Study on the Relationship Between ODC Gene Expression and Colorectal Carcinoma

Xianxi Liu, Aihua Sun, Xiaoming Wang, Shilian Liu, Yan Zhang, Bing Zhang, Haiyan Hu, Lei Gong, Hui Sun

Institute of Biochemistry and Molecular Biology, School of Medicine, Shandong University, Jinan 250012, P. R. China

ABSTRACT Objective The ornithine decarboxylase (ODC) mRNA and protein levels in colorectal cancer and normal colorectal tissue were compared in order to decipher the relationship between ODC gene expression and the phathogenesis of colorectal cancer (CRC). **Methods** Total RNA was extracted from 31 surgically resected specimens of colorectal carcinoma and normal colorectal mucosa close to the lesions. The ODC mRNA levels in the lesions and the normal surrounding mucosa were examined semi–quantitatively by the reverse transcription polymerase chain reaction (RT–PCR). The protein levels were determined by western blotting. The correlation of ODC gene expression to clinical pathological stages was determined. **Results** The ODC mRNA and protein levels in colorectal carcinoma tissues were significantly higher than that in the surrounding normal mucosa ($p<0.01$). Based on the ratio of ODC expression in neoplasm to that in contiguous normal mucosa, the 31 patients were divided into two groups: ODC over–expression group (12 cases) and ODC high–expression group (19 cases). Among the 12 patients in the ODC over–expression group, 7 patients were in Dukes C and Dukes D stages. On the contrary, in the ODC high–expression group (19 patients), only 2 cases were in Dukes C and Dukes D stages. The differences in Dukes stages between the two groups were statistically significant ($p=0.012$). **Conclusion** ODC gene expression plays an important role in colorectal carcinogenesis and the up–regulated expression of ODC mRNA and protein was related to the Dukes stages of colorectal cancer.

Key Word Colorectal carcinoma; Ornithine decarboxylase; Gene expression.

Colorectal cancer is a major health problem in China and is associated with significant morbidity and mortality. Both intracellular polyamine concentrations and ornithine decarboxylase (ODC) enzymatic activity are increased in colorectal cancer tissues [1,2]. Measurement of the polyamine contents of serum and urine has been proposed as a diagnostic marker of this cancer. It has, therefore, been suggested that ODC activity may be used as a biological marker for the tumor growth rate and biological aggressiveness[3]. There is, however, little information on the mRNA and protein status of ODC in surgical specimens of this cancer. In the current study, we confirm that the ODC mRNA and protein level is increased in human colorectal cancer tissues compared to the normal colorectal mucoca. The expression profile of this gene could be used as a diagnostic marker as well as for cancer staging.

MATERIALS AND METHODS

Tissue samples Malignant tissue and adjacent normal colon tissue were freshly obtained from 31 primary colorectal carcinomas, which were removed at operation between September, 2002 and February, 2003 at the Department of Surgery, Qilu Hospital Affiliated to Shandong University. The tissues were immediately frozen in liquid nitrogen after surgery. The mean age of the patients at diagnosis was 60 years old (range 39 to 92) and the male–to–female ratio was 15/16.

Reagent Trizol RNA extraction kit was bought from Life Technologies Inc. RT –PCR kit was bought from Takara. The primary antibody against human ODC protein was developed and provided by our laboratory.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT–PCR) Total RNA was extracted from half of the human tissue by direct homogenization in Trizol reagent according to the manufacturer´s instructions. The remaining half of the tissue was saved for protein extraction. RNA was quantified by spectrophotometry. Reverse transcription of RNA and PCR amplification of cDNA was carried out according to the manufacturers´ in-

structions. Briefly, first strand cDNA synthesis was performed in a 20μl reaction volume containing 20 units of RNase inhibitor, 1 pM of random hexamers, 10 mM of each deoxynucleotide triphosphate, 5 units of Molony murine leukemia virus reverse transcriptase, and μg of total RNA. The reaction mix was incubated for 10 min at room temperature. The program for reverse transcription step was 30 min at 55℃ and terminated by heating to 99℃ for 5 min. The primer sequences used to amplify human ODC cDNA are as follows. Forward promer: 5´–GCAGGATCCACCATGAACAACTTTGGTAA–3´ and reverses primer: 5´–GCCGAGATCTCAGAAGAAGAAACTTC–3´. This pair of primers amplifies a 120 bp fragment of human ODC exon 3. The human β–actin was used as a control. The primer sequences used to amplify β–actin are as follows. Forward primer: 5´–GCGGATGTCCACGTCACACT–3´ and reverse primer 5´–CCACTGGCATCGTGATGGAC–3´. This pair of primers amplifies a 428bp fragment of β–actin. The first strand cDNA (2.5μl) was further amplified by PCR in a final volume of 50μl with 1 x TaqMan buffer, 2.5 mM MgCl2, 200 μM of each deoxynucleotide triphosphate, 200 μM of each primer, and 1.25 units of Taq polymerase (Takara Inc.). PCR conditions for ODC and β–actin cDNA were set up as follows: 1 cycle of denaturing at 94℃ for 5 min followed by 35 cycles of 94°C for 40s, 56℃ for 40s, and 72℃ for 1 min and a final primer sequence extension step at 72℃ for 7 min. Ten μl of each amplification reaction were analyzed by electrophoresis using a 1.2% agarose gel in the presence of 5ng/ml ethidium bromide. DNA bands was quantified by Amersham gel image analysis system.

