Expression of ACP6 is an independent prognostic factor for poor survival in patients with esophageal squamous cell carcinoma

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Received December 2, 2005; Accepted February 7, 2006

Abstract. ACP6 (acid phosphatase 6, lysophosphatidic) is a lysophosphatidic acid (LPA)-specific phosphatase that hydrolyzes LPA to monoacylglycerol and is involved in lipid metabolism in the mitochondria. Its role in oncogenesis and cancer progression has not been studied. In this study, we examined the expression of ACP6 mRNA and evaluated its clinical significance in esophageal squamous cell carcinoma (ESCC). Expression of ACP6 mRNA was quantified by real-time reverse transcription polymerase chain reaction using the LightCycler in 70 esophageal ESCC specimens and their paired normal esophageal mucosa. The data were analyzed with reference to clinicopathological factors.

ACP6 mRNA expression in esophageal cancer tissue was significantly lower than that in corresponding normal esophageal mucosa (P=0.0301). Among the esophageal cancer tissues, ACP6 mRNA expression significantly correlated with local tumor invasion (T factor, P=0.0461) and lymph node metastasis (P=0.0128). Furthermore, low ACP6 mRNA expression was associated with a significantly shorter survival time compared with high expression (log-rank test, P=0.0358). In multivariate analysis, ACP6 mRNA expression emerged as a significant independent factor (P=0.0148). Impaired ACP6 expression may lead to more aggressive invasion of ESCC, and ACP6 mRNA expression level could be an independent prognostic factor for patients with ESCC.

Introduction

ESCC is the sixth most frequent cancer in Japan, and the number of deaths due to this cancer has been steadily increasing. ESCC is often diagnosed at an advanced stage and the prognosis remains poor, prompting the search for new treatment strategies. Although preoperative chemotherapy and chemoradiation therapy are currently used for patients with advanced stage ESCC, their results are not satisfactory. Even among patients with early-stage disease, we have observed many who develop locally recurrent tumors or distant metastases within a short period after curative surgery. Molecular biological studies have revealed that esophageal cancer is caused by the accumulation of multiple genetic defects in dominant oncogenes and tumor suppressor genes. We have also reported that the expression of survivin (1), DFF45/CAD (2), PTG (3), chfr (4), PPARy (5) and ERCC3 (6) correlates with the prognosis of patients with esophageal carcinoma.

Lysophosphatic acid (LPA) is a growth factor-like phospholipid present in serum and many other biological fluids. LPA mediates the most important action and cellular response, including platelet aggregation, smooth muscle contraction, regulation of cell proliferation, transcellular migration and cell survival. LPA also plays important roles in the metabolism of phospholipids inside cells, and is an intermediate lipid in the pathway of phosphatidic acid (PA) synthesis (Fig. 1). LPA and PA are synthesized on the cytosolic side of the mitochondrial outer membrane (MOM). LPA is synthesized from sn-glycerol-3 phosphate (G-3-P), which is an essential phospholipid for the function of mitochondria.

Acid phosphatase 6, lysophosphatidic (ACP6) is an LPA-specific phosphatase that hydrolyzes LPA to monoacylglycerol (MAG) (9,10). ACP6 is also called LPAP, ACPL1 and PACPL1. The structure of this enzyme shows homology to acid phosphatases including human prostatic acid phosphatase, which also has LPA phosphatase activity and degrades LPA (11). Its role in oncogenesis and cancer progression has not been studied.

In this study, we investigated the ACP6 mRNA expression level in ESCC and its paired normal esophageal mucosa by real-time reverse transcription polymerase chain reaction (RT-PCR) using LightCycler. We analyzed the results in reference to the patients' clinicopathological characteristics and effect on the prognosis of ESCC patients.
Materials and methods

Cell lines and tissue samples. Samples were obtained from 70 patients with primary esophageal squamous cell carcinomas who had undergone radical esophagectomy at the Department of Surgery II, Nagoya City University Medical School between 1996 and 2001. The study design was approved by the IRB of our university, and written consent was obtained from all patients. Tumors were classified according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus (12). There were 56 males and 14 females, and the mean age was 61.9±8.9 years (range, 47-80 years). All samples were frozen immediately in liquid nitrogen and stored at -80˚C until use. The characteristics of the 70 patients with ESCC are shown in Table I.

