significant when the P-value was less than 0.05. The StateView program was used for Kaplan–Meier analysis of survival rates, and the Mantel–Cox test was used to test the equality of survival curves.

3. Results

In this study, we investigated the major p53 alterations identified in gastric carcinomas in 113 cancer patients. Mutations were observed in 19 (16.8%) of those patients. Among them, 8 showed nonsense mutations and 11 showed missense mutations (Table 1). p53 mutations were found more frequently in the intestinal type cancers than in the diffuse type cancers (Table 2). Relations with other clinicopathological factors were not found. Kaplan–Meier overall survival curve (Fig. 1) demonstrates that patients with mutant p53 had a relatively higher survival rate than those patients with wild type p53, however, it was not significant. LOH of p53 was simultaneously analyzed in the same 113 cases. Informative analyses performed on 89 patients resulted in 31 (34.8%) cases that were judged as LOH. Ten out of fourteen patients (72%) had LOH of p53 out of the cases with p53 mutation (Table 3). LOH of PTEN was also examined in this study and was observed in 13 of 76 cases (17.1%) among the 113 analyzed cases. The clinicopathological differences due to the presence of LOH of PTEN were not pointed out (data not shown). However, we discovered that there was a strong correlation between the gene mutation of p53 and the LOH of PTEN. LOH of PTEN was observed in 7 out of 63 patients (11.1%) in the cases with normal p53s, and in 6 out of 13 patients (46.2%) in the cases with p53 mutations (Table 4). LOH of PTEN was frequently recognized in cases with the p53 gene mutation.

4. Discussion

From the results of this study, we found that in gastric cancers, the relationship between a mutation and LOH of p53 was controversial despite the fact that aberrant protein expression, gene mutation, and LOH have been, respectively, reported [13,22]. In our data, the genetic mutation of p53 was recognized in 19 patients (16.8%), and LOH of p53 was recognized in 34.8% altogether. Seventy-two percent of the patients carrying the p53 mutation showed LOH of p53. p53 mutation was more frequently recognized in intestinal

Table 1
Summary of the p53 mutated cases

Number	Exon	Mutation	Codon altered	D10S1765 (p53)	D17S796 (PTEN)
1	6	cga:tga	Arg213stop	LOH	NI
2	5	cgc:cac	Arg175His	LOH	LOH
3	6	tat:tgt,gat:tat	Tyr220Cys,Asp324Tyr	LOH	LOH
4	8	cga:tga	Arg306stop	LOH	LOH
5	7	ccc:ctc	Pro250Leu	NI	LOH
6	5	ccg:acg	Pro152Thr	ROH	NI
7	8	cgt:cat	Pro273His	LOH	LOH
8	7	tat:tgc	Tyr236Cys	LOH	NI
9	8	cga:tga	Arg306stop	ROH	ROH
10	7	ggc:agc	Gly245Ser	LOH	ROH
11	4	aag:aat,cag:tag	Lys164Asn,Gln165stop	LOH	ROH
12	7	tac:tgc	Tyr234Cys	LOH	ROH
13	6	ttg:tag	Leu201stop	NI	ROH
14	8	gac:tac	Arg281Thr	LOH	NI
15	7	tgt:tga	Cys229stop	NI	ROH
16	8	cgt:cat	Pro273His	LOH	NI
17	6	cga:tga	Arg196stop	ROH	ROH
18	5	gtc:ttc	Val157Phe	ROH	LOH
19	5	tgc:tga	Cys176stop	NI	NI

LOH, loss of heterozygosity; ROH, retention of heterozygosity; NI, not informed.

Table 2
Clinicopathological features of gastric cancers and the mutation of p53

Variable	P53		*P*-value
	Wild (n=94)	Mutant (n=19)	
Gender			
Male	65	15	NS
Female	29	4	
Age	62.7	67.1	NS
Histology			
Intestinal	32	13	0.0028
Diffused	61	5	
Serosal invasion			
Negative	40	11	NS
Positive	54	8	
Histological lymph node metastasis			
Negative	35	6	NS
Positive	59	13	
Vascular involvement			
Negative	47	10	NS
Positive	47	9	
Peritoneal dissemination			
Negative	90	18	NS
Positive	4	1	
Liver metastasis			
Negative	90	17	NS
Positive	3	2	
Stage			
I+II	41	10	NS
III+IV	53	9	

NS, not significantly.

types of gastric cancers (Table 1). Concerning colon cancers, a p53 mutation occurs earlier than LOH, and it is assumed that LOH arises after the carcinoma has progressed [23]. In gastric cancers, the relationship between mutation and LOH of p53 has rarely been argued. Our results suggest that tumor progression of gastric cancer is similar with colon cancers, especially in intestinal type gastric cancers.

Another important finding was the relationship between PTEN and p53. PTEN was identified as a novel tumor suppressor gene that has been studied in a variety of cancers. LOH and mutation of PTEN have been reported in melanoma, glioblastoma, renal cancer, lung cancer, and breast cancer [1,2,6]. To date, however, the role of PTEN in gastric carcinogenesis has not been well studied. In the present study, we examined LOH of PTEN and the mutation and performed LOH analysis of p53 in 113 patients. LOH of PTEN was found in 17.1% of the patients, which is consistent with previous reports. Surprisingly, LOH of PTEN was significantly observed at a high rate in cases with p53 mutations. LOH of PTEN was observed in 7 out of 63 patients (11.1%) in the cases with normal p53s, and in 6 out of 13 patients (46.2%) in cases with p53 mutations (Table 3).

It was reported that more than 50% of radiation-induced tumors from p53+/− and p53−/− mice showed heterozygous loss of one PTEN allele and showed one remaining mutation-free allele [18]. Other previous reports about prostate cancer in PTEN heterozygous mice also concluded that PTEN plays a haploinsufficient role in tumor development [24,25]. These reports showed that there is an important mutual relationship between p53 and PTEN. Our study also suggests that PTEN is

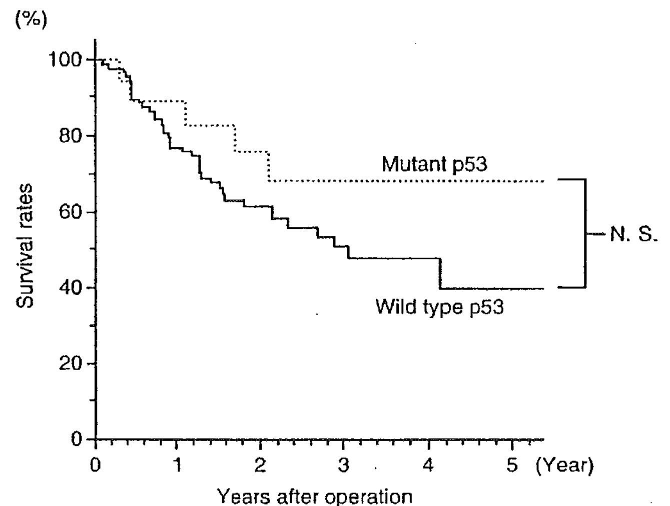

Fig. 1. Kaplan-Meier overall survival curves. The solid line demonstrates the patients who had wild type p53. The dotted line is the survival curve of the patients with mutated p53.

Table 3
Relationship between mutation and the loss of heterozygosity of p53

		p53	
		wt	mt
P53	LOH	21	10
	ROH	54	4

P<0.02. LOH, loss of heterozygosity; ROH, retention of heterozygosity; wt, wild type; mt, mutant type.

Table 4
Relationship between the mutation of p53 and the loss of heterozygosity of PTEN

		p53	
		wt	mt
PTEN	LOH	7	6
	ROH	56	7

$P<0.02$. LOH, loss of heterozygosity; ROH, retention of heterozygosity; wt, wild type; mt, mutant type.

haploinsufficient in p53 mutated gastric cancers, and it first revealed a relationship between p53 and PTEN in a clinical sample. Further study of PTEN and p53 in gastric cancer patients is necessary because the chemosensitivity or prognosis of the gastric cancer patient might be influenced by the LOH of PTEN.

In conclusion, our examination showed the importance of PTEN in the carcinogenesis of gastric cancer. The fact that gastric cancer with a p53 mutation frequently showed the loss of a PTEN allele suggests that PTEN is a haploinsufficient tumor suppressor gene and that PTEN and p53 cooperated in gastric cancer development.

Acknowledgements

We are grateful to J. Tsuchihashi, K. Miyamoto, N. Nakamura, Y. Kubota, Y. Matsumoto and T. Shishino for technical assistance. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] P.A. Steck, M.A. Pershouse, S.A. Jasser, W.K. Yung, H. Lin, A.H. Ligon, et al., Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers, Nat. Genet. 15 (1997) 356–362.

[2] J. Li, C. Yen, D. Liaw, K. Podsypanina, S. Bose, S.I. Wang, et al., PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer, Science 275 (1997) 1943–1947.

[3] H. Tashiro, M.S. Blazes, R. Wu, K.R. Cho, S. Bose, S.I. Wang, et al., Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies, Cancer Res. 57 (1997) 3935–3940.

[4] P. Guldberg, P. Thor Straten, A. Birck, V. Ahrenkiel, A.F. Kirkin, J. Zeuthen, Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma, Cancer Res. 57 (1997) 3660–3663.

[5] Y.E. Whang, X. Wu, H. Suzuki, R.E. Reiter, C. Tran, R.L. Vessella, et al., Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression, Proc. Natl Acad. Sci. USA 95 (1998) 5246–5250.

[6] S.I. Wang, J. Puc, J. Li, J.N. Bruce, P. Cairns, D. Sidransky, R. Parsons, Somatic mutations of PTEN in glioblastoma multiforme, Cancer Res. 57 (1997) 4183–4186.

[7] D.H. Teng, R. Hu, H. Lin, T. Davis, D. Iliev, C. Frye, et al., MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines, Cancer Res. 57 (1997) 5221–5225.

[8] G.L. Maxwell, J.I. Risinger, C. Gumbs, H. Shaw, R.C. Bentley, J.C. Barrett, et al., Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias, Cancer Res. 58 (1998) 2500–2503.

[9] P.L. Dahia, D.J. Marsh, Z. Zheng, J. Zedenius, P. Komminoth, T. Frisk, et al., Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors, Cancer Res. 57 (1997) 4710–4713.

[10] T. Maehama, J.E. Dixon, The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate, J. Biol. Chem. 273 (1998) 13375–13378.

[11] F.B. Furnari, H. Lin, H.S. Huang, W.K. Cavenee, Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain, Proc. Natl Acad. Sci. USA 94 (1997) 12479–12484.

[12] S.Y. Han, H. Kato, S. Kato, T. Suzuki, H. Shibata, S. Ishii, et al., Functional evaluation of PTEN missense mutations using in vitro phosphoinositide phosphatase assay, Cancer Res. 60 (2000) 3147–3151.

[13] C.M. Fenoglio-Preiser, J. Wang, G.N. Stemmermann, A. Noffsinger, TP53 and gastric carcinoma: a review, Hum. Mutat. 21 (2003) 258–270.

[14] M.B. Kastan, O. Onyekwere, D. Sidransky, B. Vogelstein, R.W. Craig, Participation of p53 protein in the cellular response to DNA damage, Cancer Res. 51 (1991) 6304–6311.

[15] A.J. Levine, p53, the cellular gatekeeper for growth and division, Cell 88 (1997) 323–331.

[16] L.A. Donehower, M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery Jr., J.S. Butel, A. Bradley, Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours, Nature 356 (1992) 215–221.

[17] V. Stambolic, D. MacPherson, D. Sas, Y. Lin, B. Snow, Y. Jang, et al., Regulation of PTEN transcription by p53, Mol. Cell 8 (2001) 317–325.

[18] J.H. Mao, D. Wu, J. Perez-Losada, H. Nagase, R. DelRosario, A. Balmain, Genetic interactions between Pten and p53 in radiation-induced lymphoma development, Oncogene 22 (2003) 8379–8385.

[19] Y. Maehara, Y. Kakeji, S. Oda, H. Baba, K. Sugimachi, Tumor growth patterns and biological characteristics of early gastric carcinoma, Oncology 61 (2001) 102–112.

