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Loss of heterozygosity (LOH) in non-small cell lung cancer: difference between adenocarcinoma and squamous cell carcinoma

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Non-small cell lung cancer;
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Loss of heterozygosity (LOH);
Smoking habit

Summary *Background*: In non-small cell lung cancer, a loss of heterozygosity (LOH) is frequently observed; however, few studies have investigated the differences in the LOH status between adenocarcinoma and squamous cell carcinoma.

Patients and methods: In a consecutive series of 49 patients with adenocarcinomas and 22 patients with squamous cell carcinomas, the LOH in tumors was analyzed using polymerase chain reaction employing 5 fluorescence-labeled dinucleotide markers (D2S123, D5S107, D10S197, D11SS904, D13S175) and an autosequencer.

Results: LOH was more frequently observed in squamous cell carcinoma (20 of 22, 90%) than in adenocarcinomas (33 of 49, 67%) ($P = 0.0348$), and the number of LOH per patient was also higher in the patients with squamous cell carcinoma (2.2 ± 1.4) than in those with adenocarcinoma (1.5 ± 1.2, $P = 0.037$). In adenocarcinomas, the number of LOH per patients correlated significantly with the pack-year index, whereas the pathological stage significantly affected the number of LOH in squamous cell carcinomas.

Conclusion: The presence of LOH is relatively uncommon in adenocarcinoma of the lung; however, the incidence of LOH tends to be associated with the smoking status.

Introduction

The incidence and mortality associated with lung cancer have both increased worldwide during the

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last several decades.[1,2] The main reason for the increase in mortality due to lung cancer lies in the increase in the number of tobacco smokers.[3–5] Loss of heterozygosity (LOH) has been reported to be a basic genetic disorder for carcinogenesis which causes allelic losses at specific chromosomal loci of several recessive oncogenes (e.g., the FHIT gene at 3p14,[6] the p53 gene at 17p13,[7,8] and the CDKN2 at 9p21).[9] It was recently reported that a LOH at chromosomes 3p14, 9p21, and 17p13 was frequently observed in the bronchial epithelium of smokers,[10] and that the frequency of LOH was significantly associated with the incidence of carcinoma in situ.[11] Therefore, it is possible that the incidence of central-type lung cancer, mainly squamous cell carcinoma, might potentially be predicted by the frequency of LOH in bronchial epithelial cells.

In adenocarcinoma of the lung, the incidence of LOH has been reported to be less frequent than in squamous cell carcinoma,[12] however, the clinical significance of LOH is still unclear.

This study was conducted in order to elucidate the relationship between the frequency of LOH and clinicopathologic factors in lung adenocarcinoma in comparison to the same findings for squamous cell carcinoma.

Patients and methods

Patients

During the period between April 2000 and September 2001, a consecutive series of 71 patients with non-small cell lung cancer (NSCLC) underwent a surgical resection at the Second Department of Surgery, Kyushu University Hospital. The histologic diagnosis of the tumors was based on the criteria of the World Health Organization,[13] and the TNM stage was determined according to the criteria revised in 1997.[14] They included 49 adenocarcinomas and 22 squamous cell carcinomas. The age and gender distribution for each cell type is summarized in Table 1. Written informed consent was obtained from each patient to permit the study of excised tissue from surgical specimens. The Institutional Review Board of our university also gave its approval.

Extraction of genomic DNA from specimens

Both cancer tissue and the corresponding normal lung tissue were obtained from surgical specimens immediately after resection. The tissue specimens were placed in liquid nitrogen until used for analysis. The remaining specimens were routinely processed for histological examinations. Frozen tissue specimens were broken up in liquid nitrogen and lysed in digestion buffer (10 mM Tris–HCl; pH 8.0,0.1 M EDTA; pH 8.0, 0.5% SDS, 20 μg/ml paracreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, and then it was dissolved in 1 × TE (10 mM Tris-Cl (pH 7.5), 1 mM EDTA).

High resolution fluorescent microsatellite analysis (HRFMA)

Five dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175 were used as markers for analysis of MSI and LOH.[15] Using genomic DNA derived from tissue specimens, the 5 microsatellite sequences were amplified by polymerase chain reaction (PCR). Oligonucleotide primers corresponding to the microsatellite sequences[15] were synthesized and purified using high performance liquid chromatography. 5′ primers were labeled with the fluorescent compound, ROX (6-carboxy-x-rhodamine) or HEX (6-carboxy-2′, 4′, 7′, 4, 7-hexachloro-fluorescein). PCR reactions were performed using TAKARA *Taq* Reagent Kits

Table 1 Status of LOH and smoking in both types of lung cancer.

Factors	Adeno[a] ($N = 49$)	Squ ($N = 22$)	P-value
Age	65.5±11.0	66.2±8.1	0.78
Male/female	23/26	20/2	<0.01
Pathological state			
I	40 (81.6%)	8 (36.4%)	
II	3 (6.1%)	7 (31.8%)	
III	6 (12.2%)	7 (31.8%)	<0.01
Number of patients with at least one LOH	33 (67%)	20 (90%)	0.03
Mean number of LOH per patient	1.5±1.4	2.2±1.2	0.037
PYI	15.8±23.3	51.7±32.3	<0.01

[a]Adeno, adenocarcinoma; Squ, squamous cell carcinoma.

