Tumor suppressor Prdx1 is a prognostic factor in esophageal squamous cell carcinoma patients

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Abstract. Peroxiredoxins (Prdxs) are a family of antioxidant enzymes that are also known as scavengers of peroxide in mammalian cells. Some reports have shown that the over-expression of Prdx1, which is one of the peroxiredoxins that is a ubiquitously expressed protein, was related to a poor prognosis in several types of human cancers. In this study, we investigated the expression levels of Prdx1 in esophageal squamous cell carcinoma by immunohistochemistry, and the correlation between the Prdx1 expression and the clinical status was elucidated. Immunohistochemical staining was performed in 114 samples which were collected from surgical esophageal cancer specimens. Cytoplasmic staining of Prdx1 was evaluated based on the following scoring criteria: Grade I, negative or weak staining; Grade II, moderate staining; and Grade III, strong staining. The percentage of patients with a Grade I expression of Prx1 was 20% (23 of 114), 44% had Grade II (50 of 114), and 36% had Grade III (41 of 114). The Prdx1 immunoreactivity showed an inverse significant correlation with T-category (P<0.0001), lymph node metastasis (P=0.048), and stage (P=0.001). In addition, the patients with tumors exhibiting a reduced Prdx1 expression had shorter overall survival (P=0.022) in comparison to the patients with tumors which had a higher Prdx1 expression. Currently, Prdx1 has been shown to act as a tumor suppressor. Our results provide strong evidence that the reduced Prdx1 expression is an important factor in esophageal squamous cancer progression and could serve as a useful prognostic marker.

Introduction

Esophageal cancer is the eighth most frequent cancer and the sixth most frequent cause of death from malignant disease in the world (1). Esophageal squamous cell carcinoma (ESCC) is the most common type in Japan (2). A large number of reports about genetic changes in ESCC have already been published, but little is known about the major tumor suppressor genes or major oncogenes in the process of tumor progression of this malignant disease. ESCC is still a fatal malignancy with a 5-year rate of 5% to 20% for advanced stage patients undergoing a curative resection (3). This miserable prognosis for ESCC involves not only the aggressive character of this tumor but also the limited number of useful markers available for diagnostic purposes.

Therefore, better markers which indicate the malignant potential of ESCC should help in the prognosis or optimal treatment of the patients suffering from this disease. In this study, we focus on Prdx1, one of the peroxiredoxins (Prdxs) belonging to a novel antioxidant family, which has been reported to be a tumor suppressor gene. Prdx1 has been thought to have an inhibitory function for both c-Abl and c-Myc, of which active forms cause several neoplasms (4,5). Neumann *et al* (6) showed, using Prdx1 knockout mice, that Prdx1 expression correlated with the reactive oxygen species and the incidence of malignant disease. Moreover, our previous study using a cDNA microarray showed that one of the novel histone deacetylase (HDAC) inhibitors, FK228, induced the Prdx1 expression in the esophageal cancer cell lines and that the expression was associated with the cell toxicity of FK228 (7). The deregulation of HDACs can cause malignant diseases. The inhibition of HDACs is an emerging new strategy in human cancer therapy (8-10). HDAC inhibitors have been shown to induce cell cycle arrest, differentiation, and apoptosis in malignant cells (9,10). From these reports and our former study, Prdx1 appears to have a possible correlation with an anti-tumor effect by producing epigenetic changes in cancer patients. On the other hand, some researchers have mentioned that this protein may be involved with cell proliferation and tumor growth in several kinds of solid cancers (11-15).

In order to contribute to the controversial discussion about the influence of Prdx1 expression in malignant tumors, we examined the expression of this protein in 114 cases of ESCC by immunohistochemistry and evaluated the correlations with the clinical parameters of ESCC to determine whether or not the expression of PrdxI in ESCC plays an important role in tumor progression.

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Table I. Characteristics of esophageal squamous cell carcinoma patients.

Variables	n
Age (years)	
Mean	62.5
Range	27-87
Sex (F/M)	95/19
Depth of invasion	
T1	45
T2	16
T3	50
T4	3
Lymph node metastasis	
N0	55
N1	59
Distant metastasis	
M0	92
M1	22
Stage	
I	26
IIa	26
IIb	18
III	22
IV	22
Histopathological grading	
1	30
2	64
3	20

Table II. Immunohistochemistry of Prdx1 expression in esophageal squamous cell cancer tissue and association with clinicopathological parameters.

