Expression of Ornithine Decarboxylase mRNA in Gastric Carcinoma

Masaki Mori, M.D.[1]
Masayuki Honda, M.D.[1]
Kenji Shibuta, M.D.[1]
Kinya Baba, M.D.[1]
Hideaki Nakashima, M.D.[1]
Masaru Haraguchi, M.D.[1]
Fumio Koba, M.D.[2]
Hiroaki Ueo, M.D.[2]
Keizo Sugimachi, M.D.[3]
Tsuyoshi Akiyoshi, M.D.[1]

[1] Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan.

[2] Department of Surgery, Oita Prefectural Hospital, Oita, Japan.

[3] Department of Surgery II, Kyushu University, Fukuoka, Japan.

Presented at the Seventh Annual Meeting of the Japanese Research Society for Gastroenterological Carcinogenesis, Oita, Japan, September 8–9, 1995.

Supported in part by the Ministry of Education, Culture, and Science of Japan.

Address for reprints: Masaki Mori, M.D., Department of Surgery, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumibaru, Beppu 874, Japan.

Received December 4, 1995; accepted December 8, 1995.

BACKGROUND. Ornithine decarboxylase (ODC) is a key rate-limiting enzyme in polyamine biosynthesis. Several studies using an enzyme assay revealed that the ODC activity was higher in tumor tissue than in normal tissue. However, there is little information on the mRNA status of ODC in surgical specimens. ODC is a transcriptional target gene of c-*myc*.

METHODS. cDNA was obtained by reverse transcription (RT) from fresh specimens of 56 surgical pairs of primary gastric carcinomas and corresponding normal tissue specimens. The ODC and c-*myc* mRNAs were subsequently detected by means of the polymerase chain reaction. The tumor/normal (T/N) ratio of ODC expression was calculated after correcting for glyceraldehyde-3-phosphate dehydrogenase as an internal control. The T/N ratio of ODC was plotted against that of c-*myc*.

RESULTS. The corrected expression levels of ODC mRNA in the tumor were greater than those of the normal mucosa in 36 of 56 cases (64%). The cases of tumor with vascular vessel invasion showed a higher T/N ratio than those without vascular invasion ($P < 0.01$). Similarly, female patients showed a higher T/N ratio than male patients ($P < 0.01$). There was a significant correlation between the expressions of both ODC and c-*myc* genes ($P < 0.05$).

CONCLUSIONS. The findings imply that (1) overexpression of ODC mRNA in tumor tissue may correlate with aggressive biologic behavior, such as vascular vessel invasion, and (2) there is an intimate correlation between ODC and c-*myc* genes.
***Cancer* 1996; 77:1634–8.**

KEYWORDS: ornithine decarboxylase, gastric cancer, reverse transcription, polymerase chain reaction, c-*myc*.

Ornithine decarboxylase (ODC) is a rate-limiting enzyme in the synthesis of polyamines that regulate DNA synthesis and is responsible for converting L-ornithine to putrescine.[1] Aberrant regulation of ODC is reported to play a role in neoplastic transformation and tumor growth.[2,3] ODC activity is increased in tumor tissue compared with normal tissue.[4–9] For example, with respect to gastric carcinoma, Okuzumi et al. determined ODC enzyme activity and reported that gastric cancer tissue had significantly elevated ODC levels over those in normal mucosa.[4] To our knowledge, however, there are few reports studying the mRNA status of ODC in human gastric carcinoma.[6]

A recent report demonstrated that the ODC gene contains a conserved repeat of the *myc* binding site in intron 1 and is a transcriptional target of c-*myc*.[10] The c-*myc* protooncogene is a key regulator of cell growth and differentiation, and enforced c-*myc* expression promotes cell cycle progression.[11] c-*myc* has been considered to be a potent transactivator of several genes, such as prothymosin-α.[12,13] ODC is the second gene that has been found to be a target gene of c-*myc*. However, there is no

study of any correlation between c-*myc* and ODC mRNA status in clinical samples of human gastric carcinoma.

In this study, we investigated the mRNA status of ODC in carcinoma and normal tissues of the stomach and attempted to clarify its clinical significance. In addition, we studied whether there was any correlation between c-*myc* and ODC mRNA status in human gastric carcinoma.