[20] S. Oda, E. Oki, Y. Maehara, K. Sugimachi, Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis, Nucl. Acids Res. 25 (1997) 3415–3420.
[21] Y. Maehara, S. Oda, K. Sugimachi, The instability within: problems in current analyses of microsatellite instability, Mutat. Res. 461 (2001) 249–263.
[22] Y. Gomyo, M. Osaki, N. Kaibara, H. Ito, Numerical aberration and point mutation of p53 gene in human gastric intestinal metaplasia and well-differentiated adenocarcinoma: analysis by fluorescence in situ hybridization (FISH) and PCR-SSCP, Int. J. Cancer 66 (1996) 594–599.
[23] K.R. Cho, B. Vogelstein, Suppressor gene alterations in the colorectal adenoma-carcinoma sequence, J. Cell Biochem. Suppl. 16G1992; 137–141.
[24] B. Tang, E.P. Bottinger, S.B. Jakowlew, K.M. Bagnall, J. Mariano, M.R. Anver, et al., Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency, Nat. Med. 4 (1998) 802–807.
[25] M.L. Fero, E. Randel, K.E. Gurley, J.M. Roberts, C.J. Kemp, The murine gene p27Kip1 is haplo-insufficient for tumour suppression, Nature 396 (1998) 177–180.

Clinical Significance of Heparin-Binding Epidermal Growth Factor–Like Growth Factor and A Disintegrin and Metalloprotease 17 Expression in Human Ovarian Cancer

Yoshihiro Tanaka,[1] Shingo Miyamoto,[1] Satoshi O. Suzuki,[2] Eiji Oki,[3] Hiroshi Yagi,[1] Kenzo Sonoda,[1] Ayano Yamazaki,[4] Hiroto Mizushima,[4] Yoshihiko Maehara,[3] Eisuke Mekada,[4] and Hitoo Nakano[1]

Abstract **Purpose:** Lysophosphatidic acid, which is enriched in the peritoneal fluid of ovarian cancer patients, plays a key role in the progression of ovarian cancer. Lysophosphatidic acid can generate epidermal growth factor receptor (EGFR) signal transactivation involving processing of EGFR ligands by ADAM (a disintegrin and metalloprotease) family metalloproteases. We aimed to investigate the clinical significance of EGFR ligands and ADAM family in the lysophosphatidic acid–induced pathogenesis of ovarian cancer.

Experimental Design: We examined the expression of EGFR ligands and ADAM family members in 108 patients with normal ovaries or ovarian cancer, using real-time PCR, immunohistochemistry, and *in situ* hybridization, and analyzed the clinical roles of these molecules. Statistical analyses of these data were done using the Mann-Whitney test, Kaplan-Meier method, or Spearman's correlation analysis.

Results: Large differences in expression were found for heparin-binding EGF-like growth factor (HB-EGF) and other EGFR ligands and for ADAM 17 and other ADAM family members. HB-EGF expression was significantly increased in advanced ovarian cancer compared with that in normal ovaries ($P < 0.01$). HB-EGF expression was significantly associated with the clinical outcome ($P < 0.01$). ADAM 17 expression was significantly enhanced in both early and advanced ovarian cancer compared with that in normal ovaries (both $P < 0.01$), although it had no clinical significance in the progression-free survival. HB-EGF expression was significantly correlated with ADAM 17 expression ($\gamma = 0.437$, $P < 0.01$).

Conclusions: Our findings suggest that HB-EGF and ADAM 17 contribute to the progression of ovarian cancer and that HB-EGF plays a pivotal role in the aggressive behavior of a tumor in ovarian cancer.

Ovarian cancer is the most common cause of death from a gynecologic malignancy in most countries (1). The high mortality is predominantly due to occult progression of the tumor in the peritoneal cavity, with the initial diagnosis usually being made at an advanced stage. Currently, ~75% of ovarian cancers are diagnosed at International Federation of Gynecology and Obstetrics stages III and IV (2). Extensive dissemination of a tumor is caused by the peritoneal fluid following the circulatory pathway in the abdominal cavity, and the peritoneal fluid acts as a rich source of growth factor activity for ovarian cancer cells (3). Thus, the dissemination of cancer cells activated by ovarian cancer–activating factors results in an exaggerated increase in peritoneal fluid, which in turn leads to tumor extension in ovarian cancer. Therefore, to develop a targeting therapy, it would be extremely useful to understand the ovarian cancer–activating factor–mediated molecular mechanisms for activating ovarian cancer cells.

Lysophosphatidic acid (LPA) is a simple phospholipid with numerous cellular effects, including growth promotion, cell cycle progression, and cytoskeletal organization (4), and is generated from precursors in membranes. LPA is elevated in the plasma and peritoneal fluid from patients with ovarian cancer in all stages, suggesting that it is a possible candidate for an ovarian cancer–activating factor (5–8). In principle, LPA-induced signaling is mediated by G protein–coupled receptors, including LPA1, LPA2, LPA3, and LPA4 (4). Recent investigations have shown that G protein–coupled receptors are able to use the epidermal growth factor receptor (EGFR) as a downstream signaling partner in the generation of mitogenic signals (9), and

Authors' Affiliations: Departments of [1]Obstetrics and Gynecology, [2]Neuropathology, [3]Surgery and Sciences, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and [4]Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Received 7/21/04; revised 12/7/04; accepted 12/15/04.

Grant support: Ministry of Health and Welfare of Japan Grant-in-Aid for Cancer Research 16591667 (S. Miyamoto) and Ministry of Education, Culture, Sports, Science and Technology Grant-in-aid for Scientific Research on Priority Areas 14032202 (E. Mekada).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Shingo Miyamoto, Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5395; Fax: 81-92-642-5414; E-mail: smiya@cis.fukuoka-u.ac.jp.

©2005 American Association for Cancer Research.

EGFR has been recognized to play a pivotal role in the progression of ovarian cancer (10, 11). According to this evidence, it can be considered that EGFR signal transactivation induced by LPA may contribute to the promotion of a tumor in ovarian cancer.

The molecular mechanisms of EGFR signal transactivation involve processing of transmembrane growth factor precursors by metalloproteases, which have been identified as members of the ADAM (a disintegrin and metalloprotease) family of zinc-dependent proteases (9). Seven-transmembrane growth factor precursors have been described as ligands for EGFR: EGF, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, transforming growth factor-α (TGF-α), betacellulin, epiregulin, and epigen (12, 13). For the metalloproteases, there have been at least 34 *adam* genes described in a variety of species, and ADAM 9, 10, 12, 17, and 19, which are ubiquitously expressed in somatic tissues, have sheddase activity (14). In particular, ADAM 9, 10, 12, and 17 are involved in the ectodomain shedding of EGFR ligands (15-21). The enhancement of EGFR signal transactivation mediated by EGFR ligands and the ADAM family is linked to the pathogenesis of hyperproliferative disorders, such as cancer. Previously, we shown that HB-EGF is involved in EGFR signal transactivation induced by LPA in ovarian cancer cell lines and that the expression of HB-EGF is attributable to tumor growth on xenografted mice using ovarian cancer cell lines (22). However, no studies have yet comprehensively examined the clinical significance of EGFR ligands and ADAM family expression in human cancers.

To investigate which molecules involved in EGFR signal transactivation are associated with human ovarian cancer, we examined the expression of EGFR ligands and ADAM family members in patients with ovarian cancer, using real-time PCR, and analyzed the clinical significance of these molecules in ovarian cancer.

Materials and Methods

Patients and surgical specimens. All 108 patients in this study had undergone surgery at the Department of Obstetrics and Gynecology, Kyushu University Hospital (Fukuoka, Japan) between January 1996 and August 2003. All the ovarian cancer specimens were obtained from 68 patients, comprising 16 cases with International Federation of Gynecology and Obstetrics stage I ovarian cancer, 10 cases with stage II, 29 cases with III, and 13 cases with stage IV. None of the patients had received chemotherapy before surgery. After dissection, half of each fresh tumor tissue specimen was immediately snap frozen in liquid nitrogen and stored at −80°C until use, whereas the other half was immediately embedded for the production of frozen or paraffin sections. Diagnosis was based on conventional morphologic examination of paraffin-embedded specimens, and tumors were classified according to the WHO classification (23). Metastases of pelvic lymph nodes in all cases and para-aortic lymph nodes in 60 cases were assessed

Table 1. Each oligonucleotide, probe sequence, or expression index of EGFR ligands or ADAM family in use of quantitative real-time PCR

	Primer sequence (5′-3′)	Probe sequence (5′-3′)
HB-EGF	TGGAGAATGCAAATATGTGAAGGA AGGATGGTTGTGTGGTCATAGGTAT	CTCCCTCCTGCATCTGCCACCC
TGF-α	GATTCCCACACTCAGTTCTGCTT CACAGCGTGCACCAACGT	CCAGCATGTGTCTGCCATTCTGGG
Amphiregulin	CCTGGCTATATTGTCGATTCA GTATTTTCACTTTCCGTCTTGTTTTG	TCAGAGTTGAACAGGTAGTTAAGCCC
Epiregulin	GGACAGTGCATCTATCTGGTGGA AGTGTTCACATCGGACACCAGTA	ACTTCACACCTGCAGTAG
Betacellulin	GCCCCAAGCAATACAAGCA GTCTCCTCTTAGGTAAAACAAGTCAACTCT	CCTTCATCACAGACACAGGAGGGCGT
ADAM 9	TGCTGAGTGTGCATATGGTGACT AAGAACCATTGCAGTACTCTGGAA	CCTCCTGGAAGGAACCGACAGTCTTTACAA
ADAM 10	TGCCAAAAGAGCAGTCTCACA TTGATGTTTCTACTTTAAATTCATCACTGA	ATGCCCATGGAAGACATTTCAACCTACG
ADAM 12	GGAAAGCAAAGAACTGATCATAAATCT TTTCGAGCGAGGGAGACATC	AAGGTCTCATTGCCAGCAGTTTCACG
ADAM 17	CAGCTGGAGTCCTGTGCATGT ACACAGCGGCCAGAAAGGT	ATGAAACTGACAACTCCTGCAAGGTGTGCT
Glyceraldehyde-3-phosphate dehydrogenase	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC

*Significant ($P < 0.05$) compared with the expression index of normal ovary.
†Significant ($P < 0.01$) compared with the expression index of normal ovary.

47

by pathologic examination using paraffin-embedded specimens after surgery. In 8 cases, metastases of para-aortic lymph nodes were evaluated by the presence or absence of lymph node swelling in an abdominal computed tomographic scan before surgery because surgical specimens were not resected. The median follow-up periods for all patients were 30.0 months (range, 2-83 months) for overall survival and 20.0 months (range, 1-63 months) for progression-free survival. After debulking surgery, all 68 patients had platinum-based chemotherapy (median, 6.0 courses; range, 1-10 courses) as first-line chemotherapy. Normal ovarian tissue specimens were obtained at surgery for benign gynecologic disorders from 40 patients, comprising 12 premenopausal cases and 28 postmenopausal cases. Informed consent was obtained from all patients in this study.

Criteria for chemotherapy response and definition of progression-free survival interval. The response to chemotherapy induction was assessed by second-look surgery, clinical and/or radiographic evaluation according to the WHO criteria, or CA125 response using Rustin et al.'s criteria (23, 24). The progression-free interval was defined as the duration from the date at surgery to the final date observed in this study (March 31, 2004) or the duration from the date at surgery to the date when progression was diagnosed, according to the proposed definitions of progression by the Gynecologic Cancer Intergroup (25).

Preparation of RNA. To ascertain the presence of cancer cells, half of each fresh tumor tissue specimen was immediately embedded in Tissue-Tek OCT compound (Sakura, Tokyo, Japan). Frozen sections were cut on the cryostat to a thickness of 6 μm and immediately stained with H&E. More than 80% of any given tumor specimen, which contained cancer cells, were used for cDNA synthesis. RNA was extracted using TRIzol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA synthesis was done with 0.8 μg total RNA using SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol.

