, Horikoshi M and Nagai R	Functional interaction between the transcription factor Kruppel-like factor 5 and poly(ADP-ribose) polymerase-1 in cardiovascular apoptosis	J Biol Chem	282	9895-9901	2007

International Congress Series 1299 (2007) 81 – 86

ELSEVIER

www.ics-elsevier.com

Systematic collection of tissue specimens and molecular pathological analysis of newly diagnosed solid cancers among atomic bomb survivors

Wataru Yasui *, Naohide Oue

Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

Abstract. To elucidate precise mechanism of radiation-induced cancers, it is important to analyze the genetic and epigenetic alterations in cancer cases among atomic bomb (A-bomb) survivors. Stored tissue samples are damaged in a certain extent, and fresh tissue samples are suitable for molecular analyses. We have established a network system comprising major hospitals in Hiroshima area, Hiroshima University and Radiation Effects Research Foundation (RERF) to conduct systematic collection and storage of fresh tissue samples of newly diagnosed solid cancers among A-bomb survivors. The project is carried out as "The Ministry of Health, Labor and Welfare of Japan Group Study on A-bomb Diseases." The subjects of this study are RERF Life Span Study (LSS) cohort members (>0 dose) who have been diagnosed with cancer of the stomach, colorectum, esophagus, breast, or lung, and have undergone surgery. As controls, LSS cohort members (=0 dose) and non-LSS members matched by sex, age, and medical institution are selected. Using the tissue samples thus collected, we are searching for genetic and epigenetic events involved in the development of solid cancers. We have developed custom-made 3-dimension oligo-DNA microarray with a total of 207 genes including those related to DNA damage response and repair. We identified 10 genes whose expression levels in the tumors were significantly different between A-bomb survivors (LSS: >0 dose) and control subjects. They might be candidate genes which participate in radiation-induced carcinogenesis and possible genetic markers for radiation-induced solid cancer.

Keywords: Systematic tissue collection; Solid cancer; Atomic bomb survivor; Molecular pathology

* Corresponding author. Tel.: +81 82 257 5145; fax: +81 82 257 5149.
E-mail address: wyasui@hiroshima-u.ac.jp (W. Yasui).

0531-5131/
doi:10.1016/j.ics.2006.09.012

1. Introduction

More than 60 years have passed since A-bombs were dropped on Hiroshima and Nagasaki. A prospective cohort study (Life Span Study: LSS) of 120,000 subjects has been conducted by Radiation Effects Research Foundation (RERF) [1]. With regard to the temporal pattern of excess cancer risks due to radiation exposure in the LSS, the excess relative risks of solid cancers including breast, colon, lung, and stomach have a long latency period, and the excess relative risks of solid cancers among those exposed when young remain high [1]. Although about half of the LSS members have already died, cancer mortality in the LSS has continued to increase with the aging of the population, and will reach its peak in 2015. Previous studies to elucidate mechanism of radiation-induced carcinogenesis mainly used formalin-fixed, paraffin-embedded archival tissues, which are not suitable for molecular analyses because of degradation of DNA, RNA and protein [2,3]. Therefore, now is the time for fresh tissue samples of these newly developed cancer cases to be systematically collected. The purpose of this project is to conduct systematic collection and storage of tissue specimens of newly diagnosed solid cancers among A-bomb survivors by constructing a network system comprising major hospitals in Hiroshima area, Hiroshima University and RERF, and to determine mechanisms of radiation-induced cancers by molecular methods. The project is carried out as "The Ministry of Health, Labor and Welfare of Japan (MHLW) Group Study on A-bomb Diseases." Here, we describe the method of systematic collection of fresh tissue samples and introduces some of the results of molecular analyses on the collected samples.

2. Systematic collection of tissue specimens of newly diagnosed solid cancers among A-bomb survivors

2.1. Subjects

The subjects of this study are LSS cohort members (>0 dose) who have been diagnosed with cancer of the stomach, colorectum, esophagus, breast, or lung, and have undergone surgery or endoscopic resection. As controls, LSS cohort members (=0 dose) and non-LSS members matched by sex, age, and medical institution are selected.

2.2. Collection and storage of tissues

At 7 major hospitals in Hiroshima Prefecture, the MHLW Study Group collects tissue specimens of patients in the LSS cohort and those not in the LSS who have been newly diagnosed with cancer. These 7 hospitals are expected to handle about 70% of newly diagnosed cancer cases in Hiroshima city from the LSS cohort, although obtained surgical specimens are further reduced for some cancers such as lung cancer.

The procedures of acquiring informed consent from study patients and collecting tissue specimens are as follows (Fig. 1):

1. Members of the MHLW Study Group in hospitals ask patients who are candidates for the study, (i.e., birth date earlier than August 6, 1945 and with cancer sites

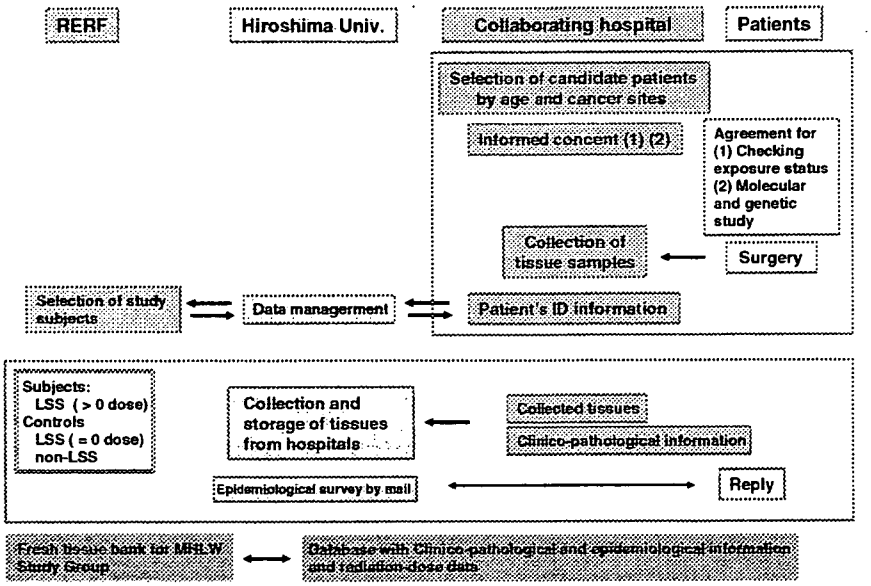

Fig. 1. Working chart for systematic collection of tissue specimens of newly diagnosed solid cancer.

described above) whether they agree to provide their name and other ID information to RERF for the purpose of selecting study patients for the MHLW Study Group (the first informed consent). At the same time, patients are asked whether they agree to the use of their tissue specimens for molecular study, using a standardized form of informed consent prepared by the MHLW Study Group (the second informed consent).

2. The lists of those candidates with agreement are sent to RERF through Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences.
3. RERF then selects study patients on the basis of radiation-exposure data, and reports the selected study patients to members of the MHLW Study Group through the Department of Molecular Pathology, without any information on radiation-exposure data or LSS cohort status attached.
4. The tissue samples of those patients who give the second informed consent are collected at the hospitals, delivered to the Department of Molecular Pathology, and stored at −80 °C under strict privacy protection.
5. A mail survey on lifestyle of study patients will be conducted using a standardized self-administered questionnaire and the lifestyle date will be stored at the Department of Molecular Pathology.
6. The Department of Molecular Pathology asks RERF for the radiation-exposure data (categorized dose levels of target organs or adjacent ones) of study patients. RERF provides these data to the Department of Molecular Pathology upon their request, on the basis of the agreement of study patients.
7. Categorized dose levels of radiation-exposure, clinical and epidemiological data of study patients are collected and stored at the Department of Molecular Pathology.
8. The collected tissue samples are anonymized (linkable) and provided by the Department of Molecular Pathology to investigators for molecular analyses.

Up to now, about 500 frozen tissue samples have been collected and stored.

2.3. Ethical issues

In accordance with the ETHICAL GUIDELINES FOR HUMAN GENOME/GENE RESEARCH enacted by the Japanese government, tissue specimens are collected and used, based on the approval of the Ethical Review Committee of the Hiroshima University School of Medicine and of ethical review committees of collaborating organizations.

3. Collection of archival tissue specimens of solid cancers among A-bomb survivors

Archival tissue specimens which were surgically resected previously, formalin-fixed paraffin-embedded and stored in the collaborating hospitals are systematically collected. Principally, under the approval from ethical review committee of each hospital, pathologist members provide name and other ID information of candidates for the MHLW Group Study, (i.e., birth date earlier than August 6, 1945 and with cancer sites) to the Department of Molecular Pathology for the purpose of selecting study patients. Selection in RERF is the same as Procedures 2 and 3 described above. The collected tissue specimens with information of radiation-exposure and clinical data are anonymized (unlinkable) and provided to investigators for molecular analysis. Up to now, about 500 archival tissue samples have been collected and stored.