MATERIALS AND METHODS

Patients and Tissue Sampling

The fresh surgical specimens included 56 primary gastric carcinomas and their paired adjacent normal gastric mucosa. Immediately after resection, the necrotic and ulcerated parts of the tumors were removed and the normal gastric mucosa was dissociated from the muscle and connective tissue. All 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 the specimens according to the previously described methods.[13,14] Briefly, each specimen was homogenized in guanidinium isothiocyanate, and the total RNA was extracted by ultracentrifugation in cesium chloride solution at 32,000 revolutions per minute for 20 hours. The concentration of RNA was measured at a wavelength of 260 nm using a spectrophotometer (DU-70; Beckman, Fullerton, CA).

Reverse Transcription-Polymerase Chain Reaction and Analysis

A reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as follows: oligonucleotide primer pairs for ODC, c-*myc*, and glyceraldehyde-3-phosphate dehydrogenase (GADDH) were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, CA) (sense ODC, 5′ - GAGCACATCCCAAAGCAAAGT - 3′; antisense ODC, 5′ - TCCAGAGTCTGACGGAAAGTA - 3′; sense c - *myc*, 5′ - AAGCTCGTCTCAGAGAAGCTG - 3′; antisense c - *myc*, 5′ - AGCCTGCCTCTTTTCCACAGA - 3′; sense GAPDH, 5′-GTCAACGGDTTTGGFCGTATT - 3′; and antisense GAPDH, 5′-AGTCTTCTGGGTGGCAGTGAT-3′[15]). The oligonucleotide primers were end-labeled with ^{32}P-adenosine 5′-triphosphate (^{32}P) (Amersham, Tokyo, Japan) at 3000 Ci/mmol using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), followed by the removal of unincorporated ^{32}P using a spin column. A polymerase chain reaction was carried out in a 25-μL volume containing 20–30 ng of cDNA template, 10 pmol each of oligodeoxynucleotide primer, 200 mM each of deoxynucleotide triphosphate, 1.5 mM magnesium chloride, 0.01% gelatin, and 1.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). The samples were overlaid with mineral oil and processed through 24 cycles consisting of 1 minute at 94 °C (denaturation), 2 minutes at 57 °C (annealing), and 2 minutes at 72 °C (elongation) for ODC and 25 cycles consisting of 1 minute at 94 °C (denaturation), 1 minute at 54 °C (annealing), and 1 minute at 72 °C (elongation) for c-*myc*. The amplification condition for GAPDH was described elsewhere.[15] Aliquots of the amplified DNA by polymerase chain reaction were mixed with a formamide gel loading buffer and were then electrophoresed on 2% agarose gels. The gels were dried, and exposed to an imaging plate. The signals were then quantitated using a Bio-Image analyzer BAS 1000 (Fuji Photo Corp., Tokyo, Japan). The mRNA expression in tumor (T) and normal (N) tissue in each pair was then estimated based on the counts obtained. The tumor-normal ratio of ODC and c-*myc* expression (T/N ratio) was calculated after a correction for that of GAPDH expression.

We determined the nucleotide sequence of polymerase chain reaction products and confirmed that both were identical to the expected fragments of cDNA of ODC and c-*myc*. A GenBank-UPD New sequences library nucleotide database search demonstrated the sequence to be specific for ODC or c-*myc*.

To check for any possible artifacts based on the possible contamination of RNA by genomic DNA during RT-PCR, a few RT-PCR reactions were performed using a truly positive sample under the same conditions but with no reverse transcription step.

Clinicopathologic Findings

The criteria for pathologic diagnosis, such as gross type, depth of tumor invasion, and vascular vessel invasion of the gastric carcinoma tissue, were based on the general rules for gastric cancer outlined by the Japanese Research Society for Gastric Cancer.[16] The histologic type was determined according to modified Lauren's classification.[17]

Statistical Methods

Associations between variables were tested by either Fisher's exact probability test or the Student's *t* test. Linear regression analysis was performed to test the relationship between mRNA expression of ODC and c-*myc*.

