Hironaka S., Boku N.	Weekly paclitaxel as second-line chemotherapy for advanced or recurrent gastric cancer.	Gastric Cancer	in press		2006
紀　貴之 滝内　比呂也	切除不能・再発胃癌に対するWeekly paclitaxel(PTX)の有効性と副作用の検討	癌と化学療法			2006 6月掲載予定
Qin B, Ariyama H, Shibata Y, Arita S, Tanaka R, Kusaba H, Baba E, Harada M, Nakano S	In vitro differential metabolism and activity of 5-fluorouracil between short-term high dose and long-term low dose treatments in human squamous carcinoma cells.	Anti-Cancer Drugs	in press		2006
Qin B, Tanaka R, Shibata Y, Arita S, Ariyama H, Kusaba H, Baba E, Harada M, Nakano S	In Vitro Schedule-dependent Interaction between Oxaliplatin and 5-Fluorouracil in Human Gastric Cancer Cell Lines.	Anti-Cancer Drugs	in press		2006
Qin B, Ariyama H, Baba E, Tanaka R, Kusaba H, Harada M, Nakano S	Activated Src and Ras Induce Gefitinib Resistance by Activation of Signaling Pathways Downstream of Epidermal Growth Factor Receptor in Human Gallbladder Adenocarcinoma Cells.	Cancer Chemotherapy Pharmacology	in press		2006
Shimizu T, Yamada Y, Yasui H, Shirao K, Fukuoka M.	Clinical Application of Immunoreactivity of Dihydropyrimidine Dehydrogenase(DPD) in Gastric Scirrhous Carcinoma Treated with S-1, a New DPD Inhibitory Fluoropyrimidine.	ANTICANCER RESEARCH	25	2997-3002	2005
Ishikura S, Ohtsu S, Shirao K, Muro K, Kagami Y, Nihei K, Mera K, Ito Y, Boku N, Yoshida S.	A phase I/II study of nedaplatin and 5-fluorouracil with concurrent radiotherapy in patients with T4 esophageal cancer: Japan Clinical Oncology Group trial (JCOG9908).	Esophagus	2	133-137	2005

Koyano S, Saito Y, Sai K, Kurose K, Ozawa S, Nakajima T, Matsumoto K, Saito H, Shirao K, Yoshida T, Minami H, Ohtsu A, Saijo N, Sawada J.	Novel Genetic Polymorphisms in the NR3C1(Glucocorticoid receptor) Gene in a Japanese Population. Drug Metabol. Pharmacokin.	Drug Metabol. Pharmacokin	20(1)	144-151	2005
Saeki M, Saito Y, Jinno H, Sai K, Kaniwa N, Ozawa S, Komamura K, Kotake T, Morishita H, Kamakura S, Kitakaze M, Tomoike H, Shirao K, Minami H, Ohtsu A, Yohida T, Saijo N, Kamatani N, Sawada J.	Genetic Polymorphisms of UGT1A6 in a Japanese Population.	Drug Metabol. Pharmacokin.	20(1)	85-90	2005
Saeki M, Saito Y, Jinno H, Sai K, Hachisuka S, Kaniwa N, Ozawa S, Kawamoto M, Kamatani N, Shirao K, Minami H, Ohatsu A, Yoshida T, Saijo N, Komamura K, Kotake T, Morishita H, Kamakura S, Kitakaze M, Tomoike H, Sawada J.	Genetic variations and haplotypes of UGT1A4 in a Japanese population.	Drug Metabol. Pharmacokin.	20(2)	144-151	2005
Kubo T, Kim S, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, Ohtsu A, Yoshida T, Saijo N, Ohono Y, Ozawa S, Sawada J.	Functional characterization of three naturally occurring single nucleotide polymorphisms in the CES2 gene encoding carboxylesterase 2(HCR-2).	DRUG METABOLISM AND DISPOSITION.	33(10)	1482-1487	2005
Shirao K.	Problems related to devising a clinical traial protocol for gastric cancer with peritoneal metastasis.	Proceedings of the society for clinical and biostatistical research.	25(1)	81-84	2005

Shirao K.	Problems and countermeasures concerning patient enrollment in a large-scale clinical trial: The national surgical adjuvant study of colorectal cancer 01 (NSAS-CC01).	Proceedings of the society for clinical and biostatistical research.	25(1)	85-88	2005
Hosokawa A, Shimada Y, Matsumura Y, Yamada Y, Muro K, Hamaguchi T, Igaki H, Tachimori Y, Kato H, Shirao K.	Small Cell Carcinoma of the Esophagus. Analysis of 14 Cases and Literature Review.	Hepato-Gastroenterology	52	1738-1741	2005
安井　久晃、白尾　国昭	放射線化学療法　補助化学療法	消化器外科	28(5)	853-858	2005
加藤　健、白尾　国昭、他	大腸癌の新しい化学療法，オキサリプラチン，アバスチン™（ベバシズマブ）	Pharma Medica	23(12)	51-56	2005
新井　達広、白尾　国昭	テガフール・ギメラシル・オテラシルカリウム配合カプセル剤	がんの薬物療法マニュアル		84-86	2005
新井　達広、白尾　国昭	疾患別標準治療　胃癌	がんの薬物療法マニュアル		174-182	2005
津田南都子、白尾　国昭	オキサリプラチン	日本病院薬剤師会雑誌	41(12)	1553-1556	2005
辻　靖，住吉徹哉，由崎直人，近藤　仁，本多加奈，高張大亮	癌化学療法におけるリスクマネジメント　外来化学療法センターの運用とリスクマネジメント	札幌市医師会医学雑誌		105-106	2005
Sato Y, Kondo H, Honda K, Takahari D, Sumiyoshi T, Tsuji Y, Yoshizaki N, Niitu Y.	A phase I/II study of S-1 plus cisplatin in patients with advanced gastric cancer: 2-week S-1 administration regimen.	Int J Clin Oncol.	10	40-44	2005
辻　靖，奥山めぐみ，近藤　仁.	大腸癌における化学療法の実際と看護	がん化学療法看護実践集		116-127	2005

村上　晶彦	急性胆道炎に対するドレナージ	外科治療 特集　消化器救急患者とその対応	93(6)	664-677	2005
Ebi H, Shigeoka Y, Saeki T, Kawada K, Igarashi T, Noriko Usubuchi, Ueda R, Sasaki Y, Minami H.	Pharmacokinetic and pharmacodynamic comparison of fluoropyrimidine derivatives, capecitabine and 5'-deoxy-fluorouridine (5'-DFUR)	Cancer Chemother. Pharmacol.	56	205-211	2005
Fujita K, Ando Y Narabayashi M, Miya T, Nagashima F, Yamamoto W, Kodama K, Araki K, Endo,H, Sasaki Y.	Gefitinib (Iressa) inhibits the CYP3A4-mediated formation of NPC, but activates that of APC from irinotecan	*Durg Metab. Dispos.*	33	1785-1790	2005
Fujita K, Ando Y, Nagashima F, Yamamoto W, Endo H, Kodama K, Araki K, Miya T, Narabayashi M, Sasaki Y.	Novel single nucleotide polymorphism of *UGT1A7* gene in Japanese	*Drug Metab. Pharmacokinet.*			2005
Fujita K, Ando Y, Nagashima F, Yamamoto W, Endo H, Kodama K, Araki K, Miya T, Narabayashi M, Sasaki Y.	Novel single nucleotide polymorphism of *UGT1A9* gene in Japanese	*Drug Metab. Pharmacokinet.*			2005
Shimizu Y, Yasui K, Fuwa N, Arai Y, Yamao K.	Late complication in patients undergoing pancreatic resection with intraoperaive radiation therapy: Gastrointestinal bleeding with occlusion of the portal system.	Journal of Gastroenterology and Hepatology	20	1235-1240	2005

