Clinical significance of the overexpression of the candidate oncogene CYP24 in esophageal cancer

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Background: By using array comparative genomic hybridization (CGH), the increased copy number of CYP24 (which encodes vitamin D 24-hydroxylase) at 20q13.2 was previously reported, leading to the identification of CYP24 as a candidate oncogene in breast cancer. CYP24 leads to abrogate growth control mediated by vitamin D.

Materials and methods: We examined CYP24 expression as well as VDR (vitamin D receptor) gene expression in 42 esophageal cancer cases using semi-quantitative RT-PCR assay. We induced CYP24 in seven esophageal cancer cell lines using 25-hydroxyvitamin D₃ [25(OH)D₃] and compared cell growth rate, measured using the 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) assay system.

Results: The overall survival rate was significantly higher in 25 cases of lower CYP24 expression than 17 cases of higher CYP24 expression (P <0.05); on the other hand, 23 cases of low VDR expression had a poorer prognosis than 19 cases of high VDR expression. Moreover, we disclosed that the inverse correlation between CYP24 and VDR expression is significant in esophageal cancer cases (P <0.05). Furthermore, the cell growth evaluated by MTT assay was greatly increased in CYP24-induced and VDR-diminished cells than non-responding cells by 25(OH)D₃ activity (P <0.01).

Conclusions: Overexpression of the candidate oncogene CYP24 is inversely correlated to vitamin D receptor expression, and may play an important role in determination of the malignant potential of esophageal cancer.

Key words: 20q13.2, esophageal cancer, oncogene, prognostic factor, vitamin D 24-hydroxylase, vitamin D receptor

Introduction

Chromosomal location 20q13.2 is particularly attractive because aberrations in this region occur in various cancers. Increased copy number was observed in 40% of breast cancer cell lines [1], colon cancer [2], pancreatic cancer [2], and head and neck cancer [3]. The amplification of chromosome 20q in cultured cells is associated with progressing tumor phenotypes, including immortalization and genomic instability, therefore identification of genes in 20q has been a common goal for numerous researchers. Albertson et al. identified the putative oncogenes ZNF217 and CYP24 (encoding vitamin D 24-hydroxylase) in 20q13.2, within a 2 Mb region of recurrent aberrations that occur in breast cancer, by using array comparative genomic hybridization (CGH) [4]. CYP24 is distally located to the putative oncogene ZNF217, and thus is a candidate gene itself. As the overexpression of CYP24 in breast cancer tissue has been reported, we investigated the clinical significance of CYP24 overexpression in other human cancers, especially in esophageal cancer which is known to be one of the malignant tumors associated with poor prognosis.

The known function of CYP24, i.e. the limitation of the biological activity of the vitamin D signaling system [5–7], supports its candidacy. In fact, CYP24 overexpression is likely to lead to abrogation of growth control mediated by vitamin D in breast cancer cases. We therefore focused on whether the significant relationship between CYP24 and VDR expression was observed in esophageal cancer in vivo and in vitro. The result may be one of the clues towards clarifying the mechanism of CYP24 in promoting malignant behavior in esophageal cancer.

This is the first report to disclose the clinical significance of the expression of the newly identified candidate oncogene CYP24 and its relationship with VDR gene in esophageal cancer.

Materials and methods

Surgical specimens and cancer cell lines

Tumors and the corresponding normal specimens from 35 male and seven female patients with esophageal cancer were made available for this study by the Department of Surgery, Saitama Cancer Center, from 1993 to 1996. The centers of the cancer and normal tissues were extracted from surgically...
resected specimens and pathologically diagnosed. Five cases were treated with carboplatin, cisplatin or vinblastine as a postoperative chemotherapy. All samples were immediately stored at –80°C until utilized. The average patient age was 62.1 years (range 40–82 years). The data of several clinicopathological variables, such as sex, depth of tumor invasion, lymph node metastasis, lymph vessel permeation, vascular vessel permeation, disease stage and overall survival, were completely reviewed for all patients (Table 1). There were 39 cases of squamous cell carcinoma, two cases of basal squamous cell carcinoma and one case of adenocarcinoma of the esophagus.

We examined the following esophageal cancer cell lines: TE7, TE12, TE13, KY70, KY110, KY150 and KY180 [8, 9]. TE12, TE13, KY70, KY110, KY150 and KY180 were derived from squamous cell carcinoma, while TE7 was from adenocarcinoma.

cDNA preparation from tissue specimens

Total RNA was extracted from –500 mg of each resected specimen according to the method of Chomczynski et al. All samples were treated in Eppendorf tubes (Eppendorf, Germany) and handled with gloves to avoid contamination with RNAase. To ensure that the RNA was not degraded, 2 μg of RNA were run on an agarose gel and stained with ethidium bromide to look for intact 28S and 18S RNAs. The 2 μg of RNA was reverse transcribed into cDNA using oligo-dT primers according to the manufacturer’s protocol (Gibco-BRL, Japan).

