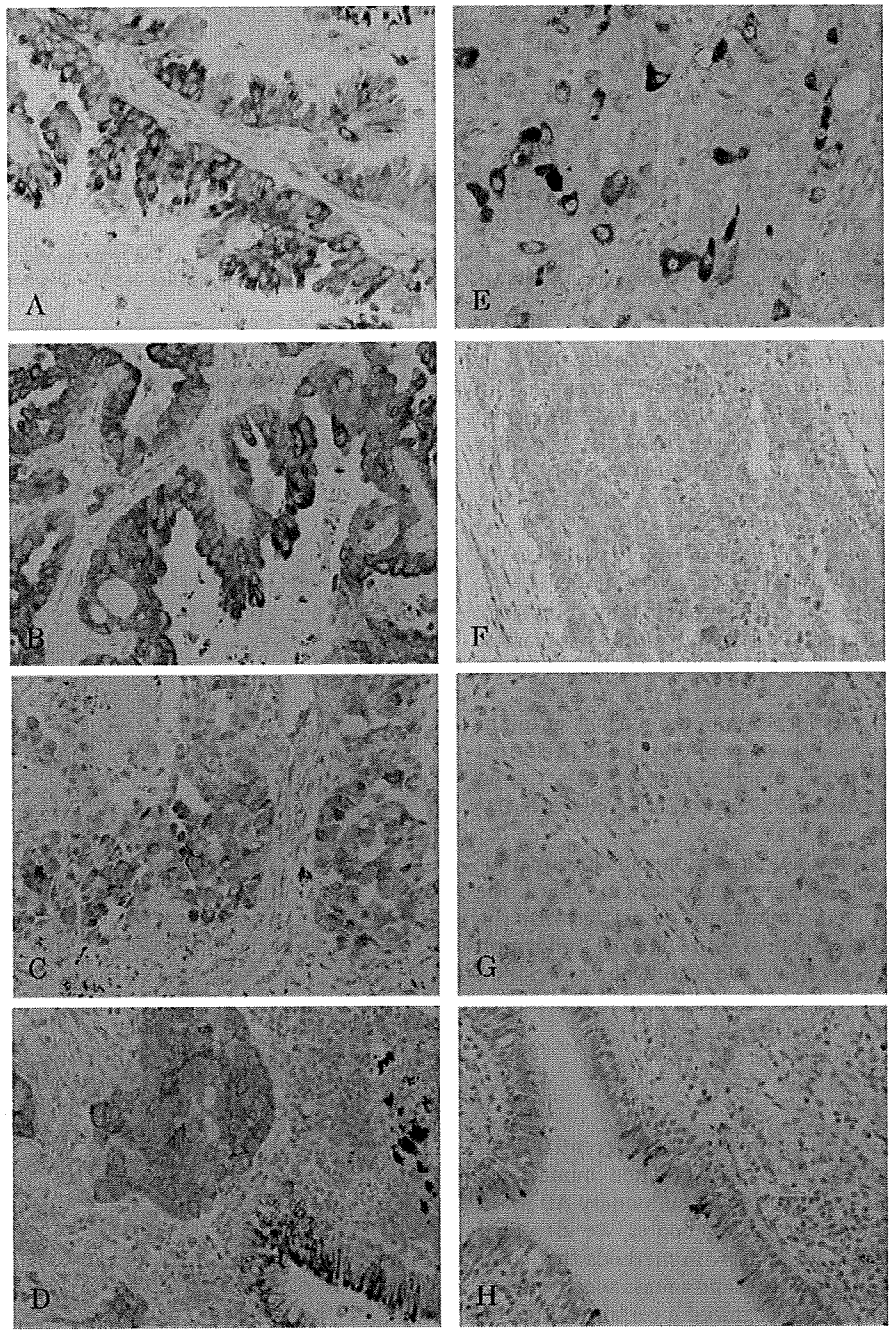

Fig. 4. Expression of RACK1 in pulmonary carcinomas. RACK1 was observed to various degrees in the cytoplasm of pulmonary carcinomas. In general, moderate to marked expression was observed both in adenocarcinomas (A–D) and large cell neuroendocrine carcinomas (E); however, no obvious staining was observed in small cell carcinomas (F) and in almost all squamous cell carcinomas (G). Moderate to marked expression of RACK1 was also observed scattered in bronchial epithelia (H) and stronger expression was also found in proliferative bronchial epithelium (D).

within the cell [3]. RACK1 has many other binding partners involved in the organization of adhesions and cell migration, including the cytoplasmic tail of β integrins [13,14], phospholipase C-γ, Ras-GAP [15], PTPμ [16], β-spectrin, dynamin [17], and Fyn [18]. These interactions support the role of RACK1 as a key scaffolding protein that mediates protein–protein interactions for the regulation of cell motility.

RACK1 can also bind and inhibit Src family kinases [19]. Src family kinases have been shown to play a key role in regulating adhesion formation, adhesion release, and cell migration. RACK1, through its interaction with PKC and Src kinases, may function as a critical adaptor protein mediating cross-talk between serine/threonine and tyrosine kinase signaling pathways. Using siRNA, Doan and Huttenlocher showed that RACK1 is required for efficient cell migration and the dynamic turnover of adhesions [20]. Moreover, it is likely that RACK1 also functions to negatively regulate cell migration since previous studies indicated that overexpression of RACK1 can be associated with reduced migration [13,21]. In this study, the expression of RACK1 was negatively correlated with tumor stage and nodal status, and we thought from previous studies and the present data that it played a role in the inhibition of migration ability. Recent studies indicated that RACK1 reduced cell-cycle progression and the growth of colon carcinoma cells by negatively regulating endogenous Src kinase activity, suggesting that RACK1 may be an attractive therapeutic target to treat cancer [22]. Furthermore, as RACK1 expression was significantly higher in

Please cite this article in press as: Nagashio R, et al. Expression of RACK1 is a novel biomarker in pulmonary adenocarcinomas. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.09.015

small (<3 cm) than in large (≧3 cm) tumors, we also considered the inhibitory activity of tumor growth by RACK1.

Although RACK1 has many bioactivities, as mentioned above, and Slager et al. [23] reported that RACK1 protein is localized exclusively in basal, non-ciliated (and non-goblet) bronchial epithelial cells, the expression of this molecule in human lung cancers remains unknown. In this study, normal bronchial epithelium cells also expressed RACK1 protein to various degrees as well as tumor cells.

Angiogenesis plays a pivotal role in many processes, including embryonic development, myocardial ischemia, the ovarian cycle, and tumor growth [24–28]. Angiogenesis is associated with marked changes in the expression of genes related to intracellular signal transduction [25]. RACK1 is a pivotal molecule directly associated with the signal transduction pathway of PKC. Specifically, PKCβ, the ligand of RACK1, is known to play an important role in angiogenesis [9,29]. Recently, VEGF-induced tumor angiogenesis and tumor growth *in vivo* were shown to be PKCβ dependent [9], and the inhibition of PKCβ significantly suppressed VEGF-induced neovacularization in mouse hepatocellular carcinoma [9]. In addition to an angiogenically active endothelium, RACK1 expression was observed in tumor cells and normal mucosa of the colon and ovarian follicles [8].

RACK1 mRNA was reported to be over-expressed in colon cancer tissues compared to non-cancerous regions using the combination of suppression subtractive hybridization and macro-DNA array [30]. Taken together, our data demonstrated that the overexpression of RACK1 was limited in lung ADs.

To clarify RACK1 expression as a useful differential diagnostic marker for pulmonary adenocarcinomas, further immunohistochemical studies with a larger number of samples, including LCNEC and SCLC cases, will be required.

Finally, the present study used a random immunization method that can select antibodies which react with only tumor cells at the time of screening and is useful to obtain many potential immunostaining antibodies in a short time.

5. Conclusion

With the aim of identifying useful early and/or differential diagnostic markers for pulmonary adenocarcinomas, we developed monoclonal antibodies by a random immunization method using A549 cells derived from a pulmonary adenocarcinoma as an immunogen, and an antibody against RACK1 showed a significantly higher positive rate for pulmonary adenocarcinomas than tumors of other histological subtypes ($p < 0.0001$). Moreover, RACK1 expression was significantly associated with the pathological stage ($p = 0.0042$), tumor size ($p = 0.0074$), and lymph node status ($p = 0.0009$) of adenocarcinoma patients. These results suggest that RACK1 is a novel differential marker for pulmonary adenocarcinomas.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research C (C19590365) from the Japan Society for the Promotion of Science, and for Research Project (No. 2008-1003) from the School of Allied Health Sciences, and 2008 Project Study from the Graduate School of Medical Sciences, Kitasato University.

References

[1] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
[2] Hirohashi S, Watanabe M, Shimosato Y, Sekine T. Monoclonal antibody reactive with the sialyl-sugar residue of a high molecular weight glycoprotein in sera of cancer patients. Gann 1984;75:485–8.
[3] Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. Proc Natl Acad Sci USA 1994;91:839–43.
[4] McCahill A, Warwicker J, Bolger GB, Houslay MD, Yarwood SJ. The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. Mol Pharmacol 2002;62:1261–73.
[5] Schechtman D, Mochly-Rosen D. Adaptor proteins in protein kinase C-mediated signal transduction. Oncogene 2001;20:6339–47.
[6] Sklan EH, Podoly E, Soreq H. RACK1 has the nerve to act: structure meets function in the nervous system. Prog Neurobiol 2006;78:117–34.
[7] Imai Y, Suzuki Y, Tohyama M, Wanaka A, Takagi T. Cloning and expression of a neural differentiation-associated gene, p205, in the embryonal carcinoma cell line P19 and in the developing mouse. Brain Res Mol Brain Res 1994;24:313–9.
[8] Berns H, Humar R, Hengerer B, Kiefer FN, Battegay EJ. RACK1 is up-regulated in angiogenesis and human carcinomas. FASEB J 2000;14:2549–58.
[9] Yoshiji H, Kuriyama S, Ways DK, Yoshii J, Miyamoto Y, Kawata M, et al. Protein kinase C lies on the signaling pathway for vascular endothelial growth factor-mediated tumor development and angiogenesis. Cancer Res 1999;59:4413–8.
[10] Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y. The AMeX method. A simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. Am J Pathol 1986;125:431–5.
[11] Lammli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[12] Nagashio R, Sato Y, Jiang SX, Ryuge S, Kodera Y, Maeda T, et al. Detection of tumor-specific autoantibodies in sera of patients with lung cancer. Lung Cancer 2008;62:364–73.
[13] Buensuceso CS, Woodside D, Huff JL, Plopper GE, O'Toole TE. The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration. J Cell Sci 2001;114:1691–8.
[14] Liliental J, Chang DD. Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. J Biol Chem 1998;273:2379–83.
[15] Chang BY, Chiang M, Cartwright CA. The interaction of Src and RACK1 is enhanced by activation of protein kinase C and tyrosine phosphorylation of RACK1. J Biol Chem 2001;276:20346–56.
[16] Mourton T, Hellberg CB, Burden-Gulley SM, Hinman J, Rhee A, Brady-Kalnay SM. The PTPmu protein-tyrosine phosphatase binds and recruits the scaffolding protein RACK1 to cell–cell contacts. J Biol Chem 2001;276:14896–901.
[17] Rodriguez MM, Ron D, Touhara K, Chen CH, Mochly-Rosen D. RACK1, a protein kinase C anchoring protein, coordinates the binding of activated protein kinase C and select pleckstrin homology domains in vitro. Biochemistry 1999;38:13787–94.
[18] Yaka R, Thomton C, Vagts AJ, Phamluong K, Bonci A, Ron D. NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1. Proc Natl Acad Sci USA 2002;99:5710–5.
[19] Chang BY, Conroy KB, Machleder EM, Cartwright CA. RACK1, a receptor for activated C kinase and a homolog of the beta subunit of G proteins, inhibits activity of Src tyrosine kinase and growth of NIH 3T3 cells. Mol Cell Biol 1998;18:3245–56.
[20] Doan AT, Huttenlocher A. RACK1 regulates Src activity and modulates paxillin dynamics during cell migration. Exp Cell Res 2007;313:2667–79.
[21] Cox EA, Bennin D, Doan AT, O'Toole T, Huttenlocher. RACK1 regulates integrin-mediated adhesion, protrusion, and chemotactic cell migration via its Src-binding site. Mol Biol Cell 2003;14:658–69.
[22] Mamidipudi V, Dhillon NK, Parman T, Miller LD, Lee KC, Cartwright CA. RACK1 inhibits colonic cell growth by regulating Src activity at cell cycle checkpoints. Oncogene 2007;26:2914–24.
[23] Slager RE, DeVasure JM, Pavlik JA, Sisson JH, Wyatt TA. RACK1, a PKC targeting protein, is exclusively localized to basal airway epithelial cells. J Histochem Cytochem 2008;56:7–14.
[24] Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931–4.
[25] Risau W. Mechanisms of angiogenesis. Nature (London) 1997;386:671–4.
[26] Battegay EJ. Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. J Mol Med 1995;73:333–46.
[27] Vernon RB, Sage EH. Between molecules and morphology, extracellular matrix and creation of vascular form. Am J Pathol 1995;147:873–83.
[28] Reymolds LP, Killilea SD, Redmer DA. Angiogenesis in the female reproductive system. FASEB J 1992;6:886–92.
[29] Zhou LY, Disatnik M, Herron GS, Mochly-Rosen D, Karasek MA. Differential activation of protein kinase C isozymes by phorbol ester and collagen in human skin microvascular endothelial cells. J Invest Dermatol 1996;107:248–52.
[30] Saito A, Fujii G, Sato Y, Gotoh M, Sakamoto M, Toda G, et al. Detection of genes expressed in primary colon cancers by in situ hybridization: overexpression of RACK1. J Clin Pathol: Mol Pathol 2002;55:34–9.

