Mutation of the nm23-H1 gene has a non-dominant role in colorectal adenocarcinoma

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Abstract. Nm23-H1 is a metastasis suppressor gene, which is has a reduced expression in patients with digestive system cancer. However, the mechanistic basis for the genetic instability remains unknown. To study the expression of the nm23-H1 gene in patients with colorectal cancer, polymerase chain reaction-single strand conformation polymorphism was used to analyze any point mutation, and immunohistochemistry was used to detect the expression of nm23-H1. Results revealed that all 63 specimens of Chinese human colorectal cancer tissues exhibit no point mutation. Among those 63 specimens, 19 (30%) exhibited positive immunostaining for the nm23-H1 protein and 44 (70%) exhibited negative immunostaining. These observations suggested that the protein and gene expression levels of nm23-H1 are reduced in colorectal cancer compared with the adjacent normal tissues, and the point mutation in the nm23-H1 gene is not the dominant cause of metastatic colorectal cancer.

Introduction

Metastasis is one of the biological features of malicious cancer, and is one the causes of mortality as a result of colon cancer. The mechanism of metastasis is always the hot-spot of molecular biology research. Previous evidence shows that during the metastasis of malicious cancer, numerous genes are involved in the control and regulation at various levels. To date, researchers have identified that numerous genes can suppress cancer metastasis, including nm23, multiple cancer inhibition suppressor gene-1 and DLC-1 (1,2). The nm23/NDP kinase is known as a non-metastasis gene, since Steeg *et al* (3) identified it from murine K-1735 melanoma cell lines in 1988, Subsequently, Biggs *et al* (4) reported that the microtubule-associated abnormal wing disc gene (Awd)/NDP kinase is critical in spindle microtubule polymerization, and the product of awd of Drosophila is 78% identical to the product of the nm23 gene of mammals. In the following decades, the nm23 gene was further investigated and it was shown that suppression of metastatic mechanism of human nm23 in various cancer types may be through a dynamin-mediated pathway (5). The nm23 gene regulates cell invasion, cell differentiation and motility via the regulation of growth factor receptor signaling (6,7). In 2013, the expression in chronic myelogenous leukemia cells was detected, and revealed that the nm23-H1 gene may inhibit K562 cell proliferation and migration (8). In order to further assess the role of the nm23-H1 gene mutation in colon cancer evolution, the present study used the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP)-silver staining method to detect changes in the nm23 gene in the colon cancer tissue, and an immunohistochemistry assay to analyze the protein expression of nm23 in patient samples.

Patients and methods

Patient samples. The present study obtained 63 specimens of human colorectal cancer tissue from Shanghai Pudong Hospital Affiliated to Fudan University (Shanghai, China), which were all diagnosed pathologically. Of these, 20 (32%) exhibited lymph gland metastasis. The pathological types classification were as follows: 3 (5%) cases of high differentiation gland cancer, 56 (89%) cases of medium differentiation gland cancer and 4 (6%) cases of low differentiation gland cancer. The present study was approved by the Ethics Committee of Shanghai Pudong Hospital Affiliated to Fudan University.

DNA-extraction. The genomic DNA extraction of fresh cancer tissue was performed using standard methods. The DNAwas isolated by digestion with Proteinase K (Takara Bio, Inc., Shiga, Japan) followed by phenol:chloroform (1:1) (Takara Bio, Inc.) extraction. Briefly, the cancer tissue was mashed up, and mixed with 10% sodium dodecyl sulfate and protein enzyme K (10 g/l) (Takara Bio, Inc.) for digestion at 55˚C for 3 h. Precipitation was performed using NaCl adn the samples were centrifuged at 81,600 x g for 20 mins. The upper

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Table I. Primers used and PCR amplification conditions.

Primer	Sequence (5'→3')	Conditions	Size of amplified products (bp)
Exons 2	TGTTTCATTCCTCTACCTGC GTTCTCCTATACTATGAAGG	95˚C for 5 min (95˚C for 45 sec, 52˚C for 45 sec, 72˚C for 60 sec) x32 72/5 min	171
Exons 3	GTTATTCTCATTCTCTGTCC CATACTTGGAGTATCCCAC	95˚C for 5 min (95˚C for 45 sec, 52˚C for 45 sec, 72˚C for 60 sec) x32 72˚C for 5 min	128
Exons 4	GACCATATCTTCTTCTGTCC CCTTGTGGCAACTAAATCAG	95˚C for 5 min (95˚C for 45 sec, 53˚C for 45 sec, 72˚C for 60 sec) x32 72˚C for 5 min	154
Exons 5	GTCTTGGTCATGTGACTATC GTGAAAAGCAATGTGGTCTG	95˚C for 5 min (95˚C for 45 sec, 53˚C for 45 sec, 72˚C for 60 sec) x32 72˚C for 5 min	141