Hosokawa A, Maeda T, Tateishi U, Satake M, Iwata R, Ojima H, Arai Y.	Hepatic Hemangioma Presenting Atypical Radiologic Findings: A Case Report.	Radiation Medicine	23	371-375	2005
Tateishi U, Uno H, Yonemori K, Satake M, Takeuchi M, Arai Y.	Prediction of Lung Adenocarcinoma Without Vessel Invasion: A CT Scan Volumetric Analysis.	Chest	Nov128	3276-3283	2005
Tateishi U, Hasegawa T, Nojima T, TakegamiT, Arai Y.	MRI features of extraskeletal myxoid chondrosarcoma.	Skeletal Radiol.	Oct 12	1-7	2005
Tateishi U, Hasegawa T, Yamamoto S, Yamaguchi U, Yokoyama R, KawamotoH, Satake M, Arai Y.	Incidence of multiple primary malignancies in a cohort of adult patients with soft tissue sarcoma.	Jpn J Clin Oncol.	Aug;35	444-452	2005
Tateishi U, Hasegawa T, Onaya H, Satake M, Arai Y, Moriyama N.	Myxoinflammatory Fibroblastic Sarcoma: MR Appearance and Pathologic Correlation.	AJR Am J Roentgenol	Jun. 184	1749-53	2005
Iinuma G, Tomimatsu H, Muramatsu Y, Moriyama N, Kobayashi T, Saito H, Maeda T, Miyakawa K, Wakao F, Satake M, Arai Y.	Recent Advances in Radiology for the Diagnosis of Gastric Carcinoma. The Diversity of Gastric Carcinoma.	Pathogenesis, Diagnosis, and Therapy.		221-232	2005
荒井保明	消化器癌肝転移に対する動注化学療法	臨牀消化器内科	20(2)	189-197	2005
荒井保明	大腸癌肝転移に対する肝動注化学療法の位置づけ	大腸疾患NOW		93-99	2005

楠本昌彦、荒井保明、他	肺腫瘤病変に対する生検の適応についての考え方－肺癌術前に確定診断は全例に必要か－	IVR	20	58-59	2005
荒井保明	序文－臨床試験技術習得のススメ	IVR	20	p42	2005
荒井保明、佐竹光夫、稲葉吉隆、新槇　剛、松枝清	癌緩和医療における Interventional radiology(IVR)	癌の臨床	51(3)	213-220	2005
荒井保明	IVR（インターベンショナル・ラジオロジー）	がん看護	10(3)	261-266	2005
荒井保明	大腸癌・肝動注化学療法	綜合臨床	54(7)	2081-2082	2005
荒井保明、山本清一郎	臨床研究に必要な統計「以前」の知識	IVR会誌	20	371-375	2005
Shimamura M, Saito A, Sakuma T, Terui Y, Ueno K, Yumoto H, Yamauchi K, Yamamura K, Mirmura H, Sano Y, Yabashi M, Tamasaku K, Nishio K, Nishino Y, Endo K, Hatake K, Mori Y, Ishizaka Y, Ishikawa T.	Element array by scanning X-ray fluorescence microscopy after cis-diamminedichloro-platinum(II) treatment.	Cancer Res.	65	4998-5002	2005
Toi M, Saeki T, Aogi K, Sano M, Hatake K, Asaga T, Tokuda Y, Mitsuyama S, Kimura M, Kobayashi T, Tamura M, Tabei T, Shin E, Nishimura R, Ohno S, Takashima S.	Late Phase II Clinical Study of Vinorelbine Monotherapy in Advanced or Recurrent Breast Cancer Previously Treated with Anthracyclines and Taxanes.	Jpn J Clin Oncol.	35	310-315	2005
Nagashima F, Boku N	Biological markers as a predictor for response and prognosis of unresectable gastric cancer patients treated with irinotecan and Cisplatin	Jpn J Clin Oncol.	35(12)	714-9	2005

安井　博史、朴　成和	進行癌（遠隔転移例）の化学療法　新規抗癌剤の可能性を含めて	胃と腸	40(7)	1015-1022	2005
Miki I, Tamura T, Nakamura T, Makimoto H, Hamana N, Uchiyama H, Shirasaka D, Morita Y, Yamada H, Aoyama N, Sakaeda T, Okumura K, Kasuga M.	Circadian Variability of Pharmacokinetics of 5-Fluorouracil and CLOCK T3111C Genetic Polymorphism in Patients With Esophageal Carcinoma.	Ther Drug Monit.	27(3)	369-374	2005
Hirasaki S, Tanimizu M, Tsubouchi E, Nasu J, Masumoto T.	Gastritis cystica polyposa concomitant with gastric inflammatory fibroid polyp occurring in an unoperated stomach.	Internal Medicine	44 巻 1 号	46-9	2005
Kohno H, Mizuno M, Nasu J, Makidono C, Hiraoka S, Inaba T, Yamamoto K, Okada H, Fujita T, Shiratori Y.	Stool decay-accelerating factor as a marker for monitoring the disease activity during leukocyte apheresis therapy in patients with refractory ulcerative colitis.	J Gastroenterol Hepatol	20 巻 1 号	73-78	2005
Nishikawa Y, Chikamori F, Murakami M, Nasu J.	Clinical application of an indwelling needle for esophageal varices in endoscopic injection sclerotherapy with simultaneous ligation.	Digestive Endoscopy	17 巻 4 号	331-333	2005
Hirasaki S, Tanimizu M, Nasu J, Shinji T, Koide N.	Treatment of elderly patients with early gastric cancer by endoscopic submucosal dissection using an insulated-tip diathermic knife.	Internal Medicine	44巻 10 号	1033-1038	2005

Moriwaki T, Hyodo I, Nishina T, Hirao K, Tsuzuki T, Hidaka S, Kajiwara T, Endo S, Nasu J, Hirasaki S, Masumoto T, Kurita A.	A phase I study of doxifluridine combined with weekly paclitaxel for metastatic gastric cancer.	Cancer Chemother Pharmacol	56 巻 2 号	138-144	2005
Nasu J, Doi T, Endo H, Nishina T, Hirasaki S, Hyodo I.	Characteristics of metachronous multiple early gastric cancers after endoscopic mucosal resection.	Endoscopy	37 巻 10 号	990-993	2005
Hirasaki S, Tanimizu M, Moriwaki T, Nasu J.	Acute pancreatitis occurring in gastric aberrant pancreas treated with surgery and proved by histological examination.	Internal Medicine.	44 巻 11 号	1169-1173	2005
那須淳一郎、平家勇司、谷水正人、佐々木晴子、山田純子、福岡しのぶ、大住省三、久保義郎、青儀健二郎、新海哲、高嶋成光.	家族歴調査のシステム化による家族性腫瘍相談室の運営.	家族性腫瘍	5 巻 1 号	57-60	2005
谷水正人、新海哲、兵頭一之介、舛本俊一、那須淳一郎、平崎照士.	【がん治療後の患者ケア 家庭医に知ってもらいたいこと】患者ケアにおけるインターネットがん情報の検索.	治療	87 巻 4 号	1635-1639	2005
梶原猛史、那須淳一郎、平崎照士、仁科智裕、片岡淳朗、日高聡、森脇俊和、壷内栄治、山内雄介、舛本俊一、谷水正人、兵頭一之介.	膵癌に伴う上部消化管病変の検討.	日本消化器内視鏡学会雑誌	47 巻 6 号	1220-1226	2005
那須淳一郎、仁科智裕、片岡淳朗、壷内栄治、梶原猛史、森脇俊和、今峰聡、谷水正人、野崎功雄、栗田啓.	早期胃癌における遠位胃切除術は残胃癌の危険因子か.	消化器科	41 巻 6 号	466-470	2005
辻　晃仁	腫瘍マーカーは実地臨床で有用か？ -胃癌の腫瘍マーカーとその活用-	成人病と生活習慣病	第 35 巻第 6 号	621-626	2005

Tanaka R, Ariyama H, Qin B, Takii Y, Baba E, Mitsugi K, Harada M, Nakano S	In vitro Schedule-Dependent Interaction between Paclitaxel and Oxaliplatin in Human Cancer Cell Lines.	Cancer Chemotherapy Pharmacology	55	595-601	2005
Tanaka R, Ariyama H, Qin B, Shibata Y, Takii Y, Kusaba H, Baba E, Mitsugi K, Harada M, Nakano S	Synergistic Interaction between Oxaliplatin and SN-38 in Human Gastric Cancer Cell Lines in Vitro.	Oncology Reports	14	683-688	2005
Tanaka R, Takii Y, Shibata Y, Ariyama H, Qin B, Baba E, Kusaba H, Mitsugi K, Harada M, Nakano S	In Vitro Sequence-dependent Interaction between Nedaplatin and Paclitaxel in Human Cancer Cell Lines.	Cancer Chemotherapy Pharmacology	56	279-285	2005