Performance of reverse transcription-PCR and real-time quantitative PCR for epidermal growth factor receptor ligands or a disintegrin and metalloprotease family members. Sense and antisense primers based on the nucleotide sequences of HB-EGF cDNA, TGF-α cDNA, amphiregulin cDNA, epiregulin cDNA, betacellulin cDNA, and EGF cDNA were used, and the PCR protocol for each EGFR ligand followed those described by Adam et al. (26) or Sorensen et al. (27). The PCR products were electrophoresed in 2% agarose gels, and the bands were visualized with ethidium bromide and photographed with a camera (Funakoshi, Tokyo, Japan). When no bands were detected, the number of amplifications was increased by 50 cycles. Real-time PCR (TaqMan PCR) was done using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously (28). The sequences of the oligonucleotide primer pairs and TaqMan probes for each EGFR ligand and ADAM family member are summarized in Table 1. Serial 1:10 dilutions of plasmid DNA containing each target cDNA (10^7-10^1 copies/μL) were analyzed and served as standard curves, from which we determined the rate of change of the threshold cycle values. The correlation coefficients of the standard curves were >0.995, thus ensuring the accuracy of our data. Plasmid DNA played the role of a positive control for each reaction. Copy numbers of the target cDNAs (HB-EGF, amphiregulin, TGF-α, epiregulin, betacellulin, ADAM 9, ADAM 10, ADAM 12, and ADAM 17) were estimated from the standard curves. All reactions for the standard and patient samples were done in triplicate, and the data were averaged from the values obtained in each reaction. To determine the mRNA levels of four EGFR ligands and four ADAM family members, we used the mRNA

Table 1. Each oligonucleotide, probe sequence, or expression index of EGFR ligands or ADAM family in use of quantitative real-time PCR (Cont'd)

Expression index (mean ± SE)		
Normal ovary (n = 40)	Ovarian cancer, stage I-II (n = 26)	Ovarian cancer, stage III-IV (n = 42)
16.5 ± 11.9	60.4 ± 101.4	137 ± 204*
0.084 ± 0.114	0.083 ± 0.064	0.072 ± 0.084
5.26 ± 4.85	7.56 ± 12.02	23.0 ± 46.9
0.035 ± 0.051	0.022 ± 0.004	0.032 ± 0.06
1.246 ± 0.154	1.858 ± 0.362	2.865 ± 1.002
108 ± 99	232 ± 454	304 ± 817
21.2 ± 14.9	26.4 ± 25.1	21.0 ± 31.0
7.84 ± 11.49	18.1 ± 37.7	23.0 ± 39.0
488 ± 445	1,500 ± 1,582†	3,192 ± 4,825†

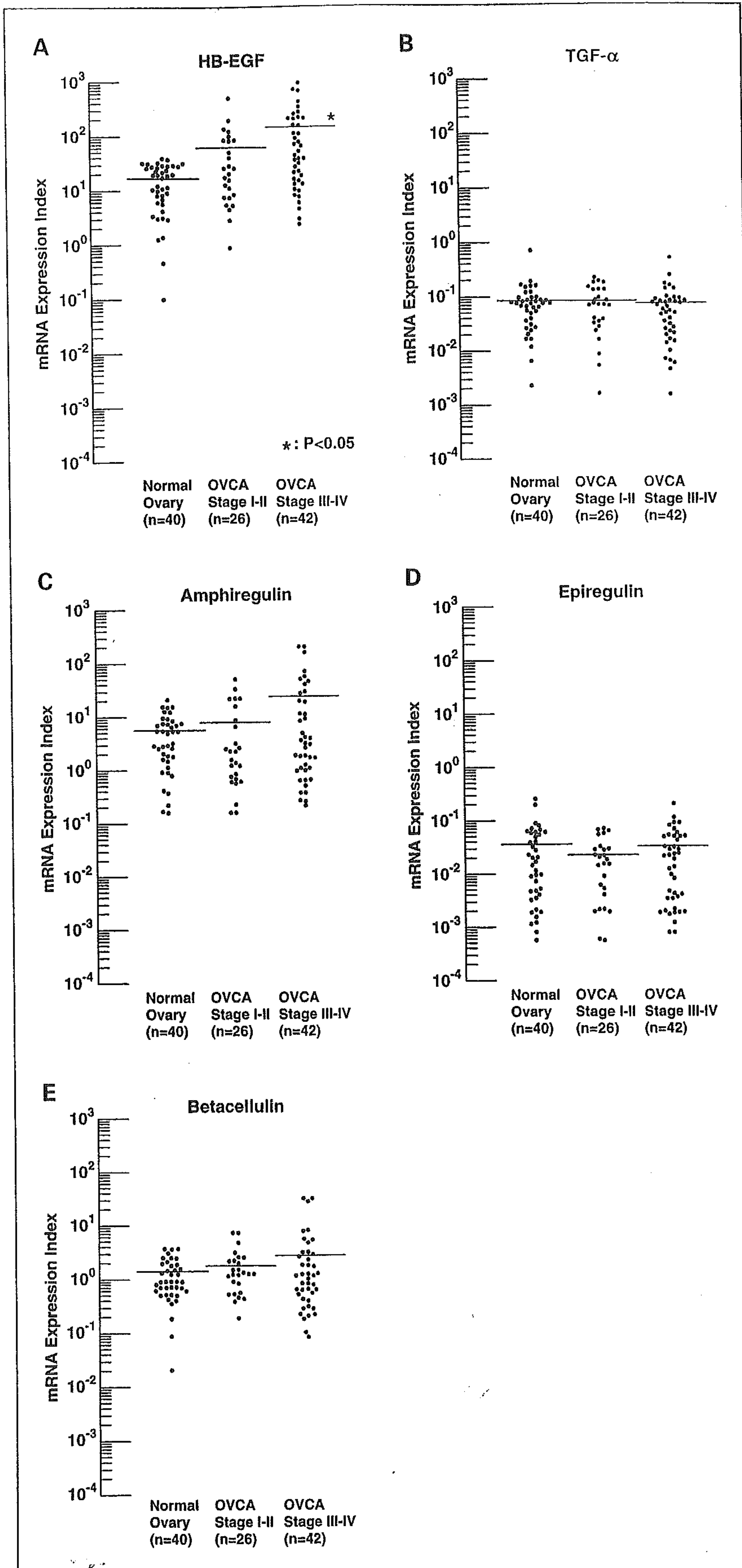

Fig. 1. Differences in expression of EGF ligands between normal ovaries and ovarian cancer (*OVCA*). mRNA expression index of HB-EGF (*A*), TGF-α (*B*), amphiregulin (*C*), epiregulin (*D*), and betacellulin (*E*) in patients with normal ovaries, early ovarian cancer (stages I-II), and advanced ovarian cancer (stages III-IV). A line indicates the mean value of the mRNA expression index for each group. *, $P < 0.05$, versus patients with normal ovaries.

expression index, which is a relative mRNA expression level standardized by glyceraldehyde-3-phosphate dehydrogenase. The mRNA expression index was calculated as follows (in arbitrary units): mRNA expression index = (copy number of each EGFR ligand or each ADAM family member mRNA / copy number of glyceraldehyde-3-phosphate dehydrogenase mRNA) × 10,000 arbitrary units. When the expression index was over the maximal value in patients with normal ovaries, it was regarded as a high expression status of the molecule under analysis.

In situ *hybridization.* *In situ* hybridization was done as described previously (29). Briefly, digoxigenin-labeled antisense and sense riboprobes were generated by *in vitro* transcription using a DIG RNA Labeling kit (SP6/T7; Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. The frozen samples were sectioned to a thickness of 6 μm. After fixation in 4% paraformaldehyde for 10 minutes, slides were immersed in 0.2 mol/L HCl for 10 minutes and then rinsed in phosphate buffer. Acetylation was done in 0.1 mol/L triethanolamine in 0.25% acetic anhydride for 15 minutes. After rinsing in phosphate buffer, sections were dehydrated in an ethanol gradient and dried. Sections were hybridized with the HB-EGF probe (diluted 1:10) at 55°C overnight. After high-stringency washing, the signal was visualized using an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics). Three examiners (Y.T., S.M., and K.S.) separately evaluated the HB-EGF mRNA staining by cell counting. At least 20 high-magnification fields were chosen randomly, and 1,000 cells in total were counted.

Immunohistochemistry. Immunohistochemistry was done on frozen sections using a goat polyclonal antibody against HB-EGF (R&D Systems, Inc., Minneapolis, MN) and on formalin-fixed, paraffin-embedded sections using a goat polyclonal antibody against ADAM 17 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Frozen sections were cut on a cryostat to a thickness of 6 μm, mounted on poly-L-lysine-coated slides, and either used immediately or stored at −80°C until needed. Paraffin-embedded sections were cut on a microtome to a thickness of 4 μm, mounted on poly-L-lysine-coated slides, and then dewaxed and rehydrated through xylene, graded ethanol solutions (100%, 90%, and 70%), and water. Briefly, the frozen and paraffin-embedded sections were subsequently immersed for 30 minutes in 0.3% H_2O_2 in absolute methanol, treated with 5% normal rabbit serum for 30 minutes, and incubated with the primary antibody against HB-EGF or ADAM 17 overnight at 4°C. The sections were then incubated with biotinylated rabbit anti-goat IgG (Nichirei

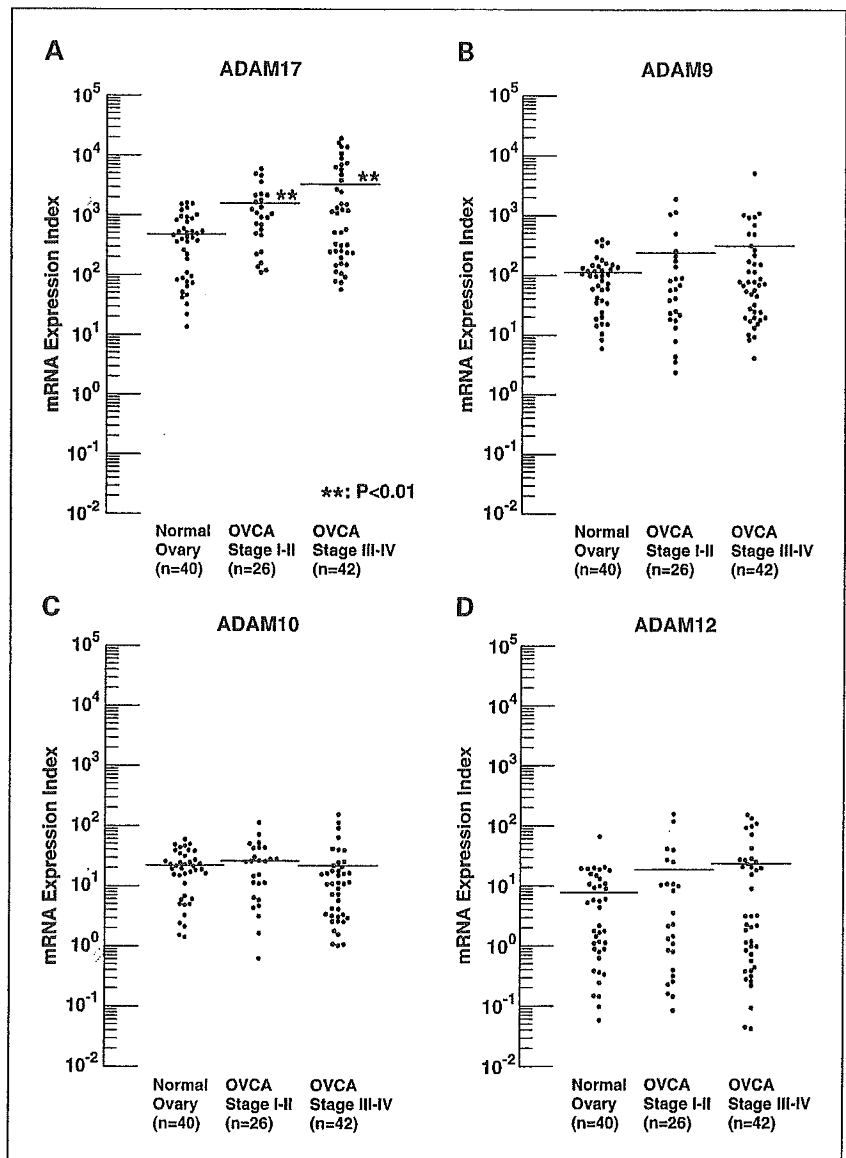

Fig. 2. Differences in expression of ADAM family members in normal ovaries and ovarian cancer. mRNA expression index of ADAM 17 (*A*), ADAM 9 (*B*), ADAM 10 (*C*), and ADAM 12 (*D*) in patients with normal ovaries, early ovarian cancer (stages I-II), and advanced ovarian cancer (stages III-IV). A line indicates the mean value of the mRNA expression index for each group. **, $P < 0.01$, versus patients with normal ovaries.

Corp., Tokyo, Japan) for 30 minutes followed by an avidin-biotin-peroxidase complex solution. The peroxidase reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.05% H_2O_2, and the sections were then counterstained in Mayer's hematoxylin, washed in tap water, dehydrated in graded ethanol, cleared in xylene, and coverslipped. Control staining was done using nonimmune goat IgG as the primary antibody. Three examiners (Y.T., S.M., and K.S.) separately evaluated the HB-EGF and ADAM 17 staining by counting the immunoreactive cells. At least 20 high-magnification fields were chosen randomly, and 1,000 cells in total were counted.

Statistical analysis. Statistical analysis was done with StatView software version 5.0 (Abacus Concepts, Berkeley, CA). The Mann-Whitney test was done to test the equality of the distribution of age and the mRNA expression index of five EGFR ligands and four ADAM family members among patients with normal ovaries, early ovarian cancer, and advanced ovarian cancer. Progression-free survival curves were estimated using the Kaplan-Meier method and analyzed by the log-rank test. Correlation between the mRNA expression indices of molecules was analyzed using Spearman's correlation analysis. Statistical significance was based on two-tailed statistical analyses, and $Ps < 0.05$ were considered statistically significant.

