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Difference in expression of two neurokinin-1 receptors in adenoma and carcinoma from patients that underwent radical surgery for colorectal carcinoma

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Abstract. Mechanisms underlying tumor progression remain a main problem in the diagnosis and treatment of patients with tumors. The present study compared the expression of full-length neurokinin-1 receptors (fl-NK-1R) and truncated neurokinin-1 receptors (tr-NK-1R) in adenoma and carcinoma from patients with colorectal carcinoma, to explore their possible contributions in adenoma-carcinoma progression. Samples were collected immediately following colorectal carcinoma surgery. Using reverse transcription-quantitative polymerase chain reaction and immunohistochemical staining, the relative mRNA and protein levels of tr-NK-1R and fl-NK-1R were compared in adenoma and carcinoma. tr-NK-1R mRNA was significantly upregulated (1.7 fold; P=0.026) in carcinoma tissues compared with adenoma tissues, while the fl-NK-1R transcription level showed no difference (P=0.438). No significant change was observed in the fl-NK-1R protein level in adenoma, carcinoma and peri-carcinoma tissues (P=0.244). However, total neurokinin-1 receptor (NK-1R) protein levels in adenoma and carcinoma tissues were significantly increased compared to peri-carcinoma tissue (P=0.026 and P=0.007, respectively). The outcomes suggested that the increase in total NK-1R protein in adenoma and carcinoma tissues is the result of the increase in tr-NK-1R levels. The present findings indicate that tr-NK-1R serves an important role in colorectal adenoma progression, with a possible role in adenoma-carcinoma progression. Thus, tr-NK-1R may be used as a marker for diagnosing and treating patients with colorectal adenomas.

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and the total number of cases in the world has been estimated to increase from 1.36 million in 2012 to 2.4 million in 2035 (1). The effects of environment and lifestyle (2) have been shown to largely affect the incidence of CRC. However, the effects of inherited as well as acquired genetic and epigenetic dysregulations (3-5) on the onset and development of CRC remain controversial. Early detection and treatment may significantly improve the survival of patients with CRC (6). Therefore, identification of new molecular mechanisms underlying CRC tumorigenesis and progression is pivotal for developing promising therapies.

Since at least two-thirds of all CRCs develop from precancerous lesions with adenomatous features (7), colorectal adenomas (AD) are considered to be precursor lesions of CRC. Fearon and Vogelstein (8) proposed that the process of histopathological transition between AD and carcinoma (CA) in patients with CRC was associated with a series of events that significantly promote growth of a clonal population of CRC cells. This multistep genetic alteration model has identified a series of key regulatory oncogenes and tumor suppressive genes that harbor either activation or loss of functional mutations, driving the progression between normal colon epithelia and CRC cells (3-10). Despite Vogelstein's model (11) and the high risk or advanced AD concepts (12,13), there are no absolute criteria to describe the AD-CA sequence. Classical morphological characteristics have failed to accurately distinguish between ADs that are potentially high risk to become malignant disease and those that are not. Therefore, improved understanding of cancer development may aid the characterization of ADs at high risk for malignant progression.

Substance P (SP) was the first identified member of the tachykinin family. It is a pro-inflammatory neuropeptide reported as a component in extracts of horse brain and intestinal tissues by von Euler and Gaddum (14), with functions on intestinal contractility and blood pressure regulation. SP is an undecapeptide synthesized by various cell types, most frequently in neurons and inflammatory cells, including human monocytes and macrophages (15). Neurokinin-1

