Increased Expression of Ornithine Decarboxylase Messenger RNA in Human Esophageal Carcinoma

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ABSTRACT

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines, which are essential for cell proliferation. The purpose of this study was to evaluate ODC expression in human esophageal cancer at the mRNA level. Sixty-four pairs of primary esophageal cancers and normal esophageal epithelia were examined by reverse transcription-PCR for ODC mRNA expression. The ODC mRNA levels were higher in primary esophageal carcinoma than in adjacent normal esophageal epithelium in 58 (90.6%) of 64 cases. The tumor:normal (T:N) ratio of ODC mRNA expression in esophageal specimens has a significant correlation with tumor-node-metastasis staging (P = 0.043), lymph node metastasis (P = 0.039), vascular vessel invasion (P = 0.035), and histology (P = 0.034) of the tumor. In well- and moderately differentiated squamous cell carcinoma, the patients with a higher T:N ratio showed a significantly poorer prognosis (P = 0.027), and multivariate analysis also confirmed that the T:N ratio has a significant correlation with poor prognosis (P = 0.043). The steady-state of ODC mRNA overexpression in esophageal carcinoma implies that the ODC gene may play an important role in tumorgenesis in squamous epithelium. Furthermore, ODC mRNA expression may be used as a prognostic marker, especially for well- and moderately differentiated squamous cell carcinoma.

INTRODUCTION

ODC plays a key role in the biosynthesis of polyamines (1–3), which are essential for cell proliferation (4, 5). The expression of ODC is transiently increased on stimulation by growth factors (1–3) but becomes constitutively activated during cell transformation induced by carcinogens (6, 7), viruses (8–10) or oncogenes (11–15). Recently, the aberrant regulation of ODC has been reported to play an important role in neoplastic transformation and tumor growth (16–18).

Several studies using enzyme assays have disclosed that the ODC activity is higher in tumor tissue than in normal tissue (19–23). High ODC activity in tumor tissue has been demonstrated primarily among poorly differentiated tumors (20, 22), and ODC activity seems to be correlated with the compartment size of aneuploidic cells in the tumor (20). It has, therefore, been suggested that ODC activity may be used as a marker for the tumor growth rate and biological aggressiveness. There is, however, little information on the mRNA status of ODC in surgical specimens of neoplasms. Yoshida et al. (23) have reported that, among several kinds of tumors, both ODC activity and mRNA in esophageal carcinoma are remarkably increased, and that a significant correlation can be noted between ODC activity and mRNA expression. In this study, therefore, we aimed to confirm the increased steady-state mRNA of ODC in carcinoma of the esophagus and to clarify its clinical significance.

MATERIALS AND METHODS

Cell Lines. Thirteen human esophageal cancer and human fibroblast cell lines were studied. Of these 13 esophageal cancer cell lines, 6 were of the TE series (TE1, -2, -4, -5, -7, -8) obtained from Dr. Nishihira at Tohoku University (Sendai, Japan), and seven were of the KY series (KY110, -140, -150, -170, -180, -200, -700) provided by Dr. Shimada at Kyoto University (Kyoto, Japan). The details of the TE series and KY series have been described elsewhere (24, 25). Three fibroblast cell lines were of the CRL series (CRL1485, -1498, -1506) obtained from the American Type Culture Collection. These cell lines were maintained in DMEM (TE series) or RPMI 1640 (KY series) containing 10% FCS and antibiotics.

Patients and Sampling. Surgical specimens of 64 pairs of primary esophageal carcinomas and adjacent normal esophageal mucosa were obtained fresh from the operating room at the Saitama Cancer Center Hospital (Saitama, Japan). Immediately after resection, the necrotic and ulcerated parts of the tumors were removed, and the normal esophageal mucosa was dissociated from the muscle and connective tissue. All of the

3 The abbreviations used are: ODC, ornithine decarboxylase; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T:N ratio, tumor:normal ratio; TNM, tumor-node-metastasis.
tissue specimens were then frozen in liquid nitrogen and kept at –90°C until the extraction of RNA.