Ⅳ. 研究成果の刊行物・別刷

Comparison of *HER2* gene amplification assessed by fluorescence *in situ* hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer

TOMONORI YANO[1,2], TOSHIHIKO DOI[2], ATSUSHI OHTSU[2], NARIKAZU BOKU[2], KAORU HASHIZUME[3], MAMORU NAKANISHI[4] and ATSUSHI OCHIAI[1]

[1]Pathology Division, Reseach Center for Innovative Oncology; [2]Division of Digestive Endoscopy and Gastrointestinal Oncology, National Cancer Center Hospital East, Chiba; [3]Medical Science Department, DakoCytomation Co. Ltd.; [4]Bioscience Division, FALCO Biosystems Ltd., Kyoto, Japan

Received July 18, 2005; Accepted September 16, 2005

Abstract. A monoclonal antibody to HER2 protein is widely used in the treatment of patients with HER2-overexpressing breast cancer and has also been found to exhibit antitumor activity in human gastric cancer cells that overexpress HER2. The purpose of this study was to evaluate the frequency of HER2 overexpression and concordance between the results for protein expression and gene amplification in both surgical and biopsy specimens of gastric cancer as assessed with two commercial kits, one for immunohistochemistry (IHC) and the other for fluorescence *in situ* hybridization (FISH). The specimens consisted of formalin-fixed, paraffin-embedded sections of biopsy specimens and surgically resected tumors from 200 cases of invasive gastric cancer that had been treated surgically at the National Cancer Center Hospital East. The lesions were analyzed with the IHC kit, and expression was graded by the United States Food and Drug Administration (FDA)-approved grading system. Gene amplification was evaluated by FISH. IHC revealed HER2 overexpression in 46 of the 200 (23%) cases. The FISH assay was technically uccessful in 199 cases (99.5%), and gene amplification was observed in 54 cases (27.1%). The concordance rate between the results obtained by IHC and FISH was 86.9%. The concordance rate between the findings in the surgically resected tumors and the 200 pre-treatment biopsy specimens was 88.7%. HER2 expression can be assessed in gastric cancer with a commercial kit as previously reported in breast cancer. Even small biopsy specimens were found to be suitable for evaluating gastric cancer for HER2 overexpression.

Correspondence to: Dr Atsushi Ochiai, Pathology Division, Reseach Center for Innovative Oncology, NCC-Kashiwa, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
E-mail: aochiai@east.ncc.go.jp

Key words: gastric cancer, HER2, immunohistochemistry, fluorescence *in situ* hybridization

Introduction

The *HER2* (also called c-erbB2) is a proto-oncogene and is located on chromosome 17q21 (1). *HER2* encodes a Mr 185,000 transmembrane glycoprotein, which is a member of the HER receptor family and possesses tyrosine kinase activity. Overexpression of HER2 protein has been described in approximately 25-30% of invasive breast cancers, and it has been used as a marker of resistance to various therapeutic modalities, and short disease-free survival (2,3). Trastuzumab (Herceptin, Genentech, Inc., South San Francisco, CA), a monoclonal antibody to the HER2 protein, is a promising agent for the treatment of breast cancer patients with a poor prognosis, and Slamon *et al* have reported that addition of Trastuzumab to the chemotherapy regimen yields a significantly higher response and prolongs time to progression and overall survival of a breast cancer patients with HER2 overexpression (4).

Various methods are available to determine the HER2 status of breast cancer, however, many of them require fresh tissue, involve a complicated procedure, and are costly. Immunohistochemistry (IHC) is widely used to evaluate protein expression in formalin-fixed, paraffin-embedded specimens, and, Southern blot hybridization is recognized as the standard method for analysis of *HER2* gene amplification, but the procedure requires a large, fresh specimen (5). Fluorescence *in situ* hybridization (FISH) can be used to analyze small formalin-fixed, paraffin-embedded specimens for gene amplification, and Press *et al* have evaluated FISH as a mean of assessing *HER2* amplification in breast cancer (3). In their study, FISH was used to test for *HER2* amplification in 140 breast cancers in which gene amplification had already been demonstrated by Southern hybridization and it was found to have a sensitivity of 98% and a specificity of 100%. IHC and FISH are widely used methods for evaluating HER2 status for breast cancer, furthermore, Food and Drug Administration (FDA) in the United States approved IHC and FISH tests to determine HER2 status for breast cancer patients: Hercep test kit (DakoCytomation Denmark A/S, Glostrup, Denmark), and PathVysion HER2 DNA probe kit (Vysis Inc., Downers Grove, IL). Many investigators have

compared IHC and FISH as means of evaluating HER2 in breast cancer (5-7). In general, correlation level between IHC and FISH results approximately 90% in IHC strong positive cases (3+), however weakly positive cases (2+) obtain only a minor association. Therefore, National Compresive Cancer Network (NCCN) guidelines for treatment of breast cancer recommend that HER2 testing should be done using IHC and/or FISH, an IHC results of 2+ should be confirmed with FISH (8).

Many investigators subsequently evaluated HER2 status in gastric cancer by IHC, and the frequency of HER2 overexpression varied widely, from 8 to 31% (9-14). The consensus of almost all reports is that the majority of positive cases are the intestinal type histologically. Methods of IHC which evaluate for HER2 status in gastric cancer have not been standardized and there is a wide range in the frequency of overexpression, furthermore, there have been few reports claiming to have demonstrated *HER2* gene amplification in gastric cancer (15-17).

In xenograft experiments, Trastuzumab has also shown antitumor activity in gastric cancer cell lines with HER2 overexpression, and synergy has been demonstrated with some cytotoxic agents (18). Results of that study encourage the clinical investigation of Trastuzumab for patients with HER2-overexpressing gastric cancer.

We usually collect small biopsy specimens endoscopically from advanced gastric cancers before initiating chemotherapy. In unresectable cases, tumor behavior before treatment has to be evaluated on the basis of these small specimens alone. However, since gastric cancer is considered to be a heterogeneous tumor, small biopsy specimens may not reflect its behavior.

Before using Trastuzumab in the treatment of gastric cancer, concordance between protein expression and gene amplification of *HER2* has to be confirmed with commercial kits as examined for breast cancer, and the feasibility of using biopsy specimens to evaluate HER2 status must also be confirmed.

The purpose of this study was to evaluate the frequency of HER2 overexpression in gastric cancer and the concordance between protein expression and gene amplification in both surgical and endoscopic biopsy specimens with two commercial kits, an IHC and a FISH.

Materials and methods

A total of 1,254 patients with primary gastric cancer underwent surgery at the National Cancer Center Hospital East (Kashiwa, Japan) between July 1992 and March 2000. Of the 1,254 patients, 261 cases were invasive intestinal-type gastric cancer. We selected 200 of 261 cases in which preoperative endoscopic biopsy samples were completely preserved in our hospital. The specimens consisted of formalin-fixed, paraffin-embedded sections of preoperative endoscopic biopsy specimens and surgically resected tumors.

All tissues were fixed with 10% buffered formalin, generally for 24 and 48 h, and paraffin-embedded. Sections 4-μm thick were cut from a paraffin block of each specimen and applied to slides for IHC, and 5-μm thick sections were cut and applied to slides for FISH.

The IHC analysis was performed with the Hercep test kit (DakoCytomation Denmark A/S) at Dako Cytomation Co. Ltd, Kyoto, Japan. FISH for *HER2* gene amplification was performed with the PathVysion HER2 DNA probe kit (Vysis Inc.) at FALCO Biosystems, Kyoto, Japan.