Semi-quantitative analysis of CYP24 and VDR

The primer sequences of CYP24, VDR [4] and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), which was utilized as an internal control, are described elsewhere [10].

In brief, CYP24 and VDR cDNA amplification was performed in 25 μl of 10× PCR buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 25 mM dNTP (dATP, dCTP, dGTP and dTTP, 100 mM each), 10 pmol each of the CYP24 and VDR primers and 1 U Taq DNA polymerase (Promega, Madison, WI). The reactions were subjected to 24 cycles for 1 min at 94°C, 1 min at 60°C and 90 s at 72°C in a 9600 thermal cycler (Perkin-Elmer Roche, Japan). GAPDH amplification was performed as described previously [10]. Amplified DNA fragments were separated on a 1.5% agarose gel con-
taining ethidium bromide. The intensities of amplified products of CYP24 and VDR were analyzed by densitometry using the Image program (version 1.4; National Institutes of Health, Bethesda, MD) [11]. The expression of CYP24 or VDR was measured and normalized with respect to GAPDH by RT-PCR. An expression ratio of CYP24 or VDR in tumor to normal (T:N) >1.0 was defined as overexpression. The statistical significance of CYP24 expression or VDR expression in clinicopathological factors and overall survival were determined by Fisher’s exact test and the Cox-Mantel test, respectively.

Induction of CYP24 expression by 25-hydroxyvitamin D3

Previously, Jones et al. reported CYP24 induction in non-small-cell lung cancer (NSCLC) [12]. Among six NSCLC cells, three NSCLC cell lines had the degradation product 24-hydroxylase (CYP24), which catalyzes 25-hydroxyvitamin D3 [25(OH)D3] (Sigma, Tokyo) to the hormone 1 alpha, 25-dihydroxyvitamin D3. Based on this study, the role of CYP24 on the biological activity of esophageal cancer cells by the induction of 25(OH)D3 was examined. Esophageal cancer cell lines TE7, TE12, TE13, KY70, KY110, KY150 and KY180 were examined to identify cells expressing CYP24, which is a degradation product. These esophageal cancer cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Approximately 8.5 × 10⁶ cells were washed twice with PBS and the medium was replaced with RPMI-1640 supplemented with 1% bovine serum albumin (BSA). The inducer, 10 µM 25(OH)D₃, and the antioxidant, 100 µM 1,2-dianilinoethane (Sigma, Tokyo) with ethanol as a solvent, were added and the cells were subsequently incubated for 24 h.

The induction of CYP24 expression and the MTT assay

We examined TE12 as a representative cell line that induced CYP24 expression and diminished VDR expression. On the other hand, KY110 was selected as a non-responding cell to the administration of 25(OH)D₃. Both cell lines were examined three times to compare cell growth rate using the 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) assay system. Both cell lines were cultured in RPMI-1640 supplemented with 10% FCS. Approximately 8.5 × 10⁶ cells were washed twice with PBS and the medium was replaced with RPMI-1640 supplemented with 1% BSA. The inducer, 10 µM 25(OH)D₃, and the antioxidant, 100 µM 1,2-dianilinoethane (Sigma, Tokyo) with ethanol as a solvent were added and the cells were incubated for 24 h.

We performed MTT assay for both cells as follows. After the incubation at 37°C for 8 days in a humid 5% CO₂ atmosphere, the plates were centrifuged at 700 g, the supernatants were discarded and 20 µl of MTT solution (10 µM) was added to all the wells according to the manufacturer’s protocol for the Cell Proliferation Kit I (Roche, Tokyo, Japan). After incubation for 4 h, 150 µl of stop solution was added to all the wells for extracting the formazan formed by MTT. The absorbance of formazan in each well was measured using a microplate reader (Bio-Rad, Japan) at a test wavelength of 570–650 µm; results were given as optimal density per milligram of protein (OD/mg protein).

Results

CYP24 and VDR expression in tumor and normal tissues

The semi-quantitative measurements of CYP24 expression in tumor and the corresponding normal tissues with the appropriate RT-PCR cycles are shown in Figure 1. CYP24 had not previously been implicated in esophageal cancer, however a relatively higher expression of CYP24 was observed in tumor tissues as compared with normal tissues in this study. In addition, CYP24 is up-regulated in malignant tissues (Figure 2). This observation led us to focus on the clinicopathological significance of CYP24 expression and its possible role in esophageal cancer progression.
venous permeation; however, there was no statistical significance among them (Table 1). In contrast, the average VDR expression is 1.16, ranging from 0.1 to 15. Lower expression of the VDR gene was observed in five of seven cases (70%) with poorly differentiated squamous cell cancer, 15 of 27 cases (56%) of high lymph node metastases, and 16 of 26 cases (62%) of high lymphatic permeation, however there was no statistical significant correlation between these clinicopathological factors and VDR expression (Table 1). Interestingly, the overall survival rate was significantly higher in 25 cases of lower CYP24 expression than 17 cases of higher CYP24 expression (P = 0.03). Nineteen cases of high VDR expression showed a tendency towards a better prognosis than 23 cases of lower VDR expression; however, statistical significance was not reached between them.