4. Molecular pathological analysis of newly diagnosed solid cancers among A-bomb survivors

Using tissue specimens thus collected, molecular analyses are subjected to be performed; those include global analysis of gene expression and genomic aberration, epigenetic deregulation, genetic abnormalities and so on. Representative research projects are as follows: Microsatellite instability (MSI) of colon cancer among A-bomb survivors; Loss of heterozygosity (LOH), mutations in p53 and K-ras, and methylation of p16 and tumor

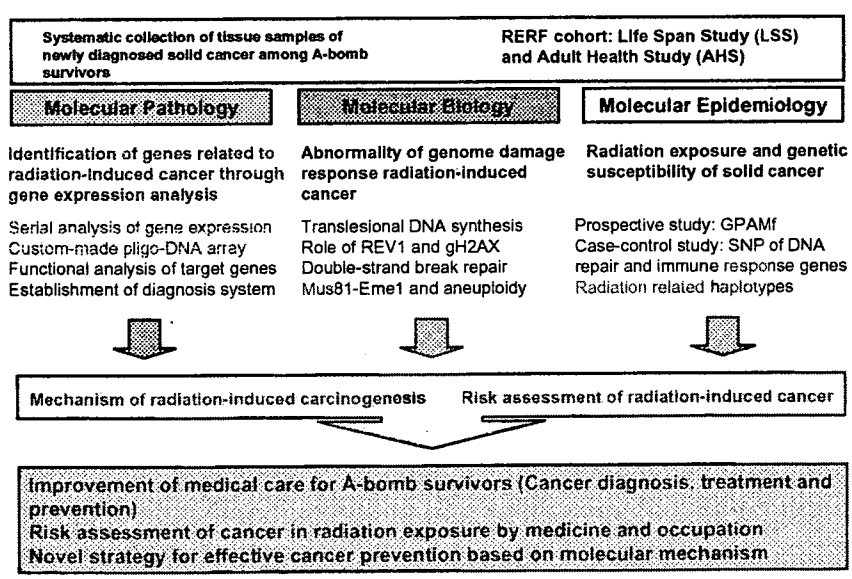

Fig. 2. Research strategy of Group Study on "Molecular analyses of radiation-induced carcinogenesis and their application to diagnosis and prevention" of MHLW Research Grant "Third Term Comprehensive 10-year Strategy for Cancer Control."

suppressor genes at 3p in lung cancer among A-bomb survivors; LOH and MSI of breast cancer among A-bomb survivors; Global analysis of gene expression in esophago-gastric cancer among A-bomb survivors; and Expression of molecules related to DNA damage response in gastric cancer among A-bomb survivors. The molecular analyses are in part carried out as Group Study on "Molecular analyses of radiation-induced carcinogenesis and their application to diagnosis and prevention" of MHLW Research Grant "Third Term Comprehensive 10-year Strategy for Cancer Control" (Fig. 2).

Genome-wide study of gene expression profile is of great advantage to uncover precise mechanism of radiation-induced carcinogenesis and identify novel genetic markers of radiation-induced cancer. Serial Analysis of Gene Expression (SAGE) is a powerful technique to allow global analysis of gene expression in a quantitative manner [4]. We have made the largest SAGE libraries of gastric cancer in the world and sequence data are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue) [5]. Then, we have developed custom-made 3-dimension oligo-DNA microarray in collaboration with Mitsubishi Rayon Co., Ltd. with a total of 207 genes including specific genes identified by our SAGE analysis, known genes related to development and progression of cancer, and genes related to DNA damage response and repair such as base excision repair, homologous recombination, double-strand breaks repair and translesional DNA synthesis. Using this custom-made microarray, we examined the gene expression profile on freshly collected tissue samples of gastric cancers among

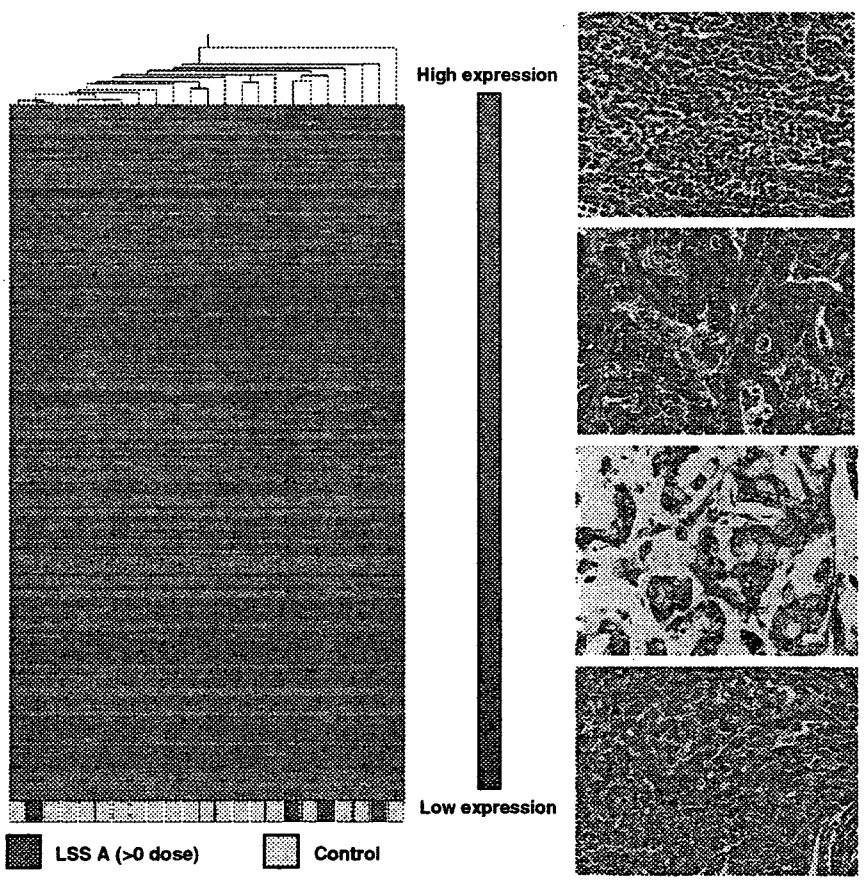

Fig. 3. Gene expression profile of gastric cancers among A-bomb survivors and controls using freshly collected tissue samples. Gene expression profiles were examined on 23 gastric cancer tissues including 4 cases of A-bomb survivor using custom-made 3-dimension oligo-DNA microarray after T7-based RNA amplification. Histological features of 4 gastric cancers among A-bomb survivor are shown.

A-bomb survivor (Fig. 3). This is the initial study of gene expression in solid cancer among A-bomb survivors which has not been possible using archival tissue samples. We could identify 10 genes whose expression levels in the tumors were significantly different between A-bomb survivors (LSS cohort members (>0 dose)) and control subjects (non-LSS members) by one-way ANOVA ($p<0.05$). Those included two genes selected by SAGE analysis and one gene related to DNA damage response. They might be candidate genes which participate in radiation-induced carcinogenesis and possible genetic markers for radiation-induced gastric cancer.

Thus, our project is the powerful way to approach the mechanism of radiation-induced carcinogenesis and identify genetic markers for radiation-induced cancer. This must contribute to not only an improvement of medical care for A-bomb survivors but also risk assessment of cancer in radiation exposure by medicine and occupation and novel strategy for effective cancer prevention based on molecular mechanism.

5. Project members

MHLW Study Group on A-bomb Diseases: W. Yasui (Representative), K. Arihiro (Hiroshima University Hospital), T. Fukuhara (Hiroshima Prefectural Hospital), M. Fujiwara (Hiroshima Red-Cross A-bomb Hospital), H. Matsuura (Hiroshima Citizens Hospital), Y. Hayashi and M. Kaneko (Asa Citizens Hospital), Y. Daimaru (Hiroshima General Hospital), K. Taniyama (Kure Medical Center), Y. Takeshima (Hiroshima University); Collaborating investigators in RERF: K. Nakachi, K. Kodama, N. Nishi, H. Katayama, S. Fujiwara, and N. Takahashi.

Research Group on "Molecular analyses of radiation-induced carcinogenesis and their application to diagnosis and prevention" of MHLW Research Grant: W. Yasui (Representative), N. Nishi, K. Nakachi, Y. Kusunoki, H. Eguchi (RERF), K. Kamiya (Hiroshima University) and K. Miyagawa (University of Tokyo).

References

[1] D.E. Thompson, K. Mabuchi, E. Ron, et al., Cancer incidence in atomic bomb survivors. Part II: Solid tumors, 1958–1987, Radiat. Res. 137 (1994) S17–S67.

[2] Y. Takeshima, T. Seyama, W.P. Bennett, et al., p53 mutations in lung cancers from non-smoking atomic-bomb survivors, Lancet 342 (1993) 8886–8887.

[3] K.S. Iwamoto, T. Mizuno, T. Ito, et al., Feasibility of using decades-old archival tissues in molecular oncology/epidemiology, Am. J. Pathol. 49 (1996) 399–406.

[4] W. Yasui, N. Oue, R. Ito, K. Kuraoka, H. Nakayama, Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implication, Cancer Sci. 95 (2004) 385–392.

[5] N. Oue, Y. Hamai, Y. Mitani, et al., Gene expression profile of gastric carcinoma: Identification of genes and tags potentially involved in invasion, metastasis and carcinogenesis by serial analysis of gene expression, Cancer Res. 64 (2004) 2397–2405.

Modern Pathology (2007) 00, 1–12

www.modernpathology.org

Gene expression profiling with microarray and SAGE identifies PLUNC as a marker for hepatoid adenocarcinoma of the stomach

Kazuhiro Sentani[1], Naohide Oue[1], Naoya Sakamoto[1], Koji Arihiro[2], Kazuhiko Aoyagi[3], Hiroki Sasaki[3] and Wataru Yasui[1]

[1]*Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan;* [2]*Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan and* [3]*Genetics Division, National Cancer Center Research Institute, Tokyo, Japan*

Gastric cancer is one of the most common malignancies worldwide. In this study, we screened for genes upregulated in gastric cancer by comparing gene expression profiles from serial analysis of gene expression and microarray and identified the palate, lung, and nasal epithelium carcinoma-associated protein (*PLUNC*) gene. Immunostaining for PLUNC in 140 gastric cancer cases revealed strong and extensive staining of PLUNC in hepatoid adenocarcinoma of the stomach, whereas 7% of conventional gastric cancer cases showed focal immunostaining of PLUNC. Gastric hepatoid adenocarcinoma is an extrahepatic tumor characterized by morphologic similarities to hepatocellular carcinoma. To investigate the utility of PLUNC immunostaining in the diagnosis of gastric hepatoid adenocarcinoma, six cases of gastric hepatoid adenocarcinoma (six primary tumors and two associated liver metastases) were studied further. PLUNC staining was observed in all six primary hepatoid adenocarcinomas. PLUNC staining was observed in both the hepatoid adenocarcinoma and tubular/papillary adenocarcinoma components of primary tumors, although PLUNC staining was preferentially localized in tubular/papillary adenocarcinoma components. Staining of PLUNC was also detected in both liver metastases. PLUNC staining was not observed in 52 cases of primary hepatocellular carcinoma or in normal adult or fetal liver. These results indicate that PLUNC is a novel marker that distinguishes gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.
Modern Pathology (2007) **0**, 000–000. doi:10.1038/modpathol.3801050

Keywords: hepatoid adenocarcinoma; hepatocellular carcinoma; PLUNC; microarray; SAGE; gastric cancer

According to the World Health Organization, gastric cancer is the fourth most common malignancy worldwide, with approximately 870 000 new cases occurring every year. Mortality due to gastric cancer is second only to that due to lung cancer.[1] Cancer develops as a result of multiple genetic and epigenetic alterations.[2–4] Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment are the major goals in this field.[5] To identify potential molecular markers for cancer and to better understand the development of cancer at the molecular level, comprehensive gene expression analysis may be useful. Among the comprehensive methods used to analyze transcript expression levels, array-based hybridization[6] and serial analysis of gene expression (SAGE)[7] are currently the most common approaches.