clear liquid layer was obtained and 700 ml/l cool alcohol was added for 20˚C for 30 mins. The samples were centrifuged at 142,800 x g for 10 mins. Phenol-chloroform and chloroform-isoamyl alcohol (Takara Bio, Inc.) were added separately and the samples were centrifuged at 132,600-153,000 x g for 5 mins. TE buffer (Takara Bio, Inc.) was added to dissolve and preserve the DNA.

PCR-SSCP. Four pairs of primers were designed, corresponding to the numbers 2-5 position exons of the nm23-H1 gene. PCR was performed, according to the stated conditions (Table I) on a Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany).

Following PCR, 10 μl PCR product was added to the sample-loading buffer (Takara Bio, Inc.), including 950 g/l formamide. The samples was denatured for 5 min at 95˚C, and was immediately put on ice. The denatured samples were separated by polyacrylamide gel electrophoresis in electrophoresis buffer (0.5 X TBE) at 70 V, 4˚C, overnight. Following running, silver staining (Wuhan Boster Company, Wuhan, China) was performed.

Immunohistochemistry. Rabbit polyclonal anti-nm23-H1 antibody and biotinylated goat anti-rabbit immunoglobulin G (each from Wuhan Boster Company) were used at concentrations of 1:50 and 1:500, respectively. The human colon tissue samples were preserved in 10% formaldehyde solution, and were dehydrated and embedded in paraffin, according to routine methods. Briefly, the 4 μm-thick paraffin sections were removed and blocked with 3% peroxide-methanol at room temperature for 20 min for endogenous peroxidase ablation. The sections were then incubated with the rabbit anti-nm23 antibody diluted in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 2 h at 37˚C. Following incubation with primary antibody, the samples were incubated with goat anti-rabbit immunoglobulin G for 30 min at 37˚C. The samples were subsequently stained with 3,3-diaminobenzidine (Wuhan Boster Company) followed by washing in PBS. Nuclear counterstaining was performed using Mayer's hematoxylin, followed by dehydration, clearing and mounted with neutral gums.

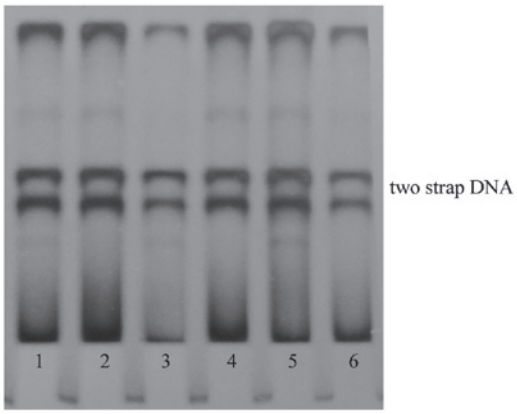

Figure 1. PCR-SSCP analysis of the colon cancer tissue (Samples 1-6). The DNA products of nm23 gene 4th exon exhibit two straps following silver staining.

Results

PCR-SSCP. The present study performed PCR-SSCP detection on the 63 cases of colon cancer tissue specimens to analyze any point mutation of the nm23 gene on the 2nd-5th exon position. Through repeated screening and operation, the results from the DNA samples revealed that the 2nd-4th exons of nm23-H1 exhibit two straps (Fig. 1), but the 5th exon displays four straps for certain specimens (data not shown). The coding protein was then analyzed and revealed that only the 5th exon sequence of the nm23-H1 gene has a polymorphism. The 360th position base exhibits a T→C replacement, but the coding amino acid AGT→AGC remains unchanged (serine), therefore, it is a synonymous mutation.