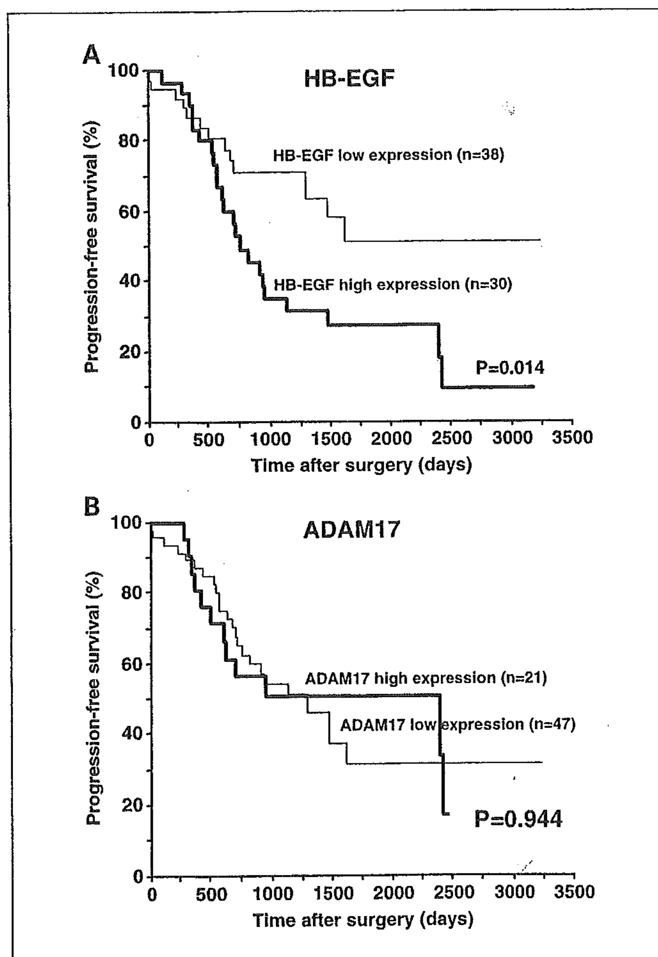

Fig. 3. Clinical significance of HB-EGF and ADAM 17 expression in ovarian cancer. Progression-free survival of 68 patients with ovarian cancer in relation to the tumor HB-EGF expression status (*A*) and tumor ADAM 17 expression status (*B*). *P*s were determined using the log-rank test.

Results

Expression of epidermal growth factor ligands in normal ovaries and ovarian cancer. Using real-time PCR, large differences in the mRNA expression index were found for HB-EGF and four other EGFR ligands between normal ovaries and ovarian cancer (Fig. 1; Table 1). For HB-EGF, the mRNA expression index was significantly elevated in advanced ovarian cancer compared with that in normal ovaries, although there was no significant difference between early ovarian cancer and normal ovaries (Fig. 1; Table 1). For TGF-α, amphiregulin, epiregulin, and betacellulin, no significant differences in the mRNA expression index were found among the three groups (Fig. 1; Table 1). No clear expression of EGF was detected in 10 patients with normal ovaries or 30 patients with ovarian cancer by reverse transcription-PCR, although EGF expression was confirmed in human placenta tissue using the same primer sets (26). Therefore, real-time PCR for EGF was not done in this study. To further investigate the expression of HB-EGF in surface normal ovarian epithelial cells, we examined the expression index of HB-EGF using 10 samples extracted by brushing normal ovarian epithelial cells. The expression index of HB-EGF mRNA was 7.6 ± 7.9 (mean ± SE), which was not significantly changed from that in samples extracted from whole normal ovaries. In a cancerous state, the expression of HB-EGF significantly increased compared with that in a normal state. These results suggest that HB-EGF contributes to the progression of ovarian cancer among the EGFR ligands.

Expression of a disintegrin and metalloprotease family members in normal ovaries and ovarian cancer. A large difference in the mRNA expression index was found for ADAM 17 and three other ADAM family members in normal ovaries and ovarian cancer (Fig. 2; Table 1). For ADAM 17, the mRNA expression index in early or advanced ovarian cancer was significantly elevated compared with that in normal ovaries, and there was no significant difference between early and advanced ovarian cancers (Fig. 2; Table 1). For ADAM 9, 10, and 12, no significant differences in the mRNA expression index were found among the three groups (Fig. 2; Table 1). To further investigate the expression of ADAM 17 in normal surface ovarian epithelial cells, we examined the expression index of ADAM 17 using 10 samples extracted by brushing normal ovarian epithelial cells. The expression index of ADAM 17 mRNA was 440 ± 380 (mean ± SE), which was not significantly changed from that in samples extracted from whole normal ovaries. In a cancerous state, the expression of ADAM 17 significantly increased compared with that in a normal state. Taken together, these results suggest that ADAM 17 is involved in the occurrence of ovarian cancer.

Clinical significance of heparin-binding epidermal growth factor–like growth factor or a disintegrin and metalloprotease 17 expression in ovarian cancer. HB-EGF expression ranged from a mRNA expression index of 10 to 39 in patients with normal ovaries. Ovarian cancer patients with a HB-EGF mRNA expression index of >40 were therefore regarded as cases with a high expression status of HB-EGF. In the progression-free survival curves, ovarian cancer patients with a high expression status of HB-EGF showed a significantly less favorable prognosis than those with a low expression status (Fig. 3*A*). Taken together, these results suggest that HB-EGF plays a significant

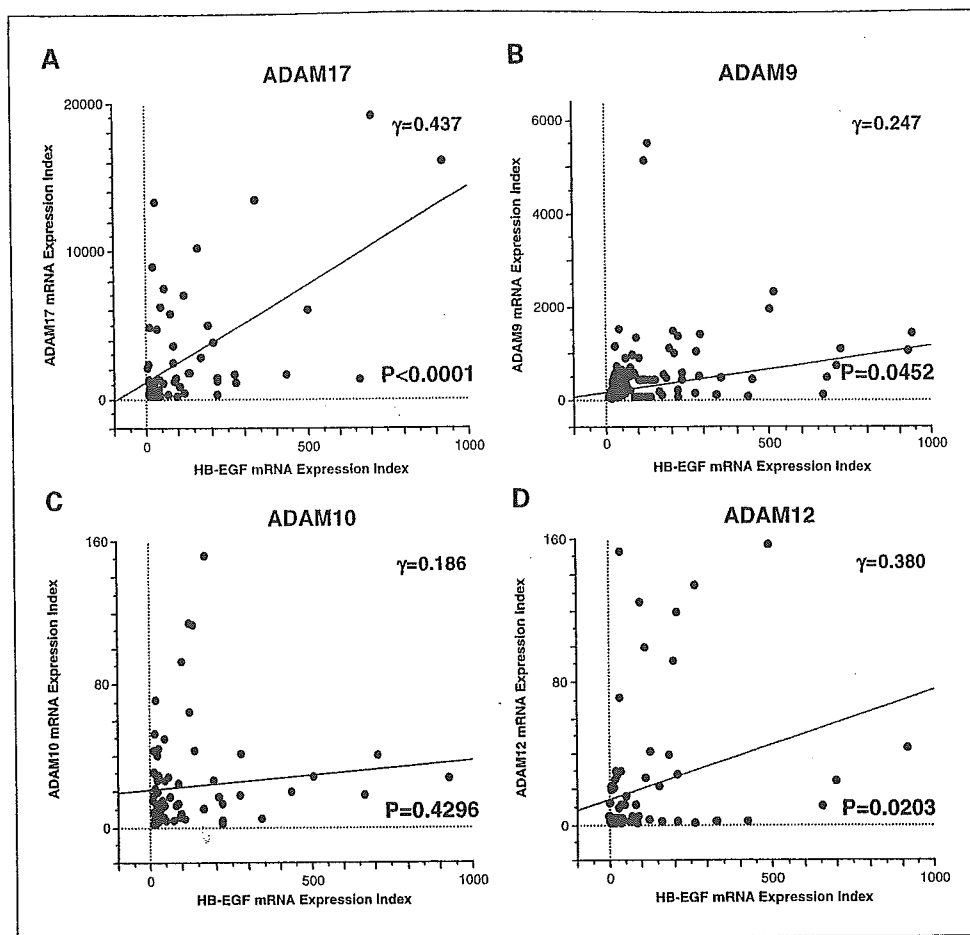

Fig. 4. Spearman's correlations between the mRNA expression indices of HB-EGF and ADAM family members in patients with ovarian cancer. The ordinate and abscissas indicate the mRNA expression index of HB-EGF and ADAM 17 (*A*), ADAM 9 (*B*), ADAM 10 (*C*), and ADAM 12 (*D*), respectively, in patients with ovarian cancer. γ indicates the Spearman's correlation coefficient.

role in the progression of ovarian cancer. ADAM 17 expression was within a mRNA expression index of 999 in patients with normal ovaries. Ovarian cancer patients with an ADAM 17 mRNA expression index of >1,000 were therefore regarded as cases with a high expression status of ADAM 17. No significant difference in the progression-free survival curves was found between ovarian cancer patients with a low and high expression status of ADAM 17 (Fig. 3*B*), suggesting that ADAM 17 is not significantly associated with the clinical outcome.

Relationships between the expression indices of heparin-binding epidermal growth factor–like growth factor and a disintegrin and metalloprotease family members in ovarian cancer. Significant correlations were found between the mRNA expression indices for HB-EGF and ADAM 9, 12, and 17 (Fig. 4*A*, *B*, and *D*). The mRNA expression index of ADAM 10 showed no significant correlation with that of HB-EGF in ovarian cancer (Fig. 4*C*). The correlation coefficient between the mRNA expression index of HB-EGF and that of ADAM 17 was increased compared with those between HB-EGF and ADAM 9 or 12, suggesting that ADAM 17 has a more significant contribution to the ectodomain shedding of HB-EGF than ADAM 9 or 12.

Localization of heparin-binding epidermal growth factor–like growth factor and a disintegrin and metalloprotease 17 proteins in normal ovarian epithelial cells and ovarian cancer cells. Abundant HB-EGF protein appeared as positive in interstitial tissues surrounding the ovarian cancer cells, whereas no definite expression of HB-EGF was observed in normal ovarian epithelial cells or interstitial tissues (Fig. 5*A* and *B*). In *in situ* hybridization, diffuse staining for HB-EGF mRNA was only found in ovarian cancer cells and not in either interstitial cells surrounding the cancer cells or normal ovarian epithelial cells (Fig. 5*C* and *D*), suggesting that HB-EGF protein is only produced by cancer cells, and not by interstitial cells, and that the proteolytic form of HB-EGF accumulates in the extracellular matrix with heparin sulfate in the interstitial tissues surrounding the cancer cells. The correlation coefficient between the mRNA expression index of HB-EGF and the *in situ* hybridization score of HB-EGF was 0.876 in ovarian cancer ($P < 0.001$). In addition, positive staining for ADAM 17 was observed in cancer cells, whereas no cells showed positive expression of ADAM 17 in normal ovarian epithelium (Fig. 5*E* and *F*). In ovarian cancer, the correlation coefficient between the ADAM 17 immunostaining and the mRNA expression index of ADAM 17 was 0.839 ($P < 0.01$).

