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Expression of Notch-signaling pathway in familial adenomatous polyposis and its clinical significance

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Abstract. – OBJECTIVE: To investigate the relationship between Notch-signaling pathway and familial adenomatous polyposis (FAP) through the expressions of Notch-1, DLL-1, and *p*-mTOR, and to explore its correlation with tumorigenesis.

MATERIALS AND METHODS: The expressions of Notch1, DLL-1, and p-mTOR were detected in 21 cases of FAP polyps, 20 cases of colorectal inflammatory polyp, and 20 cases of poorly differentiated colorectal cancer by Western blotting and reverse transcription polymerase chain reaction (RT-PCR). The protein and mRNA expressions of these genes were then compared among the FAP group, colorectal adenoma group, and colorectal cancer group.

RESULTS: The protein and mRNA expressions of Notch-1, DLL-1, and p-mTOR in the FAP group were significantly higher than those in colorectal adenoma group; furthermore, they were significantly higher in the colorectal cancer group (all $p < 0.05$).

CONCLUSIONS: Notch signaling is activated in FAP. The activated Notch-signaling pathway may play an important role in the malignant transformation and tumorigenesis.

Key Words:
Familial adenomatous polyposis, FAP, Notch1, DLL-1, p-mTOR.

Introduction

Familial adenomatous polyposis (FAP), also known as adenomatous polyposis coli (APC), is one of rare autosomal dominant genetic diseases of gastrointestinal system, characterized by early disease onset and multiple polyps. There were hundreds even thousands of colorectal polyps throughout the full colorectal system. Despite of unknown etiology, the formation of APC was mostly believed to be a result of mutations of the adenomatous polyposis gene (e.g., APC)[1]. Although the prevalence of malignant transformation from FAP to colorectal cancer was 5%, the tumorigenesis risk of FAP might be as high as 100% in FAP treatment-naive patients of about 40 years[2,3]. Currently, the diagnosis of this disease is mainly based on symptomatic, endoscopic, and molecular genetic testing evidences. Surgical resection was identified to be the main intervention against this disease. Therefore, the early diagnosis and intervention of FAP require concerns. The Notch-signaling pathway is involved in the regulation of multiple cellular biological processes, such as cell proliferation, differentiation, and survival. As demonstrated by recent evidences, Notch signal abnormalities are closely correlated to tumorigenesis. In this study, the expression profile of Notch-signaling pathway proteins was examined in FAP to elucidate the correlation between this signal pathway and the malignant transformation of FAP.

Materials and Methods

Materials

Surgical and biopsy specimens were collected from patients admitted to or undergone physical examinations in our hospital from March 2006 to March 2013, including: 21 cases of FAP patients (13 males and 8 females, aging from 16 to 43 years old, with the average age of 29.5 years, including 12 cases of tumorigenesis: 7 males and 5 females). In addition, 20 cases of colorectal inflammatory polyp and 20 cases of poorly differentiated colorectal cancer were selected, and all these case received no pre-operational radiotherapies or chemotherapies. All tissue specimens of patients with colorectal FAP were collected within 30 min following surgical resection, and were transferred into liquid nitrogen for preservation, for total protein extraction and further comparisons of expression profiles among Notch1, DLL-1, and p-mTOR.

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Reagents

Notch-1 rabbit anti-human polyclonal antibody and DLL-1 murine anti-human monoclonal antibody were purchased from Abcam Company (Combridge, UK). p-mTOR rabbit anti-human monoclonal antibodies were purchased from Cell Signaling Technology Company (Boston, MA, USA); RNAisoTM Plus, RNA PCR kit (AMV) V.3.0 were supplied by Takara (Otsu, Shiga, Japan). The primers were synthesized by Sangon, Shanghai, with the sequence depicted as follows: Notch-1 upstream primer: 5′-GGGTC-CACCAGTTTGAATGG-3′, downstream primer: 5′-GTTTGCTGGCTGCAGGTTCT-3′, with the length of 306 bp; DLL-1 upstream primer: 5′-AGGTCTTGTCGATGAAGC-3′; downstream primer: 3′-CGTGTCCTCGTAAGTATC-5′, with the length of 283 bp. β-Actin upstream primer: 5′-GACCCAGATCATGTTTGAGACC-3′, downstream primer: 5′-TAGGAGCCAGGGC-AGTAATCT-3′, with the length of 611 bp.