We previously performed SAGE of four primary gastric cancers.[8] From the SAGE data, we identified several gastric cancer-associated genes;[9] however, these alterations cannot completely explain the pathogenesis of gastric cancer. In addition, although gene expression profiles from SAGE and microarray have better correlations for genes with high-fold changes, the gene expression profiles from these methods show relatively poor correlations among genes with low-fold changes, suggesting that SAGE data may not yield a comprehensive gene expression profile.[10] In our previous study, the invasion/metastasis-associated genes identified by SAGE

Correspondence: Dr W Yasui, MD, PhD, Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: wyasui@hiroshima-u.ac.jp
Received 29 June 2007; revised 10 September 2007; accepted 14 December 2007; published online 00 00 0000

Gml : Ver 6.0
Template: Ver 1.1.8

Journal: Mpath ☐Disk used Despatch Date: 31/12/2007
Article : npg_Mpath_3801050
Pages: 1–12 OP: vimala ED: XXX CE: XXX Graphic: XXX

were quite different from those identified by microarray.[11] Therefore, we performed gene expression profiling using Affymetrix GeneChip Human Genome U133Plus 2.0 arrays of one gastric cancer sample previously analyzed by SAGE and identified several candidate gastric cancer-associated genes. Among these candidate genes, the palate, lung, and nasal epithelium carcinoma-associated protein (*PLUNC*, also known as lung-specific X protein, *LUNX*) gene is upregulated in human gastric cancer. However, little is known about the relation of PLUNC to human gastric cancer.

PLUNC was originally identified in the nasal epithelium of mouse embryo and the trachea and bronchi of adult mouse lung.[12] The human *PLUNC* gene shows a similar expression pattern, including localization to the tracheal epithelium.[13] *PLUNC* was also identified as a marker of non-small-cell lung carcinoma.[14] Immunohistochemical analysis of PLUNC in lung cancer revealed that PLUNC is commonly expressed in adenocarcinoma, mucoepidermoid carcinoma, and bronchoalveolar carcinoma and is absent from small cell carcinoma and squamous cell carcinoma.[15] Although PLUNC is a major secreted protein product in the upper respiratory tract,[15] the biologic function of PLUNC is poorly understood.

In this study, we examined the expression and distribution of PLUNC in human gastric cancer by immunohistochemistry. The relation between staining for PLUNC and clinicopathologic characteristics was examined. In addition, because we observed frequent immunostaining of PLUNC in hepatoid adenocarcinoma of the stomach, we also performed PLUNC immunostaining of primary hepatocellular carcinoma to investigate the potential utility of PLUNC immunostaining in the diagnosis of gastric hepatoid adenocarcinoma.

Materials and methods

Tissue Samples

In total, specimens from 144 cases of primary gastric cancer and 52 cases of primary hepatocellular carcinoma were collected. Patients were treated at the Hiroshima University Hospital. The histologic classification was based on the World Health Organization system. Tumor staging was according to the TNM classification system.[16] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government. For microarray analysis, one primary gastric cancer sample (Case P208T, 60-year-old man, T4N3M0, stage IV, poorly differentiated adenocarcinoma) and corresponding non-neoplastic mucosa were used. This gastric cancer sample was analyzed previously by SAGE for comprehensive gene expression profiling.[8] For quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis, four gastric cancer samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained during surgery at the Hiroshima University Hospital. We confirmed microscopically that the tumor specimens were predominantly (>50%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Samples of normal brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, peripheral leukocytes, and trachea were purchased from Clontech (Palo Alto, CA, USA). For western blot analysis, lysates from normal adult stomach and trachea were purchased from Clontech. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 144 patients who had undergone surgical excision of gastric cancer (65 women and 79 men; age range, 38–90 years; mean, 68 years). Thirty-nine of the 144 patients had early gastric cancer, and 105 had advanced gastric cancer. Early gastric cancer is limited to the mucosa or the mucosa and submucosa, regardless of nodal status. Advanced gastric cancer is a tumor that has invaded beyond the submucosa.[17] Primary gastric cancers exhibiting a hepatoid component were regarded as hepatoid adenocarcinomas. Out of 144 primary gastric cancers, 6 cases were histologically classified as hepatoid adenocarcinoma. Out of six hepatoid adenocarcinoma cases, liver metastasis samples were available for two cases. Two patients with liver metastasis were serologically negative for HBV and HCV infection and they did not show any clinical or echographic signs of cirrhosis. Both patients had no history of alcohol abuse. In addition, we used archival formalin-fixed, paraffin-embedded tissues from 52 patients who had undergone surgical excision of hepatocellular carcinoma (8 women and 44 men; age range, 44–82 years; mean, 64 years; stage I, 31 cases; stage II, 16 cases; stage III, 5 cases). Normal adult ($n=2$, 45 and 57 years) and fetal ($n=2$, 10 and 18 gestational weeks) livers were obtained at autopsy.

Microarray Analysis

One primary gastric cancer sample (P208T) and corresponding non-neoplastic mucosa were analyzed by genome-wide microarray, as described previously.[18] Here, we used Affymetrix GeneChip Human Genome U133Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Each transcript on this array is represented by a set of 11 probe pairs, called the probe set. The array contains >54 000 probe sets, representing 47 400 transcripts, including 38 500 genes. Five micrograms of total RNA was used to prepare antisense biotinylated RNA with One-cycle Target Labeling and Control Reagent (Affymetrix) as

per the manufacturer's instructions. In brief, first-stranded cDNA was synthesized with a T7-RNA polymerase promoter-attached oligo(dT) primer followed by second-stranded cDNA synthesis. This cDNA was purified and served as a template in the subsequent *in vitro* T7-transcription (IVT). The IVT reaction was carried out in the presence of T7 RNA polymerase and biotinylated UTP for cRNA production. The biotinylated cRNAs were then cleaned up and fragmented. The fragmented, biotinylated cRNA was hybridized to the array (45°C for 16 h). The procedures for staining, washing, and scanning of arrays were carried out as per the instructions in the Affymetrix technical manual. The expression value (average difference, AD) of each probe was calculated with GeneChip Operating Software Version 1.1 (Affymetrix). The mean of AD values in each experiment was 1000 to reliably compare variable multiple arrays.

Quantitative RT-PCR

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of *PLUNC* mRNA levels in human tissue samples was done by real-time fluorescence detection, as described previously.[19] *PLUNC* primer sequences were 5′-CAG TTGCCT TCT CTC CGA GG-3′ and 5′-CAT GGG ATG TTA CAC ACGCC-3′. PCR was performed with an SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), as described previously.[20] *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Western Blotting

Western blotting was performed as described previously.[21] Lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against PLUNC (goat polyclonal, dilution 1:500; R&D Systems, Abingdon, UK). Peroxidase-conjugated anti-goat IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-Actin (Sigma, St Louis, MO, USA) was also stained as a loading control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. Antigen retrieval was done by microwave heating in citrate buffer (pH 6.0) for 30 min for PLUNC, HepPar1, cytokeratin 19 (CK19), cytokeratin 20 (CK20), and polyclonal carcinoembryonic antigen (p-CEA). After peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, sections were incubated with normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min to block nonspecific antibody-binding sites. Sections were incubated with the primary antibodies against PLUNC (the same antibody used in western blotting to PLUNC, dilution 1:50), alpha-fetoprotein (AFP) (C3, 1:20; Novocastra, Newcastle, UK), HepPar1 (OCH1E5, 1:20; Dako Cytomation), CK19 (RCK108, 1:50; Dako Cytomation), CK20 (Ks20.8, 1:50; Dako Cytomation), and p-CEA (1:1000; Dako Cytomation) for 1 h at room temperature, followed by incubations with biotinylated anti-goat, anti-rabbit, or anti-mouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with 10 min incubation with the substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. The staining results were recorded in semiquantitative fashion as follows: 0, absence of staining; 1+, any tumor cell stained to 10% of tumor cells stained; 2+, 11–50% of tumor cells stained; and 3+, more than 50% of the tumor cells stained.

Statistical Methods

Associations between clinicopathologic parameters and PLUNC expression were analyzed by χ^2-test. Kaplan–Meier survival curves were constructed for PLUNC-positive and PLUNC-negative patients. Survival rates were compared between PLUNC-positive and PLUNC-negative groups. Differences between survival curves were tested for statistical significance by log-rank test.[22] A *P*-value of less than 0.05 was considered statistically significant.

