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Ornithine decarboxylase gene is overexpressed in colorectal carcinoma

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Abstract

AIM: To investigate the ornithine decarboxylase (ODC) gene expression in colorectal carcinoma, ODC mRNA was assayed by RT-PCR and ODC protein was detected by a monoclonal antibody against fusion of human colon ODC prepared by hybridoma technology.

METHODS: Total RNA was extracted from human colorectal cancer tissues and their normal counterpart tissues. ODC mRNA levels were examined by RT-PCR. ODC genes amplified from RT-PCR were cloned into a prokaryotic vector pQE-30. The expressed proteins were purified by chromatography. Anti-ODC mAb was prepared with classical hybridoma techniques and used to determine the ODC expression in colon cancer tissues by immunohistochemical and Western blotting assay.

RESULTS: A cell line, which could steadily secrete anti-ODC mAb, was selected through subcloning four times. Western blotting reconfirmed the mAb and ELISA showed that its subtype was IgG2a. RT-PCR showed that the ODC mRNA level increased greatly in colon cancer tissues ($P<0.01$). Immunohistochemical staining showed that colorectal carcinoma cells expressed a significantly higher level of ODC than normal colorectal mucosa (98.6±1.03% *vs* 5.26±5%, $P<0.01$).

CONCLUSION: ODC gene overexpression is significantly related to human colorectal carcinoma. ODC gene expression may be a marker for the gene diagnosis and therapy of colorectal carcinoma.

Key words: Ornithine decarboxylase; RT-PCR; Colorectal Cancer

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INTRODUCTION

Colorectal cancer is one of the most common cancers in the world and the second leading cause of cancer death in the USA[1,2], and the fourth rate of mortality in China[3]. Therefore, efficient diagnostic and therapeutic approaches are important for colorectal carcinoma research. Laboratory testing and screening are potentially favorable to the outcome and survival rate of patients. Studies have been directed on identifying markers for the development of colorectal cancer[4,5]. Among the biochemical alterations found in cancer cells, polyamine content is one of the most consistent changes[6]. Polyamines are highly charged cations found in all living cells. They are essential for the maintenance of cell proliferation and differentiation, and macromolecule biosynthesis by interacting with nucleic acids, proteins and membranes. Polyamine synthesis is one of the early events occurring during the G1 phase of the cell cycle and cell division[7]. Ornithine decarboxylase (ODC) is the first rate-limiting enzyme in the polyamine biosynthesis pathway[8], and plays a critical role in cell transformation[9]. ODC has been implicated as an essential promoter of cell proliferation. It has recently been postulated that the ODC gene may act as an oncogene since overexpression of this gene is essential for cell transformation. Polyamine content as well as ODC activity have been found to be increased significantly in adenocarcinoma tissues compared to paired normal tissues[10,11]. It is, therefore, suggested that ODC activity may be used as a biologic marker for the tumor growth rate and biological aggressiveness[12]. There is, however, little information on the mRNA and protein status of ODC in surgical specimens of adenocarcinoma.

In our previous studies, we constructed the human ODC expression vectors and obtained the expressed proteins[13]. In the current study, we developed an anti-ODC monoclonal antibody using the recombinant human ODC protein. Using the monoclonal antibody, we compared the ODC protein expression level in colon cancer tissues with normal colon tissues. We also compared the ODC mRNA level in colon

cancer tissues with the surrounding uninvolved mucosa. The results indicate that ODC mRNA and protein expressions in cancer tissues are much higher than those in normal tissues.

MATERIALS AND METHODS

Tissue samples

Eighty-eight paraffin colorectal tissue specimens and 62 fresh specimens were collected by colonoscopy or surgical resection. All patients had no radiation therapy or chemotherapy before surgery. Among these, 100 samples were taken from sporadic colorectal carcinoma and 50 samples of normal colon mucosa were taken from 15 cm apart from the neoplasm.

Cloning, expressing and purifying of human ODC protein

ODC cDNA was synthesized by RT-PCR using total RNA template extracted from human colon cancer tissues. An ODC gene expression vector pQE-ODC was established by inserting ODC cDNA into an expression vector pQE-30, which had a 6-His tag. The protein was purified by Ni-NTA affinity chromatography[13].