receptor (NK-1R), which is widely expressed in the human body, is the primary receptor of SP. SP can bind to NK-1R and subsequently activate the receptor, which results in phosphoinositide hydrolysis (16), calcium stabilization (17) and mitogen-activated protein kinase (MAPK) activation (18,19). Numerous studies have hypothesized that the SP/NK-1R system is involved in various cancers (20), including brain, thyroid, skin, laryngeal, breast, gastrointestinal, pancreatic and ovarian cancers (21-25). SP and NK-1R have been identified in tumor cells and intra- and peri-tumoral blood vessels (24,26,27). The activation of NK-1R was revealed to be involved in several stages of oncological progression, including proliferation, angiogenesis and cell metastasis (28). Kage *et al* (27) revealed that NK-1R is a G protein-coupled receptor (GPCR), and that it has two isoforms transcribed: A full-length and truncated form, containing 407 and 311 amino acids, respectively. The truncated transcript arises from a splice variant and does not possess the cytoplasmic C-terminal tail (29). Additional studies were performed to demonstrate the functional distinctions between the two isoforms. Patel *et al* reported that the full-length NK-1R (fl-NK-1R) mediated a slower tumor cell growth, while the truncated NK-1R (tr-NK-1R) enhanced tumor cell growth and induced the production of cytokines with growth-promoting functions (30). A previous study by Zhou *et al* (31) demonstrated that tr-NK-1R may promote tumor progression and distant metastasis in breast cancer.

In light of the present findings, the expression of two isoforms of NK-1R was evaluated in the archival formalin-fixed paraffin-embedded (FFPE) tissue of AD and CA from the same patients that underwent radical CRC resections. The present study attempted to differentiate between the two isoforms of NK-1R and explore their functions in the progression of AD-CA.

Materials and methods

Patients. A total of 15 patients (9 males and 6 females; aged 69.5±12.6 years; range 45-86 years), attending the General Surgery Department of Beijing Tong Ren Hospital (Beijing, China) for radical colectomy or rectectomy between September 2013 and August 2014, were involved in the present study. Colorectal AD samples, including AD, CA and PC tissue were collected from each patient. The patients were included in the present study if the diagnoses of the patients were reconfirmed histologically by two gastrointestinal pathologists independently and each resected sample contained AD, CA and PC tissue. The advantage of obtaining all histopathological types from the same case was that variances in patient characteristics were avoided, including genetic background, environmental effects, lifestyle, dietary and bowel habits, disease duration, and preoperative therapeutic interventions.

The present study was approved by the ethics committee of Beijing Chao-Yang Hospital, Capital Medical University. All patients provided informed consent prior to their inclusion in the present study. This article contains no personal information of any patients enrolled.

Tissue preparation and analysis. RNA extraction from FFPE samples was demonstrated to be feasible (32) and capable of yielding similar results as frozen fixed tissue, although RNA quantity and quality in those samples are partially decreased (33). A total of 15 patients with FFPE blocks from each of the three tissue areas (AD, CA and PC) were selected. Samples were cut into 10-μm thick sections and mounted on glass slides. RNA was extracted from the slides using the miRNeasy FFPE kit (catalog no. 217,504; Qiagen China Co., Ltd., Shanghai, China), according to the manufacturer's protocol. Purified RNA was then reverse transcribed using GoScript™ Reverse Transcription System (catalog no. A5000; Promega Biotech Co., Ltd., Beijing, China).

Custom polymerase chain reaction arrays (Qiagen China Co., Ltd.) containing primers for the full-length and truncated TACR1 and β-actin as the control (Table I). The amplification threshold for the samples was set at (Cq) value of ≤40. Results were calculated using the $2^{-\Delta\Delta Cq}$ method (34) and SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis.

Immunohistochemistry. The FFPE samples from the 15 aforementioned patients were cut into 5-μm thick sections, mounted on glass slides, deparaffinized and rehydrated. Slides were incubated in PBS and then probed with two primary antibodies for NK-1R: NK-1R T-20 antibody that binds to the epitope on the second extracellular loop (dilution, 1:50; catalog no. sc-5220; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and NK-1R K-18 antibody (dilution, 1:50; catalog no. sc-14116; Santa Cruz Biotechnology, Inc.) that binds to the epitope on the C terminus. The specificity of the two antibodies was tested by probing samples with or without blocking peptides. Samples were then incubated with the corresponding fluorescein-labeled secondary antibody (dilution, 1:100; catalog no. 31509; Thermo Fisher Scientific, Inc.), mounted (VECTASHIELD mounting medium; Vector Laboratories, Inc., Burlingame, CA, USA), and images were captured and photographed at x400 magnifications (Leica AF6000 Fluorescence Microscope; Leica Microsystems, Inc., Buffalo Grove, IL, USA; Fig. 1). A total of four representative fields from each slide were randomly selected for calculation, and their mean fluorescent intensities were quantified.