Results

Comparison of Gene Expression Profiles from Microarray and SAGE

The gene expression profiles obtained from the gastric cancer sample (P208T) and corresponding non-neoplastic gastric mucosa sample were compared. To identify ideal biomarkers for gastric cancer, we focused on genes that showed significantly increased expression in gastric cancer. The top 20 genes that showed higher expression in the gastric cancer sample than in the corresponding non-neoplastic gastric mucosa sample by microarray

Table 1 Comparison of gene expression profiles from microarray and SAGE

Microarray				*SAGE*[a]		
Symbol	*Intensity*		*Fold*	*Symbol*	*Tag counts*	
	P208T[b]	*Non-neoplastic mucosa*			*P208T*[b]	*Normal stomach*[c]
PLUNC	74421	31	2401	*TSG101*	41	0
MAGEA12	7492	8	937	Transcribed locus	32	0
LACRT	15873	21	756	*CTNND1, CYP20A1*	28	0
MAGEA6	15966	31	515	*BCL2L2*	50	1
MAGEA2	4266	12	356	No match	24	0
MAGEA3	18963	59	321	*TUSC3*	21	0
FN1	2818	9	313	*ELOVL5*	20	0
NTRK2	2123	7	303	*S100A9*	17	0
HOXA10	4481	16	280	*KRTHB1, PHYHD1*	16	0
COL11A1	2303	10	230	*PAWR*	16	0
FGFR2	5593	25	224	*USP7*	14	0
C20orf186	4711	21	224	*KRT7, SH3BP2*	14	0
NXF3	1820	10	182	*CTSL*	14	0
LECT1	3348	24	140	*SEC11L1, WIF1*	26	1
SPP1	288	2	144	No match	12	0
SH3BP4	1214	10	121	*TFF3*	12	0
HM74	22034	209	105	*TKT*	12	0
FLJ20300	9617	96	100	*DNAJC10*	12	0
LOC284527	676	7	97	No match	11	0
SLC19A3	1631	17	96	*DDOST*	11	0

[a]The 20 most upregulated genes in gastric cancer (P208T) compared with normal stomach by SAGE analysis was determined previously.[8]
[b]Gastric cancer sample (60-year-old man, T4N3M0, stage IV, poorly differentiated adenocarcinoma).
[c]SAGE data from normal gastric epithelia (GSM784, SAGE normal gastric body epithelial, El-Rifai *et al*[23]).

analysis are listed in Table 1. The gene showing the greatest increase in expression in the gastric cancer sample by microarray was *PLUNC*. Increased expression of the *MAGE* genes (*MAGEA2, MAGEA3, MAGEA6*, and *MAGEA12*),[24] *FN1* (encoding fibronectin 1),[25] and *FGFR2* (encoding fibroblast growth factor 2)[26] has been reported previously. The 20 genes with the greatest increase in expression in gastric cancer compared with normal stomach[23] by SAGE analysis are shown in Table 1. Surprisingly, the 20 most upregulated genes identified by microarray were quite different from those identified by SAGE, indicating that genes upregulated in gastric cancer are not always detected by SAGE. We reviewed the expression level of *PLUNC* with our SAGE data. The sequence of the SAGE tag that represents *PLUNC* was TGCCTCACCT, and this sequence appeared only three times in the P208T SAGE data. Because expression of *PLUNC* has not been investigated in gastric cancer, we decided to analyze *PLUNC* expression in gastric cancer. Quantitative RT-PCR was performed to investigate the specificity of *PLUNC* expression. As shown in Figure 1a, *PLUNC* expression was clearly detected in adult trachea and to a lesser extent in adult lung. Expression of *PLUNC* was not detected in any other normal organs, including stomach. These results are consistent with those of a previous report.[13] *PLUNC* was expressed in P208T, whereas other gastric cancer tissue samples did not express *PLUNC*.

Immunohistochemical Analysis of PLUNC in Gastric Cancer

We observed upregulation of *PLUNC* mRNA in gastric cancer tissue; however, the expression pattern of PLUNC protein in gastric cancer remains unclear. To address this issue, we performed immunostaining of PLUNC. We first tested the specificity of the anti-PLUNC antibody. Western blotting of lysates from normal adult trachea and normal stomach was performed. The anti-PLUNC antibody detected an approximately 27 kDa band in adult trachea (Figure 1b). These results are consistent with our quantitative RT-PCR data for *PLUNC*. Immunostaining of P208T revealed that PLUNC was present in cytoplasm of tumor cells but not in corresponding non-neoplastic mucosa (Figure 1c). Stromal cells showed weak or no staining of PLUNC. Staining of PLUNC was not observed in the remaining three gastric cancer samples used for quantitative RT-PCR. Taken together, these data show that this anti-PLUNC antibody specifically recognizes PLUNC protein.

We next performed immunohistochemical analysis of PLUNC in 140 human gastric cancer tissue samples. Strong cytoplasmic staining of PLUNC was found in 12 (9%) of 140 gastric cancer samples. Only tumor cells were positive for PLUNC. Stromal cells were not stained. Corresponding non-neoplastic gastric mucosa, including intestinal metaplasia,

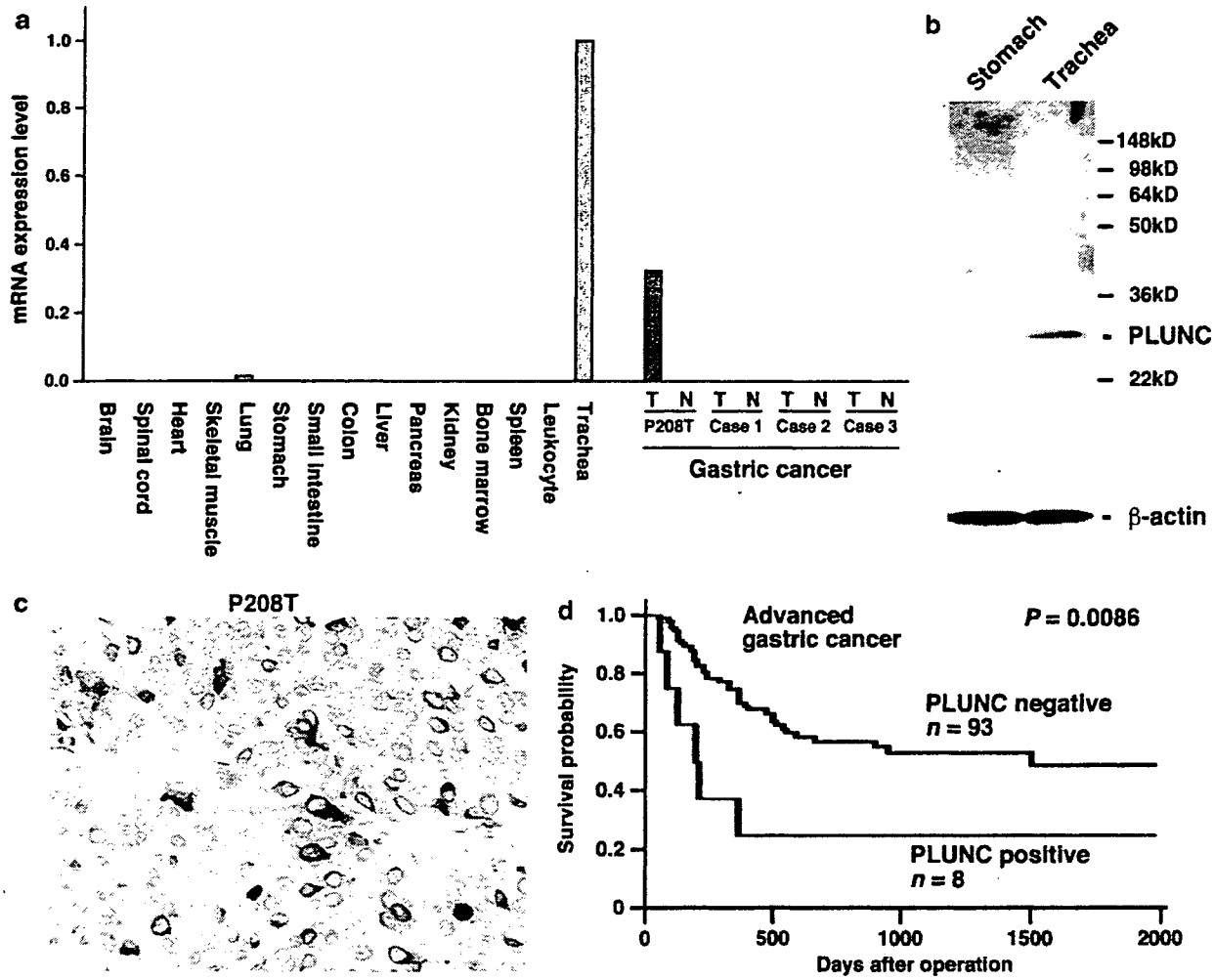

Figure 1 (a) Quantitative RT-PCR analysis of *PLUNC* in 15 normal tissues, four gastric cancer cases (T), and corresponding non-neoplastic mucosa (N). Case P208T was analyzed by SAGE and microarray. The units are arbitrary, and we calculated the level of *PLUNC* mRNA expression by standardization to 1.0 μg of total RNA from trachea as 1.0. (b) Western blot analysis of PLUNC with anti-PLUNC antibody. An approximately 27 kDa band is present in lysate of normal trachea. (c) Immunostaining of PLUNC in gastric cancer (Case P208T). Focal-positive staining for PLUNC in the cytoplasm is observed. (d) Prognostic value of PLUNC staining. Patients with gastric cancer showing PLUNC expression had a significantly worse survival rate than patients without PLUNC expression ($P = 0.0086$, log-rank test).

was not stained. In gastric cancer tissue, staining of PLUNC was heterogeneous, and among 12 PLUNC-positive gastric cancer cases, less than 1% of tumor cells were stained in 9 gastric cancer cases. In the remaining three gastric cancer cases, more than 30% of tumor cells were stained. We then analyzed the relation of PLUNC expression to clinicopathologic characteristics. No correlation was found between PLUNC expression and depth of invasion, lymph node metastasis, or tumor stage (Table 2). In contrast, among 101 advanced gastric cancer cases followed up at the hospital, patients with PLUNC-positive gastric cancer had a significantly worse survival rate than those patients with PLUNC-negative gastric cancer ($P = 0.0086$, log-rank test, Figure 1d). Interestingly, of the three gastric cancer cases with more than 30% of tumor cells stained, two cases showed hepatoid adenocarcinoma of the stomach. The remaining one case was P208T and we confirmed that P208T was not gastric hepatoid adenocarcinoma. It has been reported that gastric hepatoid adenocarcinoma shows canalicular staining pattern of p-CEA,[27] and we confirmed that P208T displayed cytoplasmic pattern of p-CEA (data not shown). PLUNC staining was found more frequently in gastric hepatoid adenocarcinoma (2/2, 100%) than in other types (well and poorly differentiated adenocarcinoma) of gastric cancer (10/138, 7%, $P = 0.0007$, χ^2-test).