Protein Extraction and Western Blot Analysis Tissue was homogenized in Tris buffer [50 mM Tris–CI (pH 7.5), 75 mM NaCl, 1% Triton, and 0.1 x SDS] and centrifuged at 5000 x g for 20 min at 4°C. The supernatants were used for western blot. One hundred and twenty–five μg of protein were electrophoresed on a 12% SDS–PAGE gel. Separated proteins were transferred onto a nitrocellulose membrane in Tris/glycine buffer [25 mM Tris base, 250 mM glycine, 0.1% SDS (pH 8.3)] at 90 V for 1 h at 4℃. Nonspecific binding sites were blocked for 1 h at room temperature with nonfat dried milk dissolved in TBS [50 mM Tris base(pH 7.5), 150 mM NaCl]. The blots were incubated overnight at 4℃ in a solution containing primary anti–ODC antibodies. The blots were washed three times with TBS followed by an incubation step with a secondary antibody against mouse IgG labeled with horseradish peroxidase (1:500 in TBS) for 1 h at room temperature. Reactive proteins were visualized with a chemiluminescence detection system.

RESULTS

Semi–quantitative PCR analysis of ODC mRNA The ODC mRNA level was evaluated by RT–PCR. The oligonucleotide primers in this study were used to amplify a 120–base cDNA fragment of the human ODC gene. All 31 colorectal cancer samples showed increased levels of ODC gene expression. In contrast, the normal tissue specimens showed no apparent increase or significantly low ODC mRNA level. The representative findings are shown in Figure 1 and summarized in Table 1. Table 2 shows the pathological data and the ODC expression profile. The cases in stages C+D also showed a significantly greater T:N ratio than those in stages A+B (*P*=0.012). There was no difference between the T:N ratio of ODC gene expression related to the other pathological factors, such as age, sex, or tumor location.

Table 1 The difference of ODC mRNA expression between cancer and normal tissues

sample	n	level of ODC mRNA $(\bar{x}\pm s)$
Normal tissues	31	19.64±4.73
Cancerous tissues	31	37.05±5.32*
Duke´s stages		
A+B	22	33.79±4.41▲
C+D	9	45.00±6.50

* *P*<0.01 vs normal mucosas close to the lesions
▲ *P*<0.05 vs C+D group

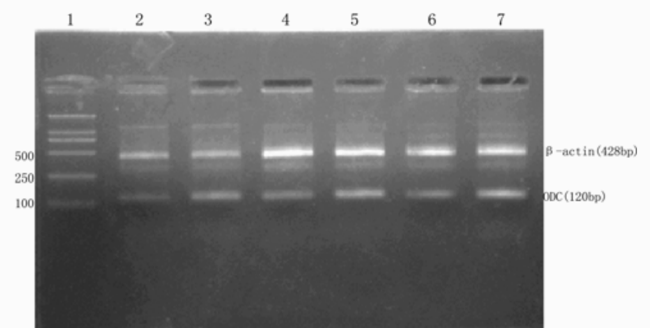

Fig.1. Representative RT–PCR analysis from colorectal tumor patients. β–actin was amplified as an internal control. Lane 1: molecular weight marker DL–2000. Lane 2,4 and 6:normal mucosa tissues. Lane 3, 5 and 7;malignant tissue samples.

Table 2 Clinico pathological data and ODC mRNA expression

Variables	ODC over-expression/ODC high-expression	Fisher´s exact test
sex		
male	6/9	
female	6/10	
histology		p=1.0
well-to-moderately	9/18	
differentiated		p=0.272
Undifferentiated	3/1	
Ducks stage		
A+B	5/17	p=0.012
C+D	7/2	

Western Blot Analysis of ODC protein To examine whether mRNA expression resulted in production of ODC protein, whole protein extracts from tumor and normal tissue were analyzed by western blot. ODC protein is expressed at low levels in the normal colorectal tissue but is markedly up-regulated in most of the tumor tissues (Fig. 2).