IHC for HER2 protein expression. The immunohistochemical analysis with the Hercep test was performed according to the manufacturer's guidelines. All reagents were included in the kit. Briefly, heat-induced epitope retrieval was performed on the deparaffinized sections in advance by immersing the slides in Epitope Retrieval Solution (10 mM citrate buffer; pH 6.0), which had been preheated to 95°C. They were then placed in a 95°C water bath for 40 min, followed by 20-min at room temperature, then endogenous peroxidase was quenched with Peroxidase Blocking Reagent. Next, the slides were incubated at room temperature for 30 min with ready-to-use rabbit polyclonal antibody to HER2 oncoprotein, and the primary antibody was detected by incubation at room temperature for 30 min with Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulins). After washing, slides were developed with Substrate Chromogen Solution at room temperature for 10 min. The expression grading and evaluation were performed in accordance with the FDA-approved system for breast cancer (19). Only membrane staining intensity and pattern were evaluated using the 0 to 3+ scale. Scores of 0 or 1+ were considered negative, a score of 2+ was weakly positive when >10% of the tumor cells showed weak to moderate complete membrane staining, and a score of 3+ was strongly positive when a strong complete membrane staining was observed in >10% of the tumor cells (Fig. 1). In the present study, we defined HER2 expression positive when tumor cell staining was evaluated as 2+ or greater with Hercep test.

FISH for HER2 gene amplification. The results of FISH for *HER2* were evaluated using a PathVysion HER2 DNA probe kit which uses a dual-color probe to determine the number of copies of both *HER2/neu* (SpectrumOrange) and CEP17 (chromosome enumeration probe 17) (SpectrumGreen). The kit was used according to the manufacturer's protocol. Briefly, an appropriate formalin-fixed paraffin-embedded tissue block from each case was selected by the pathologists, cut into 5-μm thick sections, and mounted on silane-coated slides (Dako A/S). One of the sections was stained with H&E and used for the microscopic confirmation of the invasive part of the carcinoma tissue, and other sections were used for the FISH assay. The slide for FISH was deparaffinized in Hemo-De for 10 min three times, and then dehydrated in 100% ethanol for 5 min twice. Air-dried tissue sections were treated in 0.2 N hydrochloric acid for 20 min, then washed in distilled water for 3 min. After immersion in Vysis wash buffer for 1 min, they were immersed in pre-treatment solution for 30 min at 80°C. After washes in distilled water for 3 min and immersion in Vysis wash buffer for 5 min twice, the slides were exposed to protease solution at 37°C for 20-30 min, then immersed in Vysis wash buffer for 5 min twice, and dried in air for 20 min at room temperature. The slides were subsequently immersed in 10% buffered formalin for 10 min, and then immersed in Vysis wash buffer for 5 min, twice, and dried in air for 15 min

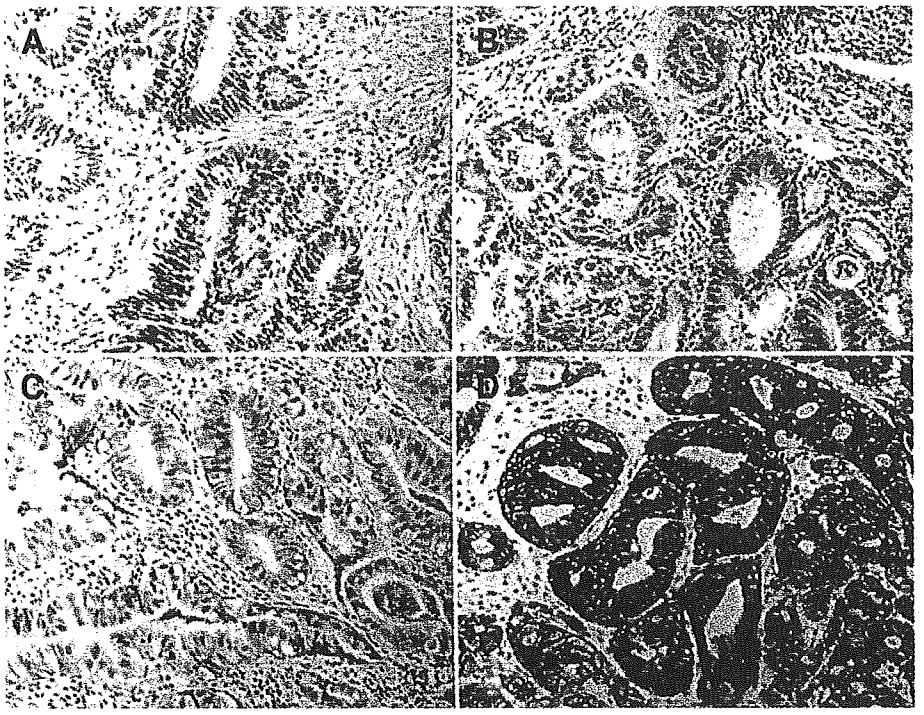

Figure 1. The expression grading and evaluation were estimated in accordance with the FDA-approved system, using the 0 to 3+ scale, score of 0 (A) or 1+ (B) were considered negative, 2+ (C) was weak positive and 3+ (D) was strong positive for HER2 overexpression.

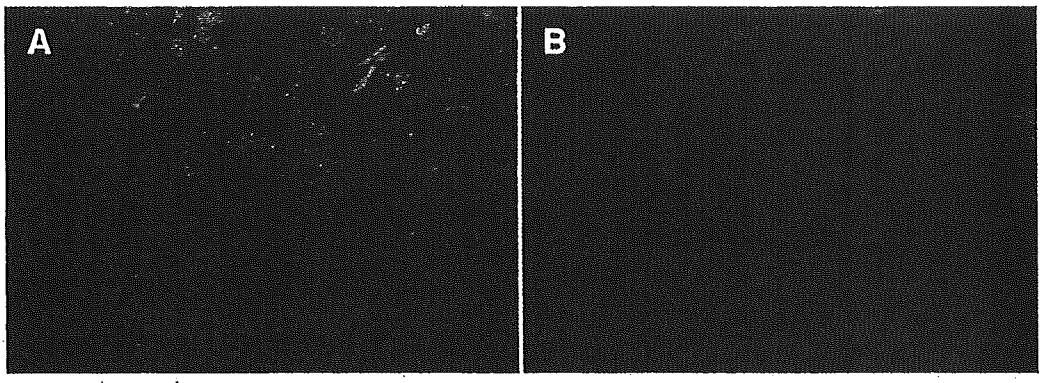

Figure 2. The ratio of *HER2* signals to CEP17 signals was determined, with ratios of <2.0 considered non-amplified (A, negative) and those ≥2.0 amplified (B, positive).

at room temperature. Hybridization was performed at 37°C for 14-18 h with a denatured DNA probe followed by immersion in pre-warmed post-wash solution at 72±1°C for 2 min. Finally, the slides were air dried in the dark and counterstained with 4,6-diamidino-2-phenylindole (DAPI).

The total numbers of the *HER2* and CEP17 signals were counted in 60 interphase tumor cell nuclei examined with a fluorescent microscopes and appropriate filters. The ratios of *HER2* signals to CEP17 signals were calculated as follows: when the ratio was <2.0, the gene was considered non-amplified and when it was ≥2.0, the gene was considered to be amplified (Fig. 2).

Statistical analysis. To assess the concordance rate between the results for protein overexpression and gene amplification and between the results for surgically resected tumor and biopsy specimens, we compared the positive and negative rates calculated for each examination. Confidence intervals were computed using the normal approximation to the binomial distribution. Positive predictive value (PPV) was calculated as the number of positive in biopsy specimens divided by the number of positive surgically resected tumors. Negative predictive value (NPV) was calculated as the number of negative biopsy specimens divided by the number of negative surgically resected tumors.

Results

Patient and lesion characteristics are shown in Table I. Median age was 66 years (range 39-88 years), and the male/female ratio was 155/45. There were 194 tubular adenocarcinomas and 6 papillary adenocarcinomas. The pathological stage was: T2/3 in 99/101, N0/1/2/3 in 54/84/47/15, and stage I/II//III/IV in 42/55/67/36 patients.

Table I. Patient and lesion characteristics.

Gender	
Male	155
Female	45
Age	
Median	66
Range	33-88
Histology	
Tubular (tub)	194
Papillary (pap)	6
Pathological TNM classification	
T-stage	
T2	99
T3	101
N-stage	
N0	54
N1	84
N2	47
N3	15
M-stage	
M0	176
M1	24
I	42
II	55
III	67
IV	36

Table II. Her2 overexpression in surgically resected tumors.

IHC score	No. Tub	No. Pap	No. Total	%
0	126	0	126	63
1+	28	0	28	14
2+	12	0	12	6
3+	28	6	34	17
Total	193	6	200	
Positive rate (%)	20.6	100		23

Table III. *HER2* gene amplification in surgically resected tumors.