Immunohistochemical Analysis of PLUNC in Hepatoid Adenocarcinoma of the Stomach

It is well known that gastric hepatoid adenocarcinoma has an aggressive clinical course and poor survival.[28,29] Its biologic behavior is mainly due to its extensive hematogenous metastasis to the liver and early and frequent involvement of the lymph nodes. Gastric hepatoid adenocarcinoma closely

Table 2 Association of PLUNC expression with clinicopathologic features of gastric cancer

	PLUNC expression (%)		P-*value*[a]
	Positive	*Negative*	
T grade			
T1	4 (10)	35	0.9157
T2/T3/T4	8 (8)	93	
N grade			
N0	5 (7)	62	0.8833
N1/N2/N3	7 (10)	66	
M grade			
M0	11 (8)	120	0.7784
M1	1 (11)	8	
Stage			
Stage I	4 (7)	56	0.6394
Stage II	3 (12)	22	
Stage III	2 (8)	23	
Stage IV	3 (10)	27	
Histologic type			
Well-differentiated adenocarcinoma	6 (7)	75	0.0007[b]
Poorly differentiated adenocarcinoma	4 (7)	53	
Hepatoid adenocarcinoma	2 (100)	0	

[a]χ^2-Test.
[b]Well-differentiated and poorly differentiated adenocarcinoma *vs* hepatoid adenocarcinoma.

mimics and is even indistinguishable from hepatocellular carcinoma. This makes differential diagnosis challenging, especially when the primary tumor is unknown and the first diagnosis has to be established by liver biopsy. Therefore, there is an urgent need for specific biomarkers of hepatoid adenocarcinoma. We performed immunohistochemical analysis of PLUNC in four additional cases of hepatoid adenocarcinoma to investigate the potential utility of PLUNC immunostaining in the diagnosis of hepatoid adenocarcinoma. Among six cases of hepatoid adenocarcinoma (two cases from immunohistochemical analysis in 140 gastric cancer cases plus four additional cases), staining of PLUNC was observed in all six primary tumors. It has been reported that most hepatoid adenocarcinoma cases contain coexistent tubular or papillary adenocarcinoma components within the tumor.[30] All six gastric hepatoid adenocarcinoma cases contained both tubular/papillary adenocarcinoma and hepatoid adenocarcinoma components. out of the six hepatoid adenocarcinoma cases, PLUNC staining was observed in both the hepatoid adenocarcinoma component (Figure 2a and b) and the tubular/papillary adenocarcinoma component (Figure 2c and d). We confirmed that hepatoid adenocarcinoma component showed a canalicular pattern of p-CEA staining (Figure 2a, inset). The PLUNC staining was preferentially found in the tubular/papillary adenocarcinoma component of the primary tumors. In some cases, even when PLUNC-positive tumor cells were found in the tubular/papillary adenocarcinoma component near the hepatoid adenocarcinoma component, PLUNC was not stained in the hepatoid adenocarcinoma component (Figure 2e).Out of the six cases of hepatoid adenocarcinoma, the hepatoid adenocarcinoma component contained 5–10% PLUNC-positive tumor cells, whereas tubular/papillary adenocarcinoma component contained $\leq$70% PLUNC-positive tumor cells (Table 3). Out of the six cases of hepatoid adenocarcinoma, liver metastases from two cases were available for immunohistochemistry and were composed of hepatoid adenocarcinoma component. PLUNC staining was observed in both liver metastases (Figure 2f). One case showed 5% PLUNC-positive tumor cells, and another case contained 20% PLUNC-positive tumor cells within the metastatic tumor. Adjacent nonneoplastic liver tissues were not stained for PLUNC (Figure 2f). We also performed immunohistochemical analysis of PLUNC in 52 cases of hepatocellular carcinoma, 2 cases of normal adult liver, and 2 cases of fetal liver; however, staining of PLUNC was not observed (data not shown). These results indicate that PLUNC is a good marker to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.

Immunohistochemical Analysis of AFP, HepPar1, CK19, and CK20 in Hepatoid Adenocarcinoma of the Stomach

Because production of AFP is usually observed in gastric hepatoid adenocarcinoma, we performed immunostaining of AFP. The results are summarized in Table 3. Although all six hepatoid adenocarcinoma cases were positive for AFP, AFP-positive tumor cells were not positive for PLUNC. AFP-positive tumor cells were observed in hepatoid adenocarcinoma components, whereas PLUNC-positive tumor cells were found mainly in tubular/papillary adenocarcinoma components (Figure 3a and b). Even when PLUNC-positive tumor cells were found in hepatoid adenocarcinoma components, PLUNC-positive tumor cells did not express AFP (Figure 3c and d).

It was previously reported that immunohistochemical analyses of HepPar1, CK19, or CK20 are useful to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.[27] HepPar1 staining is detected more frequently in hepatocellular carcinoma than in gastric hepatoid adenocarcinoma.[27] Staining for CK19 and CK20 is detected more frequently in gastric hepatoid adenocarcinoma than in hepatocellular carcinoma.[27] Therefore, we also performed immunostaining of HepPar1, CK19, and CK20 in gastric hepatoid adenocarcinoma and hepatocellular carcinoma

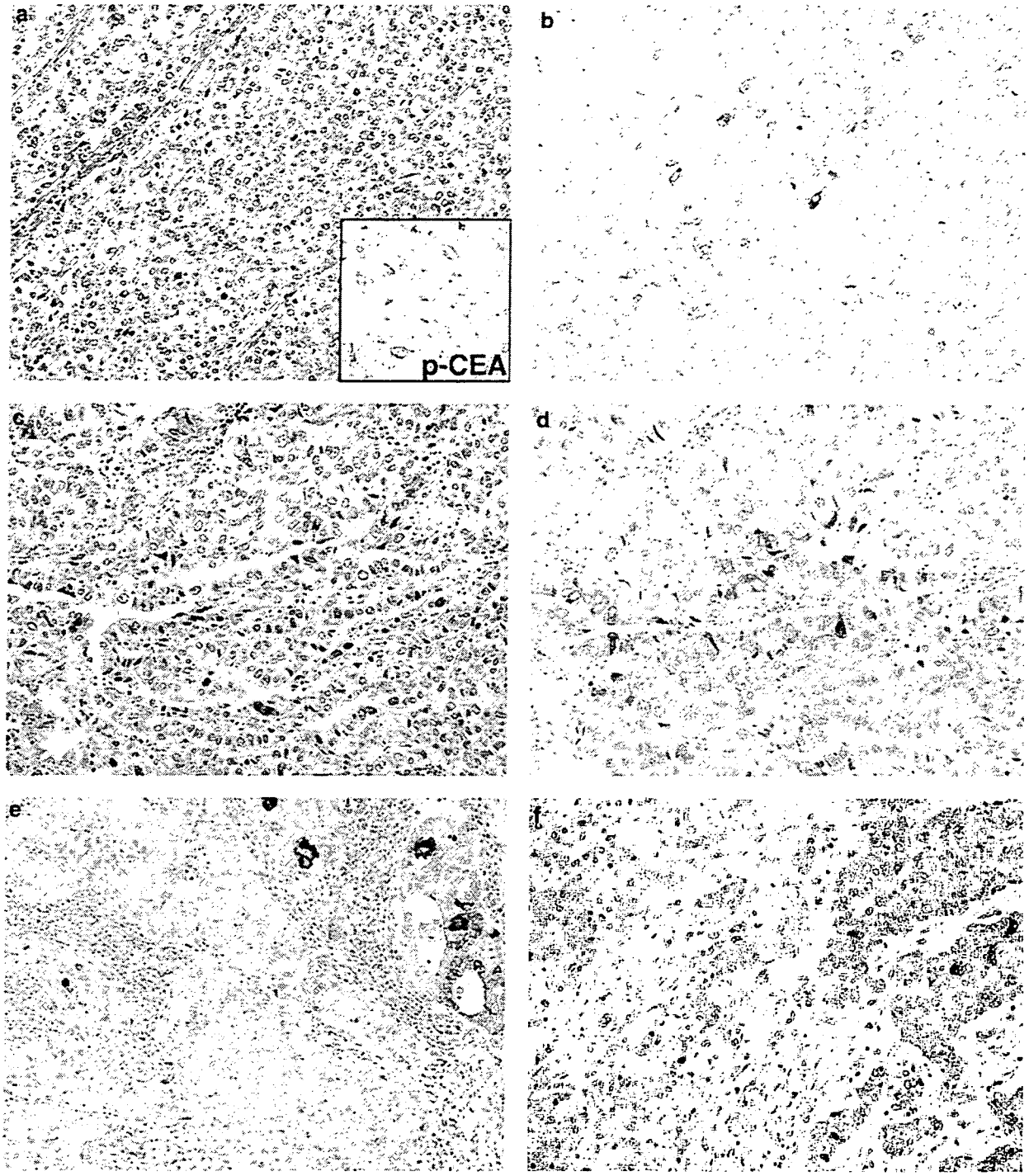

Figure 2 Immunostaining of PLUNC in hepatoid adenocarcinoma of the stomach. **(a)** H&E staining of hepatoid adenocarcinoma component of primary tumor. Tumor cells display a solid structure resembling that of hepatocellular carcinoma. Inset: hepatoid adenocarcinoma component showed a canalicular pattern. **(b)** Corresponding PLUNC staining. Focal-positive staining for PLUNC is observed. **(c)** H&E stain of tubular/papillary adenocarcinoma component of primary tumor that consists of polygonal tumor cells with large central nuclei. **(d)** Corresponding PLUNC staining. Focal-positive staining for PLUNC is observed. **(e)** The tubular adenocarcinoma component is stained by anti-PLUNC antibody, with luminal and cytoplasmic patterns, whereas the hepatoid adenocarcinoma component shows no staining. **(f)** Liver metastasis of gastric hepatoid adenocarcinoma. Focal-positive staining for PLUNC is detected, whereas adjacent non-neoplastic hepatocytes are not stained.