Parameters	Prdx1 expression level			P-value
	I	II	III	
Age				
>60 years	14	30	23	0.908
<60 years	9	20	18	
Sex				
Male	16	43	36	0.136
Female	7	7	5	
Depth of invasion				
T1 + T2	8	19	37	<0.0001
T3 + T4	15	31	4	
Lymph node metastasis				
N0	10	19	26	0.048
N1	13	31	15	
Distant metastasis				
M0	19	38	35	0.686
M1	4	12	6	
Stage				
I + II	11	24	35	0.001
III + IV	12	26	6	
Histopathological grading				
1	12	11	7	0.054
2	9	30	27	
3	1	9	8	

Materials and methods

Patients and tissue samples. A total of 114 primary esophageal squamous cancer samples were obtained at the Department of Frontier surgery, Chiba University Hospital, Chiba, Japan. All of the patients were undergoing surgery without any preoperative radiotherapy or chemotherapy. The histological diagnoses revealed that all of the patients had squamous cell carcinoma. Simultaneously normal samples from areas adjacent to the cancerous areas were obtained and they were confirmed to be normal specimens. The clinicopathological characteristics of the samples are listed in Table I. The staging of the tumors was carried out according to the TNM classification.

Immunohistochemistry. The specimens were immersed in 10% formaldehyde immediately after removal and then were embedded in a paraffin block. Blocks containing both carcinoma and the adjacent normal epithelium were chosen and two serial sections were made from the blocks. One section was stained with hematoxylin and eosin while the other section was used for immunohistochemical studies of Prdx1. Immunohistochemical staining was performed in order to detect the expression of Prdx1 with rabbit anti-human Prdx1 (Alexis Biochemicals, Lausen, Switzerland). In brief, the sections were deparaffinized and the endogenous peroxide activity was inactivated in 100% methanol containing 3% hydrogen peroxide. After the blocking of non-specific binding by treating the slides with 5% skim milk at 37˚C for 60 min, the slides were incubated at room temperature with a primary antibody at 1:150 dilution. The sections were then washed and incubated with the ENVISION+ kit (Dako, Copenhagen, Denmark) for 60 min. 3, 3-Diaminobenzidine was used as a chromogen to reveal the antigen, and the sections were then counterstained with Harris hematoxylin.

Scoring of antibody staining. The Prdx1 immunostaining was semiquantified by a visual grading system (16) in which the intensity of the staining was classified into 3 groups as follows: Grade I, negative or weak staining (Fig. 1A and B); Grade II, moderate staining (Fig. 1C and D); and Grade III, strong staining (Fig. 1E and F).