Statistical analysis. Student's t-test was used to compare TACR1 expression among different groups and normality and variance were examined prior to the comparisons. The fluorescent intensities were then analyzed using one-way analysis of variance and the normality and variance were examined prior to the comparisons. Post-hoc pairwise comparison of the means was performed using the Student Newman Keuls test (SPSS 19.0 software; IMB SPSS).

Results

mRNA levels of tr-TACR1 were increased in the CA groups. The mRNA expression of the tr-TACR1 transcript had a 1.7-fold increase in CA, compared with AD (P=0.026). However, the mRNA expression of the fl-TACR1 transcript did not show a significant difference between the AD and CA groups (P=0.438).

Protein level of truncated NK-1R was upregulated in the AD and CA groups. Two types of antibodies that probe NK-1R protein were applied in the analysis. NK-1R T-20 antibody binds to the second extracellular loop so that it can probe the two isoforms. However, the NK-1R K-18 antibody binds to the cytoplasmic

Table I. The forward and reverse sequences for the primers.

Primer	Primer sequence (5' to 3')	Size of the PCR products (base pair)
NK-1R forward (NK-1R-F[a])	CAAGCGCAAGGTGGTCAAA	227
NK-1R-Long reverse (NK-1R-R-Long[a])	TGCTTGAAGCCCAGACGG	
NK-1R (NK-1R-F[b]) forward	CAAGCGCAAGGTGGTCAAA	245
NK-1R-Short reverse (NK-1R-R-Short[b])	TGTGGCCCCTGGAGAGCT	
Actin forward	ACTTAGTTGCGTTACACCCTT	156
Actin reverse	GTCACCTTCACCGTTCCA	

[a]NK-1R-F and NK-1R-R-Long are used to detect full-length TACR1; [b]NK-1R-F and NK-1R-R-Short are used to detect truncated TACR1. PCR, polymerase chain reaction; neurokinin-1 receptor.