Discussion

Impairment of the EGF system is involved in the pathogenesis of different types of carcinomas (10, 11). Univariate and multivariate statistical analyses have confirmed that EGFR overexpression is significantly associated with a high risk of progression in ovarian cancer patients (30). Relatively high

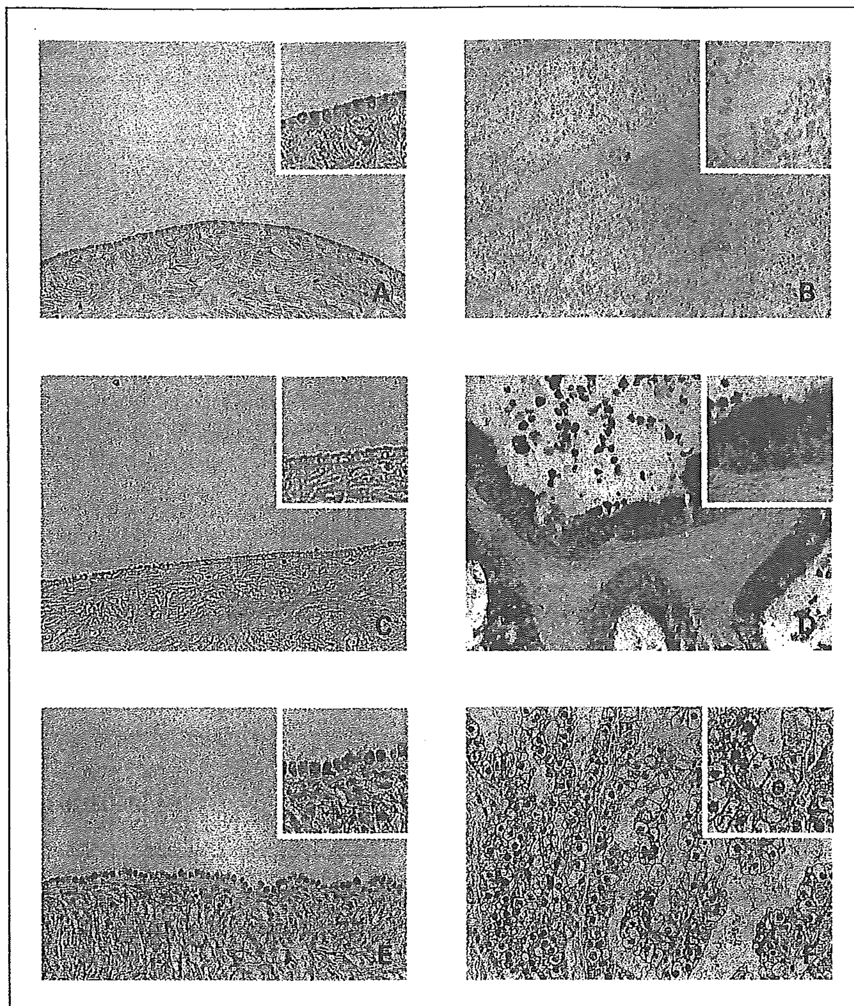

Fig. 5. Immunohistochemical staining and *in situ* hybridization of HB-EGF. No definite expression of HB-EGF protein (*A*) or mRNA (*C*) is present in a patient with normal ovaries. In a patient with advanced ovarian cancer, positive immunostaining of HB-EGF is observed in interstitial tissues surrounding the cancer cells and in some of the cancer cells (*B*). Diffuse staining of HB-EGF mRNA is only detected in the cytoplasm of cancer cells by *in situ* hybridization (*D*). No clear expression of ADAM 17 protein is found in a patient with normal ovaries (*E*). In a patient with advanced ovarian cancer, positive immunostaining of ADAM 17 is observed in cancer cells (*F*). Original magnification, ×200. *Insets*, higher-magnification view (×400).

frequencies of TGF-α and amphiregulin have been described in ovarian carcinomas, although the staining varied from weak to strong in tumors (31, 32). Ovarian cancer cells are sensitive to the diphtheria toxin, indicating the expression of pro-HB-EGF (33). No significant expression of EGF is present in normal ovaries or ovarian cancer. Thus, it remained unclear which EGFR ligands were predominantly expressed in ovarian cancer. In this study, however, abundant expression of HB-EGF was found in ovarian cancer compared with other EGFR ligands. Recently, several studies have revealed that HB-EGF is involved in a variety of cancers. In bladder cancer, HB-EGF is abundantly expressed and a significant prognostic marker for survival (34). HB-EGF expression is also associated with the clinical outcome in gastric, pancreatic, and breast cancers, in which HB-EGF expression is markedly abundant (35–37). In addition, *Helicobacter pylori* infection in human gastric carcinogenesis and the inflammatory processes associated with this type of infection have been linked to HB-EGF-dependent EGFR signal transactivation in human gastric epithelial tumor cells (38, 39). According to these studies, HB-EGF has been implicated in the occurrence and progression of human cancers. In this study, HB-EGF expression was significantly associated with the clinical outcome in ovarian cancer, suggesting that HB-EGF plays a crucial role in the aggressive behavior of a tumor in ovarian cancer.

The ADAM family has been implicated in diverse processes, including membrane fusion, cytokine and growth factor shedding, and cell migration (14). In particular, recent findings have revealed that the ADAM family is involved in cancer. ADAM 9 expression is associated with the clinical significance of human breast and pancreatic cancers (40, 41), whereas abundant ADAM 17 protein is expressed in human breast cancer (42). In human gastric carcinoma, high levels of transcripts for ADAM 10, 17, and 20 are present (43), whereas, in human liver cancer, expression of ADAM 9 and 12 is associated with tumor aggressiveness and progression (44). Thus, a few members of the ADAM family may be simultaneously associated with the acceleration and progression of human cancers. Therefore, any ADAM family members with similar functions should be examined to identify those involved in the pathogenesis of cancer. In this study, the expression of each ADAM family member involved in the ectodomain shedding of HB-EGF (15–21) was quantitatively estimated in human ovarian cancer. ADAM 17 was abundantly expressed compared with the other three ADAM family members, and its expression was enhanced in ovarian cancer. Therefore, this elevation of ADAM 17 expression in cancer might facilitate the proteolytic cleavage of EGFR ligands that are involved in the progression of cancer.

153

LPA can mediate EGFR signal transactivation through different combinations of EGFR ligands and ADAM family members. In NCI-H292 lung cancer cells, LPA transactivates EGFR through the ectodomain shedding of HB-EGF or amphiregulin, which is cleaved by ADAM 17 (45). In kidney cancer cells, EGFR transactivation is mediated by LPA, in association with HB-EGF and ADAM 10 or 17 (46). In bladder cancer cells, ADAM 15 has a role in EGFR transactivation mediated by LPA via soluble forms of amphiregulin or TGF-α (46). Thus, in the same cell system, there is a functional redundancy between EGFR ligands and ADAM family members that depends on a variety of stimuli. In ovarian cancer cells, HB-EGF and ADAM 17 were abundantly expressed compared with other EGFR ligands and other members of the ADAM family, respectively, and LPA activated EGFR through the ectodomain shedding of HB-EGF (22). In this study, the expressions of both HB-EGF and ADAM 17 were also abundant compared with those of other EGFR ligands and other members of the ADAM family in human ovarian cancer. In addition, HB-EGF protein appeared to accumulate in the interstitial tissues surrounding cancer cells and abundant ADAM 17 was also expressed in cancer cells, leading to the speculation that most HB-EGF expressed in cancer cells is quickly cleaved by ADAM 17. In fact, a large amount of HB-EGF was observed in the peritoneal fluid of ovarian cancer patients compared with the levels of amphiregulin and TGF-α (22). Taken together, these results suggest that proteolytic cleavage of HB-EGF was extensively provoked by ADAM 17 in human ovarian cancer.

This is the first study to show that both HB-EGF and ADAM 17 are significantly expressed among EGFR ligands and ADAM family members in human ovarian cancer. We have shown that tumor formation of ovarian cancer was completely blocked by *pro-HB-EGF* gene RNA interference and that the release of soluble HB-EGF is essential for tumor formation (22). Therefore, the development of therapeutic tools against HB-EGF and ADAM 17 would allow us to explore novel targeting therapy to human ovarian cancer.

Acknowledgments

We thank E. Hori and Y. Kubota for technical assistance and L. Saza and Drs. H. Baba, R. Tsunematsu, M. Tsuneyoshi, K. Sueishi, and T. Iwaki for critical comments.

References

1. Penson RT, Shannon KE, Sharpless NE, Seiden MV. Ovarian cancer: an update on genetics and therapy. Compr Ther 1998;24:477–87.
2. Friedlander ML. Prognostic factors in ovarian cancer. Semin Oncol 1998;25:305–14.
3. Mills GB, May C, McGill M, Roifman CM, Mellors A. A putative new growth factor in ascitic fluid from ovarian cancer patients: identification, characterization, and mechanism of action. Cancer Res 1988;48:1066–71.
4. Mills GB, Moolenaar WH. The emerging role of lysophosphatidic acid in cancer. Nat Rev Cancer 2003; 3:582–91.
5. Xu Y, Gaudette DC, Boynton JD, et al. Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. Clin Cancer Res 1995; 1:1223–32.
6. Westermann AM, Havik E, Postma FR, et al. Malignant effusions contain lysophosphatidic acid (LPA)-like activity. Ann Oncol 1998;9:437–42.
7. Xiao YJ, Schwartz B, Washington M, et al. Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. Anal Biochem 2001;290: 302–13.
8. Xu Y, Shen Z, Wiper DW, et al. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. JAMA 1998;280:719–23.
9. Fischer OM, Hart S, Gschwind A, Ullrich A. EGFR signal transactivation in cancer cells. Biochem Soc Trans 2003;31:1203–8.
10. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
11. Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS. Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr Relat Cancer 2003;10: 1–21.
12. Riese DJ II, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 1998;20:41–8.
13. Strachan L, Murison JG, Prestidge RL, Sleeman MA, Watson JD, Kumble KD. Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. J Biol Chem 2001;276: 18265–71.
14. Seals DF, Courtneidge SA. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 2003;17:7–30.
15. Sunnarborg SW, Hinkle CL, Stevenson M, et al. Tumor necrosis factor-α converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. J Biol Chem 2002;277:12838–45.
16. Sahin U, Weskamp G, Kelly K, et al. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol 2004;164:769–79.
17. Izumi Y, Hirata M, Hasuwa H, et al. A metalloprotease-disintegrin, MDC9/meltrin-γ/ADAM9 and PKCδ are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. EMBO J 1998;17:7260–72.
18. Umata T, Hirata M, Takahashi T, et al. A dual signaling cascade that regulates the ectodomain shedding of heparin-binding epidermal growth factor-like growth factor. J Biol Chem 2001;276:30475–82.
19. Lemjabbar H, Basbaum C. Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* in epithelial cells. Nat Med 2002; 8:41–6.
20. Yan Y, Shirakabe K, Werb Z. The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. J Cell Biol 2002;158:221–6.
21. Asakura M, Kitakaze M, Takashima S, et al. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med 2002;8:35–40.
22. Miyamoto S, Hirata M, Yamazaki A, et al. Heparin-binding EGF-like growth factor and the LPA-induced ectodomain shedding pathway is a promising target for the therapy of ovarian cancer. Cancer Res 2004; 64:5720–7.
23. WHO. WHO handbook for reporting results of cancer treatment. WHO Offset Publ 1979;48:5–45.
24. Rustin GJ, Nelstrop AE, McClean P, et al. Defining response of ovarian carcinoma to initial chemotherapy according to serum CA125. J Clin Oncol 1996;14: 1545–51.
25. Vergote I, Rustin GJ, Eisenhauer EA, et al. Re: New guidelines to evaluate the response to treatment in solid tumors [ovarian cancer]. Gynecologic Cancer Intergroup. J Natl Cancer Inst 2000;92:1534–5.
26. Adam RM, Borer JG, Williams J, Eastham JA, Loughlin KR, Freeman MR. Amphiregulin is coordinately expressed with heparin-binding epidermal growth factor-like growth factor in the interstitial smooth muscle of the human prostate. Endocrinology 1999;140:5866–75.
27. Sorensen BS, Torring N, Bor MV, Nexo E. Quantitation of the mRNA expression of the epidermal growth factor system: selective induction of heparin-binding epidermal growth factor-like growth factor and amphiregulin expression by growth factor stimulation of prostate stromal cells. J Lab Clin Med 2000;20: 91–5.
28. Ohishi Y, Oda Y, Uchiumi T, et al. ATP-binding cassette superfamily transporter gene expression in human primary ovarian carcinoma. Clin Cancer Res 2002;8:3767–75.
29. Suzuki SO, Iwaki T. Non-isotopic *in situ* hybridization of CD44 transcript in formalin-fixed paraffin-embedded sections. Brain Res Protoc 1999;4:29–35.
30. Scambia G, Benedetti Panici P, Battaglia F, et al. Significance of epidermal growth factor receptor in advanced ovarian cancer. J Clin Oncol 1992;10:529–35.
31. Morishige K, Kurachi H, Amemiya K, et al. Evidence for the involvement of transforming growth factor α and epidermal growth factor receptor autocrine growth mechanism in primary human ovarian cancers *in vitro*. Cancer Res 1991;51:5322–8.
32. D'Antonio A, Losito S, Pignata S, et al. Transforming growth factor α, amphiregulin and cripto-1 are frequently expressed in advanced human ovarian carcinomas. Int J Oncol 2002;21:941–8.
33. Morimoto H, Safrit JT, Bonavida B. Synergistic effect of tumor necrosis factor-α- and diphtheria toxin-mediated cytotoxicity in sensitive and resistant human ovarian tumor cell lines. J Immunol 1991; 147:2609–16.
34. Thogersen VB, Sorensen BS, Poulsen SS, Orntoft TF, Wolf H, Nexo E. A subclass of HER1 ligands are prognostic markers for survival in bladder cancer patients. Cancer Res 2001;61:6227–33.
35. Kobrin MS, Funatomi H, Friess H, Buchler MW, Stathis P, Korc M. Induction and expression of heparin-binding EGF-like growth factor in human pancreatic cancer. Biochem Biophys Res Commun 1994;202: 1705–9.
36. Ito Y, Takeda T, Higashiyama S, Noguchi S, Matsuura N. Expression of heparin-binding epidermal growth factor-like growth factor in breast carcinoma. Breast Cancer Res Treat 2001;67:81–5.
37. Murayama Y, Miyagawa J, Shinomura Y, et al. Significance of the association between heparin-binding epidermal growth factor-like growth factor