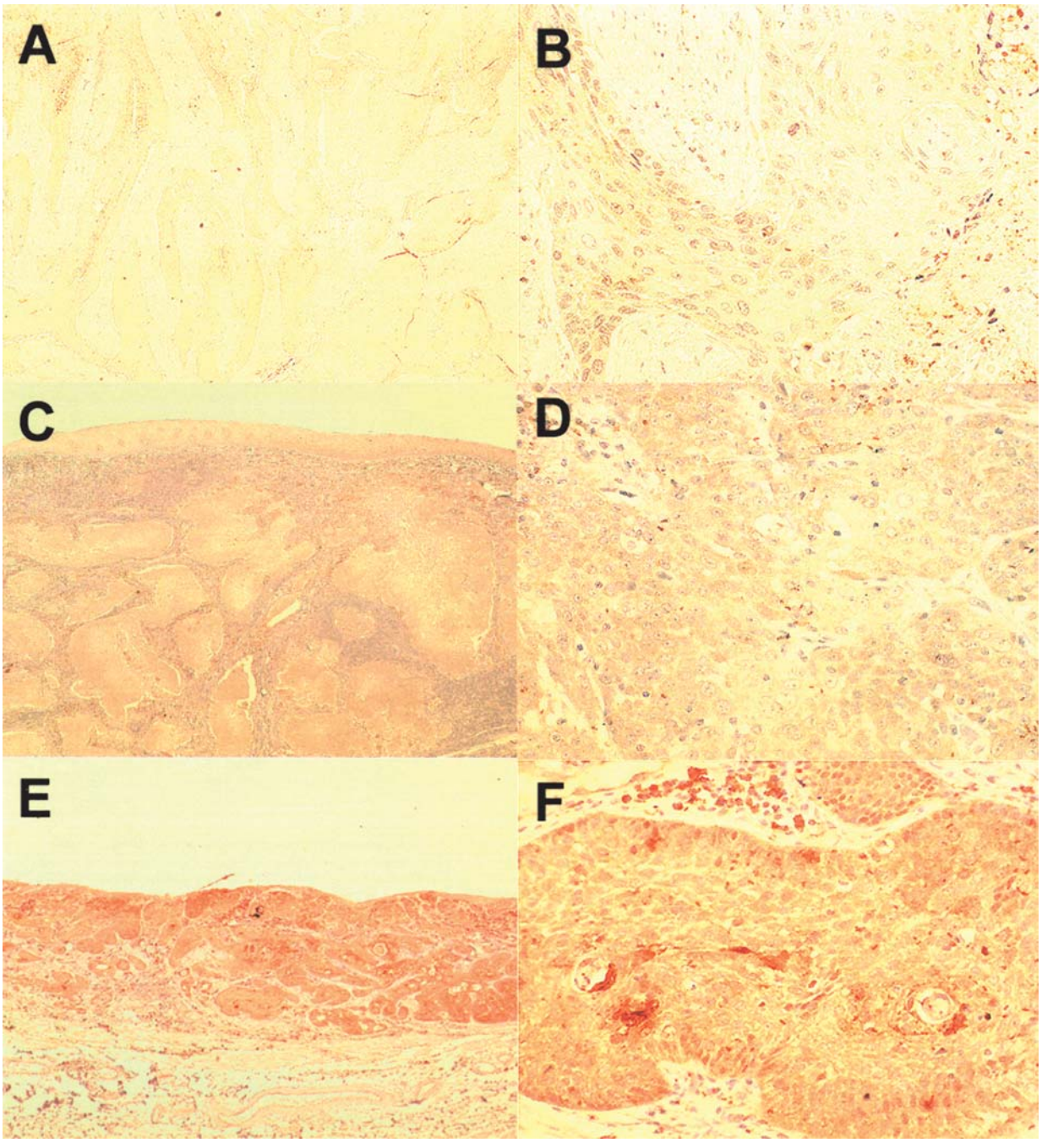

Figure 1. Immunohistochemistry of Prdx1 in esophageal squamous cell carcinoma specimens. In cancer cells, Prdx1 was demonstrated in the cytoplasm. A, weak staining of Prdx1 (x40). B, magnified view (x200). C, moderate staining of Prdx1 (x40). D, magnified view (x200). E, strong staining of Prdx1 (x40). F, magnified view (x200).

Statistical analysis. The StatView statistical package (SAS Institute Inc., Cary, NC) was used for the statistical analysis. Significant differences between the groups were analyzed using the Chi-square test. The survival data of patients were analyzed using the Kaplan-Meier estimation method and the survival curves were evaluated with the log-rank test. A value of $P<0.05$ was considered to be statistically significant.

Results

Immunohistological analysis of Prdx1 expression in ESCC. By immunohistochemistry, the Prdx1 expression in the cytoplasm was observed in 90% of the specimens examined. The percentage of patients with Grade I expression was 20% (23 of 114), 44% had Grade II (50 of 114), and 36% had Grade III (41 of 114). In order to simplify the correlation of the Prdx1 expression with the clinical features, T-categories were divided into T1 + T2 and T3 +T4 groups.

Comparing the expression level of Prdx1 with clinical features, the high expression groups included significantly more T1 + T2, N0 and stage I + II case patients. There were significant differences in the expression level of Prdx1 between the T1 + T2 and T3 + T4 groups ($P<0.0001$), between the stage I + II and stage III + IV ($P=0.001<0.01$) groups, and