Methods

Western Blot Assays

Tissue samples were sonicated. The protein concentration was determined according to the bicinchoninic acid (BCA) method. After analysis by 10% SDS-PAGE electrophoresis, the yielded proteins were transferred to nitrocellulose membranes and were blocked using the blocking solution containing 5% skim milk at room temperature for 1 h, followed by addition of Notch-1 rabbit anti-human polyclonal antibody, DLL-1 mouse anti-human monoclonal antibody and p-mTOR rabbit anti-human monoclonal antibody. With β-actin as the internal standard, the resultant mixture was cultivated at 4°C overnight. After being washed in the next day, appropriate fluorescent secondary antibody was added and the mixture was incubated in darkness for 1 h, and was stained with Oddsey system. The results were processed using the image analysis software.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For each group, extraction of total RNA was performed according to the instruction for RNA PCR kit (AMV). RT-PCR was extracted according to the instruction for RNA PCR kit (AMV) V.3.0. With β-actin as the internal standard, the extract was analyzed by 1% agarose electrophoresis at 68°C. The strips of amplified products were analyzed by the gel digital imaging system

Statistical Analysis

Statistical analyses were performed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Inter-group average comparisons were analyzed by using *t*-test. For multi-group average comparisons, One-way ANOVA was analyzed and a p value <0.05 was considered to be statistically significant.

Results

Protein Expression Profiles of Notch1, DLL-1, and p-mTOR in FAP Polyps and Their Relevance to the Pathological Characteristics

The positive rates of Notch1 protein expression were determined to be 10% (2/20), 90.48% (19/21), and 95% (19/20) for colorectal inflammatory polyp, FAP, and poorly differentiated colorectal cancer, respectively. The positive rates of Notch1 ligand protein expression were similar to those of Notch1 protein, and were determined to be 10% (2/20), 90.48% (19/21), and 100% (20/20), respectively. There were statistically significant differences in the expression profiles of both proteins between the FAP group and colorectal inflammatory polyp or poorly differentiated colorectal cancer. The expression profiles were higher than colorectal inflammatory polyp, but lower than poorly differentiated colorectal cancer ($p < 0.05$). As demonstrated by experimental evidences, the expression profiles of p-mTOR protein interacting with the Notch-signaling pathway were determined to be 20% (4/20), 85.71% (18/21), and 100% (20/20). The content of protein in the non-cancerous FAP group was significantly higher than that of colorectal inflammatory polyp, and lower than that of poorly differentiated colorectal cancer. The differences among these three groups were statistically significant ($p < 0.05$). There were 12 cases of malignant transformation out of 21 FAP patients. The expression profiles of three proteins in the group of malignant transformation were significantly higher than those of the colorectal inflammatory polyp group or the non-cancerous FAP group. These differences were statistically significant ($p < 0.05$), as shown in Figure 1 and Table I.

Table I. Comparison of Notch-l, DLL-1, and p-mTOR assays ($\bar{\chi} \pm s$)

Group	n	Notch-1	Positive rate	DLL-1	Positive rate	p-mTOR	Positive rate
Colorectal inflammatory polyp	20	0.13 ± 0.08	2 (20)	0.16 ± 0.11	2 (20)	0.21 ± 0.06	4 (20)
Poorly differentiated colorectal cancer	20	0.77 ± 0.04[a]	19 (20)	0.79 ± 0.07[a]	20 (20)	0.76 ± 0.05[a]	20 (20)
FAP	21		19 (21)		19 (21)		18 (21)
Non-cancerous group		0.51 ± 0.03[ab]	8 (21)	0.57 ± 0.03[ab]	8 (21)	0.60 ± 0.03[ab]	8 (21)
Cancerous group		0.73 ± 0.02[a]	11 (21)	0.76 ± 0.05[a]	11 (21)	0.79 ± 0.07[a]	10 (21)

Notes: Compared with the control group of colorectal inflammatory polyp.
[a]$p < 0.05$; compared with the group of poorly differentiated colorectal cancer. [b]$p < 0.05$.

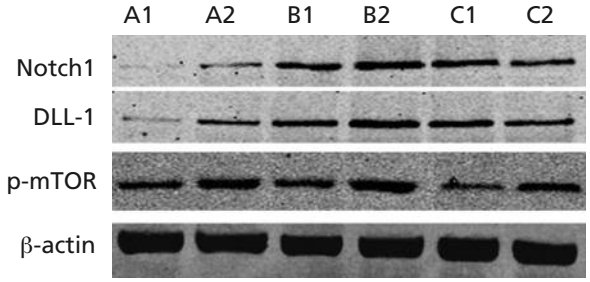

Figure 1. Expression profiles of Notch-1, DLL-1, and p-mTOR proteins in FAP quantitatively assayed by Western blot. ***A,*** Specimen of colorectal inflammatory polyp; ***B,*** Specimen of poorly differentiated colorectal cancer group; ***C,*** Specimen of FAP Group.