and CD9 in human gastric cancer. Int J Cancer 2002 ;98:505–13.
38. Romano M, Ricci V, Di Popolo A, et al. *Helicobacter pylori* upregulates expression of epidermal growth factor-related peptides, but inhibits their proliferative effect in MKN28 gastric mucosal cells. J Clin Invest 1998; 101:1604–13.
39. Wallasch C, Crabtree JE, Bevec D, Robinson PA, Wagner H, Ullrich A. *Helicobacter pylori*-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. Biochem Biophys Res Commun 2002;295:695–701.
40. O'Shea C, McKie N, Buggy Y, et al. Expression of ADAM-9 mRNA and protein in human breast cancer. Int J Cancer 2003;105:754–61.
41. Grutzmann R, Luttges J, Sipos B, et al. ADAM9 expression in pancreatic cancer is associated with tumour type and is a prognostic factor in ductal adenocarcinoma. Br J Cancer 2004;90: 1053–8.
42. Borrell-Pages M, Rojo F, Albanell J, Baselga J, Arribas J. TACE is required for the activation of the EGFR by TGF-α in tumors. EMBO J 2003;22:1114–24.
43. Yoshimura T, Tomita T, Dixon MF, Axon AT, Robinson PA, Crabtree JE. ADAMs (a disintegrin and metalloproteinase) messenger RNA expression in *Helicobacter pylori*-infected, normal, and neoplastic gastric mucosa. J Infect Dis 2002;185: 332–40.
44. Le Pabic H, Bonnier D, Wewer UM, et al. ADAM12 in human liver cancers: TGF-β-regulated expression in stellate cells is associated with matrix remodeling. Hepatology 2003;37:1056–66.
45. Hart S, Fischer OM, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor α-converting enzyme (TACE/ADAM17)-mediated trans-

155

Int. J. Cancer: **117**, 376–380 (2005)
© 2005 Wiley-Liss, Inc.

AKT phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer

Eiji Oki[1]*, Hideo Baba[1], Eriko Tokunaga[1], Toshihiko Nakamura[1], Naoyuki Ueda[1], Motonori Futatsugi[1], Kohjiro Mashino[1], Manabu Yamamoto[1], Masahiko Ikebe[1], Yoshihiro Kakeji[2] and Yoshihiko Maehara[1]
[1]*Department of Surgery and Science, Graduate School of Kyushu University, Fukuoka, Japan*
[2]*Center for the Integration of Advanced Medicine and Innovative Technology, Kyushu University Hospital, Fukuoka, Japan*

Growth factor receptor-mediated signal transduction has been implicated in conferring resistance to conventional chemotherapy on cancer cells. We describe a pathway that involves AKT/PI3K to mediate chemoresistance in gastric cancer patients. Primary gastric carcinoma tissues and corresponding normal mucosa were obtained from 76 gastric cancer patients who underwent surgery in the Department of Surgery II in Kyushu University Hospital from the years 1996–2000. AKT activation was investigated by immunostaining with a phosphorylation-specific antibody, and LOH (loss of heterozygosity) of PTEN was studied in the same samples. AKT was phosphorylated in 22 cases (28.9%) of gastric cancer cases. AKT and phosphorylated AKT were not correlated with any clinicopathological factor. We found that the gastric cancer patients who had higher AKT phosphorylation (activated AKT) seemed to have LOH of *PTEN* ($p = 0.0008$). When the chemotherapeutic sensibilities of these patients were studied in an MTT assay, it was found that the activated AKT was associated with increased resistance to multiple chemotherapeutic agents (5-fluorouracil, adriamycin, mitomycin C and cis-platinum). The results of our study indicate that AKT activation and LOH of *PTEN* plays an important role in conferring a broad-spectrum chemoresistance in gastric cancer patients. It also indicates that AKT may therefore be a novel molecular target for therapies or chemosensitivity tests that improve the outcomes of gastric cancer patients.
© *2005 Wiley-Liss, Inc.*

Key words: phosphorylation; PTEN; AKT; loss of heterozygosity; chemoresistance; gastric cancer

Chemotherapy is an important therapeutic modality for gastrointestinal cancers, although the success rate of this treatment is limited because of chemoresistance. It is now widely accepted that the apoptotic capacity of the cancer cell is important in determining its response to chemotherapeutic agents.

Several studies have suggested that the serine/threonine kinase AKT, also known as PKB, is a key molecule for protecting cells from undergoing apoptosis, and that the AKT-mediated survival-signaling pathway is an attractive target for cancer chemotherapy.[1–3]

AKT was characterized initially as the human homologue of the viral oncogene *v-AKT* from the transforming retrovirus AKT8. Three isoforms of AKT have been identified, AKT1, AKT2 and AKT3, all of which share a high degree of homology at the amino acid level. The induction of AKT activity is primarily under the control of the phosphoinositide products of PI3K. PIP2 and PIP3 bind to the PH domain of AKT, resulting in translocation of AKT to the plasma membrane area.

The encoding phosphate and tensin homologue (PTEN) gene, which is a tumor suppressor candidate, is located on chromosome 10q23 and has an extensive homology with the cytoskeletal proteins auxilin and tensin.[4,5] *PTEN* mutations have been observed frequently in various neoplasms, including glioblastoma, melanoma, prostate cancer and breast cancer.[4–10] In glioblastoma, melanoma and prostate cancer, *PTEN* mutations and allelic deletions are observed during the later stages, whereas in thyroid and endometrial cancers, *PTEN* mutation alterations are found during the early stages and include endometrial hyperplasia and benign thyroid tumors.[6–8,11–13] Germline mutations of PTEN are found in patients with Coden syndrome, a familial syndrome associated with predisposition for multiple benign hamartomas, and malignant thyroid and breast neoplasms.[14] *PTEN* encodes an enzyme with phosphatase activity toward the acidic protein substrates and the lipid second messenger, phosphatidylinositol-3,4,5-triphosphate (PIP3).[15]

Loss of the tumor suppressor gene *PTEN* is common in cancer tumors, constitutively activating AKT.[16–18] The phosphatase activity of PTEN is crucial in controlling the phosphatidylinositol-3 (PI-3) kinase signal transduction pathway and AKT activation. This indicates that PTEN plays the role of tumor-suppressor by negatively regulating the anti-apoptotic PI3-kinase/AKT signaling pathway.[16,19] In addition, the phosphatase of PTEN is a tumor suppressor whose loss triggers AKT activation.[20–23]

We investigated the activation of AKT and discovered the role of PTEN and AKT in chemosensitivities of some gastric cancer samples. To the best of our knowledge, this is the first report showing the direct correlation between AKT activation and the chemoresistance of a clinical sample.

Material and methods

Tissue samples

Primary gastric carcinoma tissues and corresponding normal mucosa were obtained from 76 Japanese patients who underwent surgery in the Department of Surgery II at Kyushu University Hospital between 1996–2000. No patient had ever received chemotherapy before surgery. Informed consent was obtained from each patient before tissue acquisition. All the tumors, examined microscopically by pathologists were diagnosed adenocarcinoma and classified according to the criteria of the Japanese Research Society for Gastric Cancer (1993). The cancer tissues and the well-separated normal gastric mucosa obtained by gastrectomy were immediately cut into two pieces. Half of the specimen was kept in liquid nitrogen for DNA extraction, and the another half was immediately put into medium (minimal essential medium; MEM) for chemosensitivity tests. The genomic DNA was prepared by proteinase K digestion and phenol/chloroform extraction, which was then followed by ethanol precipitation. In all cases, the histopathological types of the tumors were determined as adenocarcinoma. Written informed consent was obtained from each of the patients.

Immunohistochemistry

AKT (T-AKT; polyclonal rabbit anti-human AKT; Cell Signaling Technology, Beverly, MA) and phospho-AKT antibody (polyclonal rabbit anti-human phospho-AKT [Ser473]; Cell Signaling Technology) were used in our study. Immunohistochemical stain-

Grant sponsor: Ministry of Education, Science, Sports and Culture of Japan.
*Correspondence to: Department of Surgery and Science, Graduate School of Kyushu University, 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. Fax: +81-92-642-5482. E-mail: okieiji@surg2.med.kyushu-u.ac.jp
Received 6 January 2005; Accepted after revision 18 February 2005
DOI 10.1002/ijc.21170
Published online 17 May 2005 in Wiley InterScience (www.interscience.wiley.com).

Publication of the International Union Against Cancer
UICC global cancer control

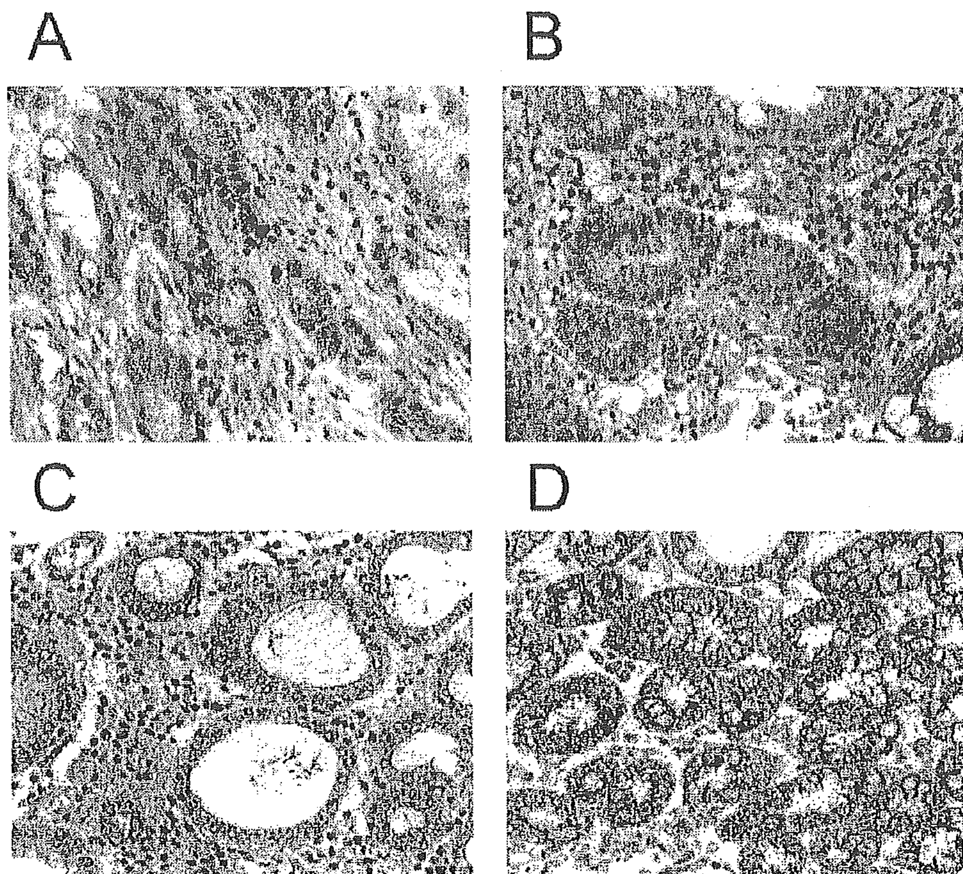

FIGURE 1 – The intensity is designated as 0 when no tumor cells stain (*a*), 1+ when 10–30% of cells stain (*b*), 2+ when 30–50% of cells stain (*c*) and 3+ when >50% of cells stain (*d*). Finally, specimens showing p-AKT expression in the cytoplasm of >50% (intensity 3+) of examined cancer cells were considered to be a positive.

ing was carried out on a Ventana ES automated immunohistochemistry instrument (Ventana Medical Systems, Inc., Tucson, AZ) with an indirect biotin-avidin system on 5-mm formalin-fixed, paraffin-embedded sections from a representative block in each case. All blocks were cut before IHC, and the slides were incubated subsequently with a biotinylated universal secondary antibody and an avidin horseradish peroxidase label (Ventana). After color development with a diaminobenzidine substrate, these slides were counterstained with hematoxylin. To confirm the specificity of the primary antibody, negative control slides were run using an isotype matched rabbit IgG (Sigma, St. Louis, MO) at the same concentration as that of the primary antibody. The immunochemicals, incubation times and final antibody concentrations and dilutions are rabbit polyclonal anti-phospho-AKT antibody, 32 minutes at 37°C, and dilution 1:10, respectively. The intensity is set up to be 0 when no tumor cells stain, 1+ when 10–30% of cells stain (weak), 2+ when 30–50% of cells stain (moderate) and 3+ when >50% of cells stain (strong). Specimens showing p-AKT expression in the cytoplasm of >50% (intensity 3+) of the examined cancer cells were considered to be a positive.