(Table 3). The overall results are summarized in Table 4. Out of six cases of gastric hepatoid adenocarcinoma, four cases showed focal positivity for HepPar1, and all four hepatoid adenocarcinoma cases showed less than 10% HepPar1-positive tumor cells. The remaining two hepatoid adenocarcinoma cases did not express HepPar1. Staining of HepPar1 was also detected in both liver metastases

Table 3 PLUNC, AFP, HepPar1, CK19, and CK20 immunoreactivity in hepatoid adenocarcinoma of the stomach

Case no.	*Organ*	*Hepatoid adenocarcinoma component*					*Tubular/papillary adenocarcinoma component*				
		PLUNC	*AFP*	*HepPar1*	*CK19*	*CK20*	*PLUNC*	*AFP*	*HepPar1*	*CK19*	*CK20*
52	Stomach	1+	2+	1+	2+	0	3+	0	1+	2+	0
126	Stomach	1+	1+	1+	2+	0	3+	0	0	3+	0
141	Stomach	1+	1+	0	1+	1+	1+	0	0	1+	0
142	Stomach	1+	1+	0	2+	0	2+	0	0	2+	1+
143	Stomach[a]	1+	2+	1+	1+	0	0	0	1+	1+	0
	Liver	1+	2+	1+	2+	0	—[b]	—	—	—	—
144	Stomach[a]	1+	2+	1+	2+	1+	1+	0	0	3+	0
	Liver	2+	3+	1+	3+	1+	—	—	—	—	—

0 indicates negative; 1+, ≤10%; 2+, 11–50%; 3+, >50%.

[a]Primary tumor.

[b]Tubular/papillary adenocarcinoma component is not found.

of hepatoid adenocarcinoma (Figure 4b). The two liver metastases of hepatoid adenocarcinoma contained less than 10% HepPar1-positive tumor cells. In contrast, most of the primary hepatocellular carcinoma cases (45 out of 52 cases, 87%) displayed strong and extensive staining (2+, 10 cases; 3+, 35 cases) of HepPar1 (Figure 4c). All six hepatoid adenocarcinoma cases showed CK19 staining (Figure 4d), and CK19 staining was observed in both liver metastases (Figure 4e). One case showed 80% CK19-positive tumor cells, and another case contained 20% CK19-positive tumor cells within the metastatic tumor. Forty-four out of 52 (85%) hepatocellular carcinoma cases showed no staining of CK19, and CK19 staining was observed in eight hepatocellular carcinoma cases (1+, 2 cases; 2+, 3 cases; 3+, 3 cases) (Figure 4f). Two out of six gastric hepatoid adenocarcinoma cases showed focal positivity for CK20, and both hepatoid adenocarcinoma cases showed less than 10% CK20-positive tumor cells (Figure 4g). CK20 staining was observed in one case of liver metastasis (Figure 4h). Forty-two out of 52 (81%) hepatocellular carcinoma cases showed no staining of CK20, and CK20 staining was observed in 10 hepatocellular carcinoma cases (1+, 3 cases; 2+, 4 cases; 3+, 3 cases) (Figure 4i).

Discussion

Gastric hepatoid adenocarcinoma frequently shows histologic features that mimic hepatocellular carcinoma. It can be very challenging to differentiate hepatoid adenocarcinoma from hepatocellular carcinoma on the basis of morphology alone, especially when a specimen is limited, such as small tissue biopsy specimens. Immunoreactivity for AFP, alpha-1 antitrypsin,[30] and Glypican 3[31] in hepatoid adenocarcinoma has been reported. Moreover, albumin mRNA has also been detected in hepatoid adenocarcinoma by *in situ* hybridization.[32] These molecules are expressed in normal adult or fetal liver and show various degrees of utility in distinguishing gastric hepatoid adenocarcinoma from conventional gastric cancer; however, these molecules are also expressed in hepatocellular carcinoma. Therefore, there is no reliable biomarker to distinguish hepatoid adenocarcinoma from hepatocellular carcinoma. In this study, we observed overexpression of PLUNC in gastric cancer with two comprehensive gene expression profiling methods, SAGE and microarray. Although only 7% of conventional gastric cancer cases showed focal immunostaining of PLUNC, extensive staining of PLUNC was observed in all six cases of hepatoid adenocarcinoma. Hepatocellular carcinoma cases did not show staining of PLUNC, whereas both cases of liver metastases of hepatoid adenocarcinoma were positive for PLUNC. These results indicate that PLUNC is a good marker to distinguish hepatoid adenocarcinoma from hepatocellular carcinoma. In this study, staining of PLUNC was not detected in normal adult and fetal liver. In gastric hepatoid adenocarcinoma, PLUNC-positive tumor cells were not stained by AFP. These findings led us to speculate that PLUNC is not involved in hepatocyte differentiation and that this is the reason why immunostaining of PLUNC can distinguish gastric hepatoid adenocarcinoma from hepatocellular carcinoma.

What are the unique features and utility of PLUNC immunostaining? It should be mentioned here that all the samples analyzed by immunostaining in the present study were surgically resected specimens. For surgical resection of a tumor, pathologic diagnosis from a biopsy specimen is essential. Because this is the first investigation of PLUNC in gastric cancer, we analyzed surgically resected specimens to investigate the precise distributions of PLUNC-positive cells. In this study, PLUNC was preferentially expressed in the tubular/papillary adenocarcinoma components of primary tumors. In gastric hepatoid adenocarcinoma, the tubular/papillary adenocarcinoma component tends to be located superficially in the primary tumor, whereas the hepatoid adenocarcinoma component tends to be located in the deeper parts of the primary tumor.[27,33] In fact, PLUNC-positive tumor cells were frequently

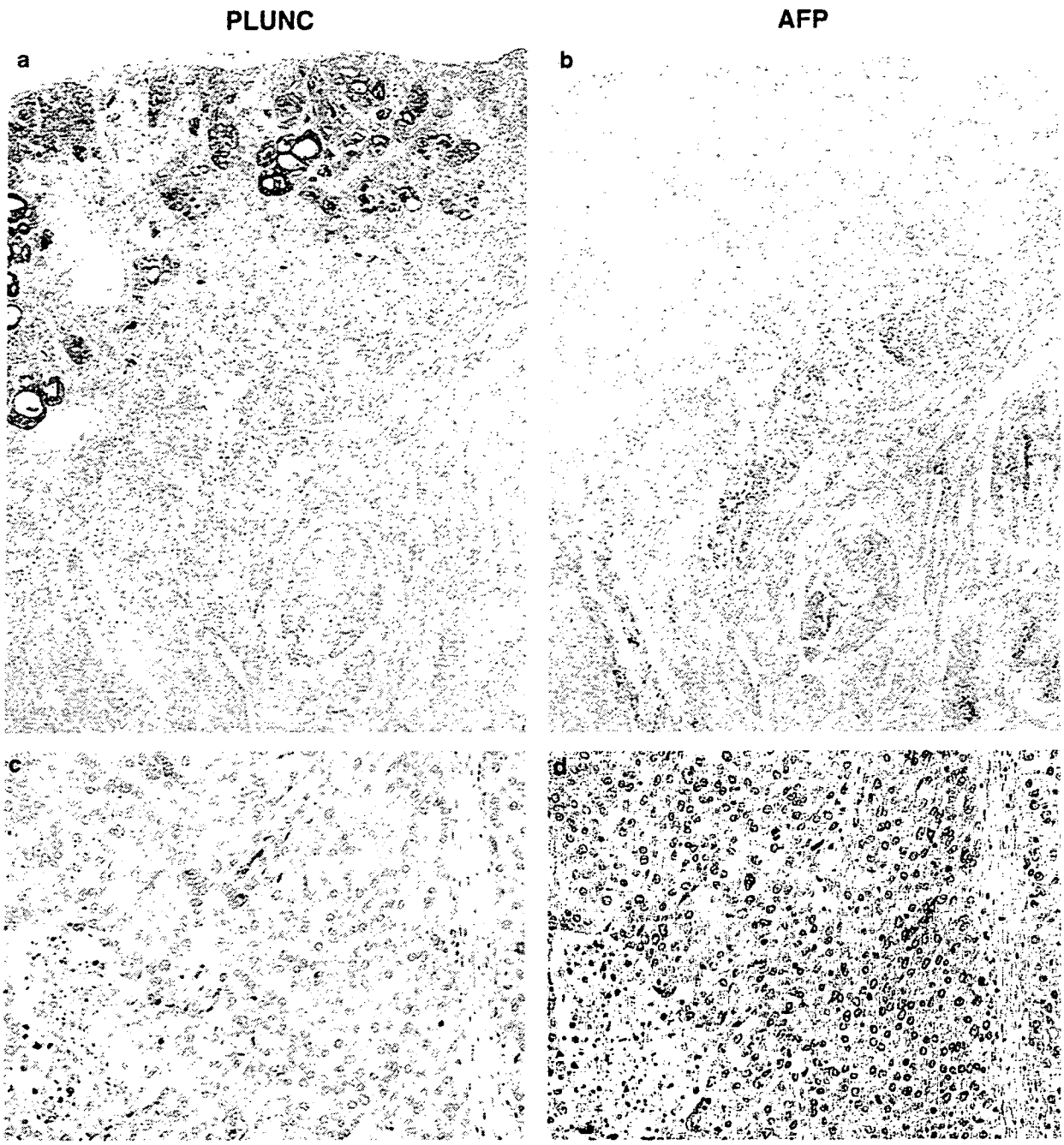

Figure 3 Immunostaining of PLUNC and AFP in hepatoid adenocarcinoma of the stomach. PLUNC staining is observed in the tubular adenocarcinoma component (**a**), whereas AFP staining is found in the hepatoid adenocarcinoma component (**b**). In the hepatoid adenocarcinoma component, PLUNC-positive tumor cells (**c**) are negative for AFP (**d**).