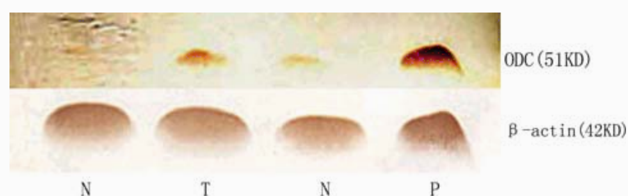

Fig. 2. Western blot analysis of tumor and surrounding normal tissues.
T: malignant tissues N: normal mucosa.

DISSCUSION

Ornithine decarboxylase is the first and rate-limiting enzyme in polyamine biosynthesis. It is responsible for converting L-ornithine to putrescine. Polyamines, i.e. putrescine, spermidine, spermine are essential for cell proliferation[5]. These plyamines are also found in both procaryotic and eukaryotic cells. ODC is aberrantly regulated in many tumor cells and virtually all tumors have higher levels of ODC activity and polyamines than normal tissues. The expression of ODC is transiently increased on stimulation by growth factors but becomes constitutively activated during cell transformation induced by carcinogens, viruses or oncogenes such as c-myc, v-src, v-raf, or an activated ras or rho A protein. Recent findings in both mouse and human tumors suggest that aberrant regulation of ODC expression plays an important role in tumor development [4,5]. Cells and transgenic animals engineered to constitutively over-express ODC have high intracellular and extracellular putrescine levels and a more cancerous phenotype, including the ability to grow in soft agar, form tumors in animals (probably via an alteration in the process of tumor promotion), and increased angiogenesis and metastasis. By using enzymatic assays our laboratory has discovered that the ODC activity is higher in tumor tissue than in normal tissue[1]. High ODC activity in tumor tissue has been demonstrated primarily among poorly differentiated tumors. ODC activity thus has been suggested to be used as a marker for tumor growth rate and biological aggressiveness. However little information on ODC mRNA level in surgical specimens of cancer is available in the literature. In the current study, we compared the ODC gene expression in colorectal carcinoma with the normal tissues adjacent to the tumor though semi-quantitative RT-PCR and western blot. Our results show that ODC mRNA level in colorectal carcinoma tissues was significantly higher than that of contiguous normal mucosa tissues. Furthermore, we found that ODC gene expression was associated with the stage of malignancy. These findings suggest that ODC may play an important role in the process of colorectal tumor promotion.

Recently, there were reports indicating the role that ODC plays in tumor invasion, metastases and angiogenesis. Kubota et al [6] have suggested that ODC is directly involved in tumor cell invasion in vitro. In his study, cancer tissues from patients with lymph node metastases showed a higher T:N ratio for ODC mRNA than those of without lymph node metastasis. Ken-ichi Mafune et al[7] has demonstrat-

ed that in stomach cancer patients cancer tissues from patients with vascular invasion showed a higher T:N ratio for ODC mRNA than those of without vascular invasion. These findings suggest that ODC may be associated with tumor invasion and metastases in in vivo. Therefore, ODC mRNA expression profile might be used as not only a marker of tumor proliferation, but also a marker of biological aggressiveness of cancer.

Angiogenesis is an essential step in tumor growth. This process is regulated by endothelial proliferation factors and their inhibitors such as endostatin. Endostatin is a carboxyl-terminal fragment of type XVIII collagen. It inhibits endothelial proliferation, angiogenesis, and tumor growth. Takahiro Nemoto et al[8] reported ODC-overexpressing cells, when transplanted into nude mice, suppressed type XVIII collagen expression and promoted neovascularization in vivo. They hypothesized that ODC overexpression may facilitate angiogenesis in tumors by suppressing endostatin expression. It was also reported that DFMO (alpha-difluoromethyl ornithine), a specific irreversible inhibitor of ornithine decarboxylase, inhibited liver metastasis of KKLS (a gastric cancer cell line) after transplanted in the stomach of nude mice. The vessel density of DFMO-treated tumors was significantly lower than that of non-treated tumors. These results suggest that DFMO plays an important role in anti-angiogenesis and apoptosis of tumor[9]. It also indicates that ODC gene expression is related to tumor metastases and angiogenesis.

Based on the ratio of ODC gene expression in neoplasm to that of contiguous mucosa, the 31 patients in our study were divided into two groups: over-expression group (12 cases) and high-expression group (19 cases). In the over-expression group patients were staged in Dukes C and Dukes D stages. On the contrary, in the high-expression group, only 2 cases were staged in Dukes C and Dukes D stages. The difference in Dukes stages between the two groups was statistically significant (p =0.012). These results indicate that ODC gene expression profile may be used to predict tumor aggressiveness and metastasis.

In conclusion, our present work supports previous observations that elevated ODC expression is associated with the invasive and aggressive behavior of tumors including CRC. It also enhances our understanding of the molecular mechanisms responsible for colorectal tumor development and progression. These findings may help to design new therapeutic strategies for the treatment of CRC and its metastases.

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