The TE series esophageal cancer cell lines were purchased from the Japanese Cancer Research Resources Bank (JCRB). The SV40-immortalized esophageal cell line, Het-1A, was purchased from the American Type Culture Collection (ATCC). Esophageal cancer TE cells were plated in tissue culture dishes and grown in RPMI-1640 medium (Sigma) with 10% fetal bovine serum (JRH Bioscience) at 37˚C in a humidified atmosphere of 95% air and 5% CO2. Het-1A cells were grown in LHC-9 serum-free medium (Biofluids, Rockville, MD) in tissue culture dishes at 37˚C in a humidified atmosphere of 95% air and 5% CO2.

RNA extraction and RT-PCR analysis. Total RNA was extracted from esophageal cancer tissue, and its corresponding normal esophageal mucosa was taken from apparently noncancerous mucosa as far away from the tumor as possible, using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Total RNA from the cell lines was similarly extracted. The concentration of total RNA was adjusted to 200 ng/ml using a spectrophotometer. Reverse transcriptional reaction was carried out at 42˚C for 90 min and 95˚C for 5 min followed by incubation at 72˚C for 15 min, using 1 μg of total RNA, 0.5 μg of oligo(dT) primer and Superscript II enzyme (Gibco BRL, Gaithersburg, MD). All samples were quantified after PCR amplification using a Lightcycler-Faststart DNA Master Sybr-Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). We used the following set of primers: forward primer ACP6-F, 5-AATGTTTGCCTTGGGAGAGA-3; and reverse primer ACP6-R, 5-AGCAGCTTTGGTAGTTGGGA-3 (the size of the product was 233 bp). The PCR protocol was initial denaturation at 95˚C for 10 min, followed by 45 cycles at 95˚C for 10 sec, annealing at 60˚C for 5 sec, and extension at 72˚C for 10 sec. The PCR product was quantified via the intensity of Sybr-Green I at 72˚C.

Statistical analysis. The relative mRNA expression levels (ACP6/GAPDH) were calculated from quantified data in
reference to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as means ± SD. Statistical analysis was performed using the Stat-View software package (Abacus Concepts, Berkeley, CA). The Wilcoxon signed-ranks test, Mann-Whitney U test, and Kruskal-Wallis test were used to evaluate the significance of differences in expression levels of ACP6 mRNA. The survival of ESCC patients after surgery was examined using the Kaplan-Meier method, and survival times were compared using the log-rank test. Multivariate analysis was performed using the Cox regression model and logistic multivariate regression model. In all analyses, P<0.05 was considered statistically significant.

Results

ACP6/GAPDH mRNA expression in 15 esophageal cancer cell lines was examined. The levels of expression in all esophageal cancer cell lines were lower than the expression in Het-1A, a normal esophageal cell line (Fig. 2). ACP6 mRNA expression was detectable in all ESCC tissue and noncancerous esophageal mucosa. The levels of expression in ESCC tissue were significantly lower than those of the corresponding normal esophageal mucosa (0.291±0.256 vs. 0.213±0.146, P=0.0301; Wilcoxon signed-ranks test) (Fig. 3 and Table II). We examined the relationship between ACP6 mRNA expression in 70 ESCC samples and the patients' clinicopathological factors (Table I). Of the 70 ESCC samples studied, there were no significant differences in ACP6 mRNA according to age, gender, tumor cell differentiation, and stage. ACP6 mRNA expression levels in patients with ESCC varied significantly according to the tumor status (T factor, P=0.0461; Kruskal-Wallis test) (Table I and Fig. 4a). ACP6 mRNA expression levels in patients with locally invasive T3-4 tumors (0.201±0.147) were significantly lower than those in less aggressive T1-2 tumors (0.296±0.120) (P=0.021; Mann-Whitney U test). ACP6 mRNA expression levels in patients who had lymph node metastasis (0.185±0.118) were significantly lower than those in ESCC patients without lymph node metastasis (0.309±0.191) (P=0.0128; Mann-Whitney U test) (Table I and Fig. 4b).

We investigated the correlation between ACP6 mRNA expression levels and the survival of ESCC patients after surgery (median follow-up, 23 months). Patients who had low ACP6 mRNA expression levels [indicated as the ratio of ACP6 mRNA expression in the tumor to that in normal esophageal mucosa (T:N ratio) <0.85, n=38] had a significantly shorter survival (13.0±1.862 months) after surgery compared with patients who had high ACP6 mRNA expression levels (T:N ratio >0.85, n=32; 31.0±10.16 months) (P=0.0358, log-rank test) (Fig. 5). Among the clinicopathological factors, univariate analysis showed that local invasiveness (risk ratio, 11.788; P<0.001), lymph node metastasis (risk ratio, 5.48; P=0.0012), and ACP6 expression (T:N ratio) (risk ratio, 1.931; P=0.0358) were statistically significant prognostic factors.