RESULTS

To approve the quantitative analysis of ODC expression using our RT-PCR method, the values of the radioactivity of the specific band for either ODC, c-*myc* or GAPDH were examined based on the various numbers of polymerase chain reaction cycles. The values of radioactivity of ODC expression increased linearly up to 24 cycles, and thus indicated a quantitative assessment in this assay. Similarly, 26 and 22 cycles were used for the amplification of c-*myc* and GAPDH, respectively. The present analysis demonstrated that gastric carcinoma and normal mucosa

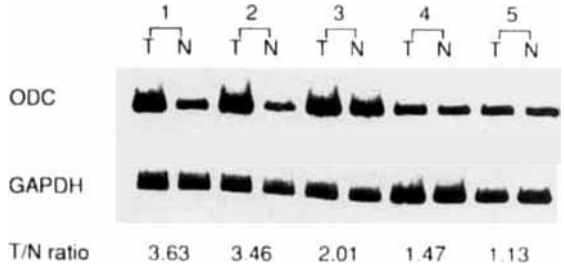

FIGURE 1. Ornithine decarboxylase (ODC) mRNA expression in five representative cases of gastric carcinoma detected by reverse transcriptase-polymerase chain reaction. T represents tumor tissue and N represents normal tissue. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal control. The T/N ratio of ODC mRNA corrected for that of GAPDH mRNA is shown at the bottom.

TABLE 1
Clinicopathologic Data and Ornithine Decarboxylase Expression

Variables	T/N ratio of ODC[a]	*P* value
Age (yr)		
60 or older (n = 42)	1.37 ± 0.81	NS
Younger than 59 (n = 14)	1.42 ± 0.90	
Sex		
Male (n = 38)	1.18 ± 0.52	$P = 0.005$
Female (n = 18)	1.78 ± 1.02	
Histology		
Intestinal (n = 24)	1.32 ± 0.59	NS
Diffuse (n = 32)	1.41 ± 0.85	
Depth of invasion[b]		
Within the wall (n = 29)	1.28 ± 0.75	NS
Beyond the wall (n = 27)	1.47 ± 0.77	
Lymph node metastasis		
Absent (n = 14)	1.18 ± 0.05	NS
Present (n = 42)	1.43 ± 0.83	
Lymphatic vessel invasion		
Absent (n = 14)	1.22 ± 0.70	NS
Present (n = 42)	1.42 ± 0.78	
Vascular vessel invasion		
Absent (n = 32)	1.12 ± 0.59	$P = 0.004$
Present (n = 24)	1.70 ± 0.84	
Stage of disease		
1, 2 (n = 27)	1.32 ± 0.75	NS
3, 4 (n = 29)	1.42 ± 0.78	

ODC: ornithine decarboxylase; NS: not significant.
[a] The tumor-normal ratio of ODC mRNA expression corrected for that of GAPDH mRNA expression.
[b] Within the wall includes tumors showing subserosal invasion.

showed variable levels of the ODC mRNA signal. The T/N ratio of ODC mRNA, which was corrected for that of GAPDH mRNA, ranged from 0.27 to 3.63, and was less than 1 in 20 cases (36%). In 64% of the cases, the expression of ODC mRNA was greater in T than in N. Figure 1 shows 5 representative cases whose T/N ratio ranged from 1.13 to 3.63. Table 1 shows the clinicopathologic

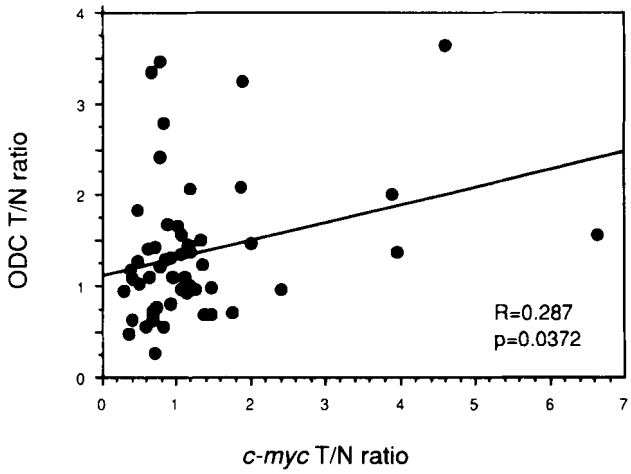

FIGURE 2. The tumor/normal ratio of ornithine decarboxylase (ODC) mRNA is plotted against the ratio for c-*myc* mRNA for each case. There is a significant correlation between the mRNA expression of these two genes ($P < 0.05$).

data and ODC expression. The female patients showed a higher T/N ratio than the male patients ($P < 0.01$). The cases of tumors with vascular vessel invasion showed a significantly higher corrected T/N ratio of ODC than those without vascular vessel invasion ($P < 0.01$). The cases of tumors with deep wall invasion, lymph vessel invasion, or lymph node metastasis also showed a higher T/N ratio than those without, but the difference was not significant.