**Extraction of Total RNA.** The total cellular RNA was extracted from cell lines or specimens according to previously described methods (26–28). Briefly, each specimen or each cell line was homogenized in guanidinium isothiocyanate, and ultracentrifuged through cesium chloride solution at 32,000 rpm for 20 h. The concentration of RNA in Tris/EDTA buffer was measured at a wavelength of 260 nm using a spectrophotometer (DU-70; Beckman, Fullerton, CA).

**RT-PCR and Analysis.** A RT-PCR was carried out as follows. Oligonucleotide primer pairs for ODC and GAPDH were synthesized on a DNA synthesizer [Applied Biosystems, Foster City, CA; sense ODC, 5’-GAGCACATCCAAAAG-CAAGT-3’; antisense ODC, 5’-TCCAGAAGCTGACGGAAATTA-3’; sense GAPDH, 5’-GTCAACGGATTGGCTGATT-3’; antisense GAPDH, 5’-AGTCTTCTGGGTGGCAGTGAT-3’ (29)]. The oligonucleotide primers were end-labeled with [32P]adenosine 5’-triphosphates (Amersham, Tokyo, Japan) at 3000 Ci/mmol using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), followed by the removal of unincorporated 32P using a spin column. A PCR was carried out in a 25-μl volume containing 20–30 ng of genomic DNA template, 10 pmol each of oligodeoxynucleotide primer, 200 mM of each deoxynucleotide triphosphate, and 1.5 units of Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). The samples were overlaid with mineral oil and processed through 24 cycles consisting of 1 min at 94°C (denaturation), 2 min at 57°C (annealing), and 2 min at 72°C (elongation) for ODC, and 22 cycles consisting of 1 min at 94°C (denaturation), 1 min at 54°C (annealing), and 1 min at 72°C (elongation) for GAPDH. The aliquots of DNA amplified by the PCR were mixed for 20 h. The concentration of RNA in Tris/EDTA buffer was measured at a wavelength of 260 nm using a spectrophotometer (DU-70; Beckman, Fullerton, CA).

**Statistical Methods.** Mean and SD were calculated for the expression value and the T:N ratio of the expression. Associations between variables were tested by either Fisher’s exact probability test or Student’s t test. Excluding nine patients who died of unrelated diseases, the survival rates were calculated by the Kaplan and Meier actuarial method. Patients were split into two groups according to their T:N ratio (more than 3), and the Cox-Mantel test was used to detect significant differences between calculated survival curves. Multivariate analysis using the Cox proportional hazards model was performed to predict the hazard function given by the significant covariants.

**RESULTS**

All of the cell lines of esophageal cancer and fibroblast and all of the specimens from 64 esophageal cancers showed the same positive band of the 2-kb mRNA. The expression of ODC mRNA was much higher in all of the esophageal cancer cell lines than that in surgical specimens of esophageal cancer and that in all of the fibroblast cell lines (Fig. 1). The present analysis demonstrates that surgical specimens of esophageal carcinoma and normal mucosa show variable levels of ODC mRNA signal. The T:N ratio of ODC mRNA, which was corrected for that of GAPDH mRNA, ranged from 9.4 to 24.53. In 58 (90.6%) of 64 cases, the expression of ODC mRNA was greater in T than in N (the T:N ratio was higher than 1.0), and in only six cases (9.4%), the T:N ratio of ODC mRNA was less than 1.0. Ten representative cases whose T:N ratios ranged from 2.2 to 24.5 are shown in Fig. 2.

Table 1 shows the clinicopathological data and the ODC mRNA signal.
The cases of tumors with lymph node metastasis showed a significantly higher T:N corrected ratio of ODC than those without lymph node metastasis \( (P = 0.039) \). The cases of tumors with vascular invasion showed a significantly higher T:N ratio than those without vascular vessel invasion \( (P = 0.035) \). The cases in stages III and IV also showed a significantly greater T:N ratio than those in stages I and II \( (P = 0.043) \). The cases of squamous cell carcinoma indicated a higher T:N ratio of ODC mRNA than those of undifferentiated carcinoma \( (P = 0.034) \). The T:N ratio was also higher in cases with more severe lymphatic vessel invasion or with deeper tumor invasion, although the differences were not significant. There was no difference between the T:N ratio of ODC expression and the other clinicopathological factors, such as age, sex, or tumor location.