(TAKARA Co. Ltd, Tokyo, Japan) and analyzed using in the Perkin–Elmer GeneAmp PCR system 9600 or 2400 (Norwalk, CT). Each 50 μl reaction mixture contained 1x reaction buffer, 350 μM of each dNTP, 10 pmol of each primer, 2.5 U of the polymerase, and 25 μg of the genomic DNA. 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, one cycle at 72 °C for 10 min. Next 0.5 U of T4DNA polymerase was added to the mixture, followed by incubation at 37 °C for 10 min. Each 1.5 μl product was mixed with 0.5 μl loading buffer (blue dextran, 25 mM EDTA), 2.5 μl of formamide, and 0.5 μl of dH_2O. To compare the electrophoretic profiles of the two samples, 1.2 ml of ROX-labeled product and 0.3 ml HEX-labeled product were mixed together. Samples were denatured and loaded onto ABI 373A sequencer (Applied Biosystems, Foster City, CA). In each case, a size marker labeled with TMRA (*N,N,N',N'*-tetramethyl-6-carboxyrhodamine) was always electrophoresed in each lane in order to standarize the mobility of the sample. The running conditions were 1500 V, 20 mA, and 30 W for 5.5 h. The data were processed using the ABI software package from GeneScan (Applied Biosystems).

Detection of LOH

The method of detecting microsatellite alteration including microsatellite instability and LOH used herein has been described previously.[15] Briefly, for the detection of LOH, the fluorescence of a peak decreased more than that of the normal control when the LOH occurred in the amplified region of the genomic DNA derived from tumor tissue specimens as shown in Fig. 1.

Statistical analysis

The number of LOH detected among the 5 markers was compared among the subgroups, which were divided according to clinicopathologic factors, and differences were analyzed using Student's *t*-test. Each statistical difference was determined to be significant when the *P*-value was below 0.05. The correlation between the number of LOH and the pack-year index (PYI) was analyzed using Spearman's test.

Results

Differences in the clinicopathologic factors including the LOH status for the two cell types are summarized in Table 1. The proportion of cases in which LOH was detected by at least one out of the 5 markers was significantly lower in adenocarcinomas (33 of 49, 67%) than in squamous cell carcinomas (20 of 22, 90%) ($P = 0.03$). The mean number of LOH per patient was also lower in adenocarcinomas (1.5 ± 1.4) than in squamous cell carcinomas (2.2 ± 1.2) ($P = 0.04$). In patients with adenocarcinomas, the proportions of male patients and those with stage II/III disease, and the value of PYI (PYI means a value calculated with a formula: the number of cigarette packs per day divided by the years of smoking) were significantly lower.

Next, we investigated whether the differences in such factors affect any differences in the frequency

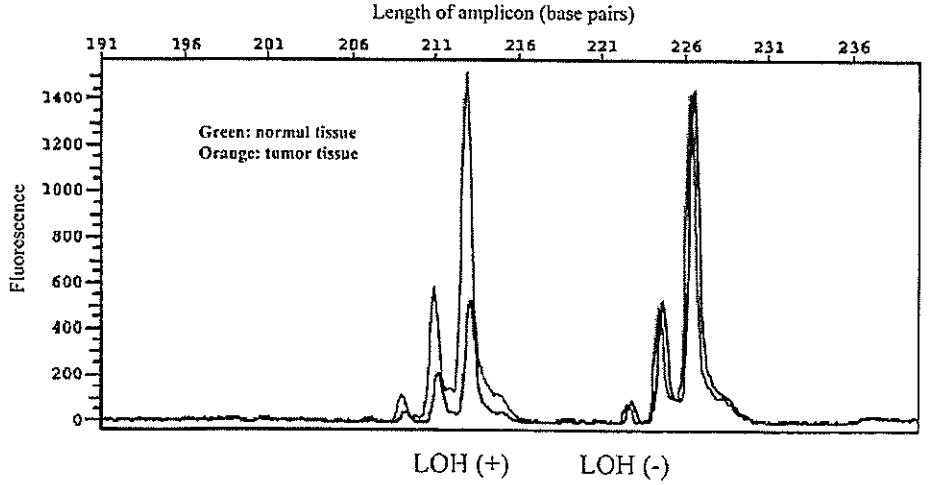

Figure 1 Detection of LOH. The fluorescence (*y*-axis) of two peaks at bp 210 and 213 decreased more than that in normal tissues, regarding the amplified product derived from the genomic DNA of tumor tissue using the marker D2S123. Green line and orange line represent normal tissue and tumor tissue, respectively.

of LOH among adenocarcinomas and squamous cell carcinomas. In patients with adenocarcinoma, the number of LOH showed a significantly higher increase with a high PYI (Table 2). The number of LOH for 10 heavy smokers (2.3±2.90) was significantly higher than in 28 non-smokers (1.178±1.19) ($P = 0.03$). No significant differences in the number of LOH for the subgroups divided by gender and pathological stage were observed. In patients with squamous cell carcinoma, those with stage IA disease showed a significantly lower number of LOH (1.00±0.82) than the other stages (stage IB or more) (2.44±1.10), whereas no differences were observed among the subgroups divided by gender and PYI (Table 3).

Discussion

In previous studies analyzing LOH in the tumor suppresser genes such as FHIT (3p14), p53 (17p13), CDKN2 (9p21), hMLH1 (3p21.3), or hMSH3 (5q11-13), the association between the incidence of LOH and tobacco exposure has been demonstrated in the bronchial epithelium of non-cancer bearing individuals and in lung cancer tissues.[10,12,16,17] Regarding adenocarcinomas, which arise from the peripheral airway, relative risk is actually associated with cigarette smoking although the risk is relatively lower than that seen in squamous cell carcinomas[18,19] although little is known about the relationship between smoking and genetic alterations in pneumocytes. The purpose of the present study was to elucidate any differences, in the LOH status between adenocarcinoma and squamous cell carcinoma, and to determine whether the incidence of LOH in adenocarcinoma was associated with the degree of tobacco exposure, using a unique automatic fluorescent system.