Table II. Univariate analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Risk ratio</th>
<th>95% CI</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Age at surgery</td>
<td></td>
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</tr>
<tr>
<td>&lt;65 years</td>
<td>1</td>
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<td></td>
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<tr>
<td>&gt;65 years</td>
<td>1.469</td>
<td>0.798-2.703</td>
<td>0.2167</td>
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<tr>
<td>Gender</td>
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</tr>
<tr>
<td>Female</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>1.107</td>
<td>0.528-2.322</td>
<td>0.7875</td>
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<tr>
<td>Histological grade</td>
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<td></td>
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</tr>
<tr>
<td>Moderate-poor</td>
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<tr>
<td>Well</td>
<td>1.603</td>
<td>0.847-3.032</td>
<td>0.1171</td>
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<td>Primary tumor</td>
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<td></td>
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<tr>
<td>T1-2</td>
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<tr>
<td>T3-4</td>
<td>11.788</td>
<td>2.835-49.019</td>
<td>&lt;0.0010</td>
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<td>Lymph node metastasis</td>
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<td>Negative</td>
<td>1</td>
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<tr>
<td>Positive</td>
<td>5.48</td>
<td>1.687-17.798</td>
<td>0.0012</td>
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<td>ACP6 expression (T/N)</td>
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<tr>
<td>High</td>
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<tr>
<td>Low</td>
<td>1.931</td>
<td>1.023-3.644</td>
<td>0.0358</td>
</tr>
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</table>

CI, confidence interval.
factors (Table II). Multivariate analysis revealed that the extent of the primary tumor (P=0.0026), lymph node metastasis (P=0.0269), and ACP6 expression (T:N ratio) (P=0.0148) were independent prognostic factors (Table III).

Discussion

The ACP6 gene encodes a 458-amino-acid protein with a molecular mass of 51.9 kDa. The ACP6 mRNA with a complete length of 1811 bp is expressed ubiquitously and most often in the kidney, heart, small intestine, muscle and liver. The ACP6 gene is located on chromosome 1 at 1q21, and immunohistochemical analysis demonstrated that ACP6 was shown to localize to the mitochondria (10). The previous report indicated that the cells that expressed ACP6 showed reduction of phosphatidylglycerol (PG) and cardiolipin (10). Cardiolipin is involved in the mitochondrial electron carrier proteins and transport and is essential for cell viability and the pathways that initiate programmed cell death (13). PG is a potential activator of the protein kinase C family (14), which is essential for subsequent responses such as cell proliferation and differentiation (15). PG and cardiolipin are synthesized mainly in mitochondria from LPA. Therefore, increased expression of ACP6 is expected to reduce PA through the hydrolysis of LPA to MAG, resulting in the reduction of cardiolipin and PG (10) (Fig. 1). ACP6 might regulate mitochondrial lipid metabolism including PG and cardiolipin. However, the
precise function of ACP6 remains unknown and will need to be investigated.

The mechanism behind down-regulation of ACP6, including DNA methylation, mutation and loss of heterozygosity (LOH), was not examined in this study. However, LOH in this chromosomal locus of ACP6 (1q21) has been detected and was suggested to harbor the putative tumor suppressor gene within this region (1q21) (16). ACP6 could be a candidate tumor suppressor gene. The promoter region of ACP6 contains CpG islands, suggesting a role of methylation in silencing ACP6 mRNA expression.

Among cancers of the digestive tract, esophageal squamous cell carcinoma (ESCC) has the poorest prognosis. Therefore, it is important to identify prognostic factors for patients with this disease. Several genes, including cyclinD1 (16), PCNA (16), MMP7 (17), p21 (18) and p97 (19), have been reported as independent prognosis factors in patients with ESCC. In this study, we have reported for the first time the relationship between ACP6 and tumor progression and suggested that ACP6 could be a good candidate prognosis marker in esophageal cancer.

Acknowledgements

The authors thank Ms. Shinobu Makino for her excellent technical assistance.

References