found in superficial areas of gastric hepatoid adenocarcinoma. Therefore, PLUNC immunostaining can be used to test gastric biopsy specimens because only superficial areas are obtained by gastric biopsy. Furthermore, among 138 conventional gastric cancer cases, less than 1% of tumor cells were stained in nine gastric cancer cases, and only one gastric cancer case (P208T) showed extensive staining of PLUNC. These results indicate that immunostaining of PLUNC can differentiate gastric hepatoid adenocarcinoma from conventional gastric cancer. Because PLUNC is frequently stained in the tubular/papillary adenocarcinoma component of gastric hepatoid adenocarcinoma, PLUNC staining can assist in diagnosing gastric hepatoid adenocarcinoma even when the hepatoid adenocarcinoma component is not found in gastric biopsy specimens.

The liver is the most common organ for metastasis by gastric hepatoid adenocarcinoma. It is difficult in some cases to distinguish a liver metastasis of hepatoid adenocarcinoma from a primary hepatocellular carcinoma with liver biopsy specimens because gastric hepatoid adenocarcinoma frequently shows histologic and immunostaining features that

Table 4 Summary of PLUNC, AFP, HepPar1, CK19, and CK20 immunostaining

	No. of positive[a] cases		
	Primary gastric hepatoid adenocarcinoma (n = 6)	*Liver metastasis of gastric hepatoid adenocarcinoma (n = 2)*	*Hepatocellular carcinoma (n = 52)*
PLUNC	6 (100%)	2 (100%)	0 (0%)
AFP	6 (100%)	2 (100%)	11 (21%)
HepPar1	4 (67%)	2 (100%)	48 (92%)
CK19	6 (100%)	2 (100%)	8 (15%)
CK20	2 (33%)	1 (50%)	10 (19%)

[a]1+, 2+, and 3+ cases were considered positive.

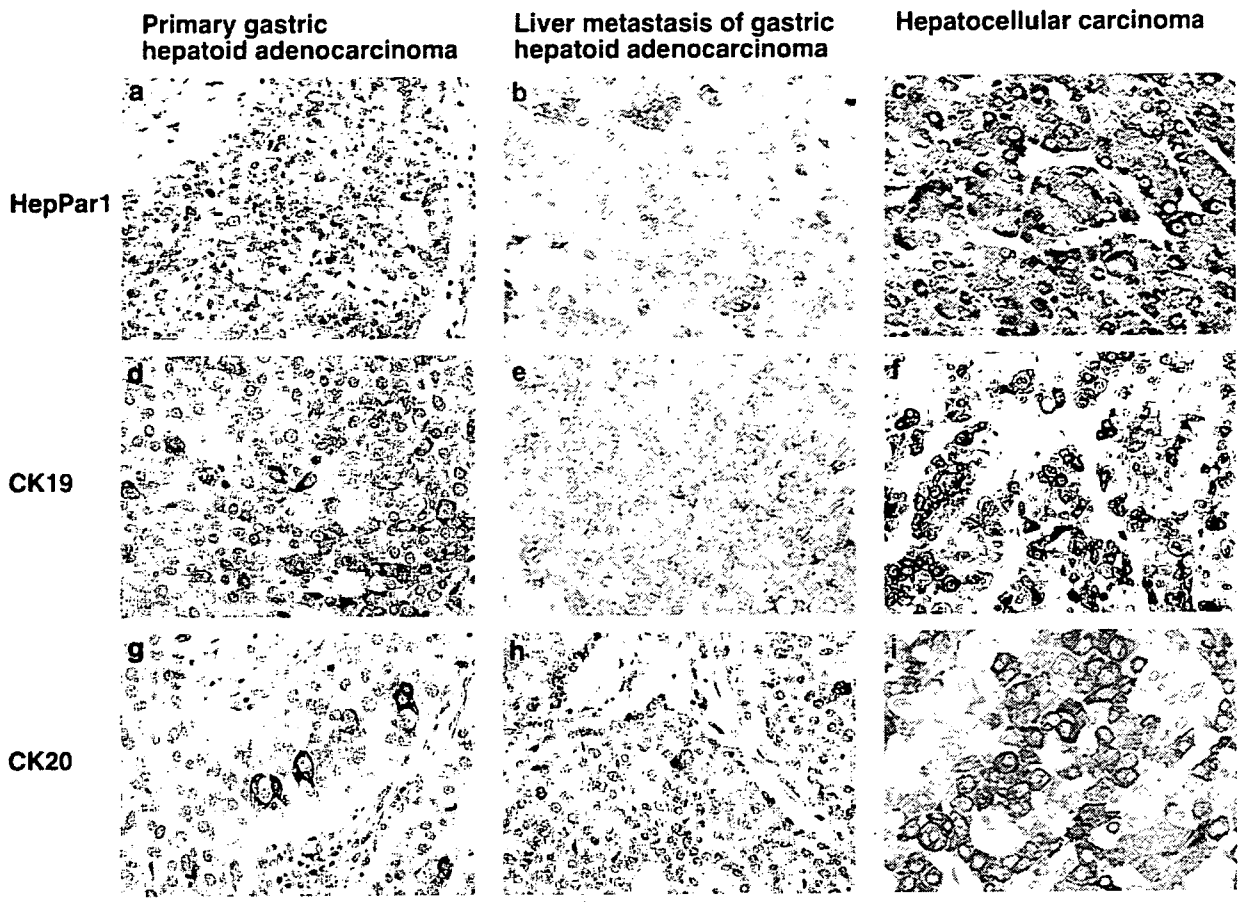

Figure 4 Immunostaining of HepPar1 (**a–c**), CK19 (**d–f**), and CK20 (**g–i**) in primary gastric hepatoid adenocarcinoma, liver metastasis of gastric hepatoid adenocarcinoma, and hepatocellular carcinoma. (**a**) In gastric hepatoid adenocarcinoma, hepatoid adenocarcinoma component shows focal-positive for HepPar1 within the primary tumor. (**b**) In liver metastasis of gastric hepatoid adenocarcinoma, focal-positive staining for HepPar1 is found. (**c**) Hepatocellular carcinoma cases show strong and extensive staining for HepPar1. (**d**) Cytoplasmic staining of CK19 is detected in hepatoid adenocarcinoma component in primary gastric hepatoid adenocarcinoma. (**e**) Diffuse CK19 staining is observed in liver metastasis of gastric hepatoid adenocarcinoma. (**f**) Some hepatocellular carcinoma cases show focal CK19 staining. (**g**) Focal CK20 staining is found in primary gastric hepatoid adenocarcinoma. (**h**) In liver metastasis of gastric hepatoid adenocarcinoma, focal-positive staining for CK20 is detected. (**i**) Some hepatocellular carcinoma cases show focal CK20 staining.

mimic those of hepatocellular carcinoma. It was previously reported that immunostaining for HepPar1, CK19, or CK20 is useful to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.[27] In fact, most primary hepatocellular carcinoma showed extensive staining of HepPar1, whereas only focal staining of HepPar1 was observed in gastric hepatoid adenocarcinoma in this study. Staining for CK19 and CK20 was detected more frequently in gastric hepatoid adenocarcinoma than in hepatocellular carcinoma in the present study. Because only six gastric hepatoid adenocarcinoma cases and two liver metastases of hepatoid adenocarcinoma were investigated in this study, it is

difficult to conclude which is the better marker. At least however, because PLUNC staining was found only in gastric hepatoid adenocarcinoma, but not in hepatocellular carcinoma, PLUNC is a specific marker to distinguish hepatoid adenocarcinoma from hepatocellular carcinoma.

In this study, the 20 genes showing the greatest increase in expression on microarray were quite different from those obtained by SAGE. Investigation of the difference between microarray and SAGE is beyond the scope of this study and will be described elsewhere.

In summary, we found that PLUNC is expressed in gastric hepatoid adenocarcinoma but not primary hepatocellular carcinoma. Although larger trials are required, this initial study shows the potential of PLUNC immunostaining to serve as a marker to distinguish metastatic hepatoid adenocarcinoma from primary hepatocellular carcinoma. In conventional gastric cancer cases, patients with PLUNC-positive gastric cancer had a significantly worse survival rate than those patients with PLUNC-negative gastric cancer; therefore, expression of PLUNC may be a key factor mediating the malignant behavior of gastric hepatoid adenocarcinoma.

Acknowledgement

We thank Ms Emiko Hisamoto and Mr Masayuki Ikeda for their excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank the Analysis Center of Life Science, Hiroshima University, for the use of their facilities. This work was supported, in part, by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports, and Technology of Japan; by the program for promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio); and by a Grant-in-Aid for the Third Comprehensive 10-Year Strategy for Cancer Control and for Cancer Research (16-15) from the Ministry of Health, Labour and Welfare of Japan.