Figure 2 shows the correlation between the T/N ratio of ODC and that of c-*myc* in each case. There was a significant correlation between the expression of ODC and c-*myc* ($P < 0.05$).

DISCUSSION

ODC enzyme activity, determined by measuring the release of CO_2 with biochemical procedures, is higher in gastric cancer tissues than in normal tissues of gastric mucosa.[4,5,18,19] This was also recognized by our determination of mRNA status by RT-PCR in this study, and the average T/N ratio of ODC corrected for that of GAPDH was 1.37.

Terashima et al. reported that ODC activity in gastric cancer tissue was significantly higher in patients who had lymph node metastasis, serosal invasion, or peritoneal dissemination than in those who did not.[18] Conversely, by the same methodology, Okuzumi et al. reported there was no significant correlation between ODC activity and any clinicopathologic factors, although the tumors with a larger size and deeper invasion showed a higher ODC activity than those with smaller size and shallower invasion, respectively.[4] The discrepancy may be due to the

difference in obtaining specimens; Terashima et al. examined biopsy specimens obtained at endoscopic studies whereas Okuzumi et al. examined specimens obtained from surgically resected stomachs. The turnover of ODC is very rapid and the ODC activity decreased rapidly in the resected specimens.[9,18] Another explanation may be heterogeneity of ODC in gastric cancer tissue. Nakanishi et al. reported that ODC activity was higher in the surface region than in the advancing marginal area of the gastric cancer.[19] The biopsy specimens are obtained from the surface region; therefore, the ODC activity in biopsy specimens may be different from that in surgical specimens.

Although there have been several reports focusing on the role of ODC in the process of malignant transformation, few reports describe the role of ODC in tumor invasion or metastasis. Kubota et al. recently compared the invasiveness of mouse mammary carcinoma cell line, FM3A, and its variant, EXOD, which overproduces ODC.[20] EXOD cells showed an approximate fivefold greater invasiveness compared with FM3A cells. Furthermore, antisense oligonucleotides of ODC suppressed EXOD cell invasion. They thus considered that ODC was directly involved in tumor cell invasion in vitro. The present study using RT-PCR demonstrates that the tumor specimens with vascular vessel invasion show a higher T/N ratio than those without vascular vessel invasion. This suggests that ODC may be associated with tumor invasion even in in vivo specimens.

A possible role of ODC in gastric carcinogenesis has been unclarified. In the rat, continuous administration of gastric carcinogen induced ODC in the gastric mucosa.[4] Okuzumi et al. suggested that repeated administration of carcinogens or tumor-promoting stimuli such as nitroso compounds or salt, which are considered carcinogens of gastric carcinoma, induced ODC in the gastric mucosa as in the rat.[4] Auvinen et al. reported that aberrant expression of ODC is not just a coincident, pleiotypic response to transformation, but a critical factor contributing to oncogenesis.[3] They believed that the increase in production of ODC was associated with changes in tyrosine phosphorylation.

The present study demonstrated that female patients showed a higher T/N ratio than male patients. Hoshino et al. also detected a higher ODC activity in female tumor tissue compared with male tumor tissue, although it was not significantly different.[21] There is currently no appropriate explanation for the higher ODC activity in females rather than in males. One possibility is that estradiol may stimulate the polyamine cascade by inducing the ODC mRNA level as shown by the experiment using breast cancer cells.[22] However, further study would be necessary to clarify this suspect.

ODC is one of the transcriptional target genes of c-*myc;* c-*myc* is a potent transactivator of the ODC promoter-reporter gene, which has the CACGTG repeat in which *myc* can bind,[10] and enforced c-*myc* expression results in constitutive expression of the ODC gene.[23] This activation may be direct as well as for prothymosin-α,[12,13] because it does not need intermediate protein synthesis. Our study demonstrated that the T/N ratios of ODC mRNA expression paralleled those of c-*myc* mRNA expression in clinical samples of gastric carcinomas. Our data support the hypothesis that the transcription of the ODC gene in human gastric carcinomas may be associated with that of c-*myc* gene, although this does not provide direct proof.