Please cite this article in press as: Nagashio R, et al. Expression of RACK1 is a novel biomarker in pulmonary adenocarcinomas. Lung Cancer (2009), doi:10.1016/j.lungcan.2009.09.015

Pathology International

Pathology International 2010 doi:10.1111/j.1440-1827.2009.02487.x

Original Article

Significant high expression of cytokeratins 7, 8, 18, 19 in pulmonary large cell neuroendocrine carcinomas, compared to small cell lung carcinomas

Ryo Nagashio,[1] Yuichi Sato,[1,2] Toshihide Matsumoto,[1,2] Taihei Kageyama,[1,2] Yukitoshi Satoh,[3] Shinichiro Ryuge,[4] Noriyuki Masuda,[4] Shi-Xu Jiang[2,5] and Isao Okayasu[2,5]

[1]*Department of Molecular Diagnostics, School of Allied Health Sciences,* [2]*Department of Cellular and Histo-Pathology, Graduate School of Medical Sciences,* [3]*Departments of Thoracic and Cardiovascular Surgery,* [4]*Respiratory Medicine and* [5]*Pathology, School of Medicine, Kitasato University, Kanagawa, Japan*

The aim of the present study was to clarify protein profiling in small cell lung carcinoma (SCLC) and pulmonary large cell neuroendocrine carcinoma (LCNEC). The proteomic approach was used, and involved cell lysate from two cell lines (N231 derived from SCLC and LCN1 derived from LCNEC), with 2-D gel electrophoresis (2-DE). In the present study, 25 protein spots with greater than twofold quantitative differences between LCN1 and N231 cells on 2-DE gels were confirmed. Within the 25 identified proteins, cytokeratins (CK) 7, 8, 18 and 19 were upregulated in LCN1 cells compared with N231 cells. The expression of CK7, 8, 18, and 19 was further studied on immunohistochemistry with 81 formalin-fixed and paraffin-embedded pulmonary carcinomas, which included 27 SCLC, 30 LCNEC, 14 adenocarcinomas, and 10 squamous cell carcinomas. Although the expression of CK7, 8, 18, and 19 was observed in all histological types, the mean immunostaining scores of CK7, 8, 18, and 19 were significantly higher in LCNEC than in SCLC ($P < 0.001$, $P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively). These data suggest that the biological characteristics of LCNEC and SCLC may be different and the expression of CK may serve as differential diagnostic markers.

Key words: cytokeratin, immunohistochemistry, large cell neuroendocrine carcinoma, lung cancer, small cell lung carcinoma, 2-D gel electrophoresis

Correspondence: Yuichi Sato, PhD, Department of Molecular Diagnostics, School of Allied Health Sciences, Kitasato University, 1-15-1, Kitasato, Sagamihara, Kanagawa 228-8555, Japan. Email: yuichi@med.kitasato-u.ac.jp

Received 22 July 2009. Accepted for publication 28 September 2009.

Neuroendocrine carcinoma is the general term for carcinomas that secrete or express various peptide hormones and biogenic amines (such as adrenocorticotropic hormone, gastrin-releasing peptide, calcitonin, and serotonin). Generally, neuroendocrine carcinomas have morphological characteristics such as organoid structures, palisading basal cell arrangement and rosette formation. In 1991, Travis *et al.* introduced the term 'large cell neuroendocrine carcinoma' (LCNEC) to describe a distinct category of high-grade neuroendocrine tumor with biological and light microscopy characteristics different from those of high-grade small cell lung carcinoma (SCLC).[1]

Morphologically, SCLC is composed of small (most cells less than the nuclear diameter of three small resting lymphocytes), round to fusiform cells with a high nuclear/cytoplasmic ratio, hyperchromatic nuclei with fine chromatin, and absent or inconspicuous nucleoli. The mitotic index is high. Although chemotherapy and radiotherapy are more effective against SCLC than the other histological types, the prognosis of SCLC patients is very poor because most tumors relapse after chemoradiotherapy. The 5 year survival rate of SCLC patients is approximately 35.7%.[2]

In contrast, however, LCNEC is also characterized by neuroendocrine morphology (rosette formation); the tumor cells are large (threefold larger in diameter than a small resting lymphocyte) and tend to be polygonal rather than fusiform, with a low nuclear/cytoplasmic ratio and prominent nucleoli. The nuclear chromatin tends to be coarse and granular. The 5 year survival rate of LCNEC patients is 40.3%, which is not significantly different to that of SCLC.[2]

Although LCNEC appears to fall between atypical carcinoid (AC) and SCLC, it is difficult to differentiate LCNEC from SCLC, and definitive discrimination points (except for

morphological characteristics) and the details of its biological behavior, including tumor aggressiveness and degree of differentiation, remain unclear.

In the present study, to clarify the biological differences of SCLC and LCNEC, we performed protein profiling using 2-D gel electrophoresis with an agarose isoelectric focusing gel in the first dimension (agarose 2-DE). Agarose 2-DE is unique in that it can analyze much larger quantities and a wider dynamic range of proteins than 2-DE with immobilized pH gradient (IPG) gel for isoelectric focusing (conventional 2-DE), and is also able to resolve high-molecular-weight proteins >100 kDa, which are difficult to resolve on conventional 2-DE.[3] We identified proteins with more than twofold quantitative differences between LCNEC cells and SCLC cells using matrix-assisted laser desorption/ionization time of flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS).

MATERIALS AND METHODS

Cell lines

LCN1, an LCNEC line, was established in our laboratory at Kitasato University.[4] The SCLC line N231 was purchased from the American Type Culture Collection (Rockville, MD, USA). The cell lines were grown in RPMI-1640 medium (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Auckland, New Zealand). Subconfluented cells were harvested and washed twice with PBS without bivalent ions, and were partly fixed in 10% formalin and embedded in paraffin for immunohistochemical staining and a sample was stored at −80°C until proteome analysis.

Tissues

Eighty-one cases of surgically resected lung cancer tissues at Kitasato University Hospital were used in the present study. They were divided into 27 SCLC, 30 LCNEC, 14 adenocarcinomas (AD), and 10 squamous cell carcinomas (SCC).

This study was approved by the Ethics Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave consent to donate their samples.

Agarose 2-DE

The solubilization of cells and quantification of cell lysates have been described in a recent study.[5] The agarose-2-DE method used in the present study was previously described by Oh-Ishi *et al.* [3] After 2-DE, the gel was visualized on Coomassie Brilliant Blue R-350 (CBB, PhastGel Blue R; Amersham Pharmacia Biotech, Uppsala, Sweden) staining. Each agarose 2-DE was performed twice. Protein patterns in gels were recorded as digitalized images using a high-resolution scanner (GT-9800; Epson, Tokyo, Japan), and the intensity of each spot was compared. Each spot was analyzed using Scion Image Beta 4.02 (Scion, Fredrick, MD, USA) and the abundance of the same proteins was compared. Protein expression levels more than twofold different between LCN1 and N231 cells progressed to in-gel digestion.

Identification of proteins differently expressed between LCN1 and N231

In-gel digestion

In brief, protein spots were excised from a 2-DE gel, destained with 50% (v/v) acetonitrile (ACN)/50 mmol/L NH_4HCO_3, dehydrated with 100% (v/v) ACN, and dried under vacuum conditions. Tryptic digestion was performed for 24 h at 37°C in a minimum volume of digestion solution that contained 20 ng/μL trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega, Madison, WI, USA) and 25 mmol/L NH_4HCO_3. After incubation, digested protein fragments eluted in solution were collected, and gels were washed once in 5% (v/v) trifluoroacetic acid/50% (v/v) ACN and collected in the same tube.

Protein identification

Tryptic peptides were spotted on a Prespotted AnchorChip 96 Set for Proteomics (Bruker Daltonik, Bremen, Germany) according to the manufacturer's recommendations. MS spectra were analyzed in an autoflex III TOF/TOF (Bruker Daltonik) in reflector mode by summarizing 1000 single spectra (5 × 200) with a 50 Hz laser in the mass range from 580 to 4000 Da applying the following instrument settings: ion source 1, 19.00 kV; ion source 2, 16.60 kV; lens, 8.55 kV; reflector 1, 21.00 kV; reflector 2, 9.70 kV; reflector detector, 1400 V; suppression up to 500 Da by deflection.

MS/MS spectra of tryptic peptides were further measured in an autoflex III TOF/TOF in MS/MS mode using the following instrument settings: ion source 1, 6.00 kV; ion source 2, 5.30 kV; lens, 3.00 kV; reflector 1, 27.00 kV; reflector 2, 11.65 kV; lift 1, 19.00 kV; lift 2, 4.20 kV; reflector detector, 1400 V.

Fragment ion spectra from MS and MS/MS were submitted to MASCOT (www.matrixscience.com/search_form_select.html) for a database search and identification of the corresponding proteins using the following database: IPI human

20 081 114 (74 049 sequences; 31 194 560 residues, www.ebi.ac.uk/IPI/Databases.html/).

Immunohistochemistry

Three micrometer-thick sections were made from 10% formalin-fixed and paraffin-embedded lung cancer tissues, deparaffinized in xylene, rehydrated in a descending ethanol series, and then treated with 3% hydrogen peroxide for 20 min. Antigen was retrieved by autoclaving in 0.01 mol/L citrate buffer (pH 6.0) with 0.1% Tween 20 at 121°C for 10 min. After blocking with 2% normal swine serum (Dako, Glostrup, Denmark) for 10 min, the sections were reacted with 1000-fold diluted mouse anti-human cytokeratin 7 (CK7; OV-TL 12/30; Dako), 250-fold diluted mouse anti-human CK8 (NCL-CK8-TS1; Novocastra, Newcastle, UK), 500-fold diluted mouse anti-human CK18 (NCL-CK18; Novocastra) or 250-fold diluted mouse anti-human CK19 (NCL-CK19; Novocastra) for 16–18 h at room temperature. After rinsing in 0.01 mol/L Tris-HCl pH 7.5, 150 mmol/L NaCl (TBS) three times for 5 min each, the sections were reacted with ChemMate Envision reagent (Dako) for 30 min at room temperature. Finally, the sections were visualized on Stable DAB solution (Invitrogen, Carlsbad, CA, USA) and counterstained with Mayer's hematoxylin.

Evaluation of immunohistochemistry

Immunohistochemistry (IHC) was scored by multiplying the percentage of positive tumor cells and staining intensity. The percentage of positive tumor cells was scored as 0 (0%), 1+ (1–25%), 2+ (26–50%), 3+ (51–75%), or 4+ (76–100%). Staining intensity was also scored as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). The Mann–Whitney *U*-test and the χ^2-test were used for statistical evaluation of IHC data. Statistical significance was considered when $P < 0.05$.

RESULTS

Detection and identification of proteins differently expressed between LCN1 and N231

More than 2000 spots were separated from the total protein of LCN1 and N231 cells using agarose 2-DE. We excised 25 protein spots with expression levels more than twofold different between LCN1 and N231 from the gel of LCN1 cells, using MALDI-TOF/TOF-MS, and identified them. Identified proteins were functionally classified into 10 enzymes, seven cytoskeletal proteins, three signal transduction factors, two nucleic acid binding proteins, and one each of the transporter and chaperone (Table 1). The expression levels of CK7, 8, 18

Table 1 Proteins differentially expressed between LCN1 and N231 cells on 2-DE

No.	Protein description	Molecular function	Molecular weight (Da)	LCN1/N231 ratio
1	Transketolase	Enzyme	67 775	4.0
2	Pyruvate kinase, M1 isozyme	Signal transduction	57 939	2.1
3	Enolase 1	Enzyme	47 150	0.4
4	Unknown	Unknown	101 979	2.1
5	Aldehyde dehydrogenase 1A1	Enzyme	54 843	12.2
6	Glyceraldehydes-3-phosphate dehydrogenase	Enzyme	36 035	2.2
7	Aldolase A	Enzyme	39 270	3.5
8	Uridine diphosphoglucose dehydrogenase	Enzyme	55 075	8.7
9	Protein disulfide isomerase	Enzyme	56 778	2.7
10	Heterogeneous nuclear ribonucleoprotein A2/B1	Nucleic acid binding	35 987	0.5
11	Phosphoglycerate kinase 1	Signal transduction	44 596	0.4
12	6-Phosphogluconate dehydrogenase, decarboxylating	Enzyme	53 122	0.3
13	Heat shock protein	Chaperone	69 977	23.9
14	Keratin 18	Cytoskeletal structural protein	48 010	17.0
15	Lamin B1	Cytoskeletal structural protein	66 348	2.0
16	Keratin 19	Cytoskeletal structural protein	44 061	3.3
17	Heterogeneous nuclear ribonucleoprotein K	Nucleic acid binding	50 958	2.6
18	Neuropolypeptide h3	Signal transduction	22 907	3.3
19	Ubiquitin carboxyl-terminal esterase L1	Enzyme	24 806	0.4
20	Phosphoglycerate mutase 1	Enzyme	28 785	2.0
21	Valosin-containing protein	Transporter	89 303	0.2
22	Ezrin (p81)(Villin 2)	Cytoskeletal structural protein	69 380	10.2
23	Villin 1	Cytoskeletal structural protein	92 677	69.8
24	Keratin, type II cytoskeletal 8 (Cytokeratin 8)	Cytoskeletal structural protein	53 656	27.0
25	Keratin, type II cytoskeletal 7 (Cytokeratin 7)	Cytoskeletal structural protein	51 399	4.6

and 19 of the LCN1 cells were 4.6-fold, 27-fold, 17-fold and 3.3-fold higher, respectively, than N231 cells, and these four CK were studied further (Fig. 1).