Squamous cell carcinomas clearly showed a higher incidence of LOH, and a higher number of LOH per patient than did adenocarcinomas. This finding was not inconsistent with the results of a previous report,[12] and one reason for this difference might be due to different levels of tobacco smoking (PYI) among the patients with the two cell types. However, a positive correlation between the PYI and the frequency of the LOH was observed in adenocarcinoma but not in squamous cell carcinoma. In patients with lung adenocarcinoma, smoking must also be a risk factor for carcinogenesis. The mean number of LOH in patients with adenocarcinoma and heavy smokers (2.3) was comparable to that of patients with squamous cell carcinoma (2.2), most of whom were smokers. The incidence of LOH was therefore strongly influenced by tobacco smoking.

In this study, the incidence of LOH in adenocarcinoma was lower than in squamous cell carcinomas (67% vs. 90%, P=0.03). This suggests that mechanisms other than LOH may also play some role in genetic disorder or the regulation of protein expression. An abnormality of mismatch repair enzymes has been reported to be a major cause of carcinogenesis in colorectal cancer.[20] Our detection system for the microalteration of genes also demonstrated microsatellite instability,[21] which may reflect a mismatch repair disorder; however, only 2 patients out of the 49 adenocarcinomas showed microsatellite instability in this study (data not shown). Recently, methylation of promotor regions for tumor suppressor genes has attracted attention as a possible cause of several types of cancers.[22] In lung adenocarcinoma, such a genetic disorder should thus be examined in this future.

More recently, Powell et al.[23] reported that LOH of 3p, 8p, 9p, 10p and 18q in lung adenocarcinoma

Table 2 The relationship between the frequency of LOH and other clinicopathologic factors in adenocarcinomas.

Variables	Number of LOH	*P*-value
Gender		
Male ($N = 23$)	1.57±1.27	0.65
Female ($N = 26$)	1.39±1.45	
Pathological stage		
IA ($N = 33$)	1.27±1.23	0.15
Others[a] ($N = 16$)	1.88±1.50	
PYI 0 ($N = 28$)	1.18±1.19	0.03
Less than 40 ($N = 11$)	1.46±1.29	
40 or more ($N = 10$)	2.30±2.90	

[a]Others included 7 stage IB, 1IIA, 2IIB and 6 IIIA (2T1N2M0 and 4T2N2M0).

Table 3 The relationship between the frequency of LOH and other clinicopathologic factors in squamous cell carcinomas.

Variables	Number of LOH	*P*-value
Gender		
Male ($N = 20$)	2.50±3.54	0.70
Female ($N = 2$)	2.15±0.93	
Pathological stage		
IA ($n = 4$)	1.00±0.82	0.02
Others[a] ($N = 18$)	2.44±1.10	
PYI Less than 50 ($N = 12$)	2.08±1.17	0.68
50 or more ($N = 10$)	2.30±1.10	

[a]Others included 4 stage IB, 3IIA, 4IIB 7 IIIA (1T1N2M0, 4T2N2M0 and 1T3N1M0).

tissues was more frequently detected in nonsmokers than in smokers, thus suggesting that lung carcinogenesis differs in among nonsmokers and smokers. The reason for such inconsistency between their results and ours might be due to the fact that the markers and detection system used by them were different from those in the present study.

In conclusion, the presence of LOH was less common in adenocarcinoma than in squamous cell carcinoma of the lung, however, its occurrence was significantly affected by a patient's tobacco smoking history for both types of lung cancer.

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特 集 腹膜転移の治療

腹腔細胞診陽性例の臨床的意義

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Evaluation of Positive Cases with Peritoneal Lavage Cytology in Gastric Cancer: Manabu Yamamoto[*1,2], Koujiro Mashino[*1,2], Kotaro Shibahara[*2], Eiji Oki[*1], Yoshihiro Kakeji[*1], Hideo Baba[*3] and Yoshihiko Maehara[*1] ([*1]*Dept. of Surgery and Sciences, Graduate School of Medical Sciences, Kyushu University*, [*2]*Dept. of Surgery, Oita Prefectural Hospital*, [*3]*Dept. of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University*)

Summary

Peritoneal dissemination with advanced gastric cancer is of significant problem. Peritoneal lavage cytology has been an effective method for the detection of early peritoneal dissemination since 1999.

The accurate evaluation of peritoneal lavage cytology is unclear except for the same prognosis of peritoneal dissemination. We examined the clinical findings and the prognosis with positive cases in peritoneal lavage cytology. The prognosis of cases with P 1 CY 1 or P 2 P 3 group was poorer than in the P 0 CY 1 or P 0, CY 1 group. We thus review the evaluation of peritoneal lavage cytology with gastric cancer in the Japanese and English literature.

In addition, we describe the diagnosis of early peritoneal dissemination using peritoneal lavage tumor markers or molecular markers of peritoneal lavage. Key words: Gastric cancer, Peritoneal lavage cytology, Peritoneal dissemination, Corresponding author: Dr. Manabu Yamamoto, Department of Surgery, Oita Prefectural Hospital, 476 Bunyo, Oita 870-8511, Japan

要旨 進行胃癌において腹膜播種再発は重要な課題である。術中腹腔細胞診は，早期に腹膜播種を発見できる有効な検査法であるものの，その意義については様々な論議があり一定した見解を得ていない。今回，当科における腹腔細胞診陽性例の臨床病理学的特徴および予後を検討した。その結果，腹膜播種および腹腔細胞診陽性例をP0CY1，P1CY0，P1CY1，P2P3(胃癌取扱い規約12版によるP1，P2，P3に従った)の4群に分け検討したところ，予後について有意ではないが，P1CY1とP2P3はP0CY1とP1CY0に比べ予後不良の傾向を認めた。最近の国内外の報告と併せ，腹腔細胞診陽性例の臨床的意義とその治療法を述べる。さらに，腹腔洗浄水中の腫瘍マーカーや遺伝子診断を用いた潜在的腹膜播種検出の有用性につき概説する。