HER2/CEP17[a] ratio	Tub	Pap	Total
Median	1.4	6.8	1.4
Range	1.0-12.3	1.5-9.7	1.0-12.3
<2	144	1	155
≥2	49	5	54
Positive rate (%)	25.3	83.3	27.1

FISH assay was technically successful in 199 (99.7%) of the 200 cases. [a]CEP17, chromosome enumeration probe 17.

HER2 overexpression in surgically resected tumors. The results for HER2 overexpression in the surgically resected tumors are shown in Table II. All 200 cases could be evaluated by IHC. Hercep test score was 0 in 126 cases (63%), 1+ in 28 (14%), 2+ in 12 (6%), and 3+ in 34 cases (17%), respectively. All 6 of papillary adenocarcinomas were 3+ (100%). Of the 200 surgically resected tumor specimens, 46 (23%) of the tumors were found to exhibit HER2 protein overexpression [95% confidence interval (CI): 17-28%].

HER2 gene amplification in surgically resected tumors. The results for *HER2* gene amplification in the surgically resected tumors are shown in Table III. FISH assay was technically successful in 199 (99.7%) of the 200 cases and the *HER2* gene was judged to have been amplified in 54 of 199 cases (27.1%, 95% CI 21-33.2%). The median number of signals per nucleus was 1.4 (range 1.0-12.3) with the median number of signals per nucleus in 54 amplified cases of 5.55. In 194 cases with tubular adenocarcinoma, FISH assay was unsuccessful in 1 case and 49 of the 193 cases (25.3%) were determined to be amplified. Five of the 6 cases (83.3%) of papillary adenocarcinoma were evaluated as amplified with the median number of signals per nucleus of 6.8 (range 1.5-9.7).

Concordance between the results of IHC and FISH in surgically resected tumors. The concordance rate between the results of IHC and FISH in the HER2-protein overexpression cases was 86.7% (58.3% for 2+, 88.2% for 3+) (Table IV). Of the 153 cases that were HER2 protein-negative by IHC, 136 cases were not showed amplification with FISH and its concordance rate was 88.8% (90.4% for 0, 82.1% for 1+). The results of the two assays were concordant in 173 of the 199 cases (86.9%, 95% CI 82.2-91.6%).

Table IV. Concordance between the results of IHC and FISH in surgically resected tumors.

IHC score	FISH Positive	FISH Negative	Concordance rate (%)
0	12	113	90.4
1+	5	23	82.1
2+	7	5	58.3
3+	30	4	88.2
			86.9

Concordance between the results for HER2 overexpression determined by IHC in surgically resected tumors and biopsy specimens. The IHC method was technically successful in all

Table V. Concordance between the results for HER2 overexpression determined by IHC in surgically resected tumors and biopsy specimens.

	Surgically resected tumors				
Biopsy specimens	Negative	Positive	Total	NPV (%)	PPV (%)
Negative	144	13	157	91.7	-
Positive	10	33	43	-	76.7
Total	154	46	200		
Concordance rate (%)	93.5	71.7	88.5		

NPV, negative predictive value; PPV, positive predictive value.

of the biopsy specimens (Table V). A total 43 cases were evaluated as positive (21.5%) and 157 cases as negative (78.5%). The concordance rate for HER2 overexpression determined by IHC in the surgically resected tumors and the biopsy specimens was 88.5% (95% CI 87.1-89.9%). Of the 154 cases in which the surgically resected tumor was evaluated as negative for HER2 overexpression by IHC, the biopsy specimen was negative in 144 cases resulted in the concordance rate of 93.5% (95% CI 91-95%). Therefore, 144 of 157 negative cases with biopsy specimens could represent those in surgically resected specimen as HER2 negative (NPV: 91.7%). Of the 46 cases in which surgically resected tumor was evaluated as positive, the biopsy specimens was also positive in 33 cases with the concordance rate of 71.7% (95% CI 58.7-84.7%). Of the 43 cases in which the biopsy specimen was positive, 33 of 43 cases could represent those in surgically resected specimen as HER2 positive (PPV: 76.7%).

Concordance between the results of FISH in surgically resected tumors and biopsy specimens. We have evaluated all 54 biopsy specimens in which gene amplification was demonstrated in the surgically resected tumor: the FISH assay was technically successful in all 54 (100%) cases, despite the small specimens. HER2 was interpreted as being amplified in 33 of these 54 cases, which resulted in the concordance rate of 62.2% (95% CI 49.3-75.1%). The median number of signals per nucleus in the 54 cases was 3.5 (range 1.1-12.2), whereas the median number of signals per nucleus in the 33 amplified cases was 7.9.

Discussion

Gastric cancer is one of the leading causes of cancer death in the world. Despite improvements in survival as a result of early detection and curative surgery, approximately 50,000 patients died of gastric cancer in Japan in 2001 (20). Unresectable advanced cancer and recurrent gastric cancer, in particular, still have a poor prognosis. Randomized trials have demonstrated that fluorouracil (5FU)-based chemotherapy improves survival and quality of life compared with the best supportive care (21-23), however, no standard treatment regimen has been established yet. New and promising agents for gastric cancer are eagerly waited.

Although there is no relevant evidence Trastuzumab has been put forward as a potential candidate in gastric cancer therapy particularly in patients with HER2 expression. However, basic data is needed to decide whether it should be further developed in the treatment for gastric cancer. This is the first large study to evaluate concordance of HER2 status between protein expression and gene amplification in both surgical and endoscopic biopsy specimens of gastric cancer using two commercial kits, an IHC and a FISH.

In this study, HER2 protein overexpression was demonstrated in 23% formalin-fixed paraffin-embedded specimens of surgically resected advanced intestinal type gastric cancers, and *HER2* gene amplification was demonstrated in 27.1%. FISH indicated gene amplification in 86.7% of the cases in which HER2 protein overexpression was detected by IHC, and the concordance rate between the results obtained by IHC and FISH was 86.9%. Takehana *et al* performed a comparative study of IHC and FISH in gastric cancer (17). In their study, IHC revealed HER2 protein overexpression in 29 (8.2%) of 352 surgically resected gastric cancer not only for histologically intestinal type, and FISH showed gene amplification in 25 (86.2%) of the cases with HER2 overexpression. Our results are similar to their analysis and to those analyzed in breast cancer (5-7).

Ridolfi *et al* reported a low frequency of gene amplification in Hercep test 2+ breast cancer cases (24). They reported that FISH demonstrated gene amplification in only 36% of the 2+ cases. They claimed that 2+ IHC reactions are uncertain, that the majority of 2+ cases are a heterogeneous group, and concluded that FISH should be performed on all 2+ cases to confirm gene amplification. Although there have been a few reports of IHC studies of Hercep test 2+ cases, frequency of gene amplification in 2+ breast cancer cases varies widely and is generally lower than in 3+ cases (5-7). According to results of clinical trials of Trastuzumab in breast cancer, its antitumor activity in IHC 2+ cases was consistently lower than in 3+ patients (25,26), therefore Trastuzumab is considered an active agent for breast cancer evaluated as 3+ by Hercep test or as positive for gene amplification by FISH. FISH demonstrated gene amplification in 7 of 12 IHC 2+ cases (58.5%) in the present study, which is clearly lower than in the 3+ cases (88.2%). Taking these results into consideration in the target population for Trastuzumab, FISH should be assessed for patients with IHC 2+ cases even in gastric cancer.

The concordance between the IHC findings in surgically resected tumors and biopsy specimens is very important in gastric cancer clinically. Small specimens of tumors can

easily be obtained endoscopically. If a satisfactory concordance rate is obtained, HER2 status can be evaluated by IHC in unresectable cases as well as cases of recurrence after gastrectomy. In the present study, 21.5% of biopsy specimens were evaluated as positive and the concordance rate between the results in the surgically resected tumors and biopsy specimens was 93.5%. Furthermore, the NPV for the surgically resected tumors was 91.7%, and the PPV was 76.7%. Therefore, we considered it is appropriate to evaluate HER2 status using Hercep test in biopsy specimen for recruiting gastric cancer patients who become candidate for Trastuzumab.