IHC of cytokeratins

The stainability of CK7, 8, 18 and 19 in N231 and LCN1 cells was generally in agreement with the results of proteome analysis (Fig. 2). In general, positive staining was observed in the cytoplasm of LCN1 cells at various levels, but not in N231 cells. To evaluate the utility of these molecules as diagnostic markers, we also stained pulmonary carcinoma tissues. CK expression was localized in the cytoplasm of carcinoma cells at various levels in each histological type, and normal bronchial cells constantly had high expression levels (Fig. 3). The stainability of normal bronchial epithelium was used as an internal control. The staining scores and positivity of CK are summarized in Table 2. CK7 was detected in 17 of 27 (63.0%) SCLC, 27 of 30 (90.0%) LCNEC, all 14 (100%) AD, and three of 10 (30.0%) SCC, and the mean staining scores of CK7 were 2.8, 6.8, 10.9, and 1.8, respectively. CK8 was detected in 26 of 27 (96.3%) SCLC, 29 of 30 (96.7%) LCNEC, all 14 (100%) AD, and all 10 (100%) SCC, and the mean staining scores of CK8 were 4.0, 7.8, 9.4, and 5.7, respectively. CK18 was detected in 26 of 27 (96.3%) SCLC, 29 of 30 (96.7%) LCNEC, all 14 (100%) AD, and seven of 10 (70.0%) SCC, and the mean staining scores of CK18 were 6.0, 8.2, 10.0, and 3.2, respectively. CK19 was also detected in 26 of 27 (96.3%) SCLC, 29 of 30 (96.7%) LCNEC, all 14 (100%) AD, and all 10 (100%) SCC, and the mean staining scores of CK19 were 4.3, 7.6, 9.4, and 7.5, respectively. Although there was no difference in the positivity, the mean staining scores of CK7, CK8, CK18, and CK19 of LCNEC were significantly higher than that of SCLC ($P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively). In generally, positivity and mean staining scores of CK in LCNEC were similar to those in AD. Furthermore, cases of more than two or three of four CK with a score of >8 for each staining were found for 18 or 14 of 28 LCNEC (64.3% or 50.0%) and only one or none of 28 SCLC (4.2% or 0%; χ^2 test, $P = 0.0000072$ or $P = 0.000052$), respectively.

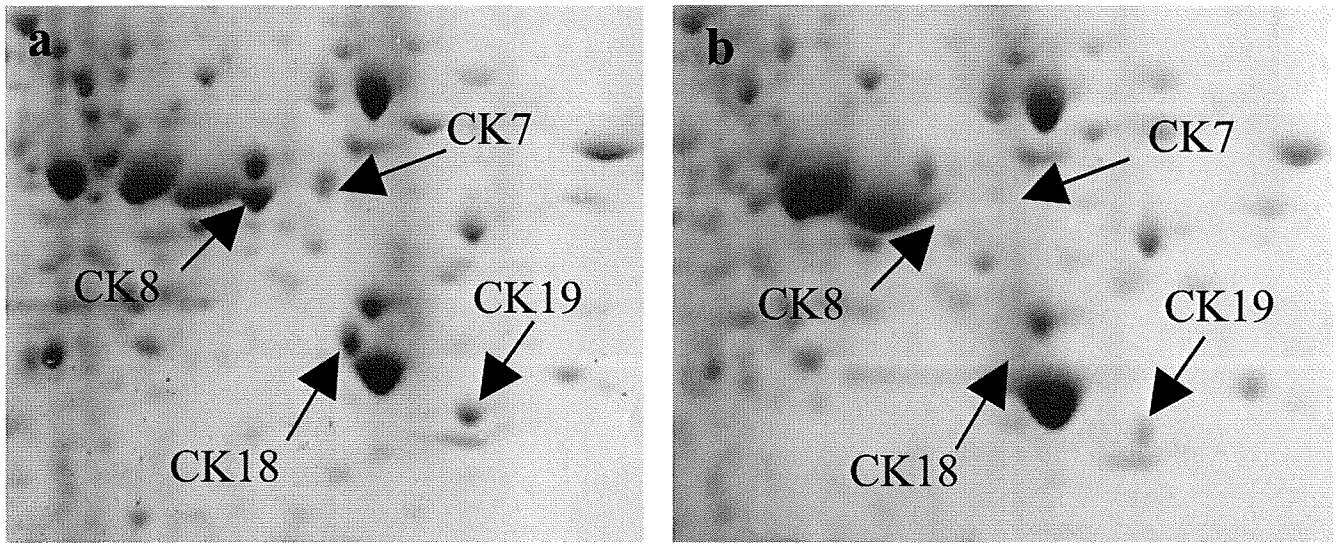

Figure 1 Differentially expressed cytokeratins (CK) between LCN1 cells and N231 cells on 2-D electrophoresis (2-DE). (**a**) LCN1 and (**b**) N231 cell lysates were separated on 2-DE. Arrows, CK upregulated in LCN1 in comparison with N231 cells.

Table 2 Expression of cytokeratin 7, 8, 18 and 19 in pulmonary carcinomas

		CK7		CK8		CK18		CK19	
	n	Score†	Positivity (%)	score	Positivity (%)	score	Positivity (%)	score	Positivity (%)
SCLC	27	2.8	63	4.0	96	6.0	96	4.3	96
LCNEC	30	6.8 *	91	7.8 *	97	8.2 **	97	7.6 *	93
AD	14	10.9	100	9.4	100	10.0	100	9.4	100
SCC	10	1.8	30	5.7	100	3.2	70	7.5	100

*$P < 0.001$, ** $P < 0.01$.
†Mean staining score.
AD, adenocarcinoma; LCNEC, large cell neuroendocrine carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma.

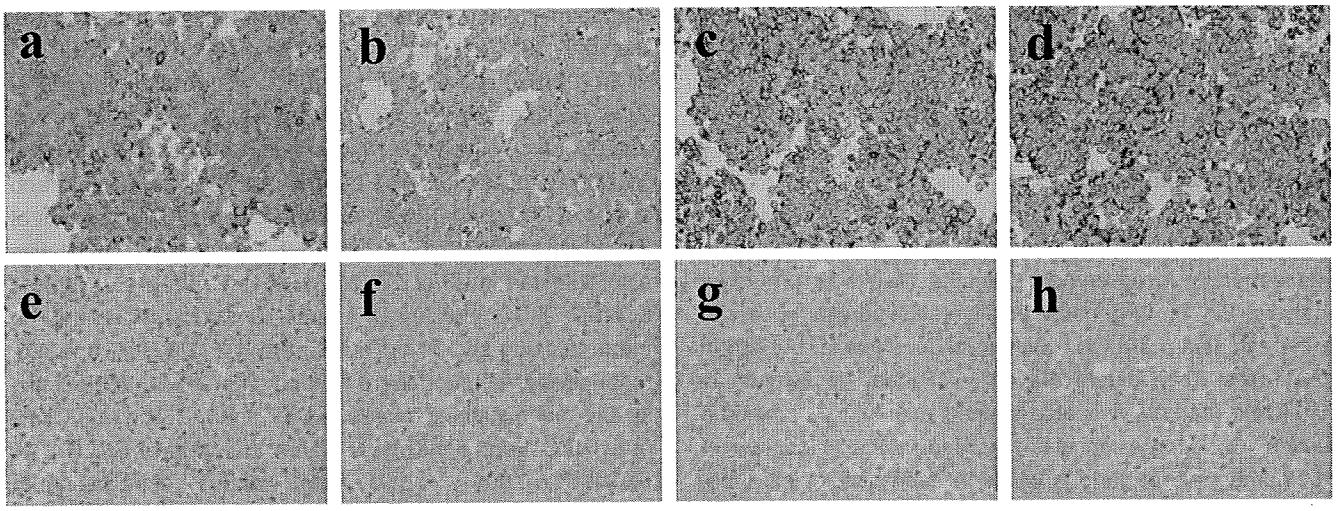

Figure 2 Expression of cytokeratins (CK) (**a**,**e**) 7, (**b**,**f**) 8, (**c**,**g**) 18 and (**d**,**h**) 19 in (**a**–**d**) LCN1 cells and (**e**–**h**) N231 cells. Expression of all CK was observed at various levels in LCN1 cells, but not in N231 cells.

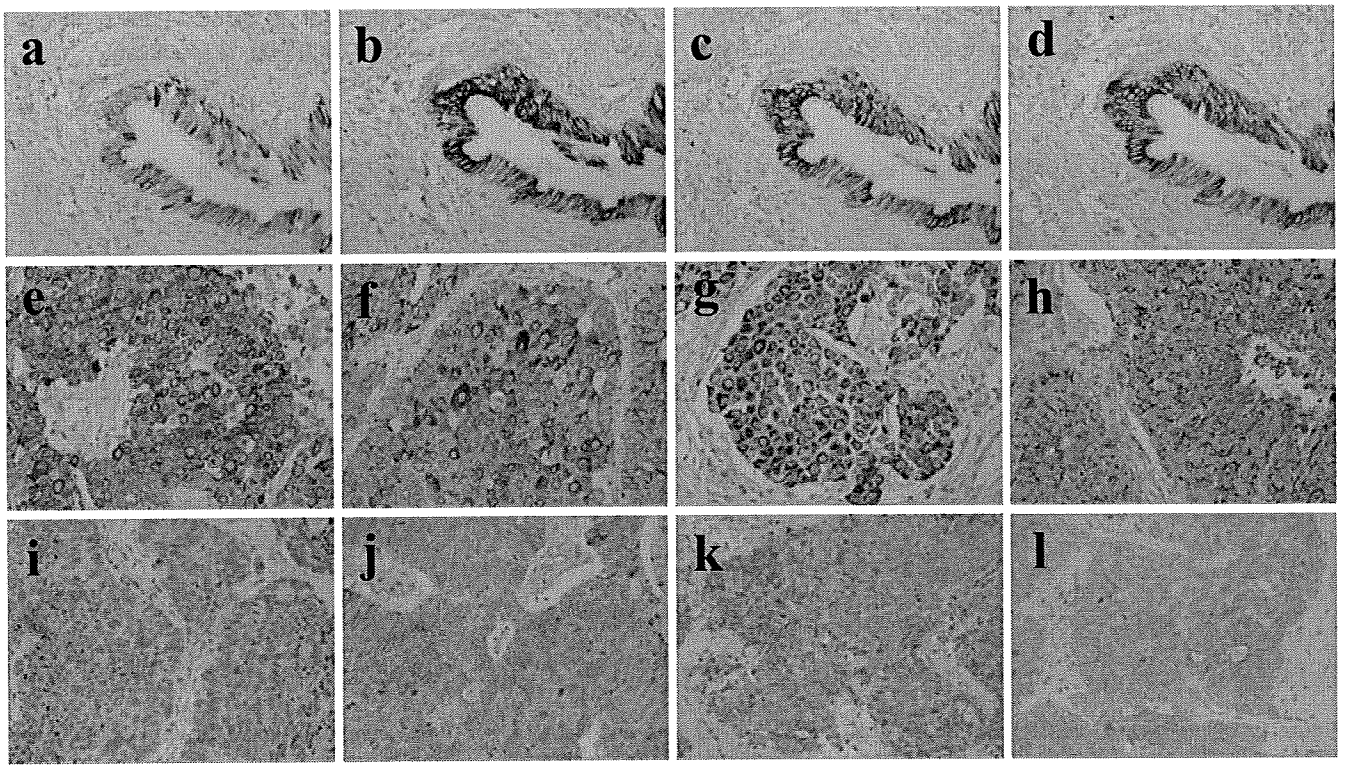

Figure 3 Expression of cytokeratins (CK) 7, 8, 18 and 19 in pulmonary carcinomas. (**a**–**d**) Normal bronchial epithelia, (**e**–**h**) large cell neuroendocrine carcinomas (LCNEC), (**i**–**l**) small cell lung carcinomas (SCLC). (**a**,**e**,**i**) CK7; (**b**,**f**,**j**) CK8; (**c**,**g**,**ki**) CK18; (**d**,**h**,**l**) CK19. Expression of all CK was observed in bronchial epithelium. For all CK expression, high mean staining scores were recognized in LCNEC in comparison with SCLC.

