Identification and Analysis of Splicing Variant of Cyclooxygenase-2 mRNA in Human Colon Cancer Tissue*

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Abstact **Objective** To explore COX-2 alternative splicing expression in human colon cancer tissue. **Methods** According to the sequence of human COX-2 gene, the special primers were designed and synthesized, then Reverse transcription polymerase chain reaction (RT-PCR) was used for investigating the expression of COX-2 in human colon cancer tissue, PCR products were cloned, sequenced and analyzed by BLAST and other bio-informatics methods. **Results** The electrophoresis of the RT-PCR product showed one short band of reported COX-2 comprising of 252bp that contains the 7 exon and the 8 exon in human normal colon tissue and colon cancer tissue, but one long new band comprising of 534bp was detected in human colon cancer tissue. Sequence analysis showed that one 282bp intron sequence was inserted between exon 7 and exon 8. Analysis of the predicted amino acid sequence indicated that an in-frame stop codon was contained in the 48-50bp of retention intron. **Conclusion** A new COX-2 splice variant (COX-2sv) that may, at best, code for a truncated COX-2 protein has been identified in human colon cancer tissue. Its biological significance remains to be further investigated.

Key Words cyclooxygenase-2; splice variant; gene cloning

There are two isoforms of cyclooxygenase(COX) that catalyze the formation of prostaglandins (PGs) from arachidonic acid. Generally, COX-1 is a housekeeping gene that is expressed constitutively in most tissues, where it maintains physiological processes. COX-2 is an immediate, early response gene that is virtually undetectable in most tissues under physiological conditions, but it may be highly inducible by numerous factors, including cytokines, LPS, growth factors, mitogens, tumor promoters etc [1]. Previous several studies using mouse models of colon cancer and the results of clinical trials have shown that COX-2 is a useful target for the prevention and treatment of colon cancer and plays an important role in the pathogenesis of colon cancer[2]. Recently, Huang et al reported that the COX-2 gene expresses an alternatively spliced mRNA COX-2 splicing variant (SV) in human myometrium[3]. Because of relationship between COX-2 and colon cancer, in this study, we determinate to investigate whether COX-2 gene alternative splicing express in human colon cancer and deduce its possible protein structure and function.

MATERIALS AND METHODS

Patient samples and Tissue collection

Twenty colon cancer tissues and adjacent normal colonic tissues were obtained from Tongji Hospital and Union Hospital, Tongji medical college , Huazhong university of science and technology. All cases were surgically resected between July 2001 and July 2002 at Tongji Hospital and Union Hospital, and diagnosed as primary advanced colon carcinoma. The patients included 15 men and 5 women ranging in age from 32 to 72 years old (mean, 53.4 years). The sample selection was restricted to consecutive cases diagnosed as stages II and III. All patients had undergone curative resection. None of the patients received chemotherapy or radiation therapy preoperatively. All colon cancer tissues after surgery was diagnosed by serum CEA level, ultrasonography, computed tomography scan. Eleven colon cancer tissues were classified as stage II, nine as stage III. Histologically, all colon cancer tissues were classified as well-differentiated adenocarcinoma. All adjacent normal colon tissues were

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verified. All sample after surgical resection was immediately rinsed in phosphate-buffered saline solution (PBS solution) which had been precooled to 4℃, homogenized on ice and then RNA was extracted.

RNA isolation

Total RNA was isolated using the Tri-Reagent protocol (GIBCO BRL, USA). All sample was homogenized in Tri-Reagent (1ml/1000mg) and centrifuged at 12,000g for 10 minutes at 4℃. The supernatant was collected and kept at room temperature for 5 minutes. Then 0.2ml chloroform per milliliter Tri-Reagent was mixed with the supernatant and centrifuged at 12,000g at 4℃ for 15 minutes. The aqueous phase was then removed, and the RNA was precipitated with isopropanol and collected by centrifugation at 12,000g at 4℃ for 5 minutes. The supernatant was removed, and the RNA pellet was washed in 75% ethanol and centrifuged at 7,500g at 4℃ for 5 minutes. The RNA pellet was then air-dried and solubilized in di-ethylpyrocarbonate-treated water.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA sample 6μl, Rnasin 1μl, oligonucleotide primers 5μl (Promega, USA), reverse transcriptase buffer- dithiothreitol 1μl, were denatured at 70℃ for 10 minutes. 5×buffer 4μl, 4×dNTPs 2μl, mouse-moloney leukaemia virus (M-MLV) reverse transcriptase (Promega, USA) 1μl were added and incubated at 37℃ for 1 hour. A reverse transcription product cDNA 5μl was then used in the amplification reaction. The PCR was preformed in 1.5 mmol/L magnesium chloride, 200μmol/L of dNTPs, 100pmol/L each primer, 10×Taq polymerase buffer 10μl, and 2.5 unit of Taq polymerase (Huamei biotech, CHN) in total 50μl volume. Reaction cycles were denaturing at 94℃ for 45 seconds, annealing at 54℃ for 1 minute, and extension at 72℃ for 1 minute for 30 cycles. Primers were designed for PCR amplification. The sense primer (5`-TGGTGCCTGGTCTGATGA TG-3`) corresponded to nucleotides 973-992 of human COX-2 cDNA sequence (Genbank accession no. NM000963). The antisense primer (5`-GCAATACGATTTTGGTACT-G-3`) corresponded to nucleotides 1206-1225. Aliquots of the PCR products (8μl) were separated by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide and quantitated under ultraviolet light.