Expression profiles of Notch-1 and DLL-1 Genes in FAP polyps and their Relevance to the Pathological Characteristics

The mRNA expression profiles of Notch-1 and DLL-1 were consistent to the results of Western blot assays. The expression profiles of FAP malignant transformation and non-cancerous FAP groups were significantly higher than those of the group of colorectal inflammatory polyp. For the non-cancerous group, the expression profile was lower than that of the group of poorly differentiated colorectal cancer. The inter-group differences were statistically significant ($p < 0.05$). Twelve of 21 FAP patients experienced malignant transformation, with the mRNA expression profiles significantly higher than those of the groups of colorectal inflammatory polyp and non-cancerous FAP groups. The differences were statistically significant ($p < 0.05$), as described in Figure 2 and Table II.

Discussion

FAP is one of rare autosomal dominant genetic diseases of gastrointestinal system, with the penetrance rate of approximate 50%. This disease has various clinical manifestations, characterized by gradual development of multiple polyps with adenomatous polyposis in appearance in the colorectal mucosa during the puberty growth period, and exponential growth with aging. More than 100 adenomatous polyps might be observed in the colon, and the entire colon might be involved. Patients experienced their clinical symptoms in their 20-30 years, with the main clinical symptoms including abdominal pain, bloody diarrhea, and intestinal obstruction. Parenteral manifestations include: (1) Upper gastrointestinal polyps, such as the stomach, duodenum, biliary system, with the small intestine involved in rare cases[4]; (2) Manifestations of eyes, soft tissue, and bone, such as congenital hypertrophy of the retinal pigment epithelium[5], which could be considered as one of characteristic manifestations of early diagnosis; osteoma of the mandible could be observed in more than 90% patients with FAP, which was also one of characteristic manifestations of this disease. The incidence of hereditary desmoid fibromatosis might be up to 6-8%; And (3) FAP patients were more likely to experience malignancies excepting for colorectal cancers, such as thy-

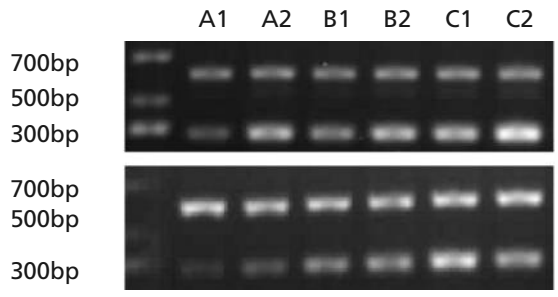

Figure 2. The gene expression profiles of Notch-1 and DLL-1 quantitatively assayed by RT-PCR. ***A,*** Specimen of colorectal inflammatory polyp; ***B,*** Specimen of poorly differentiated colorectal cancer group; ***C,*** Specimen of FAP group.

Table II. Comparison of gene expression profiles between Notch-1 and DLL-1 ($\bar{x} \pm s$)

Group	n	Notch-1	Positive rate	DLL-1	Positive rate
Colorectal inflammatory polyp	20	0.09 ± 0.01	2 (20)	0.12 ± 0.03	2 (20)
Poorly differentiated colorectal cancer	20	0.69 ± 0.02[a]	19 (20)	0.79 ± 0.11[a]	20 (20)
FAP	21		19 (21)		19 (21)
Non-cancerous group		0.48 ± 0.09[ab]	8 (21)	0.53 ± 0.04[ab]	8 (21)
Cancerous group		0.79 ± 0.11[a]	11 (21)	0.68 ± 0.06[a]	11 (21)

Notes: Compared with the control group of colorectal inflammatory polyp.
[a]$p < 0.05$; compared with the group of poorly differentiated colorectal cancer. [b]$p < 0.05$.

roid cancer, glioblastoma, medulloblastoma of central nervous system[6]. If no prompt interventions were implemented, almost all cases of this disease were likely to experience malignant transformation, with the average age of 40 years for colorectal cancer onset. Current treatments relied heavily on surgical resection, with supplement intervention of selective COX-2 inhibitors to provide anti-inflammatory and analgesic effects, as well as growth suppression of polyps[7,8]. There were 19 patients with relevant family histories enrolled to this study, with polyps mainly distributed to the stomach and colorectal sites, with adenoma as the main pathological manifestation. There were 12 patients who experienced malignant transformation. The average age at enrollment was 29.5 years, which was basically consistent with the ages and affected sites described in literature. The disease pathogenesis has not been fully understood, and the incidence of malignant transformation remained high. The progression of disease was monitored mainly based on follow-up visits. Some patients had experienced malignant transformation at diagnosis. Therefore, more detailed investigations on the pathogenesis and early intervention were of great importance.