DISCUSSION

In the post-genome era, proteome research using various techniques is increasing rapidly. Such research has increased because the expression levels of mRNA and proteins in cells or tissues are not always consistent;[6] intracellular and extra-cellular proteins usually undergo post-translational modifications, such as phosphorylation, oxidization, or the addition of carbohydrate chains,[7] and information about these modifications cannot be predicted from the gene sequence. Some studies have used proteome analyses for lung cancer. Li *et al.* identified 40 proteins for

which the expression levels differed between SCC and their non-neoplastic peripheral lung tissues on 2-DE-based analysis.[8] The identified proteins included cell-cycle and signal transduction system-related proteins, but the molecular weights of most identified proteins are ≤50 000 Da because 2-D electrophoresis with IPG (immobiline 2-DE method) cannot separate high-molecular-weight proteins efficiently. Chen *et al.* compared pulmonary AD tissues and non-neoplastic lung tissues of the same patient, and identified upregulated proteins, including antioxidant enzymes in tumor tissues, but the molecular weights of identified proteins were also only up to approximately 50 000 Da.[9] It has been reported that, compared with prokaryotes, eukaryotes have many more proteins of ≥100 000 Da that probably contain fused domains of several proteins as a result of evolution, and these proteins have come to have multiple functions.[9] Therefore, proteome analysis of humans, as eukaryotes, requires a 2-DE system that can analyze proteins with high molecular weight. Compared with the conventional immobiline 2-DE method, the agarose 2-DE method improved by Oh-ishi *et al.*, which uses agarose as a carrier in the first dimension of isoelectric focusing, can analyze high molecular weight (≥100 000 Da) and basic proteins easily with the advantage of being able to analyze 10-fold as many proteins as the conventional method.[3] Using this method, Kuruma *et al.* identified proteins that are upregulated in androgen-independent prostatic cancer, and some proteins had a molecular weight of ≥100 000 Da.[10]

In the present study we conducted proteome analysis using cell lines derived from SCLC and LCNEC by the agarose 2-DE method. The total proteins of these two cell lines were separated into approximately 2000 spots on CBB staining, and these electrophoresis patterns on gels were very similar. Twenty-five proteins had different expression levels and 15 (60%) had a molecular weight >50 000 Da. Functional classification showed that the most common were enzymes, followed by cytoskeleton proteins. The present study also confirmed the usefulness of the agarose 2-DE method. Because cellular size greatly differs between SCLC and LCNEC, we thought that the expression levels of cytoskeleton-associated proteins may also differ. We therefore focused on cytoskeletal proteins CK7, 8, 18 and 19, and further studied their expression on immunohistochemistry in clinical cases of pulmonary carcinomas.

All four CK identified in the present study have been reported as having expression primarily in the simple epithelium.[11] In contrast, most neuroendocrine tumors, including those of the lung, express CK8, 18 and 19,[12] but the differences in stainability of these CK between LCNEC and SCLC have not been extensively evaluated.

In the present study, staining scores of CK7, 8, 18 and 19 were significantly higher in LCNEC than in SCLC, suggesting that LCNEC and SCLC can be differentiated on CK7, 8, 18 and 19 staining. Although high expression levels of CK7 are observed in most AD, both the positivity and stainability of CK7 were usually low in neuroendocrine tumors, including SCLC and carcinoid, and in non-keratinizing-type SCC.[11] In the present study the staining score of CK7 was low in SCC and SCLC, in agreement with a previous report, but there are few reports on CK7 expression in LCNEC. Nitadori *et al.* performed tissue microarray analysis of surgically resected LCNEC and SCLC specimens using 48 antibodies, including CK. They demonstrated that the expression of four proteins, CK7, 8, E-cadherin and β-catenin, were significantly higher in LCNEC than in SCLC.[13] Similarly, the present study also confirmed that the positivity and staining score of CK7 were markedly higher in LCNEC, as in AD, than in SCC.

Moreover, the present study demonstrated that LCNEC can be differentiated from SCLC with high probability in cases of more than two of four CK with a score of >8 for each stain.

Lyda and Weiss have reported that most non-SCLC were positive for high-molecular-weight CK, such as 34βE12 and CK7, while most neuroendocrine carcinomas were negative for these CK.[14] According to their report, it is possible to differentiate these two types of tumor based on the stainability of CK and neuroendocrine markers, such as chromogranin A and synaptophysin,[14] but the difference in CK expression between SCLC and LCNEC was not described in their study. Lyda and Weiss also reported that the antibody designated as B72.3 was useful for differential diagnosis of non-SCLC from SCLC, for which 80% of non-SCLC were positive, while only 5% of SCLC were positive. They also reported that three of six (50%) LCNEC were positive for this marker.[14]

Giuseppe *et al.* classified neuroendocrine tumors of the lung based on their proliferative activity determined by the Ki-67 labeling index, expression levels of c-kit, p53, Rb, bcl-2 and cdk4 proteins and so on,[15] and suggested that the tumors could be divided approximately into three groups, typical carcinoid (TC), SCLC and LCNEC. These data show that TC and SCLC are genetically different, and AC and LCNEC may belong to another group rather than to the tumor group of intermediate progression from TC to SCLC;[15] the present data confirmed that study of SCLC and LCNEC. Moreover, high-grade neuroendocrine tumors are generally considered to be characterized by loss of RB protein expression. Loss of RB protein was observed in most SCLC (91%), while it occurred in only half of LCNEC, suggesting that the mechanisms for G1 checkpoint in SCLC and LCNEC differ.[15] Sturm *et al.* studied the expression of thyroid transcription factor-1 (TTF-1) in neuroendocrine tumors of the lung on IHC, and higher expression levels of TTF-1 were detected in SCLC and LCNEC than in TC and AC. Furthermore, the positivity of TTF-1 was 49% in LCNEC and 85.5% in SCLC, respectively, and the differences were significant.[16]

Recently, Ullman *et al.* studied chromosomal abnormalities in SCLC and LCNEC on comparative genomic hybridization and found that both tumors had many common chromosomal abnormalities, but 3q+ was observed in 66% of SCLC, while it occurred in only one of 18 LCNEC.[17] Thus, a gene that determines the differences in biological character between these tumors may be located in the 3q domain.

Taken together with previous studies, the present study strongly suggests that the biological characteristics of SCLC and LCNEC may differ, and CK expression was useful for the differential diagnosis of SCLC and LCNEC. Further studies of chromosomal abnormalities, gene alterations and gene expressions will facilitate the differential diagnosis of these tumors and lead to the acquisition of new markers.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research C (C19590365) from the Japan Society for the Promotion of Science, and for Research Project (No. 2008-1003) from the School of Allied Health Sciences and 2008 Project Study from the Graduate School of Medical Sciences, Kitasato University.

REFERENCES

1 Travis WD, Linnoila RI, Tsokos MG *et al.* Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. *Am J Surg Pathol* 1991; **15**: 529–53.
2 Asamura H, Kameya T, Matsuno Y *et al.* Neuroendocrine neoplasms of the lung: A prognostic spectrum. *J Clin Oncol* 2006; **24**: 70–76.
3 Oh-Ishi M, Satoh M, Maeda T. Preparative two-dimensional gel electrophoresis with agarose gels in the first dimension for high molecular mass proteins. *Electrophoresis* 2000; **21**: 1653–69.
4 Jiang SX, Kameya T, Asamura H *et al.* hASH1 expression is closely correlated with endocrine phenotype and differentiation extent in pulmonary neuroendocrine tumors. *Mod Pathol* 2004; **17**: 222–9.
5 Nagashio R, Sato Y, Jiang SX *et al.* Detection of tumor-specific autoantibodies in sera of patients with lung cancer. *Lung Cancer* 2008; **62**: 364–73.
6 Seike M, Kondo T, Mori Y *et al.* Proteomic analysis of intestinal cells expressing stabilized β-catenin. *Cancer Res* 2003; **63**: 4641–7.
7 Varshavsky A. The N-end rule: Functions, mysteries, uses. *Proc Natl Acad Sci USA* 1996; **93**: 12142–9.
8 Li C, Chen Z, Xiao Z *et al.* Comparative proteomics analysis of human lung squamous carcinoma. *Biochem Biophys Res Commun* 2003; **309**: 253–60.
9 Chen G, Gharib TG, Huang C-C *et al.* Ptoteomic analysis of lung adenocarcinoma: Identification of a highly expressed set of proteins in tumors. *Clin Cancer Res* 2002; **8**: 2298–305.
10 Kuruma K, Oh-Ishi M, Kodera Y, Maeda T, Baba S, Egawa S. Proteomic analysis of urological malignancies. *Recent Res Dev Cancer* 2002; **4**: 643–52.
11 Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathol* 2002; **40**: 403–39.
12 Miettinen M. Keratin immunohistochemistry: Update of applications and pitfalls. *Pathol Annu* 1993; **24**: 113–43.
13 Nitadori J, Ishii G, Tsuta K *et al.* Immunohistochemical differential diagnosis between large cell neuroendocrine carcinoma and small cell carcinoma by tissue microarray analysis with a large antibody panel. *Am J Clin Pathol* 2006; **125**: 682–92.
14 Lyda MH, Weiss LM. Immunoreactivity for epithelial and neuroendocrine antibodies are useful in the differential diagnosis of lung carcinomas. *Hum Pathol* 2000; **31**: 980–87.
15 Giuseppe S, Gorana T, Paola C *et al.* Gene products immunophenotyping of neuroendocrine lung tumors: No linking evidence between carcinoids and small-cell lung carcinomas suggested by multivariate statistical analysis. *Appl Immunohistochem* 2000; **8**: 49–56.
16 Sturm N, Rossi G, Lantuejoul S *et al.* Expression of thyroid transcription factor-1 in the spectrum of neuroendocrine cell lung proliferations with special interest in carcinoids. *Hum Pathol* 2002; **33**: 175–82.
17 Ullmann R, Petzmann S, Sharma A, Cagle PT, Popper HH. Chromosomal aberrations in a series of large-cell neuroendocrine carcinomas: Unexpected divergence from small-cell carcinoma of the lung. *Hum Pathol* 2001; **32**: 1059–63.

Oncogene (2009) 28, 2903–2909

www.nature.com/onc

ORIGINAL ARTICLE

Significance of RRM1 and ERCC1 expression in resectable pancreatic adenocarcinoma

H Akita[1], Z Zheng[2], Y Takeda[1], C Kim[1], N Kittaka[1], S Kobayashi[1], S Marubashi[1], I Takemasa[1], H Nagano[1], K Dono[1], S Nakamori[3], M Monden[1], M Mori[1], Y Doki[1] and G Bepler[2]

[1]*Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan;* [2]*Department of Thoracic Oncology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, FL, USA and* [3]*Department of Surgery, National Hospital Organization Osaka National Hospital, Osaka, Japan*

The identification of molecular markers, useful for therapeutic decisions in pancreatic cancer patients, is crucial for advances in disease management. Gemcitabine, although a cornerstone of current therapy, has limited efficacy. *RRM1* is a key molecule for gemcitabine efficacy and is also involved in tumor progression. We determined *in situ RRM1* and *excision repair cross complementation group 1* (*ERCC1*) protein levels in 68 pancreatic cancer patients. All had R0 resections without preoperative therapy. Protein levels were determined by automated quantitative analysis (AQUA), a fluorescence-based immunohistochemical method. The relationship between protein expressions and clinical outcomes, including response to gemcitabine at the time of disease recurrence, was determined. Patients with high *RRM1* showed significantly better overall survival than patients with low expression ($P=0.0196$). There was a trend toward better overall survival for patient with high ERCC1 ($P=0.0552$). When both markers were considered together, patients with both high *RRM1* and *ERCC1* faired the best in terms of overall and disease-free survival ($P=0.0066$, $P=0.0127$). In addition, treatment benefit from gemcitabine in patients with disease recurrence was observed only in patients with low *RRM1*. The combination of *RRM1* and *ERCC1* expression is prognostic in pancreatic cancer patients after a complete resection. On disease recurrence, only patients with low *RRM1* derive benefit from gemcitabine.
Oncogene (2009) **28,** 2903–2909; doi:10.1038/onc.2009.158; published online 22 June 2009

Keywords: pancreatic cancer; *RRM1*; *ERCC1*; AQUA; prognosis; gemcitabine

Introduction

Pancreatic cancer is one of the leading causes of tumor-related mortalities. The prognosis of patients after complete resection is poor, and more than 50% of patients develop tumor recurrence at distant or locoregional sites, with an estimated 5-year survival of only 20% (Kayahara *et al.*, 1993; Nitecki *et al.*, 1995; Staley *et al.*, 1996; Sener *et al.*, 1999; Li *et al.*, 2004). The addition of chemotherapy and radiotherapy to surgical resection is important, and gemcitabine, a pyrimidine nucleotide analogue, has become the standard chemotherapeutic agent in such programs (Burris *et al.*, 1997; Oettle *et al.*, 2007) (Rothenberg *et al.*, 1996). However, the clinical response rate to gemcitabine remains modest, mainly because of the profound chemoresistance inherent in pancreatic cancer. The selection of patients who derive a true benefit from gemcitabine could be an important stepping stone toward improvement of outcome of pancreatic cancer.

RRM1, the gene that encodes the regulatory subunit of ribonucleotide reductase, is a key determinant of gemcitabine efficacy. In various cancers, we and others have described that overexpression of the *RRM1* gene is strongly associated with gemcitabine resistance (Cao *et al.*, 2003; Rosell *et al.*, 2004; Bergman *et al.*, 2005; Bepler *et al.*, 2006; Nakahira *et al.*, 2007). However, there is no clinical study that investigated the correlation between *RRM1* protein expression and gemcitabine resistance.

On the other hand, the expression of *RRM1* was also reported to correlate with the tumorigenic and metastatic potential of lung cancer (Gautam *et al.*, 2003), and an oncogenic ras-transformed cell line with high expression of an *RRM1* transgene had reduced metastatic potential (Fan *et al.*, 1997). Furthermore, high expression of *RRM1* in transgenic mice is associated with resistance to carcinogen-induced lung tumorigenesis (Gautam and Bepler, 2006). Recently, overexpression of *RRM1* and the *excision repair cross-complementation group 1* (*ERCC1*) gene product was reported to correlate with favorable prognosis in non-small-cell lung cancer (Zheng *et al.*, 2007).