References

1 Ohgaki H, Matsukura N. Stomach cancer. In: Stewart BW, Kleihues P (eds). World Cancer Report. IARC Press: Lyon, 2003, pp 197.
2 Yasui W, Oue N, Kitadai Y, *et al.* Recent advances in molecular pathobiology of gastric carcinoma. In: Kaminishi M, Takubo K, Mafune K (eds). The Diversity of Gastric Carcinoma Pathogenesis: Diagnosis, and Therapy. Springer: Tokyo, 2005, pp 51–71.
3 Kondo T, Oue N, Mitani Y, *et al.* Loss of heterozygosity and histone hypoacetylation of the PINX1 gene are associated with reduced expression in gastric carcinoma. Oncogene 2005;24:157–164.
4 Kurayoshi M, Oue N, Yamamoto H, *et al.* Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Res 2006;66:10439–10448.
5 Yasui W, Oue N, Ito R, *et al.* Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. Cancer Sci 2004;95:385–392.
6 Lockhart DJ, Dong H, Byrne MC, *et al.* Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 1996;14:1675–1680.
7 Velculescu VE, Zhang L, Vogelstein B, *et al.* Serial analysis of gene expression. Science 1995;270:484–487.
8 Oue N, Hamai Y, Mitani Y, *et al.* Gene expression profile of gastric carcinoma: identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. Cancer Res 2004;64:2397–2405.
9 Aung PP, Oue N, Mitani Y, *et al.* Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene 2006;25:2546–2557.
10 Ishii M, Hashimoto S, Tsutsumi S, *et al.* Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. Genomics 2000;68:136–143.
11 Oue N, Aung PP, Mitani Y, *et al.* Genes involved in invasion and metastasis of gastric cancer identified by array-based hybridization and serial analysis of gene expression. Oncology 2005;69(Suppl 1):17–22.
12 Weston WM, LeClair EE, Trzyna W, *et al.* Differential display identification of plunc, a novel gene expressed in embryonic palate, nasal epithelium, and adult lung. J Biol Chem 1999;274:13698–13703.
13 Bingle CD, Bingle L. Characterisation of the human plunc gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern. Biochim Biophys Acta 2000;1493:363–367.
14 Iwao K, Watanabe T, Fujiwara Y, *et al.* Isolation of a novel human lung-specific gene, LUNX, a potential molecular marker for detection of micrometastasis in non-small-cell lung cancer. Int J Cancer 2001;91:433–437.
15 Bingle L, Cross SS, High AS, *et al.* SPLUNC1 (PLUNC) is expressed in glandular tissues of the respiratory tract and in lung tumours with a glandular phenotype. J Pathol 2005;205:491–497.
16 Sobin LH, Wittekind CH (eds). TNM Classification of Malignant Tumors, 6th edn. Wiley-Liss Inc: New York, 2002, pp 65–68.
17 Hohenberger P, Gretschel S. Gastric cancer. Lancet 2003;362:305–315.
18 Nishigaki M, Aoyagi K, Danjoh I, *et al.* Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays. Cancer Res 2005;65:2115–2124.
19 Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996;6:995–1001.
20 Kondo T, Oue N, Yoshida K, *et al.* Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res 2004;64:523–529.
21 Yasui W, Ayhan A, Kitadai Y, *et al.* Increased expression of p34cdc2 and its kinase activity in human

gastric and colonic carcinomas. Int J Cancer 1993;53:36–41.
22 Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966;50:163–170.
23 El-Rifai W, Moskaluk CA, Abdrabbo MK, *et al.* Gastric cancers overexpress S100A calcium-binding proteins. Cancer Res 2002;62:6823–6826.
24 Inoue H, Mori M, Honda M, *et al.* The expression of tumor-rejection antigen 'MAGE' genes in human gastric carcinoma. Gastroenterology 1995;109:1522–1525.
25 David L, Nesland JM, Holm R, *et al.* Expression of laminin, collagen IV, fibronectin, and type IV collagenase in gastric carcinoma. An immunohistochemical study of 87 patients. Cancer 1994;73:518–527.
26 Ueki T, Koji T, Tamiya S, *et al.* Expression of basic fibroblast growth factor and fibroblast growth factor receptor in advanced gastric carcinoma. J Pathol 1995;177:353–361.
27 Terracciano LM, Glatz K, Mhawech P, *et al.* Hepatoid adenocarcinoma with liver metastasis mimicking hepatocellular carcinoma: an immunohistochemical and molecular study of eight cases. Am J Surg Pathol 2003;27:1302–1312.
28 Chang YC, Nagasue N, Abe S, *et al.* Alpha fetoprotein producing early gastric cancer with liver metastasis: report of three cases. Gut 1991;32:542–545.
29 Ishikura H, Kishimoto T, Andachi H, *et al.* Gastrointestinal hepatoid adenocarcinoma: venous permeation and mimicry of hepatocellular carcinoma, a report of four cases. Histopathology 1997;31:47–54.
30 Ishikura H, Kirimoto K, Shamoto M, *et al.* Hepatoid adenocarcinomas of the stomach. An analysis of seven cases. Cancer 1986;58:119–126.
31 Hishinuma M, Ohashi KI, Yamauchi N, *et al.* Hepatocellular oncofetal protein, glypican 3 is a sensitive marker for alpha-fetoprotein-producing gastric carcinoma. Histopathology 2006;49:479–486.
32 Supriatna Y, Kishimoto T, Uno T, *et al.* Evidence for hepatocellular differentiation in alpha-fetoprotein-negative gastric adenocarcinoma with hepatoid morphology: a study with *in situ* hybridisation for albumin mRNA. Pathology 2005;37:211–215.
33 Kishimoto T, Nagai Y, Kato K, *et al.* Hepatoid adenocarcinoma: a new clinicopathological entity and the hypotheses on carcinogenesis. Med Electron Microsc 2000;33:57–63.

CE: Diganta ED: geetha Op: ns PAS:200842

ORIGINAL ARTICLE

Immunohistochemical Staining of Reg IV and Claudin-18 is Useful in the Diagnosis of Gastrointestinal Signet Ring Cell Carcinoma

Kazuhiro Sentani, MD, Naohide Oue, MD, PhD,* Takashi Tashiro, MD, PhD,† Naoya Sakamoto, MD,* Takashi Nishisaka, MD, PhD,‡ Toshiyuki Fukuhara, MD, PhD,‡ Kiyomi Taniyama, MD, PhD,§ Hiroo Matsuura, MD, PhD,‖ Koji Arihiro, MD, PhD,¶ Atsushi Ochiai, MD, PhD,# and Wataru Yasui, MD, PhD**

Abstract: Signet-ring cell carcinoma (SRCC) is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation and a crescent-shaped nucleus displaced toward one end of the cell. Identification of an SRCC's primary site is important for better planning of patient management because the treatment and prognosis differs markedly depending on the origin of the SRCC. In the present study, we analyzed the immunohistochemical characteristics of 94 cases of SRCC, including 21 cases of gastric SRCC, 16 of colorectal SRCC, 10 of breast SRCC, and 47 of pulmonary SRCC, with antibodies against Reg IV and claudin-18, which we previously identified as gastric cancer-related genes. We also tested known markers cytokeratin 7, cytokeratin 20, MUC2, MUC5AC, caudal-related homeobox gene 2 (CDX2), thyroid transcription factor-1, mammaglobin, gross cystic disease fluid protein 15, and estrogen receptor. All 21 cases of gastric SRCC and 16 cases of colorectal SRCC were positive for Reg IV, and the remaining SRCCs were negative. Eighteen of 21 (86%) gastric SRCCs and 6 of 16 (38%) colorectal SRCCs were positive for claudin-18, whereas another SRCCs were negative. In conclusion, Reg IV staining and claudin-18 staining can aid in diagnosis of gastrointestinal SRCC.

Key Words: signet-ring cell carcinoma, Reg IV, claudin-18, gastric cancer

(*Am J Surg Pathol* 2007;00:000–000)

Signet-ring cell carcinoma (SRCC) is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation and a crescent-shaped nucleus displaced toward one end of the cell. SRCC can arise in almost every organ and can present with distant metastases. In general, the prognosis of patients with SRCC regardless of the site of origin is poor.[14,17,28,33,35,38] SRCCs are morphologically identical irrespective of the primary site of origin or metastatic status. In some cases, the primary site of origin may be difficult to determine even after complete clinical and radiologic workups. This is a clinically significant problem because without such information, proper treatment and determination of prognosis may be delayed. Immunohistochemical profiling may aid in directing the workup of metastatic SRCC of an unknown primary site, and several markers are known. SRCC of the prostate can be differentiated from that of other sites by expression of prostate-specific antigen.[18] Similarly, SRCC of the lung expresses sensitive and specific markers, such as thyroid transcription factor-1 (TTF-1), that may help differentiate lung carcinoma from other tumors.[21] However, these SRCCs are not as common as SRCC of the stomach, colorectum, and breast, which comprise more than 90% of SRCC tumors.[2,11,14] To date, there has been no report describing useful markers for differentiating these tumors.

We previously performed serial analysis of gene expression of 4 primary gastric cancers[24] and identified several gastric cancer-related genes.[3] Of these genes, we found that *regenerating islet-derived family member 4* (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with gastric cancer. Expression of Reg IV is limited to stomach, small intestine, colon, and pancreas in normal tissues.[25]

From the *Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences; ‡Department of Pathology and Laboratory Medicine, Hiroshima Prefectural Hospital; ‖Department of Pathology, Hiroshima City Hospital; ¶Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima; †Pathology Division, Hyogo Prefectural Kakogawa Hospital, Hyogo; §Institute for Clinical Research, National Hospital Organization Kure Medical Center and Chugoku Cancer Center, Kure; and #Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Chiba, Japan.

Supported, in part, by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan and in part by a Grant-in-Aid for the Third Comprehensive 10-Year Strategy for Cancer Control and for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University.

Reprints: Wataru Yasui, MD, PhD, Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan (e-mail: wyasui@hiroshima-u.ac.jp).