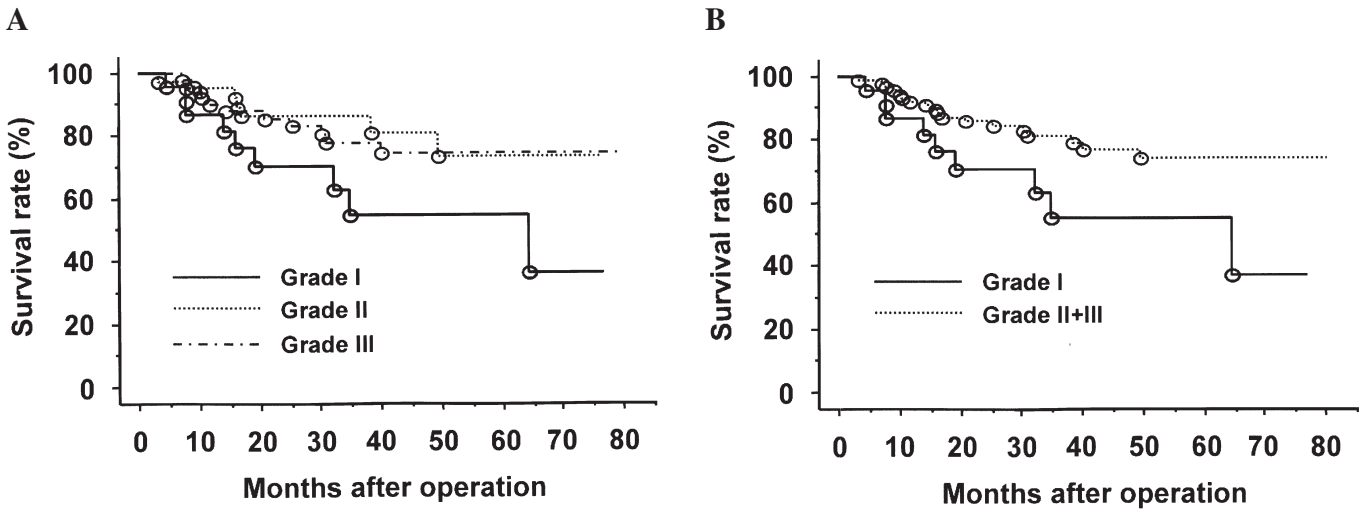

Figure 2. Kaplan-Meier analysis for long-term survival in esophageal squamous cell cancer patients, stratified according to Prdx1 expression. A, comparison of overall survival curves for patients with Prdx1 weak, moderate, and strong tumors (P=0.069). B, comparison of overall survival curves for patients between Prdx1 weak + moderate and strong tumors (P=0.022).

between the N0 and N1 groups (P=0.048<0.05). No significant difference in Prdx1 expression was observed with respect to other factors, such as age, sex, histological grade, and M factor (Table II).

Survival analysis. The follow-up data of surgically treated ESCC patients was available. For the analyses of the immunohistochemistry results, the tumors with a high Prdx1 expression were tested against the tumors with a low Prdx1 expression. A statistical analysis with the log-rank test did not reveal a significant association between the Grade I, Grade II, and Grade III groups (Fig. 2A, P=0.069). Although the expression grades were subclassified into 2 groups, Grade I + Grade II and Grade III, a significant association was observed between those groups regarding survival (Fig. 2B, P=0.022).

Discussion

In mammals, the Prdx proteins comprise a highly conserved family of six proteins. All of the Prdx proteins contain a conserved cysteine residue in the N-terminal region that is the active site of catalysis (17). In particular, Prdx1 is thought to be involved in the redox regulation of the cells and reduce peroxide (18).

Prdx1 has been thought to act as a tumor suppressor. This idea was derived from the observation that Prdx1 should play a central role in the proliferative signals of two crucial oncoproteins, c-Abl and c-Myc (4,5). Mu *et al* showed that Prdx1 overexpression could mimic the growth-promoting activity of c-Myc (5). Egler *et al* also demonstrated that the Prdx1 knockout induced ras transformation and the c-Myc target genes had altered expression levels in Prdx1 knockout mice (19). Moreover, the Prdx1 knockout mice generated malignancies in the intestine, lymphomas, and sarcomas with a high frequency (6). In addition, we previously reported that FK228, one of the novel HDAC inhibitors, induced growth inhibition and apoptosis in ESCC (7). HDAC plays a fundamental role in regulating gene expression and chromatin assembly (9,10). Because HDAC inhibitors have different biochemical and biological properties that induce acetylation of the histones, which are the key proteins in the nucleosome and chromatin structure, they are one of the epigenetic drugs that are considered to be most promising as an anti-cancer agent (8-10). Furthermore, HDAC inhibitors may achieve some of the anti-tumor effects through the reaction of a new type of dormant tumor suppressor gene. From these reports and our previous study, we determined by siRNA technology that the activation of Prdx1 expression is necessary for the effect of FK228 in ESCC. These results imply that Prdx1 is a good candidate for a tumor suppressor.

On the other hand, several reports have suggested that the overexpression of Prdx1 is associated with cancer development (11-15). In fact, Prdx1 was originally isolated from a ras-transformed human mammary epithelial cell, which has 3-fold higher expression of Prdx1 in comparison to an untransformed cell (20). However, so far few reports have attempted to elucidate the molecular mechanism of Prdx1 which correlates with tumor viability. Recently, Zhang *et al* (21) showed the role of Prdx1 in protecting cells from ionizing radiation-induced cell death, but this ability was the result of the scavenging function of Prdx1 and it may not be related to tumor progression.

Prdx1 gene mutations may contribute to carcinogenesis or tumor growth. However, Gisin *et al* (22) examined whether the Prdx1 gene is mutated in human hepatocellular carcinoma, and no mutations or polymorphisms of the Prdx1 gene were found.

In the present study, we detected Prdx1 expression by immunohistochemistry in tumors from 90% of 114 ESCC patients. An inverse correlation between the Prdx1 expression and T-category or stage suggested that Prdx1 may be a key molecule involved with tumor suppression in ESCC. Furthermore, the prognoses of the high-expression Prdx1 cases were significantly better than those of the low-expression cases. We are now planning our new clinical trial using one of the HDAC inhibitors for esophageal cancer. The Prdx1 expression

pattern may play an important role in examining the sensitivity of the HDAC inhibitor before treatment.

In conclusion, we have shown here for the first time the correlation between the Prdx1 expression and the clinicopathological parameters in ESCC. We are confident that our results will help in better predicting the prognosis of patients with ESCC.

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