Gene cloning and sequencing

The PCR products were then collected by agarose and cloned into vectors. Blue-white selection was performed to further amplification and identification. Sequencing of the RT-PCR products were performed with the Sanger Chain Termination Sequencing Method. The cDNA sequences were analyzed with basic local alignament search tool (BLAST) and Lasergene-DNA star software in Genbank database. Further the possible new COX-2 protein was analyzed with bio-informatics methods.

RESULTS

PCR amplification results

Two distinct mRNA bands (250bp and 530bp) were detected in human colon cancer tissue with the above-mentioned primers using RT-PCR, while there is only 250bp band in normal colon tissue(Fig 1).

Sequencing results

The two independent mRNA bands were isolated from human colon cancer and analyzed by DNA sequencing(Fig.2). The 250bp band exactly matched the published COX-2 cDNA sequence (accession number: NM000963), whereas the other 530bp band had a 282bp insertion between the reported seventh and eighth exon sequences. This 282bp sequence was found in the reported COX-2 genomic DNA (accession number: AL033533) and matched the AG/GT rule suggesting that it was produced by the alternative splicing. This retained intron contains an in-frame stop codon at 48~50bp. To further analyze the predicted amino acid sequence indicates that the alternative spliced cDn -t -op u l C

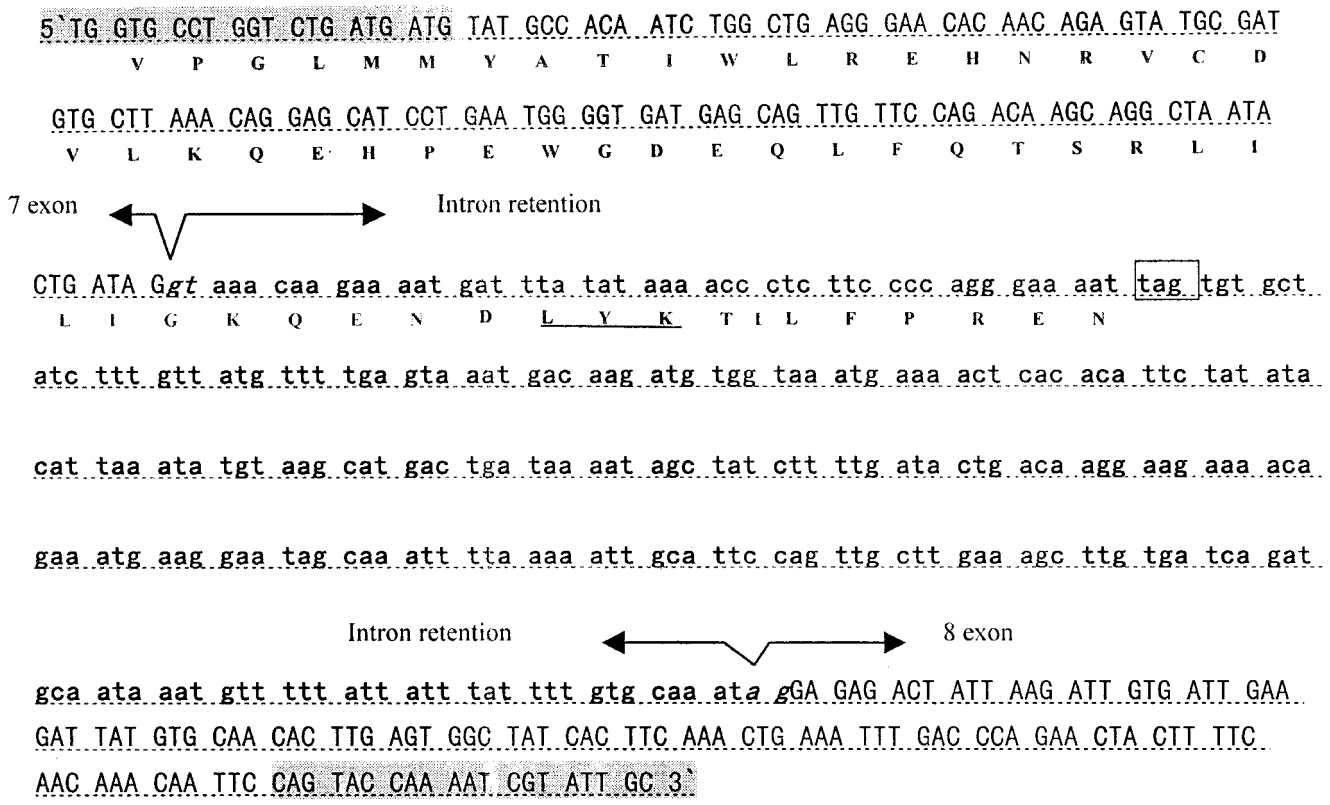

Fig 2. Nucleotide and polypeptide sequence of 530bp clone fragment

The primers are indicated with shadow. The retention is intron designated by lowercase letter. There is an in-frame stop code at 50bp (shown as an box). Splice site as italic letter. the new active-site tyrosine is shown as underline.

DISCUSSION

Colon cancer is currently one of the major causes of death from cancer in developing countries and, thus the research for pathogenesis of colon cancer has become very important. Accumulating evidence has suggested that cancer cells expressing higher levels of COX-2 may obtain a survival advantage that eventually facilitates the tumor development and progression. Many epidemiological and experimental studies have demonstrated that inhibition of cyclooxygenase (COX) is an effective measure in reducing the risk of colon carcinogenesis [4]. Although these studies have established a direct relationship between COX-2 expression and colon cancer cell survival, the precise mechanism between COX-2 and colon cancer has seldom been investigated and remains elusive. Recently studies have shown some different results between COX-2 and colon cancer. Concomitant increases in COX-2 protein and prostaglandins have not been detected in all colon neoplasia or cell lines [5]; Toyota et al. found that methylation of CpG islands near exon 1 silenced