REFERENCES

1. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res* 1988;48:759–74.
2. Shantz LM, Pegg AE. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res* 1994;54:2313–6.
3. Auvinen M, Paasinen A, Andersson LC, Holtta E. Ornithine decarboxylase activity is critical for cell transformation. *Nature* 1992;360:355–8.
4. Okuzumi J, Yamane T, Kitao Y, Tokiwa K, Yamaguchi T, Fujita Y, et al. Increased mucosal ornithine decarboxylase activity in human gastric cancer. *Cancer Res* 1991;51:1448–51.
5. Berdinskikh NK, Ignatenko NA, Zaletok SP, Ganina KP, Chorniy VA. Ornithine decarboxylase activity and polyamine content in adenocarcinomas of human stomach and large intestine. *Int J Cancer* 1991;47:496–8.
6. Yoshida M, Hayashi H, Taira M, Isono K. Elevated expression of the ornithine decarboxylase gene in human esophageal cancer. *Cancer Res* 1992;52:6671–5.
7. Clifford M, Morgan D, Yuspa SH, Soler AP, Gilmour S. Role of ornithine decarboxylase in epidermal tumorigenesis. *Cancer Res* 1995;55:1680–6.
8. Hietala OA, Yum KY, Pilon J, O'Donnell K, Holroyde CP, Kline I, et al. Properties of ornithine decarboxylase in human colorectal adenocarcinomas. *Cancer Res* 1990;50:2088–94.
9. Westin T, Edstrom S, Lundholm K, Gustafsson B. Evaluation of ornithine decarboxylase activity as a marker for tumor growth rate in malignant tumors. *Am J Surg* 1991;162:288–93.
10. Bello-Fernandez C, Packham G, Cleveland JL. The ornithine decarboxylase gene is a transcriptional target of c-*myc*. *Proc Natl Acad Sci USA* 1993;90:7804–8.
11. Evan GI, Wylie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, et al. Induction of apoptosis in fibroblasts by c-*myc* protein. *Cell* 1992;69:119–28.
12. Eilers M, Schirm S, Bishop JM. The Myc protein activates transcription of the α-prothymosin gene. *EMBO J* 1991;10:133–41.
13. Mori M, Barnard GF, Staniunas RJ, Jessup JM, Steele GD Jr, Chen LB. Prothymosin-α mRNA expression correlates with that of c-*myc* in human colon cancer. *Oncogene* 1993;8:2821–6.
14. Mori M, Staniunas RJ, Barnard GF, Jessup JM, Steele GD Jr, Chen LB. Significance of carbonic anhydrase expression in human colorectal cancer. *Gastroenterology* 1993;105:820–6.

15. Mori M, Mimori K, Inoue H, Barnard GF, Tsuji K, Nanbara S, et al. Detection of cancer micrometastases in lymph nodes by the reverse transcriptase-polymerase chain reaction. *Cancer Res* 1995;55:3417–20.
16. Japanese Research Society for Gastric Cancer. The general rules for gastric cancer study in surgery and pathology. *Jpn J Surg* 1981;11:127–39.
17. Mori M, Sakaguchi H, Akazawa K, Tsuneyoshi M, Sueishi K, Sugimachi K. Correlation between metastatic site, histological type, and serum tumor markers of gastric carcinoma. *Hum Pathol* 1995;26:504–8.
18. Terashima S, Teranishi Y, Hoshino Y, Ogata M, Ito F, Konno A, et al. Ornithine decarboxylase activity in stomach cancer [in Japanese with English abstract]. *Jpn J Cancer Clin* 1994;40:847–50.
19. Nakanishi I, Sowa M, Kato Y, Chung Y, Otani S. Heterogeneity of ornithine decarboxylase activity in gastric cancer tissue [in Japanese with English abstract]. *Oncologia* 1993;26:697–701.
20. Kubota S, Yamada T, Kamei S, Seyama Y. Ornithine decarboxylase is directly involved in mouse mammary carcinoma cell invasion in vitro. *Biochem Biophys Res Commun* 1995;208:1106–15.
21. Hoshino Y, Teranishi Y, Terashima S, Ogata M, Itoh F, Konno A, et al. Ornithine decarboxylase activity in gastric cancer. [in Japanese with English abstract]. *J Jpn Res Soc Gastroenterol Carcinogenesis* 1993;5:329–32.
22. Thomas T, Thomas TJ. Estradiol control of ornithine decarboxylase mRNA, enzyme activity, and polyamine levels in MCF-7 breast cancer cells: therapeutic implication. *Breast Cancer Res Treat* 1994;29:89–201.
23. Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-*myc* expression in an IL-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 1992;6:1915–22.