LOH analysis

LOH of PTEN was analyzed in our study using a DNA sequencer with 2 microsatellite markers. The oligonucleotide primers were synthesized and then purified by HPLC. The sequences of the primers for PCR are D10S1765-forward, 5′-CAATGGAACCAAATGTGGTC, and D10S1765-reverse, 5′-AGTCCGATAATGCCAGGATG. The sequences of the primers for PCR are D10S1173-forward, 5′-CATGCCAAGACTGAAACTCC, and D10S1173-reverse, 5′-AAACCCCAATGCCATAATGG. The PCR reactions using genomic DNA were carried out using a TAKARA GeneAmp PCR Reagent Kit and were run in the Perkin-Elmer GeneAmp PCR system 9700 (Perkin-Elmer, Norwalk, CT). The thermal conditions of the system were as follows: one cycle at 95°C for 4 min; 35 cycles at 95°C for 0.5 min, 55°C for 0.5 min, 72°C for 0.5 min and one cycle at 72°C for 10 min. The DNA derived from the cancer tissues were amplified with ROX-labeled 5′ primer and cold 3′ primer, whereas the DNA from the normal tissues were amplified with HEX-labeled 5′ primer and cold 3′ primer. The running condition of the Perkin-Elmer Genetic Analyzer 310 (Perkin-Elmer) was described previously.[24,25] The data were processed by the ABI software GeneScan. In any case where the peak value of one-gene loci diminished >30% in the carcinoma, it was judged as having LOH. When it was judged as having ROH by at least one marker, it was not assumed LOH.

Chemosensitivity test

The succinate dehydrogenase inhibition (SDI) test was conducted as described previously by Takeuchi *et al.*[26] The cell viability was estimated based on succinate dehydrogenase (SD) activity, and was determined using [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] (MTT). Chemosensitivity rate was calculated as follows: chemosensitivity rate (%) = 100 − (T–B)/(U–B) × 100% (T, treated cell: absorbance determined when tumor cells are exposed to drugs; U, untreated cell: absorbance of untreated cells; B, blank: absorbance when neither the drug nor MTT was added). When chemosensitivity rate was negative value, it was assumed zero.

Statistical methods

The statistical significance was determined using the Student's *t*-test, the χ^2 test with Yates correlation factor, and was also confirmed with a 2-tailed Fisher exact test. The difference was considered significant when the *p*-value was <0.05.

Results

AKT expression in gastric cancer cases

Immunostaining of phosphorylated AKT (p-AKT) and non-activated AKT (T-AKT) was carried out for 76 gastric cancer sam-

TABLE I – CLINICOPATHOLOGICAL FEATURES OF GASTRIC CANCERS AND AKT ACTIVATION

Variable	p-AKT(+) $n = 22$	p-AKT(−) $n = 54$	p-value[1]
Gender			
Male	16	37	NS
Female	6	17	
Age	65.3	62.4	NS
Histology			
Differentiated	12	20	NS
Undifferentiated	10	33	
Serosal inversion			
Negative	10	26	NS
Positive	12	28	
Histological lymph node metastasis			
Negative	10	18	NS
Positive	12	36	
Vascular involvement			
Negative	13	25	NS
Positive	9	29	
Peritoneal dissemination			
Negative	22	51	NS
Positive	0	3	
Liver metastasis			
Negative	21	53	NS
Positive	1	1	
Stage			
I+II	12	24	NS
III+IV	10	30	

NS, not significant.

ples. The intensity of immunohistochemistry was classified in 4 grade scales as described in the paragraph titled methods (Fig. 1). When only Grade 3, the highest intensity of all 4 grades, was judged to be positive, 22 of 79 cases (28.9%) were found to be positive for p-AKT antibody and 25 of 79 cases (32.9%) were found to be positive for T-AKT antibody. There was weak correlation between p-AKT and T-AKT immunoreactivity, however clinicopathological correlation was not found between both AKT(data not shown) and p-AKT (Table I).

LOH of PTEN in gastric cancer cases and relation with AKT

It is possible that phosphorylated AKT increased in appearance because of increased expression of non-activated AKT. Therefore, to clarify the significance of AKT activation, aberration of PTEN was investigated. Abnormality of PTEN expression and genetic change has been reported occasionally, but LOH of *PTEN* was more often reported. As a routine investigation, LOH of *PTEN* was the most sensitive way to find abnormality of PTEN. Both LOH analysis of PTEN and immunostaining of phospho-AKT were completed in the same sample for patients. There was no relationship found between LOH of *PTEN* and clinicopathological factor (data not shown). Tables II and III illustrate the relationship between the LOH of *PTEN* and AKT. Analysis of LOH of *PTEN* was completed in a total of 68 of 76 cases immunostained with AKT. It shows that gastric cancer patients having LOH of *PTEN* are likely to have higher activated AKT ($p < 0.05$), although there is no correlation between the clinicopathological features and non-activated AKT. In short, allelic loss of *PTEN* was one of reasons for activation of AKT.

Chemosensitivity of gastric cancer and phosphorylation of AKT

Phosphorylation of AKT leads to inhibition of cell death. Particularly, it was questioned whether the reaction of the chemotherapeutic agent that induced apoptosis decreased. Therefore, sensitivity of an anticancer drug was investigated *via* MTT assay using a fresh specimen of the Gastric cancer patient that was immunostained by AKT.

The chemotherapeutic sensibilities of these patients were investigated using an MTT assay. Gastric cancer tissues were exposed for 3 days to anti-tumor drugs, such as adriamycin (ADM), 5-fluorouracil (5-FU), mitomycin C (MMC) and cisplatin (CDDP). These anti-tumor drugs were tested at 10× the peak of the plasma concentration. The results were 5-FU, 100 μg/ml; ADM, 4 μg/ml; MMC, 10 μg/ml; and CDDP, 20 μg/ml.

TABLE II – RELATIONSHIP BETWEEN LOH OF PTEN AND PHOSPHORYLATED AKT

p-AKT status		PTEN[1] LOH	PTEN[1] ROH
p-AKT	Positive	8	10
	Negative	7	43

[1]LOH, loss of heterozygosity; ROH, retention of heterozygosity. $p = 0.0081$.

TABLE III – RELATIONSHIP BETWEEN LOH OF PTEN AND AKT EXPRESSION

T-AKT status		PTEN[1] LOH	PTEN[1] ROH
T-AKT	Positive	7	32
	Negative	4	25

[1]LOH, loss of heterozygosity; ROH, retention of heterozygosity.

Figure 2 demonstrates that gastric cancer cases with activated AKT exhibit increased resistances to multiple chemotherapeutic agents. The sensitivity of 5-FU was diminished significantly in the groups having been activated when compared to the groups having no activated AKT ($p = 0.0071$) (Fig. 2*a*). AKT phosphorylation is also related to sensitivity with adriamycin, mitomycin C and cis-platinum (Fig. 2*b*). Sensitivity for the anticancer drug deteriorated in the case where AKT was phosphorylated.

Discussion

The PI-3K pathway, which is regulated by tyrosine kinase receptor activity, has been shown to play a critical role in promoting cell proliferation and inhibiting cell death.[27,28] AKT, the cellular homologue of the viral oncogene v-AKT, plays a major role in mediating these PI-3K effects.[2,22] The phosphatase PTEN usually inhibits AKT activation.

Several reports have shown that PTEN inactivation was associated with the poor outcomes of patients.[29,30] These reports, however, did not elucidate whether or not the increased activity of AKT could be directly related to the resistance to chemotherapy.

Based on the information presented in our study, there are 2 reasons to investigate the aberration of PTEN. Initially, we elucidated the importance of PTEN for gastric carcinogenesis. A secondary reason was to confirm the phenomenon of phosphorylation of AKT in gastric cancers because there was the possibility that we observed only elevated expression of non-activated AKT. We chose the LOH analysis to investigate the aberration of PTEN because the relationship between phosphorylation of AKT and PTEN was reported, and it is quite difficult to assess the diminished expression of PTEN using immunohistochemistry as the mutation of *PTEN* was rarely reported in sporadic gastric cancers.

We found that gastric cancer patients who showed LOH of *PTEN* seemed to have higher AKT phosphorylation. We also demonstrated that the specimens of gastric cancer patients who showed AKT phosphorylation had increasing resistance to the 4 chemotherapeutic agents, which included those used currently on gastric cancer patients. This is comparable with our earlier studies that demonstrated a relation between LOH of *PTEN* and poor clinical outcomes in gastric cancer patients (in submission). Expressing constitutively active AKT was uniformly resistant to 4 chemotherapeutic agents with different action mechanisms. These 4 agents were 5-fluorourocil (an antimetabolite), CDDP (a platinum

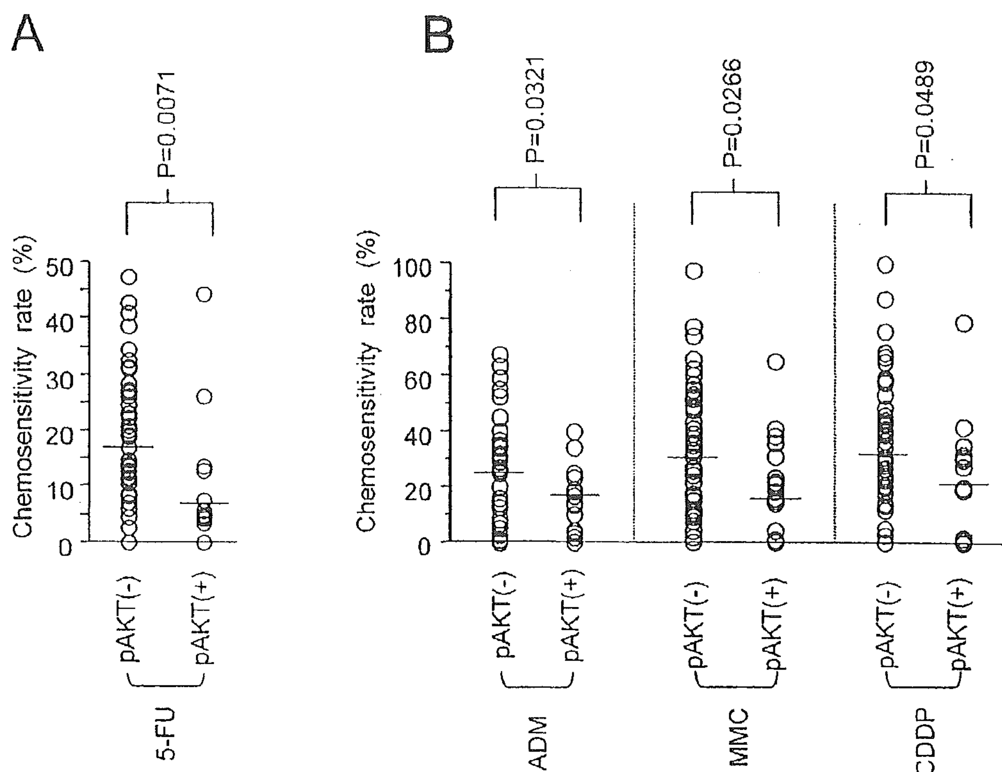

FIGURE 2 – Dot plots show presence of phosphorylated AKT and the anticancer drug sensibility of a gastric cancer specimen. 5-fluorouracil (*a*), adriamycin, mitomycin C, and cis-platinum (*b*). The chemosensitivity was assessed by an MTT assay. The dots indicate the chemosensitivity, and the horizontal bars show the average of each sample. Chemosensitivity rate was calculated as follows: chemosensitivity rate (%) = 100 − (T−B)/(U−B) × 100%. T, treated cell: absorbance determined when tumor cells are exposed to drugs; U, untreated cell: absorbance of untreated cells; B, blank: absorbance when neither the drug nor MTT was added. When Chemosensitivity rate was negative value, it was assumed zero.

compound), adriamycin (an alkylating agent) and mitomycin C (an alkylating agent). Most chemotherapeutic agents can induce apoptosis because of their cytotoxic effect. These results suggest that AKT-mediated resistance to chemotherapy is likely because of overall AKT anti-apoptotic activity. Furthermore, the activation of the PI-3K signaling cascade leads to multi-drug resistance.