はじめに

胃癌の腹膜播種は再発形式のなかで最も多く，その治療は困難を極め予後不良である。腹膜播種の経路としては漿膜面からの播種性転移が最も多いとされ，血行性，リンパ行性も考えられている。肉眼上の腹膜播種巣以外に術中腹腔洗浄細胞診を施行することで，腹膜播種を早期に診断することができると考えられている。そのため，胃癌取扱い規約13版より進行胃癌の手術施行時には，術中腹腔洗浄細胞診が施行されるようになり，現在多く施設で行われている。

I．胃癌に対する腹腔細胞診施行の経緯

1961年にMooreら[1]が悪性腫瘍に対して腹腔細胞診の意味を報告し，その後1980年に山岸ら[2]が進行胃癌の腹腔遊離癌細胞の意義を報告している。そのなかで腹膜

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播種と細胞診陽性率は腹膜播種が進むにつれて陽性率が高くなり，症例が少ないものの細胞診陽性症例はすべて腹膜播種を発症したと述べている。その後，1990年代に入り数か所の施設で腹腔細胞診が施行され，そのデータが蓄積された。寺本ら[3]は，術中細胞診陽性例の解析にて細胞診陽性例の予後がP1（胃癌取扱い規約12版に従った）症例の予後と同等であったと報告している。また，白川ら[4]は細胞診陽性例ではP0とP1症例の予後は差を認めなかったものの，漿膜浸潤胃癌では細胞診陽性例と陰性例で予後の相違を認めた。Bando ら[5]は，1,297例の胃癌患者において腹腔細胞診を行ったところ，腹腔細胞診陽性例は全体の2%であり，予後は腹腔細胞診陽性例で陰性例に比べ有意に不良であった。また，腹膜播種を認めた症例のうち，腹腔細胞診陽性例は陰性例に比べ明らかに予後不良であった。多変量解析において腹腔細胞診陽性は独立した予後因子であった。これらの報告により，胃癌取扱い規約13版では，腹腔細胞診陽性例は腹膜播種（P1）と同等の予後因子であるとしてstage IVに分類された。

II. 進行胃癌における腹腔細胞診の現況

当科において1996年から1999年までに胃癌にて手術を施行した228例に対し，すべての症例に腹腔細胞診を施行し，その結果腹腔細胞診陽性率は26例（11.5%）であった。臨床病理学的検討では，腹腔細胞診陽性例は原発巣の深達度，リンパ節転移，肉眼的腹膜播種，リンパ管浸潤，脈管浸潤と相関を認めた（表1）。腹膜播種および腹腔細胞診陽性例をP0CY1，P1CY0，P1CY1，

表1 腹腔細胞診陰性および陽性例の臨床病理学的特徴

因子	陽性例 (26例)	陰性例 (202例)	p value
年齢	60.5±13.6	61.4±12.2	NS
性別			
男性	13	115	NS
女性	13	87	
組織型			
分化型	7	89	NS
未分化型	19	113	
深達度			
T1	1	114	
T2	4	56	<0.0001
T3	21	32	
リンパ節転移			
+	24	67	<0.0001
−	2	135	
肉眼的腹膜播種			
P0	8	196	
P1	12	4	<0.0001
P2, 3	6	2	
リンパ管浸潤			
+	18	80	
−	2	118	<0.0001
不明	6	4	
脈管浸潤			
+	9	43	
−	11	155	<0.02
不明	6	4	

腹膜播種の程度は胃癌取扱い規約第12版に従った（P1，P2，P3）。
NS: not significant

表2 腹膜播種および細胞診陽性例における背景因子の比較

因子	(A) P0CY1	(B) P1CY0	(C) P1CY1	(D) P2, P3	p value
年齢	62.6±15.8	43.5±4.8	53.6±11.8	62.5±12.3	(A) (B) (B) (D) <0.02
性別					
男性	4	2	5	8	NS
女性	4	2	3	4	
組織型					
分化	3	1	1	5	NS
未分化	5	3	7	7	
深達度					
T1	1	0	0	0	
T2	2	0	2	2	NS
T3	5	4	6	10	
リンパ節転移					
+	7	3	7	11	NS
−	1	1	1	1	

腹膜播種の程度は胃癌取扱い規約第12版に従った（P1，P2，P3）。
NS: not significant

P2P3の4群に分け検討した（胃癌取扱い規約12版によるP1, P2, P3に従った）。臨床病理学的背景因子を表2に示した。各々の背景因子には年齢以外には差を認めなかったものの，予後について検討を行ったところP1CY1とP2P3はP0CY1とP1CY0に比べ予後不良の傾向を認めた（図1）。

さらにわれわれは，腹腔洗浄中の腫瘍マーカー(CEA, CA 125, CA 19-9）を測定した。その結果，腹膜播種もしくは細胞診陽性症例に腹腔洗浄中のCEAがよく相関し，腹腔洗浄中のCEA高値群が，低値群に比べ有意に予後不良であり(図2)[5]，腹膜播種再発が有意に多く認められた。腹膜播種もしくは細胞診陽性症例に対する多変量解析では，漿膜浸潤，腹腔洗浄中のCEAおよびCA 125が独立した因子であった[6]。

III. 考察—今後の方向性—

腹腔細胞診陽性例の予後に関して，太田ら[7]は肉眼的腹膜播種症例に比べP0CY1症例が予後良好であったと報告している。また，谷澤ら[8]は逆に腹膜播種P1, P2, P3に比べP0CY1症例の3年生存率が低かったと報告した。さらに宮代ら[9]は，腹腔細胞診陽性例を癌細胞の密度によって2群に分け検討したところ，高密度群が低密度群に比べて予後不良であったと述べている。また，