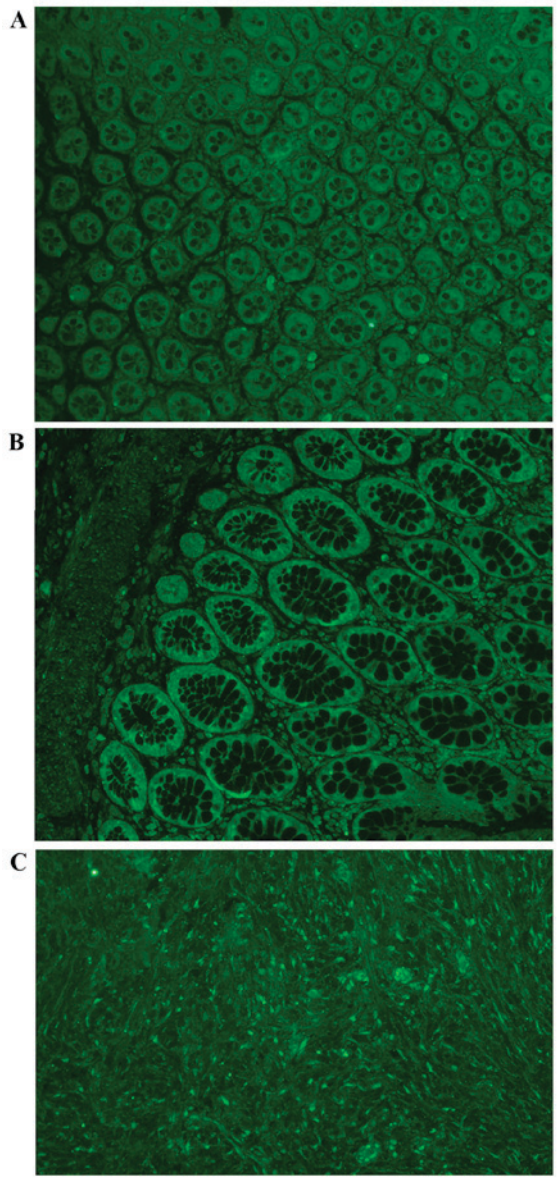

Figure 1. Contrast of immune fluorescence intensity of total NK-1R protein in epithelia from CA and AD compared with PC tissue. The representative fluorescence images of (A) PC epithelium (B) AD and (C) CA showed total NK-1R protein levels (exposure time, 250 ms; magnification, x40). Total NK-1R protein levels in AD and CA were significantly increased compared with PC tissues (P=0.026 and P=0.007, respectively). AD, adenoma; CA, carcinoma; PC, peri-carcinoma.

C-terminal tail and therefore only binds with fl-NK-1R. NK-1R T-20 antibody staining demonstrated an increase in fluorescence intensity in the two groups (P=0.016). Multiple mean protein level comparisons revealed that total NK-1R was significantly increased in the AD and CA group compared with the PC group (P=0.026 and P=0.007, respectively). The K-18 antibody showed no change in fluorescence intensity among the groups (P=0.244), indicating that the protein levels of fl-NK-1R did not change. Therefore, the increase in total NK-1R protein in AD and CA was the result of an increase in tr-NK-1R, rather than fl-NK-1R. The present finding indicated that the tr-NK-1R may perform an important role in the epithelial transition towards malignancy and provides new evidence of AD-CA in the development and progression of colorectal cancer.

Discussion

Colorectal ADs are commonly acknowledged as precursor lesions of CRC, as are the ADs developed in other organs. In 1990, Fearon and Vogelstein (8) proposed a theory for the development and progression of AD-CA, but the accurate progression and mechanism remains unknown. In the present study, the expression of tr-NK-1R and fl-NK-1R was examined in human colorectal cancer. We used the colorectal specimen from a group of patients who underwent radical surgery for CRC in the General Surgery Department of Beijing Tong Ren Hospital between September 2013 and August 2014 (Beijing, China). Since each resected specimen contained tissues in various stages of transition between AD and CA, the two types of NK-1R mRNA and protein levels were compared in areas of the AD with precancerous dysplasia, CA, as well as the normal tissue adjacent to the CA. It was demonstrated that the truncated (but not the full-length) isoform of NK-1R was significantly increased in AD and adenocarcinoma samples compared with the PC tissue (P=0.016). Additionally, using RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR), a 1.7-fold increase in tr-NK-1R mRNA in CA was identified compared with AD. Using immunohistochemistry, an increase of 30 and 40% in tr-NK-1R protein was revealed in AD and CA tissue, respectively, compared with PC tissue (P=0.026 and P=0.007, respectively). These outcomes suggest that protein upregulation was not simply due to the increased transcription of tr-TACR1. Thus, the mechanisms

underlying the tr-NK-1R increase need to be further studied. The present results indicated that tr-NK-1R may be significant in the progression of colorectal epithelium transition between hyperplasia and malignancy, which may provide a new basis for understanding the mechanism of AD-CA progression.

The NK-1R was initially cloned from rat brain in an electrophysiological study of receptor expression in Xenopus oocytes and cross-hybridization with a known bovine NK-2R (35,36), a clone of 3,408 nucleotides encoding the 407 residues of GPCR. Although the degree of similarity of NK-1Rs between different species is relatively high (94.5% identity between rat and human), there are different key residues that are sufficient to affect the overall interaction with antagonists (35). A splice variant of human NK-1R has an exon 5 deletion, which yields a truncated NK-1R with 311 residues that lacks the majority of the intracellular C-tail (37). The truncated NK-1R was identified in human monocytes and macrophages (38), brain regions (cortex, cerebellum) (39) and colonic epithelial cells of colitis-associated cancer (40).