Preparation of monoclonal antibody

BALB/c female mice were immunized with the prepared ODC protein directly administered into the spleen by about 20 μg/mouse and reimmunized every two weeks intraperitoneally by about 5 μg/mouse and administered intravenously 5 d before the mice were killed. A spleen cell suspension was prepared as shown by Gerhard *et al*[14]. BALB/c (sp2/0-Ag14) myeloma cells were mixed with immune spleen cells at 1∶10. The antibodies in the supernatant of cell clones were tested by ELISA and the positive hybridoma cells were recloned four times in HAT medium by limiting dilution. The subtype of mAb was analyzed with ELISA.

Testing mAb by Western blotting

Standard ODC protein and purified ODC protein were separated by standard SDS-PAGE techniques and transferred to a cation nylon membrane. The proteins were detected using the anti-ODC antibody purified from ascites. Immunoreactive proteins were detected using HRP-goat anti-mouse IgG.

Immunohistochemical test by anti-ODC antibody

Tissues were fixed in 96% ethanol for 6 h at 4 ℃, embedded in paraffin, and cut into 5-μm thick sections. The sections were deparaffinized in xylol, rehydrated through graded ethanol, washed with PBS-Tween, and incubated for 2 h at room temperature in a humidified chamber with 100 μL of the anti-ODC mAb at 1∶1 000 dilution. The slides were washed and incubated with HRP-labeled rabbit anti-mouse IgG (Dako) diluted in PBS with 100 g/L BSA for 1 h at room temperature. After being washed, the HRP was visualized by development with chromogenic agents.

The staining intensity was graded as follows: -, no staining; +, weak staining; ++, moderate staining; and +++, strong staining.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from normal and cancer tissues, respectively. The method of RNA extraction was similar to the TRIzol RNA extraction protocol (Life Technologies Inc.). The concentration of RNA extracted was determined at wavelength of 260 nm using a U-2000 spectrophotometer (HITACH Ltd, Tokyo, Japan). The sequences of ODC primers were as follows: up-stream primer: 5'-GCAGG-ATCCACCATGAACAACTTTGGTAA -3', down-stream primer: 5'- GCCGAGATCTCAGAAGAAGAAACTTC -3'. This pair of primers could span a 120-bp fragment of human ODC exon 3. Human β-actin was used as a control. Ten microliters of each amplification reaction were analyzed by electrophoresis using 1.2% agarose gel in the presence of 5 ng/mL ethidium bromide. DNA was detected under UV light.

RESULTS

Determination of anti-ODC mAb by enzyme linked immunosorbent assay and Western blotting

ELISA showed that the mAb could immunobind to recombinant human ODC and standard ODC protein (Sigma). ELISA showed that the immunoglobulin produced by the positive clone was the IgG2a type.

Western immunoblotting showed that the mAb bound to the ODC protein (Figure 1).

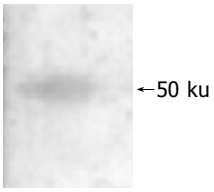

Figure 1 mAb binding to 50 ku ODC protein as shown by Western blotting.

Detection of ODC in colorectal carcinoma by immunohistochemical staining

Tumor histotype and grade of differentiation were defined according to the WHO criteria[15]. Immunohistochemical staining of human colorectal carcinoma using ODC mAb demonstrated staining in cytoplasm. Density staining in deep brown color could be seen in most of the carcinoma tissues while only a few faint stainings were in the normal tissues (Figure 2). The staining intensity showed that ODC protein expression in cancer tissue was much higher than that in normal tissue, but there was no significant difference among different histologic grades of the tumor (Table 1). There was no difference between Duck's stages A/B and C/D.

ODC mRNA expression analysis

RT-PCR showed that the expressed ODC mRNA in colorectal carcinoma tissues was significantly higher than that in normal tissues ($P<0.01$). There was no difference between male and female cases. Among the 27 well-to-moderately differentiated cancer samples, 9 were over-expressed and 18 were highly-expressed. Three of four