Many investigators have examined that HER2 overexpression could be a predictor of survival outcome in gastric cancer (10-12,14,27-29). Brien *et al* tested 61 cases for gene amplification by performing FISH on sections of paraffin-embedded gastric cancer tissue, and 43% of the cases were positive (28). The multivariate analysis in their study showed that pathological stage and *HER2* gene amplification are independent prognostic factors of survival. Allgayer *et al* confirmed the importance of HER2 status as a prognostic factor in a prospective study of gastric cancer (29). They demonstrated a significant association between level of expression of HER2 and shorter disease-free and overall survival and concluded that HER2 is a promising target for anti-invasive therapy also in gastric cancer. In the present study, there are 36 stage IV patients (18%) who would be target population for systemic chemotherapy. Of the 36 surgically resected tumor specimens, 12 (33%) of the tumors were found to exhibit HER2 protein overexpression, 2+ in 4 (11%), and 3+ in 8 cases (22%), and *HER2* gene amplification were demonstrated in 15 cases (41%). These results might indicate higher tendency of HER2 overexpression and gene amplification in stage IV than in earlier stages, though this should be confirmed in large populations.

HER2 protein overexpression and *HER2* gene amplification can be assessed with commercial kits for breast cancer even in gastric cancer. Even small endoscopic biopsy specimens are suitable for evaluating HER2 overexpression in gastric cancer. Satisfactory concordance rates could be achieved between Hercep test and Pathvision. Development of Trastuzumab for gastric cancer will progress according to the result of this study therefore it could be possible to determine adaptation with Hercep test and PathVysion even in gastric cancer.

Acknowledgements

This study was supported by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. We thank Mr. Tsutomu Yamamoto, Chugai Pharmaceutical Co., Ltd., for his assistance in the preparation of this study. We also thank Mrs. Mari Takahashi and Miss Yuki Yanagisawa for their technical assistance.

References

1. Akiyama T, Sudo C, Ogawara H, Toyoshima K and Yamamoto T: The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232: 1644-1646, 1986.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire L: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182, 1987.
3. Press MF, Bernstein L, Thomas PA, Meisner LF, Zhou JY, Ma Y, Hung G, Robinson RA, Harris C, Naggar AE, Slamon DJ, Philips RN, Ross JS, Wolman SR and Folm KJ: Her-2/neu gene amplification characterized by fluorescence *in situ* hybridization: poor prognosis in node-negative breast carcinomas. J Clin Oncol 15: 2894-2904, 1997.
4. Slamon DJ, Jones BL, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J and Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344: 783-792, 2001.
5. Hoang MP, Salin AA, Ordonez NG and Sneige N: HER-2/neu gene amplification compared with Her-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. Am J Clin Pathol 113: 852-859, 2000.
6. Jacob TW, Gown AM, Yaziji H, Barnes MJ and Schnitt SJ: Comparison of fluorescence *in situ* hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. J Clin Oncol 17: 1974-1982, 1999.
7. Lebeau A, Deimling D, Kaltz C, Sendelhofert A, Iff A, Luthardt B, Untch M and Lohrs U: HER-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence *in situ* hybridization. J Clin Oncol 19: 354-363, 2001.
8. NCCN Practice Guidelines in Oncology. Version 1.2004, 04-19-04. National Comprehensive Cancer Network, Inc.
9. Falck VG and Gullick W: c-erbB-2 oncogene product staining in gastric adenocarcinoma, An immunohistochemical study. J Pathol 159: 107-111, 1989.
10. Tateishi M, Toda T, Minamisono Y and Nagasaki S: Clinicopathological significance of c-erbB-2 protein expression in human gastric cancer. J Surg Oncol 49: 209-212, 1992.
11. Uchino S, Tsuda H, Maruyama K, Kinoshita T, Sasako M, Saito T, Kobayashi M and Hirohashi S: Overexpression of c-erbB-2 protein in gastric cancer. Cancer 72: 3179-3184, 1993.
12. Motojima K, Furui J, Kohara N, Izawa K, Kanematsu T and Shiku H: *erb*B-2 expression in well-differentiated adenocarcinoma of the stomach predicts shorter survival after curative resection. Surgery 115: 349-354, 1994.
13. McCulloch PG, Ochiai A, O'Dowd GM, Nash JG, Sasako M and Hirohashi S: Comparison of the molecular genetics of c-erb-B2 and p53 expression in stomach cancer in Britain and Japan. Cancer 75: 920-925, 1995.
14. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, Matsuda M, Sakaguchi T, Hirao T and Nakano H: The prognostic significance of amplification and overexpression of c-met and c-erbB-2 in human gastric carcinomas. Cancer 85: 1894-1902, 1999.
15. Ishikawa T, Kobayashi M, Mai M, Suzuki T and Ooi A: Amplification of the c-erbB-2 (HER-2/neu) gene in gastric cancer cells. Am J Pathol 151: 761-768, 1997.
16. Sato T, Abe K, Kurose A, Uesugi N, Todoroki T and Sasaki K: Amplification of the c-erbB-2 gene detected by FISH in gastric cancers. Pathol Int 47: 179-182, 1997.
17. Takehana T, Kunitomo K, Kono K, Kitahara F, Iizuka H, Matsumoto Y, Fujino AF and Ooi A: Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence *in situ* hybridization and enzyme-linked immunosorbent assay. Int J Cancer 98: 833-837, 2002.
18. Kaori F Ouchi, Sekiguchi F and Tanaka Y: Antitumor activity of trastuzumab (HERCEPTIN^{R}) in human gastric cancer models. Eur J Cancer 1 (Suppl 5): S294, 2003.
19. Jacobs TW, Gown AM, Yaziji H, Barnes MJ and Schnitt SJ: Specificity of hercep test in determining HER-2/neu status of breast cancers using the United States food and drug administration-approved scoring system. J Clin Oncol 17: 1983-1987, 1999.
20. The Editorial Board of Cancer Statistics in Japan. Cancer Statistics in Japan, p36, 2003.
21. Murad AM, Santiago FF, Petroianu A, Rocha PRS, Rodrigues MAG and Rausch M: Modified therapy with 5-fluorourasil, doxorubicin, and methotrexate in advanced gastric cancer. Cancer 72: 37-41, 1993.
22. Glimelius B, Hotfmann K, Haglund U, Nyren O and Sjoden PO: Initial of delayed chemotherapy with best supportive care in advanced gastric cancer. Ann Oncol 5: 189-190, 1994.
23. Pyhonen S, Kuitumen T, Nyandoto P and Kouri M: Randomized comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus best supportive care alone in patients with non-resectable gastric cancer. Br J Cancer 71: 587-591, 1995.

24. Ridolfi RL, Jamehdor MR and Arber JM: Her-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence *in situ* hybridization approach. Mod Pathol 13: 866-873, 2000.
25. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Leiberman G and Slamon DJ: Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17: 2639-2648, 1999.
26. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novothy WF, Burchmore M, Shak S, Stewart SJ and Press M: Efficacy and safety of Trastuzumab as a single agent in first line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 20: 719-726, 2002.
27. Yonemura Y, Ninomiya I, Ohoyama S, Kimura H, Yamaguchi A, Fushida S, Kosaka T, Miwa K, Miyazaki I, Endou Y, Tanaka M and Sasaki T: Expression of c-erbB-2 oncoprotein in gastric carcinoma. Immunoreactivity for c-erbB-2 protein is an independent indicator of poor short-term prognosis in patients with gastric cancer. Cancer 67: 2914-2918, 1991.
28. Brien TP, Depowski PL, Sheehan CE, Ross JS and McKenna BJ: Prognostic factors in gastric cancer. Mod Pathol 11: 870-877, 1998.
29. Allgayer H, Babic R, Gruetzner KU, Tarabichi A, Schiidberg FW and Heiss MM: c-*erb*B-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. J Clin Oncol 18: 2201-2209, 2000.

Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab

Yasuhito Terui,[1,2] Takuma Sakurai,[1,3] Yuko Mishima,[1] Yuji Mishima,[2] Natsuhiko Sugimura,[2] Chino Sasaoka,[2] Kiyotsugu Kojima,[2] Masahiro Yokoyama,[1] Nobuyuki Mizunuma,[1] Shunji Takahashi,[1] Yoshinori Ito[1] and Kiyohiko Hatake[1,2,4]

[1]Division of Clinical Chemotherapy, and [2]Olympus Bio-Imaging Laboratory, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo 135-8550, and [3]Material Research Laboratory, Morinaga Milk Industry, Zama, Kanagawa 228-8583, Japan

(Received August 29, 2005/Revised October 4, 2005/Accepted October 17, 2005/Online publication December 18, 2005)

Recently, anti-CD20 (rituximab) and anti-Her2/neu (trastuzumab) antibodies have been developed and applied to the treatment of malignant lymphoma and breast cancer, respectively. However, bulky lymphoma is known to be resistant to rituximab therapy, and this needs to be overcome. Fresh lymphoma cells were collected from 30 patients with non-Hodgkin's lymphoma, the expression of CD20 and CD55 was examined by flow cytometry, and complement-dependent cytotoxicity (CDC) assays were carried out. Susceptibility to CDC with rituximab was decreased in a tumor size-dependent manner ($r = -0.895$, $P < 0.0001$), but not in a CD20-dependent manner ($r = -0.076$, $P = 0.6807$) using clinical samples. One complement-inhibitory protein, CD55, contributed to bulky lymphoma-related resistance to CDC with rituximab. A decrease in susceptibility to CDC with rituximab was statistically dependent on CD55 expression ($r = -0.927$, $P < 0.0001$) and the relationship between tumor size and CD55 expression showed a significant positive correlation ($r = 0.921$, $P < 0.0001$) using clinical samples. To overcome the resistance to rituximab by high expression of CD55 in bulky lymphoma masses, small interfering RNA (siRNA) was designed from the DNA sequence corresponding to nucleic acids 1–380 of the CD55 cDNA. Introduction of this siRNA decreased CD55 expression in the breast cancer cell line SK-BR3 and in CD20-positive cells of patients with recurrent lymphoma; resistance to CDC was also inhibited. This observation gives us a novel strategy to suppress bulky disease-related resistance to monoclonal antibody treatment. (*Cancer Sci* 2006; 97: 72–79)

In recent years, monoclonal antibodies have been used increasingly to treat patients with malignancies such as lymphoma and breast cancer.[1–3] In particular, the anti-CD20 antibody, also called rituximab, is usually very effective for treatment of malignant lymphoma, and most patients can receive rituximab as monotherapy or combination chemotherapy.[4,5] However, in some cases with bulky mass and at stage IV, lymphoma cells become resistant to rituximab treatment.[6,7] Apart from the number of tumor cells being greater in these cases, how this resistance occurs has not yet been clarified.

Recently, some researchers have reported four mechanisms for the action of rituximab: (i) inhibition of proliferation; (ii) induction of apoptosis; (iii) complement-dependent cytotoxicity (CDC); and (iv) antibody-dependent cellular cytotoxicity (ADCC).[7,8] Because CDC could more rapidly and efficiently act on the target cells attacked by rituximab, CDC may be the most important of the mechanisms of rituximab.

The role of complementary regulatory proteins in the modulation of rituximab efficacy has been addressed, and several surface membrane proteins regulate the deposition of active complement proteins on cellular membranes to prevent cell lysis. Regulators of the complement system play an important role in CDC, and CD46, CD55 and CD59 are well known to inhibit the complement system.[9] Among these inhibitors, CD55 and CD59 seem to be the most important.[10] No differences in the expression of CD59 molecules have been reported between normal B cells and malignant B cells, whereas CD55 expression was shown to be different among individual patients with B-cell malignancy.[11] Nevertheless, *in vitro* susceptibility to rituximab-induced CDC could not be predicted by the level of these proteins in chronic lymphocytic leukemia (CLL) cells, and *in vivo* susceptibility could not be predicted in follicular lymphoma (FL) and CLL patients.[12,13] In contrast, some researchers have reported direct correlations among CDC, CD55 and CD59 using B-cell lines.[14]

CD55, also known as decay accelerating factor, is a major regulator of the alternative and classical pathways of complement activation and is expressed on all serum-exposed cells. CD55 is a 70-kDa glycoprotein, which is a glycosylphosphatidylinositol (GPI)-anchored protein.[15] CD55 can bind the complex of C3a and Bb, which is in the classical pathway, and it blocks the cascade of the complement system. A functional disorder of CD55 in blood cells causes paroxysmal nocturnal hemoglobinuria (PNH).[16] In these cases, the cascade of the complement system can not be controlled, and CDC activity is enhanced mainly against red blood cells. CD55 can enhance dissociation between C3 convertase and C4bC2/C3bBb complexes, and then inhibit the cascade of the

[4]To whom correspondence should be addressed. E-mail: khatake@jfcr.or.jp.

doi: 10.1111/j.1349-7006.2006.00139.x

complement system. While it is true that CD55 levels are low to absent in PNH, the disease is caused by phosphatidylinositol glycan-A (PIGA) gene mutations that lead to a failure to assemble GPI anchors. Hence, all GPI-anchored proteins are missing in this disease.

Previous researchers have shown that certain conditions for cancer cells, such as hypoxia, poor nutrition and bulky mass, make them chemoresistant.[17,18] When gastric cancer cells were exposed to hypoxia, hypoxia inducible factor (HIF)-1 was induced and the cells were resistant to Cis-platin (CDDP).[18] When lymphoid cells were able to resist doxorubicin (adriamycin), expression of nuclear factor (NF)-κB and its transcription activity were enhanced in doxorubicin (adriamycin)-resistant cells.[17]

Because CDC activity is especially important for rituximab therapy and CD55 may function as a mostly important inhibitor of CDC, it is possible that a decline in CDC activity by CD55 molecules may cause resistance to rituximab. CDC correlates directly with the expression of CD20 antigen in malignant B cells, and *in vitro* susceptibility to rituximab-mediated CDC depends primarily on CD20 protein expression. However, there have yet been no reports about the relationship between tumor size and sensitivity to CDC or between tumor size and CD55 expression.

More recently, small interfering RNA (siRNA) has been developed and applied to knock down target gene expression.[19] For example, the nuclear factor of activated T cells (NFAT) and NF-κB were shown to be constitutively active in large B-cell lymphoma cells, and downregulation of NFATc1 and NF-κB in malignant B-cell lymphoma with siRNA inhibited lymphoma cell growth.[20] Although many researchers tried siRNA for genes of membrane proteins such as growth factor receptors,[21] there have been no successful reports describing siRNA for complement inhibitors.

To clarify the resistance to rituximab and overcome the resistance, especially with regard to bulky mass unresponsiveness and efficacy for re-treatment, we examined the relationship between CDC activity and rituximab, and CD55 expression in our patients, using siRNA for CD55 to treat CDC with rituximab.

Materials and Methods

Cell lines

Human malignant B-cell lines as well as Daudi and Raji cells (ATCC) were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) with 10% fetal calf serum (FCS) at 37°C. The cell lines were used as sensitive and resistant controls in CDC with anti-CD20 antibody. The human breast cancer cell lines MCF7 and SK-BR3 (ATCC) were cultured in Dulbecco's minimal essential medium (DMEM; Gibco) with 10% FCS.

Complement-mediated cytotoxicity assay

Cells were washed once with fresh complete medium, and anti-CD20 antibody (rituximab; Roche, Basel, Switzerland) or anti-Her2/neu antibody (trastuzumab; Roche) was added at a concentration of 20 μg/mL. Cells were incubated at 37°C for 1 h, and then human AB blood serum from healthy volunteers with informed consent was added at 20% (v/v). After incubation at 37°C for 1 h, propidium iodide (PI; Sigma, St Louis, MO, USA) was added and CDC assays were carried out by flow cytometry with FACscan (Becton Dickinson, San Jose, CA, USA). For CDC assays using a microplate reader, Daudi, Raji and SKBR3 cells were seeded at 1×10^5 cell/mL in each well, and then rituximab or trastuzumab (20 μg/mL) and normal AB serum (20% [v/v]) were added. The reaction was incubated at 37°C for 1 h, and the cells were washed with phosphate-buffered saline (PBS) at least three times. Ten microliters of Calcein-AM (2 μg/mL) (Dojindo, Kumamoto, Japan) was added to each well and mixed thoroughly. After incubation at room temperature, fluorescence intensity was measured at 485 nm/535 nm wavelengths with a microplate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

Surface markers

Cells were washed once with PBS, and were then were stained with phycoerythrin (PE)-conjugated anti-CD20, and fluoresceinisothiocyanate (FITC)-conjugated anti-CD55 (Becton Dickinson). Flow cytometry was then carried out using FACscan. The intensities of C20 and CD55 expression were normalized compared with a control. For confocal laser scanning microscopy, rituximab and trastuzumab were labeled with Alexa Fluor 594 (Molecular Probes; Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. In brief, 100 μg of antibody was labeled with Alexa Fluor 594 for 20 min after alkalization with carbonate. The mixture was put into a spin column and spun down at 1500*g*, and the flow-through was collected as Alexa Fluor 594-conjugated antibody.