AKT is an important regulator of cell survival and apoptosis. Upon its activation, AKT can suppress apoptosis by interacting with and phosphorylating several key downstream effectors. For example, AKT phosphorylates the proapoptotic Bcl-2 partner Bad, which binds to and blocks the activity of Bcl-x, a cell survival factor.[31] Expressions of these proteins are altered in various human tumors and these abnormal expressions may contribute to tumor cell resistance and to chemotherapeutic agent-induced apoptosis. AKT can also inactivate initiation caspase-9[32] and repress the Forkhead transcription factor FKHRL-1,[33] which regulates the apoptosis-inducing Fas ligand expression. In addition, AKT phosphorylates IκB, which promotes IκB degradation, thereby increasing the activity of NF-κB that is a well-known cell survival factor.[34,35] Previous *in vitro* studies have reported that several chemotherapeutic agents such as 5-FU, paclitaxel, vinblastine, vincristine, daunomycin and doxorubicin could activate NF-κB, and that this response can result in noticeable suppression of the apoptotic potential. Constitutive activation of NF-κB is observed in various malignant cells, and this implies that activated NF-κB induced by AKT may result in cancer cell chemoresistance. Furthermore, there is an increasing number of substrates that are also phosphorylated by AKT.[36]

In our study, the relationship between *in vitro* chemosensitivity and activation of AKT was analyzed. The effect of adjuvant chemotherapy should have been analyzed to elucidate importance of AKT. It is important, however, that adjuvant therapy should not be done after curative gastrectomy in Japan. The clinical importance of AKT activation couldn't be assessed in this timeframe because over 50% of the people we analyzed in our study had undergone chemotherapy *via* a different method. In our study, a phosphospecific antibody was used for the detection of activated AKT. The results showed a significant correlation between the activated AKT and SD (succinate dehydrogenase) activity obtained from an MTT assay using the tissues from a gastric cancer patient. Immunostaining is a simple method and it is possible to confirm AKT activation from it by using biopsy samples before treatment with chemotherapeutic agents for clinical patients. We believe that immunostaining of phospho-AKT might be a promising predictor of cancer chemotherapy in the future. The AKT involvement in human cancer oncogenesis and chemoresistance indicates that AKT is an important target for cancer therapy, and that an AKT inhibitor might be a promising chemotherapeutic agent for a gastric cancer patient. Furthermore, our results indicate that the AKT pathway was constitutively active in patients whose *PTEN* showed a loss of heterozygosity. This may explain why the outcomes are poor among the patients who have LOH of *PTEN*.

In summary, we have demonstrated that AKT activation and LOH of *PTEN* plays an important role in the sensitivities of chemotherapeutic agents in gastric cancer patients. Our results suggest that clinical trials for an appropriate chemotherapy combination with conventional drugs and a new generation of signal transduction inhibitors that inhibit the PTEN/AKT pathwayshould be considered for treating gastric cancer in the near future.

Acknowledgements

We are grateful to J. Tsuchihashi, K. Miyamoto, N. Nakamura, Y. Kubota, Y. Matsumoto and T. Shishino for technical assistance.

References

1. Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, Cordon-Cardo C, Pelletier J, Lowe SW. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature 2004;428:332–7.
2. West KA, Castillo SS, Dennis PA. Activation of the PI3K/Akt pathway and chemotherapeutic resistance. Drug Resist Updat 2002;5:234–48.

3. Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L, Schmidt M, Mills GB, Mendelsohn J, Fan Z. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. Oncogene 2003;22:3205–12.
4. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15:356–62.
5. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943–7.
6. Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH. Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res 1997;57:3935–40.
7. Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res 1997;57:3660–3.
8. Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL, Said JW, Isaacs WB, Sawyers CL. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. Proc Natl Acad Sci USA 1998;95:5246–50.
9. Wang SI, Puc J, Li J, Bruce JN, Cairns P, Sidransky D, Parsons R. Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res 1997;57:4183–6.
10. Teng DH, Hu R, Lin H, Davis T, Iliev D, Frye C, Swedlund B, Hansen KL, Vinson VL, Gumpper KL, Ellis L, El-Naggar A, et al. MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res 1997;57:5221–5.
11. Maxwell GL, Risinger JI, Gumbs C, Shaw H, Bentley RC, Barrett JC, Berchuck A, Futreal PA. Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias. Cancer Res 1998;58:2500–3.
12. Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C, Eng C. Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. Cancer Res 1997;57:4710–3.
13. Wang Z, Ikeguchi M, Maeta M, Kaibara N. The correlation between the expression of p53 protein and DNA ploidy in patients with gastric cancer that has invaded the serosa. Anticancer Res 1997;17:3701–5.
14. Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. Nat Genet 1997;16:333–4.
15. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998;273:13375–8.
16. Byun DS, Cho K, Ryu BK, Lee MG, Park JI, Chae KS, Kim HJ, Chi SG. Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma. Int J Cancer 2003;104:318–27.
17. Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C. Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. Am J Pathol 2001;158:2097–106.
18. Kanamori Y, Kigawa J, Itamochi H, Shimada M, Takahashi M, Kamazawa S, Sato S, Akeshima R, Terakawa N. Correlation between loss of PTEN expression and Akt phosphorylation in endometrial carcinoma. Clin Cancer Res 2001;7:892–5.
19. Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X, Wu H. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA 1999;96:6199–204.
20. Zhou M, Gu L, Findley HW, Jiang R, Woods WG. PTEN reverses MDM2-mediated chemotherapy resistance by interacting with p53 in acute lymphoblastic leukemia cells. Cancer Res 2003;63:6357–62.
21. Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther 2002;1:707–17.
22. Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H, Haga N. Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. Cancer Sci 2003;94:15–21.
23. Tsuruo T. Molecular cancer therapeutics: recent progress and targets in drug resistance. Intern Med 2003;42:237–43.
24. Oda S, Oki E, Maehara Y, Sugimachi K. Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis. Nucleic Acids Res 1997;25:3415–20.
25. Maehara Y, Oda S, Sugimachi K. The instability within: problems in current analyses of microsatellite instability. Mutat Res 2001;461: 249–63.
26. Takeuchi H, Baba H, Inutsuka S, Takahashi I, Kusumoto H, Maehara Y, Sugimachi K. Antitumor chemosensitivity differs between clinical sarcoma and adenocarcinoma tissues. Anticancer Res 1994;14:169–71.
27. Simstein R, Burow M, Parker A, Weldon C, Beckman B. Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system. Exp Biol Med (Maywood) 2003;228:995–1003.
28. Krystal GW, Sulanke G, Litz J. Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. Mol Cancer Ther 2002;1:913–22.
29. Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. Mod Pathol 2001;14:672–6.
30. Lee JI, Soria JC, Hassan KA, El-Naggar AK, Tang X, Liu DD, Hong WK, Mao L. Loss of PTEN expression as a prognostic marker for tongue cancer. Arch Otolaryngol Head Neck Surg 2001;127:1441–5.
31. del Peso L, Lucas L, Esteve P, Lacal JC. Activation of phospholipase D by growth factors and oncogenes in murine fibroblasts follow alternative but cross-talking pathways. Biochem J 1997;322:519–28.
32. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998;282:1318–21.
33. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857–68.
34. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999;401:82–5.
35. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999;401:86–90.
36. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002;14:381–95.

Genetic Polymorphism in Cytochrome *P*450 7A1 and Risk of Colorectal Cancer: The Fukuoka Colorectal Cancer Study

Tomoko Hagiwara,[1] Suminori Kono,[1] Guang Yin,[1] Kengo Toyomura,[1] Jun Nagano,[1] Tetsuya Mizoue,[1] Ryuichi Mibu,[2] Masao Tanaka,[2] Yoshihiro Kakeji,[3] Yoshihiko Maehara,[3] Takeshi Okamura,[4] Kouji Ikejiri,[5] Kitaroh Futami,[6] Youichi Yasunami,[7] Takafumi Maekawa,[8] Kenji Takenaka,[9] Hitoshi Ichimiya,[10] and Nobutoshi Imaizumi[11]

Departments of [1]Preventive Medicine, [2]Surgery and Oncology, and [3]Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka; [4]Department of Gastroenterological Surgery, National Kyushu Cancer Center, Notame, Minami-ku, Fukuoka; [5]Division of Surgery, National Kyushu Medical Center, Jigyohama, Chuo-ku, Fukuoka; [6]Department of Surgery, Fukuoka University Chikushi Hospital, Oaza-zokumyoin, Chikushimo-shi; [7]The First and [8]Second Departments of Surgery, Fukuoka University School of Medicine, Nanakuma, Jonan-ku, Fukuoka; [9]Division of Surgery, Fukuoka City Hospital, Yoshizuka-honmachi, Hakata-ku, Fukuoka; [10]Division of Surgery, Hamanomachi General Hospital, Maizuru, Chuo-ku, Fukuoka; and [11]Division of Surgery, Fukuoka Red Cross Hospital, Ogusu, Minami-ku, Fukuoka, Japan

Abstract

Bile acids have long been implicated in the etiology of colorectal cancer, but epidemiologic evidence remains elusive. Cholesterol 7α-hydroxylase (*CYP7A1*) is the rate-limiting enzyme in the synthesis of bile acids from cholesterol in the liver, and thus may be an important determinant of bile acid production. We examined the association between the *CYP7A1 A-203C* polymorphism and colorectal cancer. The *CYP7A1 A-203C* polymorphism was determined by the PCR-RFLP method in 685 incident cases of colorectal cancer and 778 controls randomly selected from a community in the Fukuoka area, Japan. The *CC* genotype was slightly less frequent in the case group, and the adjusted odds ratio for the *CC* versus *AA* genotype was 0.88 (95% confidence interval, 0.65-1.20). In the analysis by subsite of the colorectum, a decreased risk associated with the *CYP7A1 CC* genotype was observed for proximal colon cancer, but not for either distal colon or rectal cancer. The adjusted odds ratios (95% confidence intervals) of proximal colon cancer for the *CC* genotype were 0.63 (0.36-1.10) compared with the *AA* genotype, and 0.59 (0.37-0.96) compared with the *AA* and *AC* genotypes combined. A decreased risk of proximal colon cancer in relation to the *CC* genotype of *CYP7A1 A-203C*, which probably renders less activity of the enzyme converting cholesterol to bile acids, is new evidence for the role of bile acids in colorectal carcinogenesis. (Cancer Res 2005; 65(7): 2979-82)

Introduction

Colorectal cancer is one of the most common cancers in the world, accounting for nearly 10% of all incident cases of cancer (1). Japan has experienced a rapid increase in mortality from colorectal cancer in the past 50 years (2), and is currently among the countries with the highest incidence rates worldwide (3). Bile acids have long been implicated in the etiology of colorectal cancer. Primary bile acids such as cholic and chenodeoxycholic acids are excreted in the liver, and are degraded to secondary bile acids, mainly deoxycholic and lithocholic acids, by bacteria in the intestinal lumen. Animal studies showed that secondary bile acids promoted chemically induced colorectal cancer (4, 5), and recent *in vitro* studies have identified several molecular mechanisms of deoxycholic acid promoting colorectal carcinogenesis (6, 7).

Despite these experimental observations, epidemiologic evidence remains elusive regarding the role of bile acids in colorectal carcinogenesis. Fecal levels of secondary bile acids as well as of total bile acids are higher in populations at high risk of colorectal cancer (8, 9). Several case-control studies reported higher levels of secondary bile acids in the feces or sera in patients with colorectal cancer or adenomas as compared with those without these lesions (10–13), but the findings were not replicated in other studies (14–16). A prospective study reported a suggestive increase in the risk of colorectal cancer associated with a high ratio of serum deoxycholic to cholic acids (17). Another epidemiologic evidence is the increased risk of proximal colon cancer in individuals having the gallbladder removed (18, 19). Cholecystectomy results in increased fecal excretion of secondary bile acids, probably due to increase in the bile acid pool in the enterohepatic circulation and increased degradation of primary bile acids in the gut (20, 21).

Recent studies (22, 23), but not all (24), showed that a common genetic polymorphism of cholesterol 7α-hydroxylase (*CYP7A1 A-203C*) was associated with plasma total and low-density lipoprotein cholesterol concentrations, suggesting lower activity of the enzyme in individuals with the variant C allele. *CYP7A1* is the rate-limiting enzyme in the synthesis of bile acids from cholesterol in the liver, and thus may be an important determinant of not only plasma cholesterol levels but also bile acid production. This article examined the association between the *CYP7A1 A-203C* polymorphism and colorectal cancer in order to further clarify the role of bile acids in colorectal carcinogenesis.

Materials and Methods

A case-control study was designed to examine the relation of lifestyle factors and genetic susceptibility to the risk of colorectal cancer. Cases were recruited from eight large hospitals in the study area (Fukuoka City and three adjacent areas), and controls were randomly selected in the community by frequency-matching to the distribution of incident cases with respect to sex and 10-year age class. The study protocol was approved

Requests for reprints: Tomoko Hagiwara, Department of Preventive Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-6112; E-mail: thagi@med.kyushu-u.ac.jp.
©2005 American Association for Cancer Research.

261