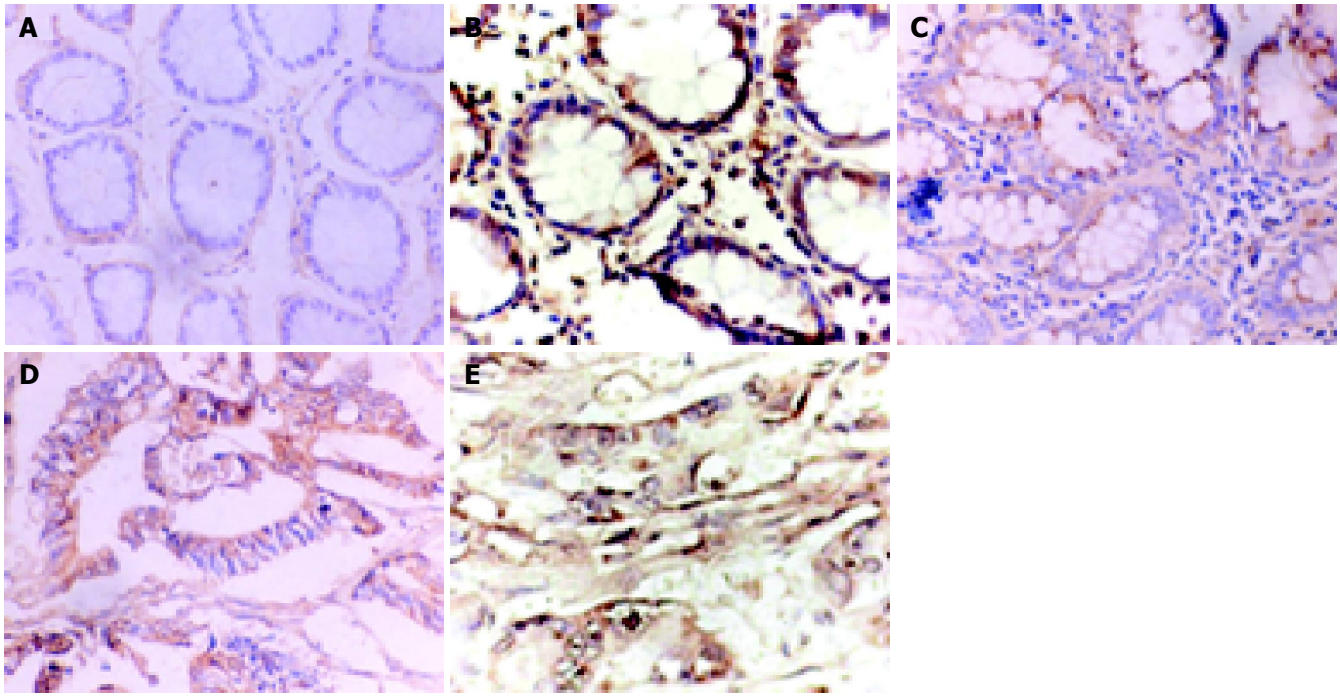

Figure 2 Immunohistochemical staining of colon tissues. **A**: Normal colon tissue obtained 15 cm apart from neoplasm; **B**: Tissue from well-differentiated adenocarcinoma; **C**: Tissue from moderately differentiated adenocarcinoma; **D**: Tissue from poorly differentiated mucinous adenocarcinoma; **E**: Tissue from undifferentiated carcinoma.

Table 1 **Immunohistochemical assay of ODC protein in colorectal carcinoma**

Histological grade and Duck's stage	–	+	++	+++	Total	Positive rate (%)	P^b
Normal	18	1	0	0	19	5	
Grade 1 well differentiated	1	2	4	9	16	93.75	<0.01
Grade 2 moderately differentiated	0	0	7	21	28	100	<0.01
Grade 3 poorly differentiated	0	0	8	7	15	100	<0.01
Grade 4 undifferentiated	0	1	6	3	10	100	<0.01
Duck'SA/B	1	1	11	17	31	96.7	<0.01
Duck'sC/D	0	1	14	23	38	100	<0.01

[b]P<0.01 *vs* normal tissue.

undifferentiated samples were over-expressed (Figure 3 and Table 2). χ^2 test showed that there was no difference among the different histologic grades, but there was a significant difference between Duck's stages AB/CD (P<0.05).