The present study was designed to evaluate the protein expression of *RRM1* and *ERCC1* in pancreatic cancer by automated quantitative analysis (AQUA). We describe the relationship between *RRM1* and *ERCC1* expression, the association between the expression of these proteins and prognosis, as well as the response to

Correspondence: Dr Y Takeda, Department of Surgery, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita-city, Osaka 5650871, Japan.
E-mail: ytakeda@gcsurg.med.osaka-u.ac.jp
Received 24 November 2008; revised 23 March 2009; accepted 14 May 2009; published online 22 June 2009

gemcitabine therapy. To our knowledge, this study is the first to examine both the prognostic and predictive aspects of *RRM1* in the same clinical samples.

Results

RRM1 and ERCC1 expression characteristics

We constructed a tissue microarray using triplicate 0.6-mm cores from formalin-fixed and paraffin-embedded specimens of the primary tumor. Immunostaining showed a granular nuclear pattern for *RRM1*, and a fine granular pattern for *ERCC1* (Figure 1). Next, we used AQUA to analyse the expression levels of *RRM1* and *ERCC1* in specimens obtained from 68 patients. The scores of *RRM1* ranged from 116 to 1644 (median, 539; mean, 546) for all specimens, and the scores of *ERCC1* ranged from 55 to 1469 (median 382, mean 412).

The average score of triplicate tissues from each patient was used for analysis of the association between staining and clinical parameters. The AQUA scores for *RRM1* did not correlate significantly with those of *ERCC1* ($r=0.172$, $P=0.1610$) (Figure 2). The median values of *RRM1* and *ERCC1* expression levels were used to divide the patients into high and low expression groups. There were no significant differences between patients with high and low tumoral *RRM1* expression or high and low tumoral *ERCC1* expression with respect to age, sex, histopathological type (well/mod/poor), tumor size, tumor location (head/body/tail), pathological depth of tumor (pT1/T2/T3), the total number of resected lymph nodes, pathological lymph node metastasis (negative/positive) and the number of metastatic

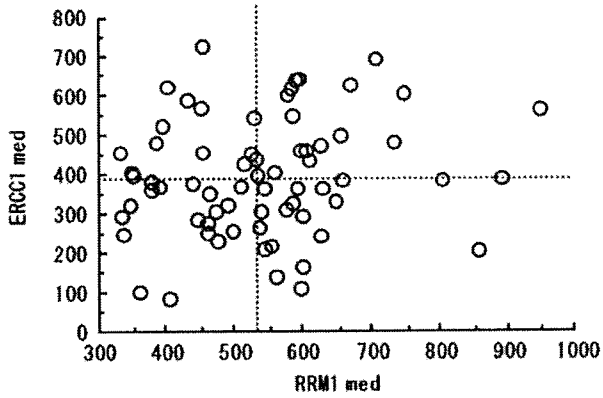

Figure 2 Relationship between automated quantitative analysis (AQUA) scores of *RRM1* and *excision repair cross-complementation group 1* (*ERCC1*) expression. *RRM1* expression did not correlate with that of *ERCC1* ($r=0.172$, $P=0.161$).

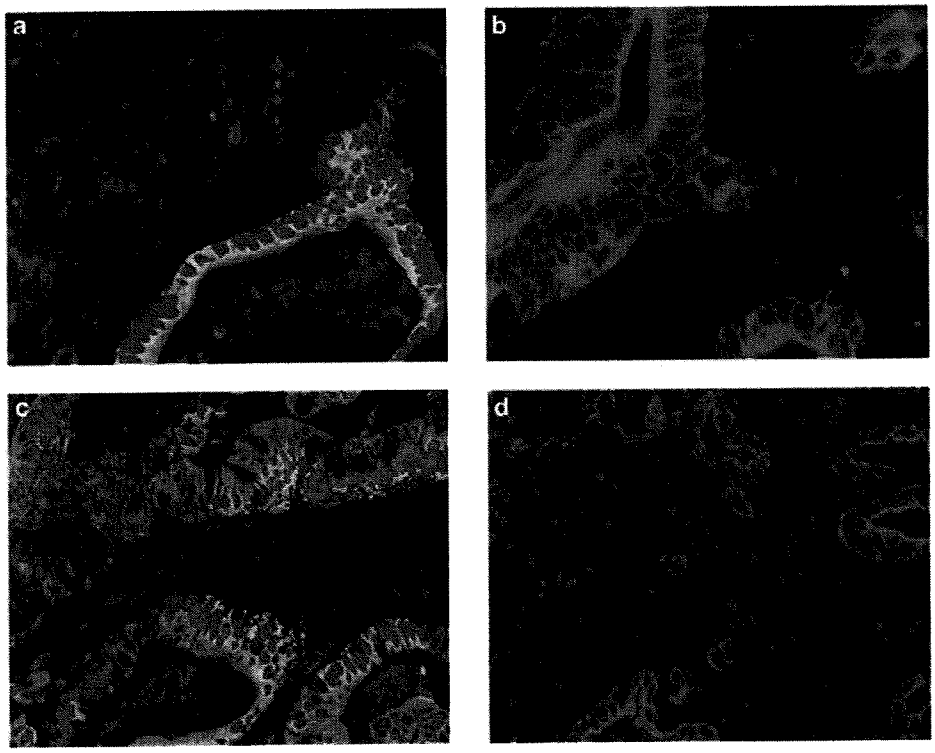

Figure 1 Staining for *RRM1* and *excision repair cross-complementation group 1* (*ERCC1*) proteins. (**a**) *RRM1*-positive sample. Note the granular nuclear pattern. Nucleus, blue; cytoplasm, red; *RRM1*, green; and merged, light blue to light green. (**b**) *RRM1*-negative sample. Nucleus, blue; and cytoplasm, red. (**c**) *ERCC1*-positive sample. Note the fine granular pattern in the nucleus. Nucleus, blue; cytoplasm, red; *ERCC1*, green; and merged, light blue to light green. (**d**) *ERCC1*-negative sample. Nucleus, blue; and cytoplasm, red.

Table 1 Relationship between protein expression levels and clinicopathological factors

	RRM1 expression level			*ERCC1 expression level*		
	High	*Low*	P-*value*	*High*	*Low*	P-*value*
Age (years) (mean ± s.d.)	66.8 ± 7.6	64.4 ± 7.9	0.220	64.6 ± 7.7	66.6 ± 7.8	0.283
Sex (male/female)	15/19	18/16	0.628	15/19	18/16	0.628
Histopathology (well/mod/poor)	17/14/3	9/18/7	0.102	12/19/3	14/13/7	0.237
Tumor size (cm) (mean ± s.d.)	27.4 ± 9.3	26.7 ± 8.2	0.752	25.2 ± 8.2	28.9 ± 8.9	0.077
Tumor location (head/body/tail)	27/6/1	27/4/3	0.497	27/4/3	27/6/1	0.497
pT (T1/T2/T3)	1/1/32	1/0/33	0.602	1/1/32	1/0/33	0.602
Total number of resected lymph node	34.4 ± 12.9	30.3 ± 13.6	0.243	30.8 ± 10.6	34.3 ± 15.7	0.330
PN (positive/negative)	12/22	17/17	0.327	18/16	11/23	0.141
Total number of metastatic lymph node	1.6 ± 1.9	1.0 ± 1.7	0.202	1.1 ± 1.7	1.5 ± 1.9	0.315
Gem therapy (+/−)	14/20	14/20	0.999	13/21	15/19	0.806

Abbreviation: *ERCC1, excision repair cross-complementation group 1.*

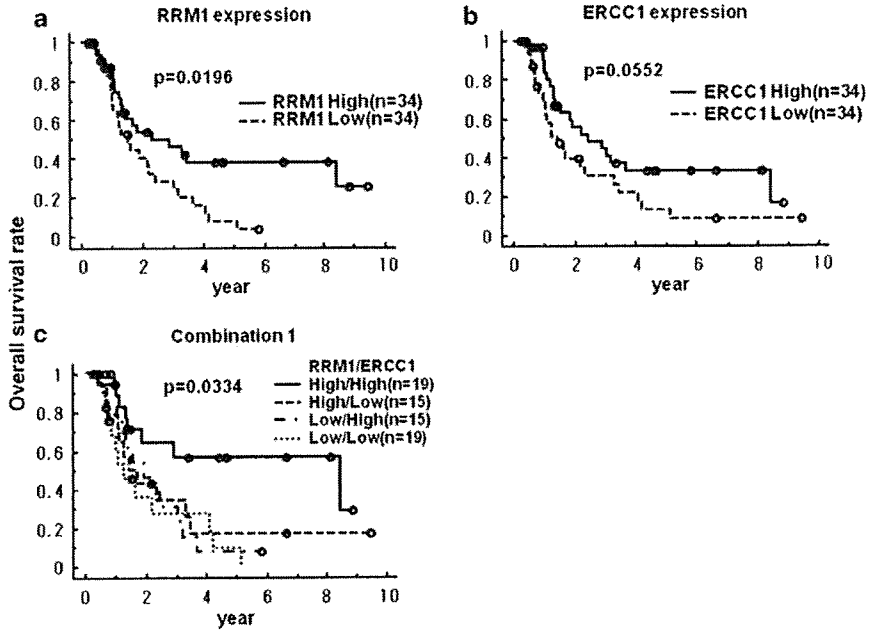

Figure 3 Relationship between *RRM1* and *excision repair cross-complementation group 1* (*ERCC1*) expression levels and overall survival rate. (**a**) Relationship between *RRM1* and overall survival is significant (3-year survival; 46.3 versus 28.6%, $P=0.0196$). (**b**) Relationship between *ERCC1* and overall survival is marginal ($P=0.0552$). (**c**) Relationship between the combination of *RRM1* and *ERCC1* expression levels in the same tumor and overall survival rate. Only high expression levels of *RRM1* and *ERCC1* in the same tumor related with the improvement of overall survival rate ($P=0.0334$).

lymph nodes, and whether or not gemcitabine was used as chemotherapy (Table 1).

Relationship between RRM1/ERCC1 expression and prognosis

The median overall survival of all patients was 16.3 months (4.3–113) and the median disease-free survival was 10.3 months (2–106). The Kaplan–Meier overall survival estimates were significantly better for patients with high *RRM1* expression compared with those having low *RRM1* expression levels (3-year survival; 46.3 versus 28.6%, $P=0.0196$) (Figure 3a). Likewise, patients with high *ERCC1* expression had a better overall survival than those with low levels of expression; although this difference was only marginally significant ($P=0.0552$) (Figure 3b). When we divided the 68 patients into four groups; that is, high tumoral expression of both proteins (High/High, $n=19$), high expression of only *RRM1* (High/Low, $n=15$), high expression of only *ERCC1* (Low/High, $n=15$) and low expression of both proteins (Low/Low, $n=19$); only patients of the High/High group had a significantly better prognosis than the others (3-year survival; 56.7 versus 30.5%, $P=0.0066$) (Figure 3c, Supplementary Figure 1).

With regard to disease-free survival, high *ERCC1* expression levels were significantly associated with better outcome (3-year survival; 30.2% for high versus

23.1% for low, $P=0.0454$). There was no significant difference in disease-free survival between the high and low *RRM1* expression groups (Supplementary Figures 2A and B). With respect to the combination of *RRM1* and *ERCC1*, only the High/High group showed a significantly better disease-free survival compared with the other groups (3-year survival, 43.2 versus 19.2%, $P=0.0127$) (Supplementary Figures 2C and D).

Univariate and multivariate analysis of factors associated with prognosis

We investigated the prognostic significance of various clinicopathological factors in pancreatic cancer patients who underwent radical resection. Univariate analysis showed that only the pathological type and absence or presence of lymph node metastases, were prognostically significant for disease-free survival ($P=0.034$, 0.025, respectively), and both parameters had marginal significance for overall survival ($P=0.078$, 0.084, respectively) (Table 2). Multivariate analysis identified the *RRM1* expression level as the only independent determinant of overall survival (hazard ratio (HR) 1.89, $P=0.046$), and none of the parameters tested was selected by the analysis as a significant prognostic factor in disease-free survival.

RRM1 expression and response to gemcitabine

Of all the 68 patients, 28 received therapy with single-agent gemcitabine. In 23 patients, this treatment was initiated at the time of tumor recurrence. To elucidate the relationship between *RRM1* expression level and gemcitabine therapy, we used survival after recurrence, which represented the period from starting gemcitabine therapy or other therapies in 50 patients with relapse, until death. First, we examined the survival benefit of gemcitabine. The 23 patients who were treated with gemcitabine had a significantly better survival than those who did not ($P=0.0074$) (Supplementary Figure 3). After dividing patients that were treated with gemcitabine into high and low *RRM1* expression groups, only patients with low *RRM1* expression benefited from gemcitabine therapy ($P=0.0010$) (Figure 4b). The survival of patients with high *RRM1* expression treated with gemcitabine was not significantly better than of those not treated with gemcitabine ($P=0.3309$) (Figure 4a). The interaction term between *RRM1* expression and gemcitabine treatment was significant for survival after recurrence ($P=0.0109$).