Laser scanning confocal microscopy and phase-contrast microscopy

To see CDC activity on living cells, pictures were taken by a CDC camera with phase-contrast microscopy after the CDC assay with rituximab or trastuzumab. The cells were also stained with Alexa Fluor 594-labeled rituximab or Alexa Fluor 594-labeled trastuzumab and FITC-labeled anti-CD55 antibody, and serum was added to the culture medium. The stained cells were observed in real time under a confocal laser scanning microscopy system (Olympus, Tokyo, Japan).

Collection of clinical samples

Fresh lymphoma cells were collected from the lymph nodes of 30 patients with non-Hodgkin's lymphoma (11 cases of diffuse large B-cell type, 10 cases of marginal zone cell type, five cases of follicular cell type, two cases of small lymphocytic type, one case of B-cell immunoblastic type, and one case of diffuse small cell type) after receiving informed consent. In brief, the lymph nodes were resected surgically and specimens were broken into small pieces with scissors and ground between two glass slides. The cells were collected after centrifugation and washed with RPMI-1640 containing 10% FCS. Cell counting and viability were assessed by toluidine-blue exclusion dye test, and CD19-positive cells were isolated using a magnetic cell sorting (MACS) system. The isolated cells were stained with FITC-conjugated anti-CD19, PE-conjugated anti-CD20, and FITC-conjugated anti-CD55 antibodies and flow cytometry was then carried out.

Vector and siRNA for CD55

CD55 cDNA in Ultimate open reading frame (ORF) clones (clone *ID*: IOH3209) was purchased from Invitrogen, and amplified by polymerase chain reaction (PCR) (forward, 5′-CGCGGATCCGCGATGACCGTCGCGCGG-3′; and reverse, 5′-TCCCCCGGGGGACTAAGTCAGCAAGCC-3′). The PCR product was subcloned into the pEGFP-C1 vector (Clontech, Mountain View, CA, USA). To generate double-stranded RNA for CD55, three parts of the DNA sequence, corresponding to nucleic acids 1–380, 381–817 and 821–1146 in the CD55 cDNA, were amplified by PCR. These sequences were named CD55-N, CD55-M and CD55-C, respectively. RNA transcription was then performed with this DNA template to generate sense and antisense single-stranded RNA. After production of double-stranded RNA, a reaction with the Dicer enzyme was carried out using a BLOCK-iT Dicer RNAi kit (Invitrogen). For siRNA for CD55, the siRNA was transfected into Raji and SK-BR3 cells using Lipofectamine 2000 (Invitrogen). In brief, 0.75 ng of siRNA and 5 μL of Lipofectamine 2000 in Optimen medium were mixed and incubated at room temperature for 20 min. The mixture was added to culture medium with SK-BR3 cells and fresh lymphoma cells, and the cells were incubated at 37°C for 72 h and 24 h, respectively. To see downregulation of CD55 expression, the CD55-transfected cells were stained with FITC-conjugated anti-CD55 antibody, and then expression of CD55 was observed without fixation of the cells at the same intensity of emission and excision as under laser scanning confocal fluorescent microscopy.

Statistical analysis

Correlation of susceptibility to CDC with tumor size, CD20 expression and CD55 expression were tested using the Spearman rank correlation coefficient. Statistical comparisons were carried out using two-sided Student's *t*-tests. All statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA).

Results

Negative correlation between tumor size and susceptibility to CDC with rituximab

Rituximab is known to be effective at the early stages of indolent and aggressive lymphomas, but the effect of rituximab declines in some patients with bulky disease and a large number of lymphoma cells. According to this fact, we investigated whether susceptibility to CDC is dependent on the size of the tumor. The diameter of extirpated lymph nodes, CDC assay and CD20 expression were examined in fresh samples from 30 patients with lymphoma, as described in 'Materials and Methods'. As shown in Fig. 1a, the relationship between susceptibility to CDC and size of extirpated lymph nodes showed a significant negative correlation ($R = -0.895$, $P < 0.001$). In contrast, the relationship between susceptibility to CDC and CD20 expression, and between size of extirpated lymph nodes and CD20 expression, did not reveal significant correlations, as shown in Fig. 1b,c ($R = -0.076$, $P = 0.6807$ and 0.072, $P = 0.6979$, respectively). This suggests that susceptibility to CDC is dependent on the size of the lymphoma tumor, and that expression of CD20 does not contribute to susceptibility to CDC with rituximab in non-Hodgkin's lymphoma.

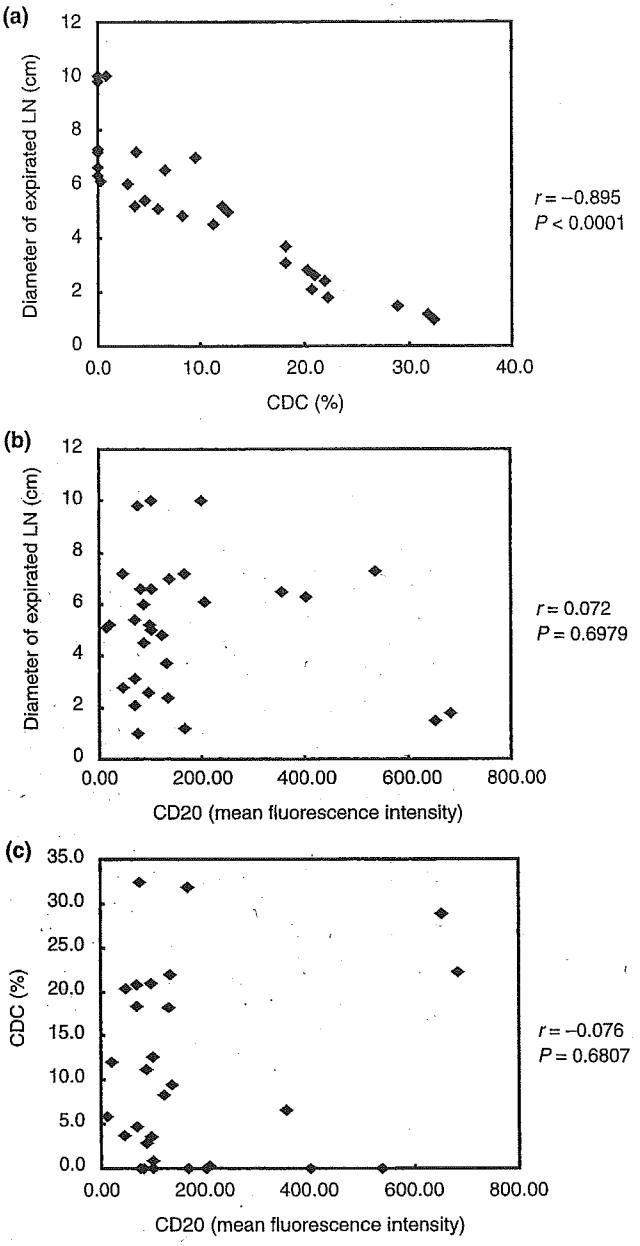

Fig. 1. Relationships between the size of extirpated tumors, susceptibility to complement-dependent cytotoxicity (CDC), and CD20 expression. The size of tumors from 30 patients with non-Hodgkin's lymphoma was measured and the cells were collected. After isolation of CD19-positive cells, FACScan analysis was carried out with anti-CD20 antibody, and CDC assay with rituximab was performed. Intensity of CD20 expression was normalized compared with a control. (a) Scatter plot and correlation analysis for size of extirpated tumor versus susceptibility to CDC. (b) Scatter plot and correlation analysis for size of extirpated tumor versus mean fluorescence intensity of CD20. (c) Scatter plot and correlation analysis for mean fluorescence intensity of CD20 versus susceptibility to CDC. All correlations were tested using the Spearman rank correlation coefficient.

doi: 10.1111/j.1349-7006.2006.00139.x