Fig. 3A shows the cumulative survival rate of the two groups: one with a T:N ratio below 3 \( (n=23) \) and the other with a T:N ratio of more than 3 \( (n=32) \). There was no significant difference in the survival rates between the two groups. However, among 39 patients with well- or moderately differentiated squamous cell carcinoma, patients with a T:N ratio for ODC mRNA below 3 \( (n=23) \) had a better survival rate than those with a higher T:N ratio \( (n=16; P = 0.027; \text{Fig. 3B}) \).

The prognostic values of ODC mRNA expression and other clinicopathological factors were examined by multivariate analysis using the Cox proportional hazards model. We fit a series of proportional hazards regression models that included the T:N ratio as a prognostic factor in addition to age, sex, tumor location, TNM staging, lymph node metastasis, depth of invasion, lymphatic vessel invasion, and blood vessel invasion. In all of the esophageal cancer patients, there was no factor that was significant as an independent prognostic indicator for overall survival. However, in well- and moderately differentiated squamous cell carcinoma, multivariate analyses confirmed that a higher T:N ratio of ODC mRNA expression and a higher incidence of blood vessel invasion was associated with poor prognosis \( (P = 0.043 \text{ and } P = 0.019, \text{respectively; Table 2}) \).
DISCUSSION

In this study we determined that ODC overexpression in esophageal cancer tissue at the mRNA level occurs at an incidence of 90.6%. We have carried out Northern blot or RT-PCR analyses using the same samples and different probes (32–34) and have also reported various mRNAs that are overexpressed in those human tumors (26–28). However, nothing seems to be more constantly overexpressed in tumors than this ODC gene. A previous study of ours has indicated that ODC is overexpressed in 36 (64%) of 56 stomach cancers (35), which is lower than the incidence in esophageal cancers. Yoshida et al. (23) have also reported that among several kinds of tumors, both ODC activity and mRNA in esophageal carcinoma were remarkably increased. Because ODC is associated with cell proliferation, esophageal cancer may be one of the fastest growing tumors with poor prognosis.

Accumulations of certain genomic alterations in proto-oncogenes may be crucial to carcinogenesis in human esophageal cancer. These proto-oncogenes include amplifications of c-myc, human epidermal growth factor receptor, hst-1/int-2, and cycline D (36–40). Furthermore, Auvinen et al. (16) have indicated that aberrant expression of ODC is not just a coincident, pleiotypic response to transformation but a critical factor contributing to oncogenesis. They also suggest that the ODC gene should be recognized as a member of the growing family of cellular proto-oncogenes. Clifford et al. (41) have reported that ODC overexpression by itself is not sufficient to induce tumors in normal cells but that increased expression of ODC enhances tumor progression in premalignant cells. It has also been reported that a high level of ODC activity can be detected in endoscopic specimens from the main tumor, with a moderate increase in specimens from the dysplastic epithelium (23). Our data showed that ODC mRNA expression is high in squamous cell carcinoma but not in undifferentiated carcinoma, although undifferentiated carcinoma usually exhibits more aggressive behavior than squamous cell carcinoma. Therefore, the steady-state of ODC mRNA overexpression in esophageal tumor, especially in squamous cell carcinoma, suggests that the ODC gene may play an important role in tumorigenesis and/or de-differentiation, especially in the squamous epithelium of the esophagus.