Discussion

Ribonucleotide reductase, composed of the regulatory subunit *RRM1* and the catalytic subunit *RRM2*, is a key enzyme involved in DNA synthesis, catalyzing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides (Wright *et al*., 1990; Hurta and Wright, 1992). *ERCC1*, a structure-specific DNA repair endonuclease responsible for the 5′ incision, has a key role in the removal of adducts from genomic DNA

Table 2 Prognostic factors for postoperative survival by Cox's proportional hazard model

	Univariate analysis				*Multivariate analysis*			
	DFS		*OS*		*DFS*		OS	
	HR	P-*value*	*HR*	P-*value*	*HR*	P-*value*	*HR*	P-*value*
Histology (poor, mod/well)	1.91	0.034	1.75	0.078	1.77	0.066	1.56	0.172
PN (positive/negative)	2.00	0.025	1.76	0.084	1.73	0.107	1.50	0.256
RRM1 expression (low/high)	1.55	0.129	2.04	0.022	1.39	0.265	1.89	0.046
ERCC1 expression (low/high)	1.75	0.048	1.78	0.059	1.42	0.265	1.54	0.194

Abbreviations: DFS, disease-free survival; *ERCC1*, excision repair cross-complementation group 1; HR, hazard ratio and OS, overall survival.

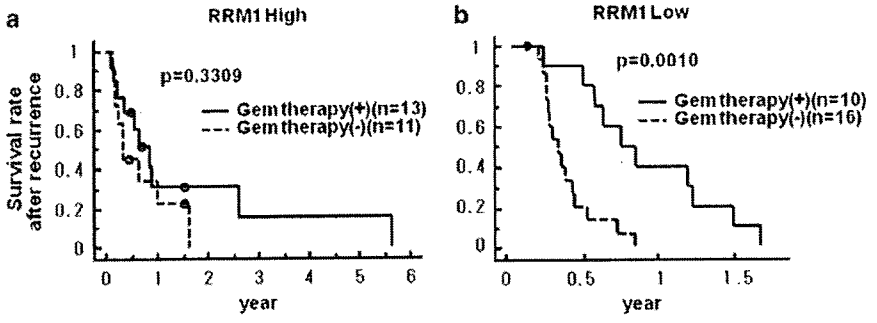

Figure 4 Relationship between survival after recurrence and patients treated with or without gemcitabine (**a**) in high *RRM1* expression group, and (**b**) in low expression group. Only patients with low *RRM1* expression benefited from gemcitabine therapy ($P=0.0010$).

through the nucleotide excision repair pathway (Reardon *et al.*, 1999; Niedernhofer *et al.*, 2004; Ceppi *et al.*, 2006). *RRM1* is reported to influence cell survival, probably through interaction with the *phosphatase and tensin homolog* (*PTEN*), which is an inhibitor of cell proliferation, and suppresses cell migration and invasion by reducing the phosphorylation of focal adhesion kinase (Gautam *et al.*, 2003; Bepler *et al.*, 2004). In lung cancer, the expression levels of *RRM1* and *ERCC1* are significantly correlated (Bepler *et al.*, 2006; Ceppi *et al.*, 2006).

Gemcitabine is the first line cytotoxic agent for treatment of patients with advanced pancreatic cancer, and it is the only agent with proven benefit in a large adjuvant clinical trial (Oettle *et al.*, 2007). However, it is estimated that only 25% of patients benefit from gemcitabine (Burris *et al.*, 1997). *RRM1* expression appears to be the key determinant of gemcitabine resistance (Dumontet *et al.*, 1999; Goan *et al.*, 1999; Jung *et al.*, 2001). This is partially due to expansion of the dNTP pool, which competitively inhibits the incorporation of gemcitabine triphosphate into DNA (Plunkett *et al.*, 1996). Another mechanism is the direct interaction between *RRM1* and gemcitabine with *RRM1* acting as a 'molecular sink' for gemcitabine (Davidson *et al.*, 2004; Bergman *et al.*, 2005). *ERCC1* is reported to be associated with the repair of cisplatin-induced DNA adducts in ovarian cancer (Li *et al.*, 2000), gastric cancer (Metzger *et al.*, 1998), colorectal cancer (Shirota *et al.*, 2001), lung cancer (Olaussen *et al.*, 2006) and esophageal cancer (Joshi *et al.*, 2005; Kim *et al.*, 2008).

Quantitative analysis of gene expression in pancreatic cancer is challenging because it contains more stromal tissue than other cancers (Sato *et al.*, 2004; Bachem *et al.*, 2005; Infante *et al.*, 2007), which makes laser microdissection a necessity to obtain gene expression of tumor tissue (Giovannetti *et al.*, 2006). Quantitative analysis of the *RRM1* protein had been difficult because of technical limitations. However, an automated, quantitative *in situ* assessment of protein expression was developed recently (Camp *et al.*, 2002), and applied for objective and practical evaluation of *RRM1* and *ERCC1* protein expression levels in tumor specimens (Zheng *et al.*, 2007). In this study, we used the above mentioned technology for gene expression analysis in pancreatic cancer specimens.

We found that the expression levels of *RRM1* and *ERCC1* affected the clinical outcome similar to that described in non-small-cell lung cancer (Zheng *et al.*, 2007). Patients with high levels of expression of both proteins had the best prognosis, including both disease-free survival and overall survival. However, once treatment with gemcitabine was initiated at the time of recurrence, it was only the group of patients with low levels of *RRM1* that benefited significantly from this intervention. In other words, patients with high tumoral *RRM1* levels may as well be treated with other agents, such as S-1 or oxaliplatin plus 5-fluorouracil plus leukovorin (CONKO-003), instead of gemcitabine (Ueno *et al.*, 2005; Okusaka *et al.*, 2008; Saif, 2008). In contrast, patients with low tumoral *RRM1* levels showed improved survival following treatment with gemcitabine (Moore *et al.*, 2007; Boeck and Heinemann, 2008). Many clinical trials of anticancer drugs, including molecular targeting agents, did not result in the improvement of outcome when conducted in unselected groups of patients (Heinemann *et al.*, 2006; Herrmann *et al.*, 2007; Cascinu *et al.*, 2008). However, if patients can be divided into groups with high or low likelihood of benefit from gemcitabine, a more rational design of future trials becomes available (Simon *et al.*, 2007). We believe that future treatment strategies for pancreatic cancer should be more precise and tailored to individual patients, and *RRM1* may be one of the candidate molecules for the stratification. We found that *RRM1* and *ERCC1* were not significantly coexpressed in pancreatic cancer, which is different from several previous reports in non-small-cell lung cancer (Ceppi *et al.*, 2006; Zheng *et al.*, 2007). This discrepancy may be explained by differences in tissue of origin and mechanisms of carcinogenesis between pancreatic cancer and lung cancer.

It is important to carry out prospective tailored therapeutic trials in pancreatic cancer with the goal of improving the clinical outcome, and it is our opinion that *RRM1* and *ERCC1* could play an important role in the design of such trials.

Materials and methods

Patients

Between January 1992 and March 2008, 166 patients underwent surgery for pancreatic cancer at Osaka University Hospital. We excluded 84 patients for the following reasons: (1) tumors were not resectable in 26 patients because of liver metastases or peritoneal carcinomatosis, (2) surgery resulted in R1 (residual microscopic cancer) or R2 (residual macroscopic cancer) resections in 21 patients, (3) chemotherapy or chemoradiotherapy was provided preoperatively to 37 patients and (4) lack of neutral-buffered formalin-fixed and paraffin-embedded tumor blocks or/and clinical follow-up information for study purposes in 14 cases. As the natural history of variant pancreatic neoplasms differs from the usual pancreatic ductal adenocarcinoma, patients with intraductal papillary mucinous neoplasms, mucinous cystic adenocarcinomas and medullary adenocarcinomas were excluded from this study. Supplementary Table 1 summarizes the characteristics of the 68 patients who were enrolled in this study. They included 33 men and 35 women with a mean age of 60.7 ± 7.8 years ($\pm$ s.d.). All patients had R0 (no residual cancer) resections by pancreaticoduodenectomy in 54 patients, distal pancreatectomy in 12 patients and other resections in 2 patients. The histopathological grading showed poorly, moderately, and well-differentiated adenocarcinoma in 10, 32 and 26 patients, respectively. The UICC-TNM classification was 2, 1 and 65 patients with pT1, pT2 and pT3; 29, 33 and 6 patients with pN0, pN1 and pM1lym; and 1, 1, 27, 33 and 6 patients with stage IA, IB, IIA, IIB and IV, respectively. None of the patients had received neoadjuvant therapy preoperatively. All 68 patients were followed until disease recurrence and/or death. The median follow-up period was 16.3 months (range, 4.3–113), the 5-year survival rate was 23.4%, and the recurrence of disease was observed in 50 patients. Treatment with gemcitabine was carried out in 28 patients; 5 patients

received it as adjuvant chemotherapy and 23 patients received it after disease recurrence. Radiation therapy was not carried out during all the follow-up period.

Immunofluorescence and automated quantitative analysis

We carried out immunostaining after constructing a tissue microarray. Immunofluorescence combined with AQUA was used to assess *in situ* expression of the target molecules as described previously (Zheng *et al.*, 2007). Antigens were retrieved by incubating the tissue in a microwave oven. Optimal concentrations of antisera and antibodies were used to detect *RRM1*, *ERCC1* and cytokeratin. The antiserum to *RRM1* was generated from rabbits and affinity-purified (R1AS-6) as described previously (Zheng *et al.*, 2007). Commercially available antibodies were used for the analysis of *ERCC1* (Ab-2 clone 8F1, MS-671-R7, Laboratory Vision Corporation, Fremont, CA, USA) and cytokeratin (antihuman pancytokeratin AE1/AE3, M3515 and Z0622, Dako Cytomation, Glostrup, Denmark) (Zheng *et al.*, 2007). They were visualized with the use of fluorochrome-labeled secondary antibodies. The final slides were scanned with SpotGrabber (HistoRx, New Haven, CT, USA), and images were analysed with AQUA (version 1.6, PM-2000, HistoRx). The AQUA scores ranged from 0 (no expression) to 3000 (maximal observed expression).

Statistical analysis and ethical issues

Data are expressed as mean ± s.d. Differences in continuous values were evaluated by the Student's *t*-test (Table 1). The Fisher's exact probability test was used to compare discrete variables (Table 1). We evaluated correlations between AQUA scores of *RRM1* and *ERCC1* by Pearson's correlation coefficient (Figure 2). Disease-free and overall survival rates were estimated by the Kaplan–Meier method and compared using the log-rank test (Table 1, Figures 3 and 4). Cox's proportional hazard regression model with stepwise comparisons was used to analyse independent prognostic factors (Table 2). The predictive value of *RRM1* was studied by testing the interaction between *RRM1* expression and gemcitabine treatment in the same Cox model. A P-value <0.05 was used to indicate statistical significance.

This study was analysed by the statistical expert in our laboratory and the study protocol was approved by the Human Ethics Review Committee of Osaka University, and a signed consent form was obtained from each subject.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was partially supported by National Institutes of Health (NIH) grant R01-CA129343 to GB and by a grant-in-aid for cancer research from the Ministry of Culture and Science of Japan.

References

Bachem MG, Schunemann M, Ramadani M, Siech M, Beger H, Buck A *et al.* (2005). Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology* **128**: 907–921.

Bepler G, Kusmartseva I, Sharma S, Gautam A, Cantor A, Sharma A *et al.* (2006). RRM1 modulated *in vitro* and *in vivo* efficacy of gemcitabine and platinum in non-small-cell lung cancer. *J Clin Oncol* **24**: 4731–4737.

Bepler G, Sharma S, Cantor A, Gautam A, Haura E, Simon G *et al.* (2004). RRM1 and PTEN as prognostic parameters for overall and disease-free survival in patients with non-small-cell lung cancer. *J Clin Oncol* **22**: 1878–1885.

Bergman AM, Eijk PP, Ruiz van Haperen VW, Smid K, Veerman G, Hubeek I *et al.* (2005). *in vivo* induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. *Cancer Res* **65**: 9510–9516.

Boeck S, Heinemann V. (2008). Second-line therapy in gemcitabine-pretreated patients with advanced pancreatic cancer. *J Clin Oncol* **26**: 1178–1179; author reply 1179.

Burris III HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR *et al.* (1997). Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **15**: 2403–2413.

Camp RL, Chung GG, Rimm DL. (2002). Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* **8**: 1323–1327.

Cao MY, Lee Y, Feng NP, Xiong K, Jin H, Wang M *et al.* (2003). Adenovirus-mediated ribonucleotide reductase R1 gene therapy of human colon adenocarcinoma. *Clin Cancer Res* **9**: 4553–4561.

Cascinu S, Berardi R, Labianca R, Siena S, Falcone A, Aitini E *et al.* (2008). Cetuximab plus gemcitabine and cisplatin compared with gemcitabine and cisplatin alone in patients with advanced pancreatic cancer: a randomised, multicentre, phase II trial. *Lancet Oncol* **9**: 39–44.

Ceppi P, Volante M, Novello S, Rapa I, Danenberg KD, Danenberg PV *et al.* (2006). ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. *Ann Oncol* **17**: 1818–1825.

Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA. (2004). An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. *Cancer Res* **64**: 3761–3766.

Dumontet C, Fabianowska-Majewska K, Mantincic D, Callet Bauchu E, Tigaud I, Gandhi V *et al.* (1999). Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. *Br J Haematol* **106**: 78–85.

Fan H, Huang A, Villegas C, Wright JA. (1997). The R1 component of mammalian ribonucleotide reductase has malignancy-suppressing activity as demonstrated by gene transfer experiments. *Proc Natl Acad Sci USA* **94**: 13181–13186.

Gautam A, Bepler G. (2006). Suppression of lung tumor formation by the regulatory subunit of ribonucleotide reductase. *Cancer Res* **66**: 6497–6502.