The relationship between CYP24 and VDR in esophageal cancer cases and esophageal cancer cell lines

Table 2 shows the inverse correlation between expression of CYP24 and VDR in 42 esophageal cancer cases. In 17 cases of high expression of the CYP24 gene, 13 (76%) cases indicated high VDR gene expression, while in 25 cases of low CYP24 expression, 15 (60%) cases indicated high VDR gene expression; there was a significant correlation between them. However, determination of the precise relationship between CYP24 and VDR genes certainly required larger numbers of cases.

In Figure 4, the induction of CYP24 expression was observed in TE7, TE12, KY70, KY150, and KY180 by 25(OH)D3. TE13 originally expressed CYP24 in the corresponding parent cells, and KY110 showed neither CYP24 nor VDR expression. We therefore selected TE12 as a representative cell line with increased CYP24 expression as well as diminished VDR expression, while KY110 was used for further experiments as a non-responding cell to the administration of 25(OH)D3.

Comparison of cell growth between CYP24-induced and VDR-reduced cells (TE12) and a non-responding cell (KY110)

In Figure 5, cell growth of the TE12 cell with CYP24 induction gradually increased more significantly than either the control or

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**Table 2. Relationship between the expression of the CYP24 and VDR genes by semi-quantitative RT-PCR**

<table>
<thead>
<tr>
<th>CYP24 expression</th>
<th>VDR expression</th>
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<tbody>
<tr>
<td>High (17)</td>
<td>Low (23)</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Low (25)</td>
<td>15</td>
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**Figure 3.** Overall survival in CYP24 and VDR. (A) Statistical significance between 17 cases of high CYP24 expression and 25 cases of low CYP24 expression using the Cox-Mantel test (P = 0.03). (B) Nineteen cases of high VDR expression showed a tendency towards a better prognosis than 23 cases of lower VDR expression; however, statistical significance was not reached between them.

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**Figure 4.** CYP24 induction by 25(OH)D3 to examine the alteration of cell viability in esophageal cancer cells. P, parent cell line; C, control cell line (parent cell with ethanol which was utilized as a solvent, but inducer 25(OH)D3 was not added); D, CYP24-induced cell line by the administration of 25(OH)D3. VDR expression was also analyzed for comparison with CYP24 expression.
parent cell. Statistical significance between them was reached at day 8 (P <0.01). On the other hand, KY110, a representative non-responding cell after the administration of 25(OH)D3, showed no significant change in cell growth.

Discussion

Vitamin D is considered to protect against malignancies by dietary intake in colorectal cancer [13, 14] and breast cancer [6]. As in the present study of esophageal cancer, Launoy et al. identified that vitamin D is one of the independent protective factors among dietary factors influencing the risk of this malignancy [15]. Therefore, the abrogating vitamin D-mediated growth control is believed to play a protective role in the pathogenesis of solid cancer. CYP24, a vitamin D hydroxylase, has been recognized as one of the regulators of the biological effect of the vitamin D signaling system [16]. Miller et al. reported that growth inhibition of human prostatic cancer cells was correlated not only with the number of vitamin receptors per cell, but also with the inverse proportion of the 24-hydroxylase activity [17].

In this study, we have shown that higher CYP24 expression and/or lower VDR expression indicates a poor prognosis, deep invasion of tumor, increased incidence of high lymphatic permeation or venous permeation. We consider that up-regulated CYP24 expression in cancer tissues and/or down-regulated VDR expression may play important roles in the progression of esophageal cancer rather than having carcinogenic activity on esophageal cancer. Interestingly, we have also clarified that vitamin D expression is inversely correlated with CYP24 expression, therefore we speculate that the disruption of the vitamin D regulating system may promote esophageal cancer progression, which is caused by the overexpression of the putative oncogene CYP24. As shown in Figure 5, growth of esophageal cancer cells increased under the high expression of CYP24 and diminished expression of VDR; however, KY110 cells with neither CYP24 nor VDR expression showed no change after the administration of 25(OH)D3. Therefore, the present results also suggest that the disruption of the vitamin D regulating system with diminished expression of the VDR gene due to overexpression of the oncogene CYP24 promote or enhance the malignant potential of esophageal cancer.

We are the first in the world to have disclosed the significance of overexpression of the candidate oncogene CYP24 in esophageal cancer. As stated above, CYP24 is considered a prognostic factor in esophageal cancer, and we expect that it can be used as a powerful new molecular target in gene therapy, to either cure or protect against esophageal cancer.

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References