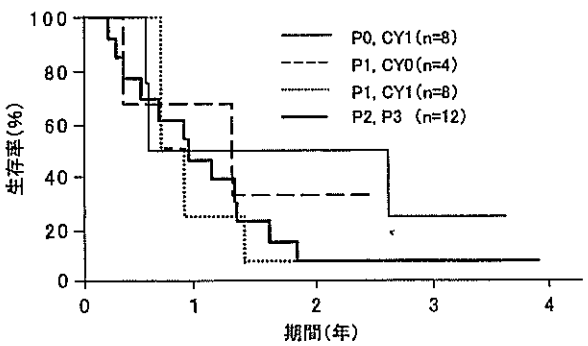

図1 腹膜播種および腹腔細胞診陽性例の予後比較

Hayesら[10]は漿膜浸潤を認めた場合，細胞診陽性と陰性では，その予後が大きく異なると結論付けている。今回のわれわれの検討では，P2P3に比べP0CY1もしくはP1CY0が予後良好の傾向を認めた。P2P3に比べP1CY0, P0CY1は予後良好であることは確かであるが，P1CY1がP2P3もしくはP1CY0, P0CY1に比べどの程度の予後となるかは今後の報告を待たなければならない。しかし，現時点でCY1というだけで手術を行わない理由にはならないと考えられる。

腹腔洗浄細胞診中の腫瘍マーカーを測定した報告は，1991年にAsaoら[11]が腹腔洗浄細胞診中のCEAを測定した結果，根治手術を施行された症例のなかで，腹腔洗浄細胞診中のCEA高値群は低値群に比べ予後不良であり，腹腔洗浄細胞診中のCEA高値群で腹膜播種再発を多く認めたと報告している。その後，われわれは腹腔洗浄細胞診中のCEAおよびCA 125が，腹膜播種の予測因子として重要であると報告した[6]。最近では，腹膜播種の早期診断は分子生物学的手法を用いて解析している施設がある。藤原ら[12]は，腹腔洗浄細胞診を用いて定量的遺伝子診断を行っている。漿膜浸潤胃癌に対して腹腔洗浄液の遺伝子診断（CEAとサイトケラチン20をマーカーとした）を行い，陽性例に術前化学療法を施行し2/3の症例で陰性化したという。さらにKoderaら[13]は，腹腔洗浄液の遺伝子診断（CEAをマーカーとした）を用いて予後に対する多変量解析を行ったところ，腹腔洗浄液の遺伝子診断のほうが腹腔洗浄細胞診よりも強く予後因子に関与していると述べている。また米村ら[14]は，同様の方法で異なったマーカー（MMP-7）を使用し，腹腔細胞診陽性かつMMP-7陽性の場合に，腹膜播種再発を起こす可能性が高いとしている。今後はさらに特異性の高いマーカーを検索する必要がある。

胃癌再発形式は，腹膜播種性転移が最も高頻度である。しかし，その予防・治療に関しては満足した結果が得られていない。米村ら[15]は，peritonectomyを行った後，腹

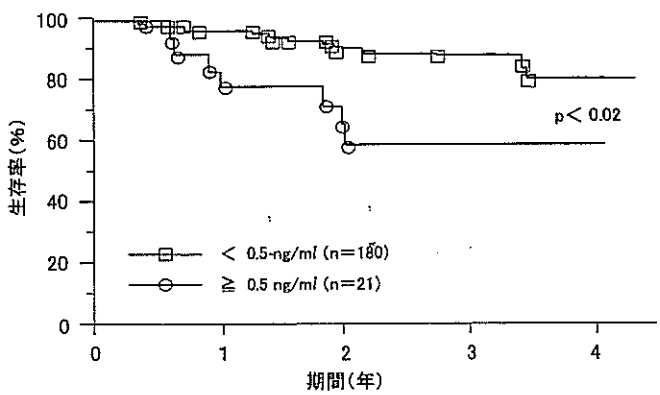

図2 腹腔洗浄水中のCEA高値群と低値群の予後比較[5]

腔内の温熱化学療法と全身化学療法を行い良好な成績を報告している。また，伏田ら[16]はpaclitaxelを腹腔内投与し，その効果を検討中である。われわれは，原則的に全身化学療法を施行している。特にTS-1とtaxane系化学療法剤は，他の化学療法剤に比べ腹膜播種に対して効果が高いため，それらの化学療法剤を中心に治療を行っている。また，われわれは二次治療としてweekly paclitaxel療法を行い，奏効率は17.6%であるものの腹膜播種による腹水消失が42.9%と高率であった[17]。細胞診陽性のみの症例に対する現時点での治療法としては，やはり化学療法が第一選択と考えられる。化学療法には，全身投与もしくは腹腔内投与がある。Fujimotoら[18]は，細胞診陽性例にMMCとOK-432を腹腔内投与し，その有効性を報告した。しかし，一般的には腹腔内投与のみでは腹水コントロール効果はあるものの予後向上にはならないと考えられている。また，稲田ら[19]はTS-1を含め全身化学療法を施行し，長期生存例が得られた症例が存在したことを報告した。現時点で有効性が確立していないが，細胞診陽性のみの症例に対するTS-1の効果は高いと考えられる。

まとめ

腹腔細胞診陽性症例の臨床的意義を述べてみた。細胞診陽性症例は腹膜播種の早期の段階であり，その予後もP1と同等である。細胞診陽性症例に対する治療は全身化学療法と考えられるが，近年新規抗癌剤の登場により細胞診陽性例の予後向上の可能性が期待できるようになってきた。

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

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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

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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.