DISCUSSION

Polyamines, such as putrescine, spermidine and spermine, play an important role in cell proliferation, differentiation, and transformation. It has been proposed that urinary or blood measurement might be a useful, non-invasive diagnostic marker of colon cancer[16]. Since the intracellular polyamine pool could be regulated by external factors, such as uptake and excretion, the research outcome was disappointed. As the first and rate-limiting enzyme, ODC is the most extensively studied enzyme in polyamine metabolism. ODC protein is 50 ku as a monomer and about 100 ku when the active dimer is formed. ODC synthesis is dramatically induced by different growth stimuli, such as hormones, growth factors, carcinogens, viruses and oncogenes. The regulation can occur at the levels of transcription, translation and protein degradation. The alterations in enzymes can occur very quickly and change polyamine level in the end. ODC gene is considered as an immediate early gene, and contains response elements for several transacting factors, including a cAMP[17] response element, a possible insulin response element[18] and several Sp1 binding sites. In addition, ODC gene expression has been tightly linked to transformation by activated *ras*, *v-src and myc*[19]. So, recently, ODC gene has been postulated as an oncogene, which is essential for cell transformation[9,20,21]. It has been demonstrated that both ODC and polyamine content analysis in biopsy specimens can be very useful in the diagnosis of malignancy and prognosis of breast cancer[22]. As to colorectal cancer ODC was found in a very limited amount in quiescent cells and its activity was found to be increased significantly in colon adenocarcinoma and prostate tissue compared to normal tissue from the same patients[23,24]. Polyp is a benign neoplasia with a high risk of developing into colorectal cancer. Increased ODC activity and polyamine concentrations have

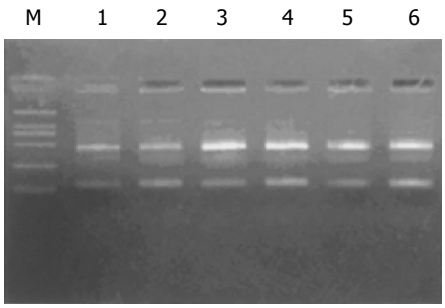

Figure 3 ODC mRNA expression assay by RT-PCR. M: molecular weight marker DL-2000. Lanes 1, 3 and 5: normal mucosa tissues. Lanes 2, 4 and 6: malignant tissue samples. β-actin was amplified as an internal control.

also been observed in the colon of presymptomatic familial adenomatous polyposis patients[22].

In the present study, we compared the ODC gene expression in human colorectal carcinoma with that in normal colon mucosa. ODC mRNA was extracted from human colon cancer tissues and ODC mRNA was detected by RT-PCR. The results showed that the ODC mRNA level in colorectal carcinoma was significantly higher than that in contiguous normal colon mucosa. Furthermore, we found that ODC gene expression was associated with the stage of malignancy. These findings suggest that the increase of ODC mRNA may play an important role in the process of colorectal tumor progression. ODC cDNA was inserted into an expression vector and the ODC gene expression vector pQE-ODC was established. The vector with a 6-His tag, which made the expression of ODC protein with about 95% purity, was used as a good immunogenic agent to immunize the BALB/c mice. Monoclonal anti-ODC antibody was produced by cell hybrids between hypoxanthine phosphoribosyl transferase-deficient myeloma cells and spleen cells of immunized mice. The antibody was the IgG2a type.

ELISA and Western blotting showed that the mAb could combine the standard ODC (EC4.1.1.17) protein. Staining of colorectal tissues with ODC mAb showed a significant difference between normal and tumor mucosae. The deep brown staining could be found in gland cells of the tumor, while there was no staining or weak staining in stoma cells. The results suggested that there was a high concentration of ODC protein in tumor cell cytoplasm. These changes did not correlate with gender, histologic grade or Duck's stage, which confirmed that increase of ODC protein was both an early event and a late event in patients with colon cancer. RT-PCR showed that the ODC mRNA was much higher in colon tumor tissues than in normal tissues. The ODC mRNA expression was higher in Duke's stages C/D than A/B. In conclusion, changes in ODC mRNA and protein content play an important role in the pathology of human colorectal carcinoma, and ODC gene expression may be a diagnosis and therapeutic marker of human colorectal carcinoma.

Table 2 **ODC mRNA expression in normal and colorectal tumor tissues**

Samples	ODC mRNA expression (*n*)			Total (*n*)	*P*
	Low	Over	high		
Normal	31	0	0	31	<0.01
Tumor	0	19	12	31	
Gender					
Male	0	9	6	15	>0.05
Female	0	10	6	16	
Histologic grade					
Well-to-moderately differentiated	0	18	9	27	>0.05
Undifferentiated	0	1	3	4	
Duck's stage					
A/B	0	17	5	22	<0.05
C/D	0	2	7	9	

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