COX-2 expression in colorectal cancers and colorectal adenomas [6]; Some studies suggest that COX-2 exerts its anti-proliferative effect independently of cyclooxygenase activity [7]; nonsteroidal anti-inflammatory drugs induces apoptosis through a target other than COX-2 by controlling the major antiapoptotic PDK1/Akt/PKB pathway [8]. All these results will make the research of relationship between COX-2 and colon cancer more complication.

In this study, according to human COX-2 DNA sequences, we firstly isolate and identify COX-2 splice variant successfully in human colon cancer tissues using the special primers. We have described the character of COX-2 splice variant. It is the seventh intron retention, we named COX-2 splice variant as COX-2b. Further analyzing, the alternatively spliced COX-2 mRNA contains an in-frame stop codon in its retained intron, it may encodes a truncated protein. In the originally reported COX-2 mRNA, the first to the seventh exon en-

codes the EGF like domain, the eighth to the tenth exon contain active tyrosine site and aspinin-acetylation site[9]. As the retained intron is located between the seventh and eighth exon, it results in the express of a protein consisting of 323 aa of the EGF like domain of the reported COX-2 protein and additional 16 aa that contain a new tyrosine active site. The encoded protein lacks the obvious aspinin-acetylation site. Simmons et at reported the expression of a catalytic variant of COX-2 has been shown in vitro in transformed macrophages treated with high dosed of NSAIDs (apart from aspirin) for 48h. This enzyme (again identified by an antibody against COX-2) is sensitive to inhibition by paracetamol but less sensitive to other NSAIDs compared with COX-2 induced by lipopolysaccharide. And aspirin does not inhibit the NSAIDs induced variant enzyme [10]. The puzzling finding shown that the composition of the aspirin acetylation active site of this variant enzyme has changed. Whether this variant enzyme that they found and we deduced is the same is to be confirmed. Undoubtedly, the COX-2b finding has very important significance in human colon cancer tissues to illustrate relationship between the COX-2 polymorphous expressing and the pathogenesis of colon cancer.

In a word, we have described a novel alternatively spliced variant of COX-2 in human colon cancer tissues, which we have named COX-2b. However, the function of COX-2b in tumorigenesis has not been established and only represents 80% of colon cancer patients expressed COX-2b mRNA. Studies are underway to further our understanding of the molecular mechanisms of COX-2b and the significance of COX-2b in colon cancer. The further studies will enhance our overall understanding of the COX related diseases.

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