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†

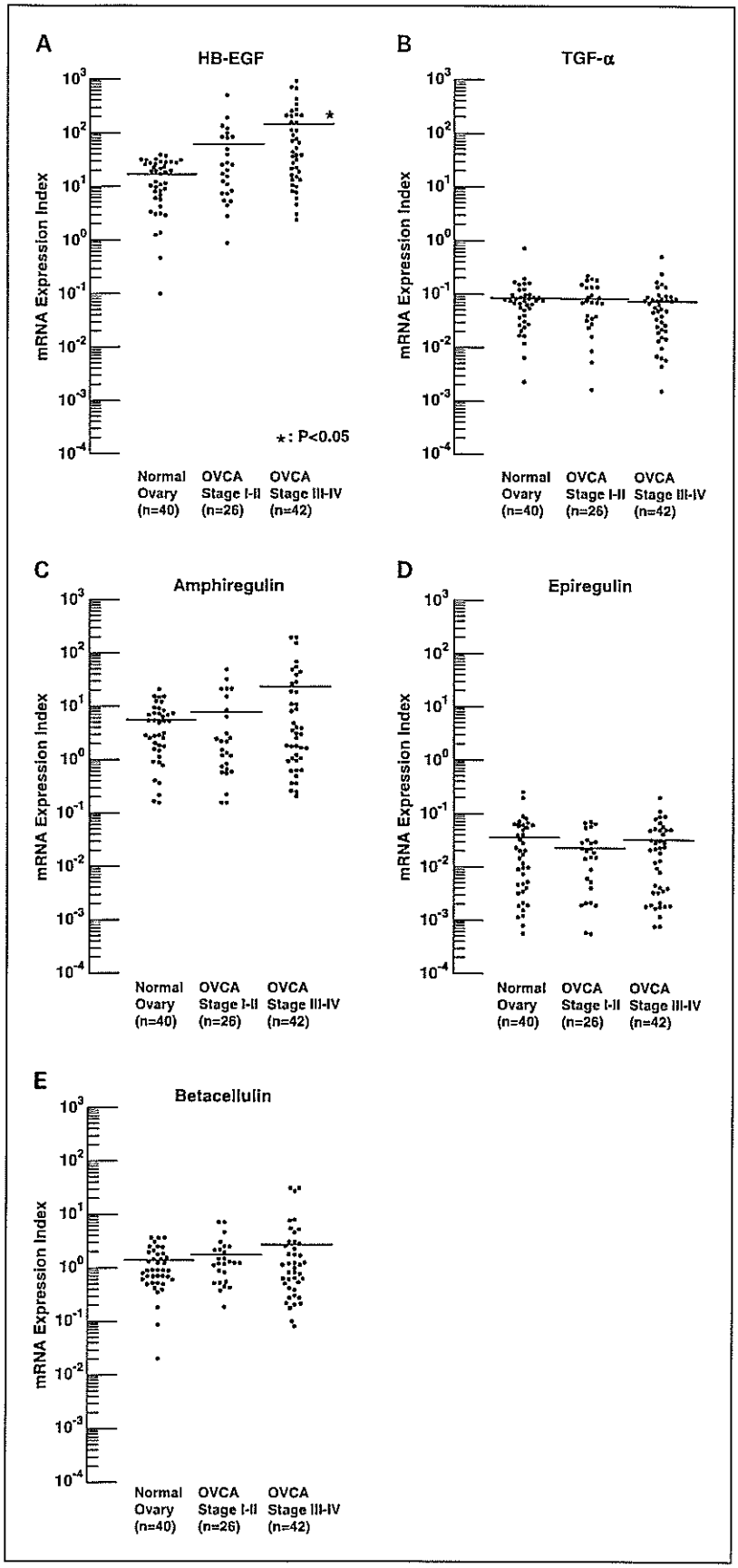

Fig. 1. Differences in expression of EGF ligands between normal ovaries and ovarian cancer (*OVCA*). mRNA expression index of HB-EGF (*A*), TGF-α (*B*), amphiregulin (*C*), epiregulin (*D*), and betacellulin (*E*) in patients with normal ovaries, early ovarian cancer (stages I-II), and advanced ovarian 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