Notch-signaling pathway is one of the classic signaling pathways in the process of biological evolution, and is involved in the regulation of various cellular biological processes, such as cell proliferation, differentiation, and survival[9-11]. As demonstrated in recent studies, expression abnormalities were closely correlated to the genesis and development of tumors[12,13]. Some studies suggested its dysfunction could prevent cell differentiation, resulting in malignant transformation of undifferentiated cells[13]. Therefore, the correlation between this signaling pathway and tumors has continuously attracted concerns.

Notch is a transmembrane receptor, with its signaling pathway highly conserved. There were two conservative family ligands Delta and Jagged involved in the process of cell differentiation. Currently disclosed ligands toward Notch receptors include: Delta-like-1 (DLL-1), DLL-3, DLL-4, Jagged-1, and Jagged-2[14]. After Notch receptor stimulation, intracellular fragments released by automatic cleavage were transferred to the nucleus to regulate the downstream gene expression profiles of Hesl, CycljnDl, CyclinA, Bcl-2, and Bcl-x, and to regulate the proliferation and apoptosis of tumor cells[15]. Notch-1 receptor and DLL-1 are two proteins mostly investigated and their vital roles in the Notch-signaling pathway have been concerned. Positive expression profiles of Notch receptors and ligands were observed in various tumor cells, and stimulated Notch was associated with malignant transformation of normal cells[16].

p-mTOR is the active form of mTOR (mammalian target of rapamycin), belonging to the serine/threonine kinase family. It is one of PI3K (PIKK)-related kinases, responsible for regulation of eukaryotic cell cycle and promotion of cell growth and proliferation. Sustained activation of mTOR signaling could induce downstream gene expression; promote transformation from G1 to S phase, resulting in excessive cell proliferation and tumor formation[17]. Therefore, the mTOR-signaling pathway is the central step for the regulation of cell growth, and its abnormal activation was associated with uncontrolled cell growth. Some evidences demonstrated that mTOR activation plays a vital role in the Notch-l-mediated survival signaling pathway, and suppression of mTOR activities is associated with reduction of some biological effects of Notch-1 in cancer cells[18].

FAP is not a congenital disease, but most patients experienced adolescence-onset. The number and size of polyps increased in an age-dependent pattern, finally resulting in the evolution

from adenoma to adenocarcinoma. Discovery of disease-related variables and early intervention on relevant pathogenesis had a great supporting role in clinical applications. Therefore, specimens of FAP polyps were collected in this study and changes of the expression profiles of Notch-1, DLL-1, and p-mTOR in the Notch-signaling pathway were examined to determine the role of this signaling pathway during the course of malignant transformation. As demonstrated by the results of this study, the expression profiles of Notch-1 and DLL-1 gene and proteins, as well as the content of p-mTOR protein, were higher in the specimens of patients with FAP polyps than in those of controls with colorectal inflammatory polyp, especially more significant increase of carcinomatous changes, suggesting the presence of Notch signal pathway activation in FAP, which could induce the malignant transformation from normal cells, resulting in the adenocarcinoma transformation of FAP. As shown by statistical analyses, Notch-1 was positively correlated to the expression profiles of DLL-1 and p-mTOR, suggesting increase of phosphorylated mTOR protein levels in response to Notch-signaling pathway activation, which provided further evidences for the activation state of this signaling pathway.

Conclusions

Despite of its low incidence, patients with FAP were generally concomitant with serious clinical symptoms, with a high tumorigenesis rate in late stages. Therefore, early clinical intervention is of great importance for the treatment of this disease. As a member of novel class of anti-tumor targets, Notch was currently becoming a highlight in this field. In this study, for this hyperplastic polyp of FAP, changes of expression profiles of Notch-signaling pathway proteins and genes were examined to extend investigations on the Notch in precancerous stages and to find intervention regimens toward early FAPs. This signaling pathway was correlated to the mTOR-signaling pathway and any effects on mTOR and relevant upstream signaling proteins could also have indirect effects on the Notch-signaling pathway. Notch receptor played different roles in different stages of tumorigenesis and its connection to the different downstream effectors was not known. Whether suppression of this signaling pathway is associated with cytotoxicity of normal tissues needs further investigation. Such series of questions related to this signaling pathway required further studies.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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