Gautam A, Li ZR, Bepler G. (2003). RRM1-induced metastasis suppression through PTEN-regulated pathways. *Oncogene* **22**: 2135–2142.

Giovannetti E, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S *et al.* (2006). Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res* **66**: 3928–3935.

Goan YG, Zhou B, Hu E, Mi S, Yen Y. (1999). Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. *Cancer Res* **59**: 4204–4207.

Heinemann V, Quietzsch D, Gieseler F, Gonnermann M, Schonekas H, Rost A *et al.* (2006). Randomized phase III trial of gemcitabine

plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. *J Clin Oncol* **24**: 3946–3952.

Herrmann R, Bodoky G, Ruhstaller T, Glimelius B, Bajetta E, Schuller J *et al.* (2007). Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. *J Clin Oncol* **25**: 2212–2217.

Hurta RA, Wright JA. (1992). Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. *J Biol Chem* **267**: 7066–7071.

Infante JR, Matsubayashi H, Sato N, Tonascia J, Klein AP, Riall TA *et al.* (2007). Peritumoral fibroblast SPARC expression and patient outcome with resectable pancreatic adenocarcinoma. *J Clin Oncol* **25**: 319–325.

Joshi MB, Shirota Y, Danenberg KD, Conlon DH, Salonga DS, Herndon II JE *et al.* (2005). High gene expression of TS1, GSTP1, and ERCC1 are risk factors for survival in patients treated with trimodality therapy for esophageal cancer. *Clin Cancer Res* **11**: 2215–2221.

Jung CP, Motwani MV, Schwartz GK. (2001). Flavopiridol increases sensitization to gemcitabine in human gastrointestinal cancer cell lines and correlates with down-regulation of ribonucleotide reductase M2 subunit. *Clin Cancer Res* **7**: 2527–2536.

Kayahara M, Nagakawa T, Ueno K, Ohta T, Takeda T, Miyazaki I. (1993). An evaluation of radical resection for pancreatic cancer based on the mode of recurrence as determined by autopsy and diagnostic imaging. *Cancer* **72**: 2118–2123.

Kim MK, Cho KJ, Kwon GY, Park SI, Kim YH, Kim JH *et al.* (2008). Patients with ERCC1-negative locally advanced esophageal cancers may benefit from preoperative chemoradiotherapy. *Clin Cancer Res* **14**: 4225–4231.

Li D, Xie K, Wolff R, Abbruzzese JL. (2004). Pancreatic cancer. *Lancet* **363**: 1049–1057.

Li Q, Yu JJ, Mu C, Yunmbam MK, Slavsky D, Cross CL *et al.* (2000). Association between the level of ERCC-1 expression and the repair of cisplatin-induced DNA damage in human ovarian cancer cells. *Anticancer Res* **20**: 645–652.

Metzger R, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K *et al.* (1998). ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* **16**: 309–316.

Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S *et al.* (2007). Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* **25**: 1960–1966.

Nakahira S, Nakamori S, Tsujie M, Takahashi Y, Okami J, Yoshioka S *et al.* (2007). Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. *Int J Cancer* **120**: 1355–1363.

Niedernhofer LJ, Odijk H, Budzowska M, van Drunen E, Maas A, Theil AF *et al.* (2004). The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. *Mol Cell Biol* **24**: 5776–5787.

Nitecki SS, Sarr MG, Colby TV, van Heerden JA. (1995). Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? *Ann Surg* **221**: 59–66.

Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K *et al.* (2007). Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *Jama* **297**: 267–277.

Okusaka T, Funakoshi A, Furuse J, Boku N, Yamao K, Ohkawa S *et al.* (2008). A late phase II study of S-1 for metastatic pancreatic cancer. *Cancer Chemother Pharmacol* **61**: 615–621.

Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad V *et al.* (2006). DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med* **355**: 983–991.

Plunkett W, Huang P, Searcy CE, Gandhi V. (1996). Gemcitabine: preclinical pharmacology and mechanisms of action. *Semin Oncol* **23**: 3–15.

Reardon JT, Vaisman A, Chaney SG, Sancar A. (1999). Efficient nucleotide excision repair of cisplatin, oxaliplatin, and Bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. *Cancer Res* **59**: 3968–3971.

Rosell R, Danenberg KD, Alberola V, Bepler G, Sanchez JJ, Camps C *et al.* (2004). Ribonucleotide reductase messenger RNA expression and survival in gemcitabine/cisplatin-treated advanced non-small cell lung cancer patients. *Clin Cancer Res* **10**: 1318–1325.

Rothenberg ML, Moore MJ, Cripps MC, Andersen JS, Portenoy RK, Burris III HA *et al.* (1996). A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. *Ann Oncol* **7**: 347–353.

Saif MW. (2008). New developments in the treatment of pancreatic cancer. Highlights from the '44th ASCO Annual Meeting'. Chicago, IL, USA. May 30–June 3, 2008. *JOP* **9**: 391–397.

Sato N, Maehara N, Goggins M. (2004). Gene expression profiling of tumor-stromal interactions between pancreatic cancer cells and stromal fibroblasts. *Cancer Res* **64**: 6950–6956.

Sener SF, Fremgen A, Menck HR, Winchester DP. (1999). Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985–1995, using the National Cancer Database. *J Am Coll Surg* **189**: 1–7.

Shirota Y, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD *et al.* (2001). ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. *J Clin Oncol* **19**: 4298–4304.

Simon G, Sharma A, Li X, Hazelton T, Walsh F, Williams C *et al.* (2007). Feasibility and efficacy of molecular analysis-directed individualized therapy in advanced non-small-cell lung cancer. *J Clin Oncol* **25**: 2741–2746.

Staley CA, Lee JE, Cleary KR, Abbruzzese JL, Fenoglio CJ, Rich TA *et al.* (1996). Preoperative chemoradiation, pancreaticoduodenectomy, and intraoperative radiation therapy for adenocarcinoma of the pancreatic head. *Am J Surg* **171**: 118–124; discussion 124–5.

Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. (2005). An early phase II study of S-1 in patients with metastatic pancreatic cancer. *Oncology* **68**: 171–178.

Wright JA, Chan AK, Choy BK, Hurta RA, McClarty GA, Tagger AY. (1990). Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase, and the significance to DNA synthesis. *Biochem Cell Biol* **68**: 1364–1371.

Zheng Z, Chen T, Li X, Haura E, Sharma A, Bepler G. (2007). DNA synthesis and repair genes RRM1 and ERCC1 in lung cancer. *N Engl J Med* **356**: 800–808.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

WJGO World Journal of Gastrointestinal Oncology

Online Submissions: http://www.wjgnet.com/1948-5204office
wjgo@wjgnet.com
doi:10.4251/wjgo.v2.i3.1

World J Gastrointest Oncol 2010 March 15; 2(3): 1-000
ISSN 1948-5204 (online)

EDITORIAL

Carcinogenesis and chemoprevention of biliary tract cancer in pancreaticobiliary maljunction

Akihiko Tsuchida, Takao Itoi

Akihiko Tsuchida, Third Department of Surgery, Tokyo Medical University, Tokyo 160-0023, Japan
Takao Itoi, Fourth Department of Internal Medicine, Tokyo Medical University, Tokyo 160-0023, Japan
Author contributions: Tsuchida A and Itoi T contributed equally to this work.
Correspondence to: Akihiko Tsuchida, MD, PhD, Associate Professor of the Third Department of Surgery, Tokyo Medical University, Shinjuku-ku, Tokyo 160-0023, Japan. akihikot@tokyo-med.ac.jp
Telephone: +81-3-33426111 Fax: +81-3-33404575
Received: June 22, 2009 Revised: July 13, 2009
Accepted: July 20, 2009
Published online: March 15, 2010

Abstract

Pancreaticobiliary maljunction (PBM) is a high risk factor for biliary tract cancer. In PBM, since the pancreatic duct and bile duct converge outside the duodenal wall beyond the influence of the sphincter of Oddi, pancreatic juice and bile are constantly mixed, producing a variety of harmful substances. Because of this, the biliary mucosa is repeatedly damaged and repaired, which causes an acceleration of cell proliferative activity and multiple gene mutations. Histological changes such as hyperplasia, metaplasia, and dysplasia ultimately result in a high incidence of carcinogenesis. In a nationwide survey by the Japanese Study Group on PBM, coexisting biliary tract cancer was detected in 278 of the 1,627 registered cases of PBM (17.1%). Of these cases, in those with dilatation of the extrahepatic bile duct, cancer was often detected not only in the gallbladder but also in the bile ducts. More than 90% of cancer cases without dilatation of the extrahepatic bile duct develop in the gallbladder. Standard treatment for PBM is a cholecystectomy and resection of the extrahepatic bile duct. However, cholecystectomy alone is performed at nearly half of institutions in Japan. Conversely, reports of carcinogenesis in the remnant bile duct or pancreas after diversion surgery are steadily increasing. One of the causes for this is believed to be an accumulation of gene mutations which were present before surgery. Anticancer drugs are ineffective in preventing such carcinogenesis following surgery, thus the postoperative administration of chemopreventive agents may be necessary.

Key words: Chemoprevention; Gallbladder cancer; Bile duct cancer; Carcinogenesis; Pancreaticobiliary maljunction

Peer reviewer: Hans Chung, MD, FRCPC Sunnybrook Odette Cancer Centre, 2075 Bayview Avenue, T-Wing Toronto ON, M4N 3M5, Canada

Tsuchida A, Itoi T. Carcinogenesis and chemoprevention of biliary tract cancer in pancreaticobiliary maljunction. *World J Gastrointest Oncol* 2010; 2(3): 1-000 Available from: URL: http://www.wjgnet.com/1948-5204/full/v2/i3/1.htm DOI: http://dx.doi.org/10.4251/wjgo.v2.i3.1

INTRODUCTION

Pancreaticobiliary maljunction (PBM), namely an anomalous arrangement of the pancreaticobiliary duct or an abnormal junction of the pancreaticobiliary ductal system, is a high risk factor for biliary tract cancer[1]. In PBM, the main pancreatic duct and common bile duct anatomically converge outside the duodenal wall, causing a reciprocal reflux of pancreatic juice and bile, which produces carcinogenic substances such as activated pancreatic enzymes and secondary bile acid, resulting in repeated damage and repair of the biliary mucosa, which contributes to pro-inflammatory prostaglandins and various gene mutations. This in turn causes histological changes such as hyperplastic epithelium (hyperplasia), metaplastic epithelium (metaplasia), and dysplastic

epithelium (dysplasia), ultimately resulting in biliary carcinogenesis[2]. Accordingly, when PBM is diagnosed the standard treatment consists of cholecystectomy and resection of the dilated extrahepatic bile duct to prevent carcinogenesis[1]. However, for PBM without dilatation of the extrahepatic bile duct, cholecystectomy alone is often performed since the incidence of bile duct cancer is low in such cases. This course of treatment is still controversial[3,4]. While the risk of carcinogenesis is mitigated considerably through preventive standard surgeries such as these, reports of carcinogenesis in the remnant bile duct and pancreas following surgery have been on the rise in recent years[5]. One of the causes for this is believed to be an accumulation of gene mutations which were present prior to surgery. In this paper, we attempt to elucidate the various carcinogenic processes in PBM and its treatment options, as well as the prevention of postoperative carcinogenesis.

EPIDEMIOLOGY

PBM is frequently reported in Asia, particularly in Japan and Taiwan, and it is known as an Asian disease[6,7]. Coexisting PBM is found in nearly all cases of congenital bile duct dilatation, and is also found in Western countries[8]. Funabiki *et al*[9] have suggested that the number of cases diagnosed might rise if there was increased interest in the diagnostic criteria for PBM in Western countries. Hasumi *et al*[6] conducted a survey on the incidence of PBM and biliary tract cancer at 133 facilities in Japan, and showed that 414 of the 12,399 patients (3.3%) on whom hepatobiliary surgery was performed had PBM. They furthermore reported that 10.4% of the patients with gallbladder cancer (80/769) and 4.4% of those with bile duct cancer (32/735) had coexisting PBM. In a nationwide survey carried out by the Japanese Study Group on PBM over 10 years from 1990 to 1999, the aggregate number of 1,627 PBM cases were examined in detail[10]. As a whole, biliary tract cancer was detected in 278 of the 1,627 cases (17.1%). Of these, in the 1,239 cases of PBM with dilatation of the extrahepatic bile duct, there were 131 cases (10.6%) with coexisting biliary tract cancer, which was located in the gallbladder in 85 cases, the bile duct in 44 cases, and of unknown origin in 2 cases. On the other hand, of the 388 cases of PBM without dilatation of the extrahepatic bile duct there were 147 cases (37.9%) with coexisting biliary tract cancer, which was located in the gallbladder in 137 cases and in the bile duct in 10 cases. The results show that "with dilated PBM there is often carcinogenesis in the bile duct besides in the gallbladder, and with undilated PBM there is carcinogenesis in the gallbladder more than 90% of the time." These results are believed to originate from the short exposure period to carcinogenic substances and their low concentration, which is due to the moderate degree of bile stasis within the bile duct with undilated PBM[11].