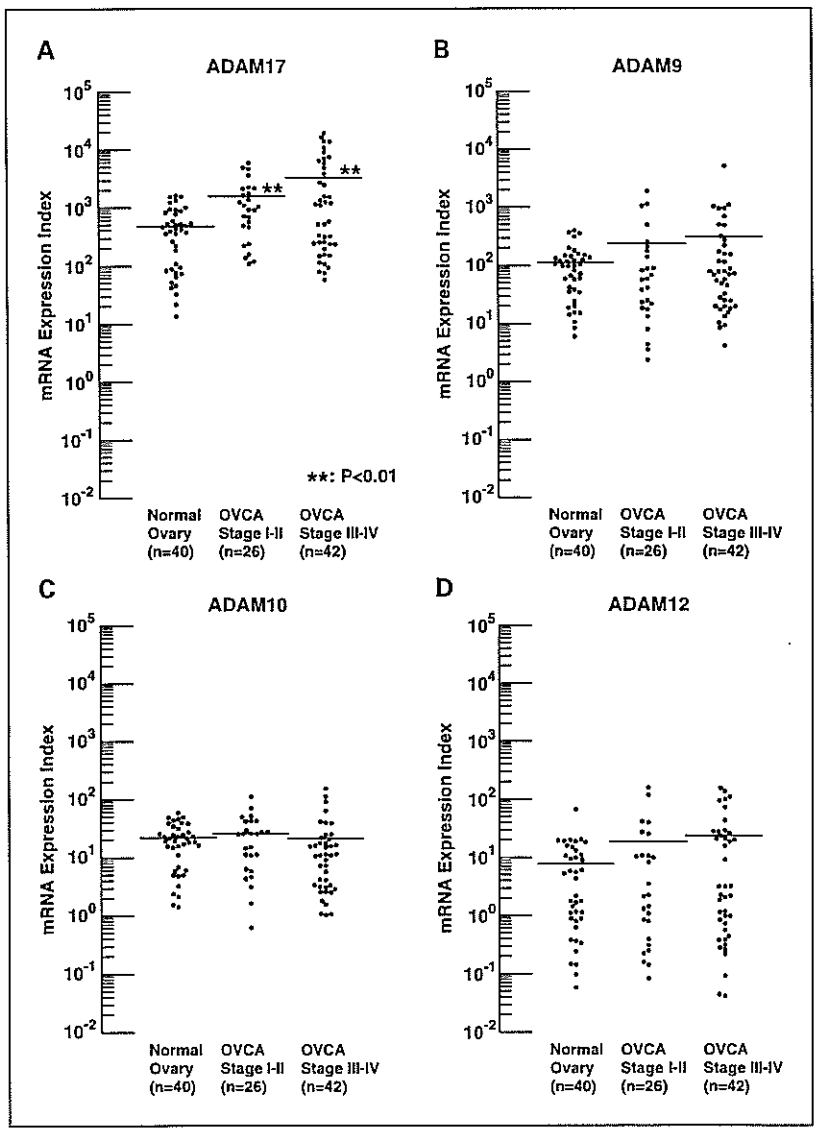

Fig. 2. Differences in expression of ADAM family members in normal ovaries and ovarian cancer. mRNA expression index of ADAM 17 (*A*), ADAM 9 (*B*), ADAM 10 (*C*), and ADAM 12 (*D*) in patients with normal ovaries, early ovarian cancer (stages I-II), and advanced ovarian cancer (stages III-IV). A line 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.

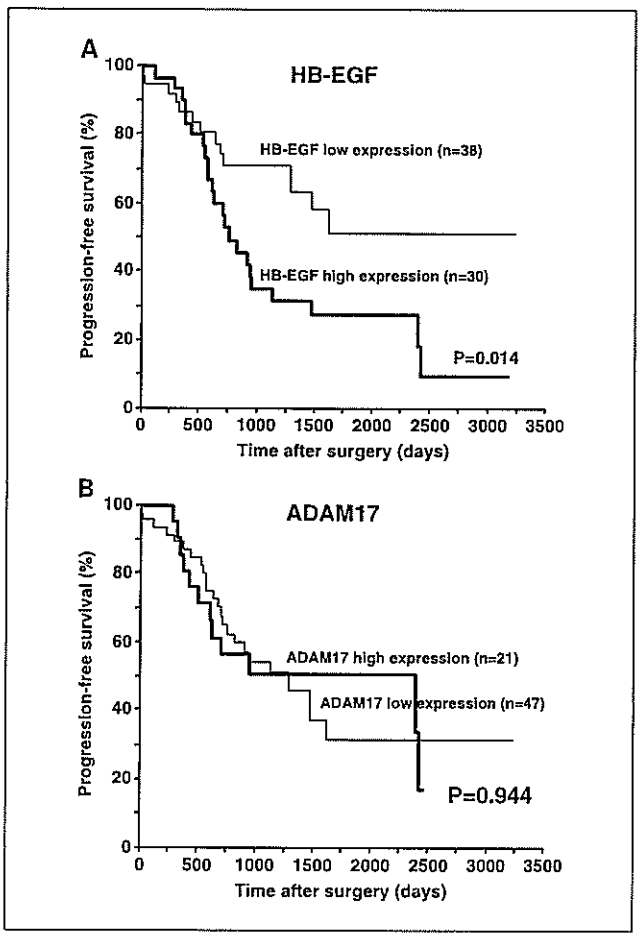

Fig. 3. Clinical significance of HB-EGF and ADAM 17 expression in ovarian cancer. Progression-free survival of 68 patients with ovarian cancer in relation to the tumor HB-EGF expression status (*A*) and tumor ADAM 17 expression status (*B*). *Ps* were determined using the log-rank test.