Neurokinin receptor signaling has been well studied using rodent animal cells (41-45). Activation of the NK-1R at the membrane triggers G protein-mediated signaling events: Activation of phospholipase C, leading to inositol triphosphate formation, which stabilizes intracellular Ca^{2+} and eventually activates protein kinase C; activation of adenylate cyclase, leading to stimulation of protein kinase A; and activation of phospholipase A2 and production of arachidonic acid. Activation of NK-1R in HEK293 cells causes a sharp change in cell morphology, including the generation of blebs in the plasma membrane, which includes changes in the Rho-associated protein kinase system and phosphorylation of the myosin regulatory light chain (46).

Signal transduction was studied in NK-1R-transfected NCM460 human colonocytes (47). Previous studies revealed that NK-1R activates the epidermal growth factor receptor (EGFR) by a mechanism involving G protein-dependent activation of members of the disintegrin and metalloproteinase domain-containing proteases (47,48). EGFR dimerizes, phosphorylates and assembles a SHC/Grb2 complex, which leads to the activation of MAPK signaling (47,48). Due to the numerous interactions among pathways, the details of activation vary between cell types. Once activated, ERK1/2 translocates to the nucleus, inducing mitosis and preventing cell apoptosis (19). Therefore, the mechanism may partially mediate the ability of the NK-1R to promote healing of the inflamed colonic epithelium (47,49). However, it may also explain the chronic inflammation in colonic epithelium and the development of colorectal AD initiating carcinogenesis once the cellular balance between proliferation and apoptosis is broken.

GPCR signaling is terminated by removing agonists from the extracellular fluid, which restricts the capacity of the receptor to couple to the signaling machinery. Following stimulation with SP, subsequent responses usually fade and then recover. G protein coupled receptor kinases (GRKs) and β-arrestins mediate desensitization of NK-1R. GRK2, GRK3 and GRK5 can bind and phosphorylate NK-1R (50-52). NK-1R is phosphorylated, which subsequently promotes high-affinity interactions with β-arrestins at the plasma membrane and in endosomes. Conversely, β-arrestins release NK-1R from G proteins and desensitize G protein-mediated signaling. However, the truncated NK-1R is resistant to phosphorylation and does not interact with β-arrestins (53). This mechanism may suggest another important reason for the termination of NK-1R signaling that the increase of tr-NK-1R in AD leads to the transition to malignancy and the appearance of adenocarcinoma in the present study.

Multiple observations support the involvement of SP and NK-1R in the proliferation and tumorigenesis of colonic epithelium. In NCM460 colonic epithelial cells, SP activates multiple pathways that are associated with cancer cell proliferation (47). NK-1R is detected in SW-403 colorectal cancer cells, and NK-1R antagonist L-733,060 impedes proliferation with or without exogenous SP, indicating the possibility of an autocrine mechanism (54). tr-NK-1R is preferentially upregulated in patients with colorectal AD who develop colorectal adenocarcinoma, suggesting the functional role of the tr-NK-1R in the histopathological transformation. The diminished desensitization and endocytosis of tr-NK-1R may amplify its tumorigenic potential.

Previously, the tumor microenvironment was identified as an integral part of tumor growth and survival (28). The microenvironment of tumors includes any given interaction of the tumor cell with its surroundings, including molecular and cellular structures. Thus, the interaction of tr-NK-1R with its ligand may perform an important role in the tumor microenvironment. tr-NK-1R, or molecules associated with its downstream signaling pathways, may prove to be useful as diagnostic markers in the identification of patients with colorectal CA. Diminishing the expression or activity of tr-NK-1R may be a potential therapeutic strategy to prevent dysplasia of the AD from progressing to CA, or even for treating CRC directly. Additional studies to differentiate between the function of tr-NK-1R and fl-NK-1R in the occurrence and development of colorectal cancer may provide new effective antitumor therapeutic strategies in the clinic.

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