Very little is known of the molecular events responsible for human esophageal tumor progression. Although there have been several reports focusing on the role of ODC in the process of malignant transformation, few reports have described the role of ODC in tumor invasion and metastases. Kubota et al. (42) have indicated that ODC is directly involved in tumor cell invasion in vitro. In this investigation, the cases with lymph node metastases showed a higher T:N ratio for ODC mRNA than those without lymph node metastasis. Our previous study (35) has demonstrated that in stomach cancer the cases of tumors with vascular vessel invasion show a higher T:N ratio for ODC mRNA than those without vascular vessel invasion. These findings suggest that ODC may be associated with tumor invasion and metastases in in vivo specimens. Thus, this ODC mRNA expression might be used as not only a nonspecific marker of tumor proliferation, but also a marker of biological aggressiveness. Very high ODC mRNA expression in earlier-stage disease may suggest a biologically aggressive tumor with a high risk of recurrence. However, undifferentiated carcinoma, which always shows aggressive behavior, exhibits low ODC mRNA expression. Thus, our data suggest that ODC may be a useful marker in patients with well- to moderately differentiated squamous cell carcinoma, as shown in Fig. 3B. Even in well- to moderately differentiated cancer, there are undifferentiated or poorly differentiated components because of tumor heterogeneity. These minor components may sometimes affect the prognosis of patients, and this is a possible reason why patients with lower ODC in well- to moderately differentiated cancer show almost the same survival curve as the higher ODC group in the earlier years after surgery (Fig. 3B). Additional studies will enable us to correlate the expression of ODC mRNA in tumor cells with survival statistics to postulate its role as a marker of poor prognosis, especially for well- to moderately differentiated squamous cell carcinoma.

Because ODC plays an important role in biosynthesis of polyamines, the level of ODC would be expected to reflect the general level of cell proliferation in a tissue. There are other markers of proliferating cells in cancer, such as Ki-67, MIB-1, and proliferating cell nuclear antigen, which are usually determined by immunohistochemical examination. Compared with our mRNA-based study, immunostaining of these markers is much more popular and much less labor-intensive. However, it is difficult to interpret results objectively because immunostaining is not a quantitative study. By using the RT-PCR technique, it is much easier to evaluate ODC mRNA expression in tissue. This is one of the reasons why we are performing the mRNA-based study. There are also several clinicopathological factors that are effective in evaluating the malignant potential of esophageal carcinomas. These factors, however, are usually obtained postoperatively by a pathological examination of the resected specimen. Our previous study (34) showed that the expression of various kinds of mRNA as determined by Northern blotting may also be effectively used for endoscopically biopsied specimens. Using the RT-PCR technique, it is much easier to evaluate mRNA expression in smaller specimens from endoscopic biopsy, and information on the status of the mRNA expression can be obtained preoperatively. This is another reason why we are performing the mRNA-based study. In fact, the ODC mRNA expression could be obtained in 91 of 100 biopsied specimens from the esophagus. The ODC mRNA expression

Table 2  Multivariate Cox proportional hazard analysis in 39 patients with well-differentiated or moderately differentiated squamous cell carcinoma

<table>
<thead>
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<th>Variables</th>
<th>Coefficient</th>
<th>SE</th>
<th>Relative risk</th>
<th>P</th>
</tr>
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<tr>
<td>Blood vessel invasion</td>
<td>1.716</td>
<td>0.734</td>
<td>5.564</td>
<td>0.019</td>
</tr>
<tr>
<td>(low or high)</td>
<td></td>
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<td></td>
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<tr>
<td>ODC mRNA expression</td>
<td>0.115</td>
<td>0.057</td>
<td>1.122</td>
<td>0.043</td>
</tr>
</tbody>
</table>

4 K. Mafune, Y. Tanaka, M. Mori, K. Takubo, and M. Makuuchi, unpublished data.
may be a useful marker for providing information preoperatively on the malignant potentiality of esophageal carcinoma. In this study, the ODC mRNA expression was significantly lower in patients without lymph node metastasis, and, for example, if we can ascertain that there is no lymph node metastasis in tumors with submucosal invasion, only an endoscopic mucosal resection would be needed instead of an esophagectomy.

REFERENCES

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