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

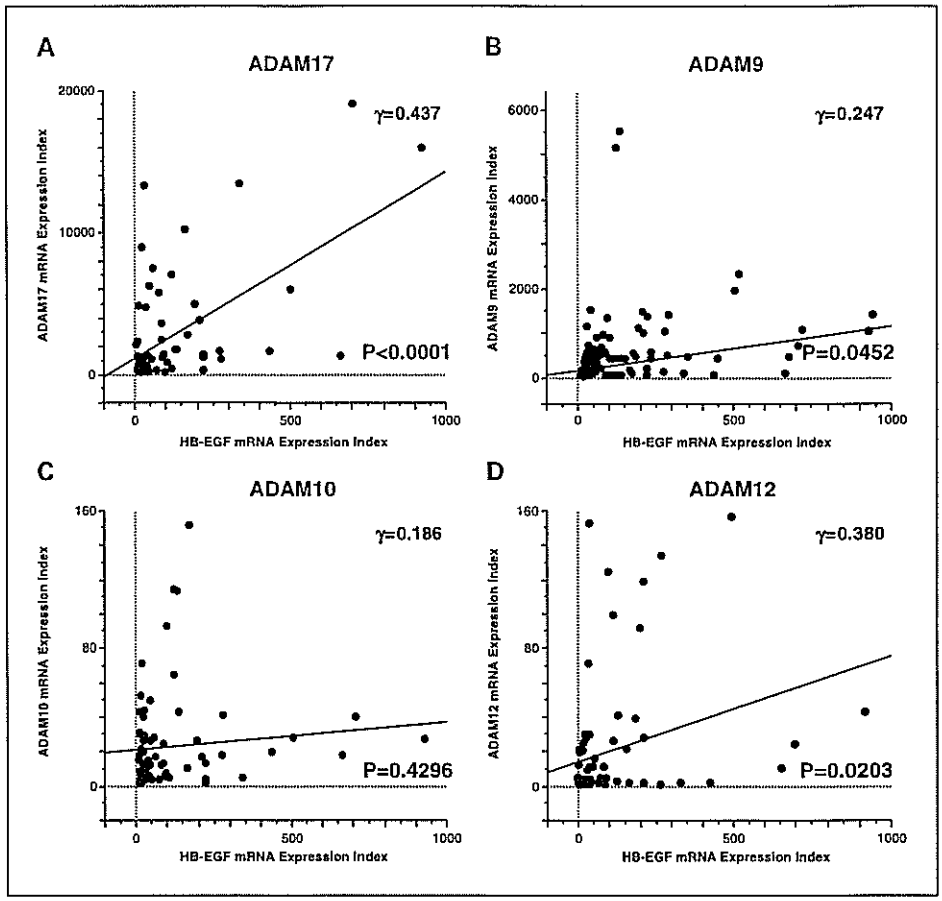

Fig. 4. Spearman's correlations between the mRNA expression indices of HB-EGF and ADAM family members in patients with ovarian cancer. The ordinate and abscissas indicate the mRNA expression index of HB-EGF and ADAM 17 (*A*), ADAM 9 (*B*), ADAM 10 (*C*), and ADAM 12 (*D*), respectively, in patients with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

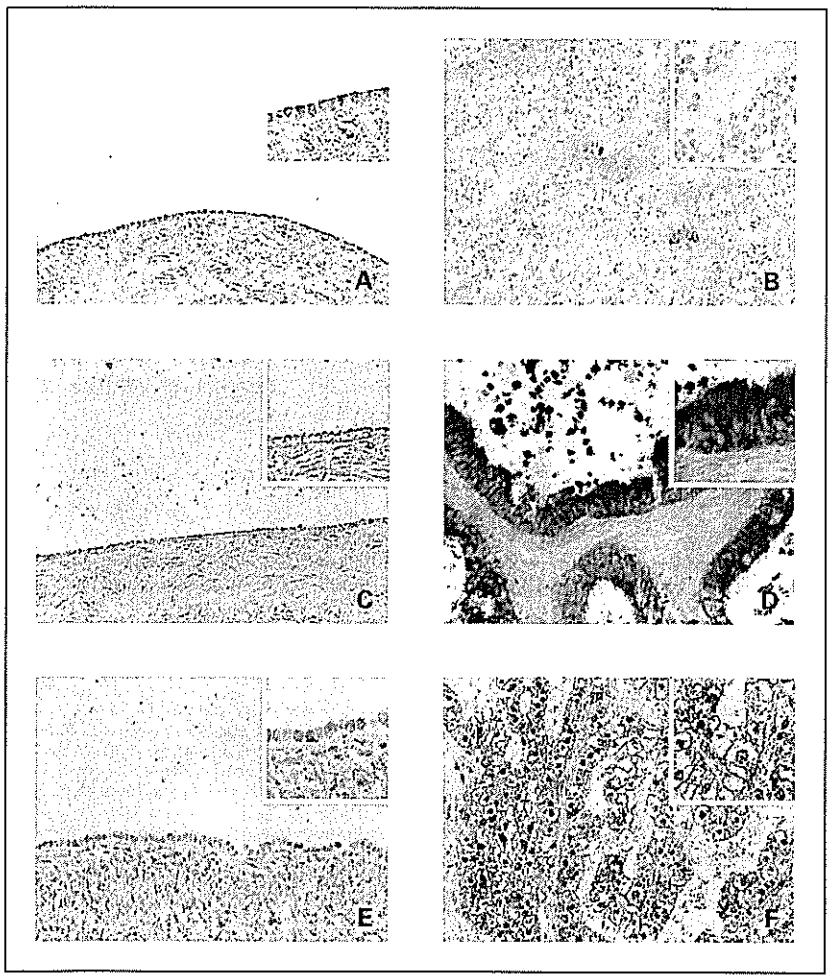

Fig. 5. Immunohistochemical staining and *in situ* hybridization of HB-EGF. No definite expression of HB-EGF protein (*A*) or mRNA (*C*) is present in a patient with normal ovaries. In a patient with advanced ovarian cancer, positive immunostaining of HB-EGF is observed in interstitial tissues surrounding 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.

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.

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Genetic mutual relationship between PTEN and p53 in gastric cancer

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Abstract

Both PTEN (encoding phosphate and tensin homologue) and p53 are known as cancer suppressor genes, and they are assumed that their gene mutations and loss of heterozygosity (LOH) occur frequently in various types of carcinoma. In the present study, we investigated both the p53 mutation and LOH of PTEN in 113 gastric cancer patients. We observed the LOH of PTEN in 11.1% of the patients with normal p53s and 46.2% of the patients with p53 gene mutations. The result that LOH of PTEN was frequently observed in the cases with p53 gene mutations and other data in this study suggested that both PTEN and p53 have complimentary roles in gastric carcinoma development.

Keywords: PTEN; p53; Gastric cancer; Loss of heterozygosity

1. Introduction

The encoding phosphate and tensin homologue (PTEN) gene, a tumor suppressor candidate, is located on chromosome 10q23 and has an extensive homology with the cytoskeletal proteins auxilin and tensin [1,2]. PTEN mutations have been frequently observed in various neoplasms, including glioblastoma, melanoma, prostate cancer, and breast cancer [1–7]. In glioblastoma, melanoma, and prostate cancer, PTEN mutations and allelic deletions are observed during the late stages, while in thyroid and endometrial cancers, PTEN mutation alterations are found during the early stages and include endometrial hyperplasia and benign thyroid tumors [3–6,8,9].

PTEN encodes an enzyme with phosphatase activity towards the acidic protein substrates and the lipid second messenger, phosphatidylinositol-3,4,5-triphosphate (PIP3) [10]. The phosphatase activity of PTEN is crucial in controlling the phosphatidylinositol-3

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