CARCINOGENIC PROCESS

Pathophysiology

A mixture of pancreatic juice and bile is constantly being produced with PBM, and when bacterial infections and an increase in intrapressure in either the pancreatic duct or the bile duct are also present, pancreatic enzymes easily become activated. All pancreatic enzymes are detected at extremely high levels within the bile of PBM patients[9]. Among the activated pancreatic enzymes, amylase and lipase have little damaging action on the biliary epithelium, but trypsin activates Ca^{2+} along with phospholipase A2. Among the pancreatic juices, phospholipase A2 has a particularly powerful destructive action on the pancreatic duct and biliary epithelium, and also converts the lecithine within the bile into lysolecithine and free fatty acids that have a strong damaging action on cell membranes[12]. Furthermore, bile acid also has tissue damaging action, and it has been posited that this promotes phospholipase A2 activity, especially when the damage from secondary bile acid itself is added in. However, Shimada *et al*[13] have suggested that secondary bile acid does not play a major role in PBM carcinogenesis. As these substances are harmful to tissue, the biliary mucosa suffers long-term damage, the cell cycle accelerates, and various changes to the epithelium and DNA damage occur. Most previous studies have shown that the proliferative activity of gallbladder mucosa with PBM was higher than that of gallbladder mucosa without PBM, regardless of whether or not cancer was present[14-16]. In addition, studies by Hanada *et al*[14] and ourselves[17], on gallbladder mucosa in PBM cases showed that there is a significant acceleration of cell proliferative activity, and the thickness of the membrane was thicker than that in cases without coexisting PBM. In addition, Tanno *et al*[18] and Tokiwa *et al*[19] reported a high incidence of hyperplastic changes in the membrane that were already present in infants, and that they possessed activity values that were largely equivalent to the cell proliferative activity in the gallbladder mucosa in adults. Since there is a possibility of this easily developing cancer occurring if factors promoting carcinogenesis are at work in this process, the PBM biliary epithelium which is constantly being exposed to harmful substances can be said to be in a precancerous state.

Pathological findings

While various histopathological findings, such as hyperplasia, metaplasia, and dysplasia, have been detected in gallbladder mucosa with PBM, the most characteristic change is hyperplasia[14]. Other than PBM, although hyperplasia in the gallbladder mucosa of cholelithiasis or noncancerous lesions for routine gallbladder cancer have been detected, these are localized and moderate in degree[20]. Conversely, hyperplasia is detected in almost all parts of the gallbladder mucosa with PBM. Metaplastic change is a serious pathological change related to the development of gallbladder cancer without coexisting

PBM[21]. However, PBM is characterized by the fact that a low frequency of metaplastic epithelium occurs in less than 10%, and metaplastic change is lower than in patients with cholelithiasis. The incidence of dysplasia in noncancerous epithelium of gallbladder cancer patients with PBM is more than double that of gallbladder cancer patients without PBM[14]. In a previous study by our group[22], a high incidence of hyperplastic change was detected in infancy, and although the incidence is lower from adolescence onwards than in infancy, it is still high. Conversely, metaplasia and dysplasia were rarely seen in infancy, and only detected from adolescence onwards. Furthermore, dysplasia was most often discovered in the mucosa surrounding gallbladder cancer. Thus, hyperplastic epithelium can be present from the early stages of infancy or at birth, whereas metaplasia and dysplasia appears with age. Although it is unclear whether hyperplastic epithelium itself is a precancerous state, this strongly suggests that a hyperplasia-dysplasia-carcinoma sequence exists in the PBM carcinogenic process[2,9].

Gene mutation

Analyses have been conducted on the various oncogenes, tumor suppressor genes, *etc.* in resected specimens of PBM patients. In previous studies, the incidence of K-*ras* mutation in gallbladder cancer of PBM patients was 33%-83%, which is higher than in non-PBM gallbladder cancer patients[14,22-25]. Furthermore, there is a high incidence of K-*ras* mutations in benign epithelium with PBM. Iwase *et al*[23] reported detecting K-*ras* mutations in 36% of cases with hyperplasia. Matsubara *et al*[25] reported mutations in 31.6% of inflammatory epithelium, and in 47.6% with both hyperplasia and metaplasia. We detected K-*ras* mutations in 64% of cases with hyperplasia, in 28% with metaplasia, and in 17% with dysplasia[22]. Furthermore, Tomishige *et al*[26] reported that K-*ras* mutations were detected in PBM patients one month after birth, which suggests that genetic mutation occurs at an early phase of life. Since K-*ras* mutations are detected in noncancerous epithelium and hyperplasia, these epithelia seem to be in a genetically precancerous state, and represent an early event in multistep carcinogenesis.

To detect p53 in PBM patients, the "detection of gene mutations using PCR-SSCP and the direct sequence method" and methods for viewing the "overexpression of the p53 protein using anti-p53 monoclonal antibodies" have been reported, but gene mutation analysis has almost never been performed. Hanada *et al*[27] analyzed exon 5-8 of p53 using PCR-SSCP and reported the detection of an abnormal band on exon 7, 8 in 3 of the 6 cases (50%) of stage I gallbladder cancer with coexisting PBM. Matsubara *et al*[25] detected p53 gene mutation in 34.8% of cases with an inflammatory epithelium, in 47.6% with both hyperplasia and metaplasia, and in 60% with cancer, stating that these were mainly exon 5, 6, 8. However, Nagai *et al*[28] reported that p53 gene mutation was not detected in cases of hyperplasia and dysplasia, but that the gene mutation was observed in 4 of 26 cases (16%) of cancer. It is still unclear whether p53 gene mutation is present in noncancerous epithelia. Conversely, with regard to the overexpression of the p53 protein, Hanada *et al*[27] stated that overexpression was observed in 4 of the same 6 cases (67%) of stage I gallbladder cancer mentioned above. Moreover, in our study and in those of other researchers, there was a 62-100% positivity rate for cancer, but noncancerous lesions were all negative. However, Matsubara *et al*[25] stated that overexpression was observed in 8.3% of cases with an inflammatory epithelium, in 33.3% with both hyperplasia and metaplasia, and in 80% with cancer. Since their criteria for immunostaining counted anything stained as positive, even if the staining was negligible, it is thought that this resulted in higher positive rates than in other studies. These results clearly indicated that overexpression of the p53 protein was largely negative in benign epithelium in cases with PBM, but that gene mutations occur with a high frequency. Since the mutation of p53 is regarded as a late event in carcinogenesis within the adenoma-carcinoma sequence for cancer of the large intestine, it may also occur in relatively later stages of the carcinogenic process for PBM as well.

Microsatellite instability (MSI) is reflected in abnormalities of DNA repair genes, and is an important factor leading to carcinogenesis. Nagai *et al*[29] reported the detection of MSI in 16 of 23 cases (69.6%) of gallbladder mucosa in PBM patients. Of these, it was detected in 8 cases (50%) of mutations in the transforming growth factor type II receptor, in 2 cases (12.5%) of mutations in the insulin-like growth factor type II receptor, in 4 cases (25%) of LOH. In addition, Nagai *et al*[28] reported 0% MSI in PBM patients with hyperplasia, 57.1% with dysplasia, and 52% with cancer. This suggests that MSI is similar to p53 mutations in that it comes into play as a late event in the carcinogenic process for PBM.

There have been additional studies on abnormalities in cancer related genes and cell cycle related factors involved in the carcinogenic process for PBM. However, since only a limited number of cases were analyzed, further investigation is required.

CHEMOPREVENTION

The standard treatment for PBM when there is coexisting cancer is to perform surgery according to the stage of the cancer, and to perform diversion surgery for the prevention of carcinogenesis when there is no coexisting cancer. However, cases of carcinogenesis in remnant bile duct and the pancreas have been steadily increasing, even when preventative diversion surgery has been performed[5]. Furthermore, a cholecystectomy alone is performed at nearly half of institutions in Japan for cases without dilatation of the extrahepatic bile duct[1], thus there is a slight possibility of carcinogenesis in the remnant bile duct. Since these cases did not have

coexisting cancer prior to surgery, the use of adjuvant therapy for cancer and likewise anticancer drugs would not be indicated for the prevention of postoperative carcinogenesis. Ordinary anticancer drugs inhibit the DNA synthesis of cancer cells, but are unable to suppress the growth of inflammatory lesions and precancerous lesions.

Recently, progress has been made in research to suppress carcinogenesis by using a variety of chemical agents. Non-steroidal anti-inflammatory drugs, which are COX-2 inhibitors, and VK2, which is a therapeutic agent for osteoporosis, are the most promising medicines among these agents. Animal experiments showed that COX-2 inhibitors suppress carcinogenesis[30] and that, epidemiologically, long-term users of aspirin showed a 40% decrease in mortality rate due to colon cancer compared to the natural control[31]. Furthermore, it was also revealed that COX-2 has a strong correlation on cell growth, carcinogenesis, invasion, and metastasis at the cellular level[32,33]. Similar results have been also reported for various tumors other than colorectal tumors, suggesting that COX-2 has potential for use in chemoprevention and is a target for treatment. At the same time, VK2 is not only used in the clinical treatment of osteoporosis and other ailments, but is known to exhibit anti-tumor effects *in vitro* and *in vivo*[34,35]. In clinical studies on malignant tumors, the administration of VK2 induced a decrease in the number of blastic cells in a patient with post-myelodysplastic syndrome (MDS) (AML)[36]. In addition, there is a similar report that mature neutrophils increased, and anemia and a decrease in blood platelets improved in a patient with MDS in which the ratio of blastic cells declined due to VK2 administration[37]. Furthermore, female patients with viral cirrhosis of the liver in the VK2-treated group had a significantly lower onset of hepatocellular carcinoma than the control group[38]. In addition, a significant suppressant effect on the incidence of relapse was detected in cases treated with VK2 among patients with hepatocellular carcinoma after curative treatment[39]. In a large number of clinical trials for patients with osteoporosis, neurological disease, or for postmenopausal women, no severe adverse events were reported with long-term VK2 treatment[40-43]. Thus, it may be safely used as a chemopreventive agent.

Using the Syrian golden hamster PBM carcinogenesis model developed by Tajima *et al*[44], we examined whether COX-2 inhibitors and VK2 could suppress carcinogenesis, and found that early stage gallbladder cancer appeared in approximately 30% of animals in the control group with the carcinogenic substance N-nitrosobis (2-oxopropyl) amin (BOP). In contrast, in the COX-2- and VK2-treated group, carcinogenesis was suppressed through the suppression of cell growth in the gallbladder mucosa, respectively[45]. Furthermore, compared to the control group, the incidence of dysplasia, a precancerous lesion, declined in the treated group, suggesting that both agents suppress the cell cycle in PBM gallbladder mucosa. Based on the results of these experiments, further studies on the clinical efficacy of potential chemopreventive agents for PBM are warranted.

CONCLUSION

PBM is a high risk factor for biliary tract cancer, and several patients reportedly had hyperplastic changes and gene abnormalities in the biliary mucosa at birth. Since the first steps have already been taken towards carcinogenesis at the fetal stage, preventive surgeries must be performed immediately once a diagnosis has been made. In addition, it is essential to reduce the risk of carcinogenesis by using chemoprevention in order to prevent postoperative carcinogenesis.

ACKNOWLEDGMENTS

The authors are indebted to Professor J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

REFERENCES

1 **Tashiro S**, Imaizumi T, Ohkawa H, Okada A, Katoh T, Kawaharada Y, Shimada H, Takamatsu H, Miyake H, Todani T. Pancreaticobiliary maljunction: retrospective and nationwide survey in Japan. *J Hepatobiliary Pancreat Surg* 2003; **10**: 345-351

2 **Tsuchida A**, Itoi T, Aoki T, Koyanagi Y. Carcinogenetic process in gallbladder mucosa with pancreaticobiliary maljunction (Review). *Oncol Rep* 2003; **10**: 1693-1699

3 **Aoki T**, Tsuchida A, Kasuya K, Endo M, Kitamura K, Koyanagi Y. Is preventive resection of the extrahepatic bile duct necessary in cases of pancreaticobiliary maljunction without dilatation of the bile duct? *Jpn J Clin Oncol* 2001; **31**: 107-111

4 **Kusano T**, Takao T, Tachibana K, Tanaka Y, Kamachi M, Ikematsu Y, Nishiwaki Y, Kida H, Waki S, Uchimura M, Furukawa M. Whether or not prophylactic excision of the extrahepatic bile duct is appropriate for patients with pancreaticobiliary maljunction without bile duct dilatation. *Hepatogastroenterology* 2005; **52**: 1649-1653

5 **Tsuchida A**, Kasuya K, Endo M, Saito H, Inoue K, Nagae I, Aoki T, Koyanagi Y. High risk of bile duct carcinogenesis after primary resection of a congenital biliary dilatation. *Oncol Rep* 2003; **10**: 1183-1187

6 **Hasumi A**, Matsui H, Sugioka A, Uyama I, Komori Y, Fujita J, Aoki H. Precancerous conditions of biliary tract cancer in patients with pancreaticobiliary maljunction: reappraisal of nationwide survey in Japan. *J Hepatobiliary Pancreat Surg* 2000; **7**: 551-555

7 **Chang LY**, Wang HP, Wu MS, Huang HT, Wang HH, Lin CC, Lin JT. Anomalous pancreaticobiliary ductal union--an etiologic association of gallbladder cancer and adenomyomatosis. *Hepatogastroenterology* 1998; **45**: 2016-2019

8 **Tuech JJ**, Pessaux P, Aube C, Regenet N, Cervi C, Bergamaschi R, Arnaud JP. Cancer of the gallbladder associated with pancreaticobiliary maljunction without bile duct dilatation in a european patient. *J Hepatobiliary Pancreat Surg* 2000; **7**: 336-338

9 **Funabiki T**, Matsubara T, Miyakawa S, Ishihara S. Pancreaticobiliary maljunction and carcinogenesis to biliary and pancreatic malignancy. *Langenbecks Arch Surg* 2009; **394**: 159-169