Immunohistochemistry. nm23-H1 is predominantly expressed in the cytoplasm of the colorectal cancer gland. Among

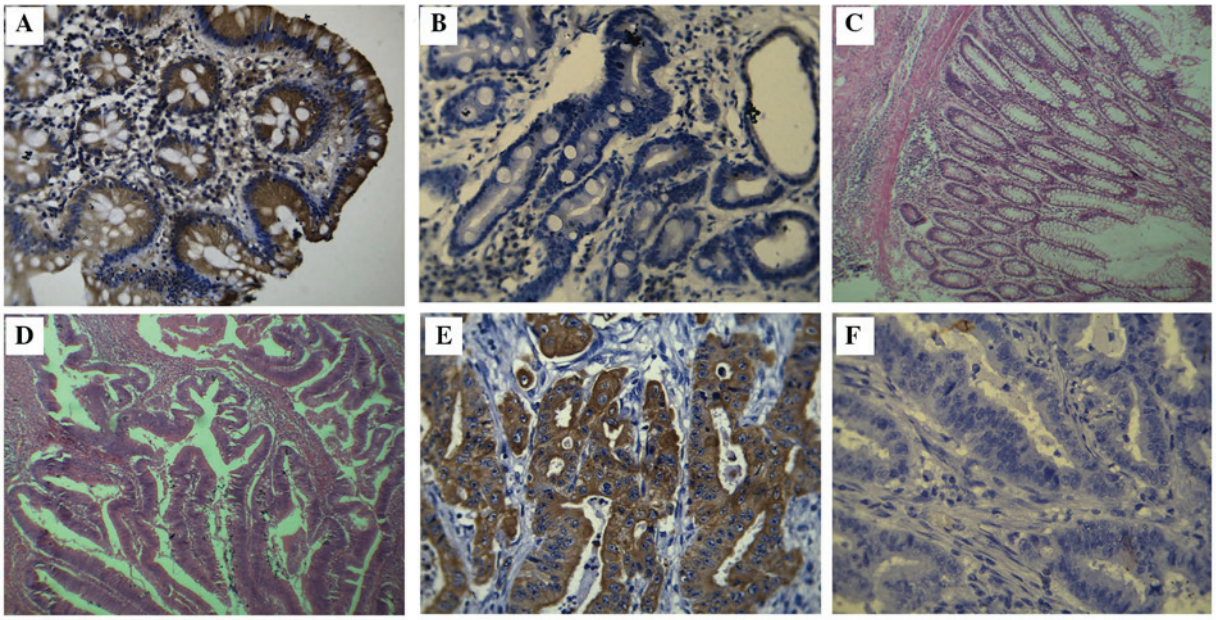

Figure 2. Microscopic assessment of colon tissue, both cancerous and non-cancerous (magnification, x400). (A) IH revealed positive cytoplasmic expression of nm23 in epithelial cells in normal colon mucosa. (B) IH revealed negative expression of nm23 in epithelial cells in colon mucosa. (C) H&E staining revealed normal colon mucosa and (D) colon adenocarcinoma. (E) IH revealed positive cytoplasmic expression of nm23 in epithelial cells in colon cancer. (F) IH revealed negative expression of nm23 in epithelial cells in colon cancer. IH, immunohistochemistry; H&E, hematoxylin and eosin.

the 63 colorectal adenocarcinoma specimens, 19 (30%) exhibited positive immunostaining for the nm23-H1 protein, 44 (70%) exhibited negative immunostaining, while the adjacent normal tissues were 72% positive staining (Fig. 2).

Discussion

The nm23 gene has been regarded as a metastatic suppressive gene in various tumor types (9). It is an important cancer metastasis suppressor gene. The nm23-H1 gene cDNA has a total length of 534 bp and 152 amino acids. The nm23 gene family has two homologous genes, nm23-H1 and nm23-H2, and both are located at 17q22, encoding a protein with a molecular weight of 18 and 17 kDa, respectively. The nm23 gene encodes a nucleoside diphosphate kinase (NDPK). Both nm23-H1 and nm23-H2 encode, the A and B subunits of NDPK, respectively. They are 88% identical in their amino acid sequences. NDPK activity is responsible for the synthesis of most cellular nucleoside triphosphates, other activities include cell proliferation, differentiation and development.

DR-nm23 cDNA was cloned in 1997. It is highly homologous to the putative metastasis suppressor nm23-H1 gene and the closely associated nm23-H2 gene (10,11). The expression of DR-nm23 in colorectal cancer tissue was significantly lower compared with that in adenoma and normal tissue. Therefore, the expression status of DR-nm23 may act as a potential prognostic factor in patients with colorectal cancer, and it may be involved in the regulation of differentiation of colorectal cancer cells (12). At the invasive front of colon carcinoma, nm23-H1 levels were also reduced or lost, and its downregulated expression was closely associated with the invasion and metastasis of colorectal cancer (13). The protein expression of nm23 was assessed in the colorectal carcinoma tissue by immunohistochemistry. This revealed that the protein expression was higher in carcinoma tissue compared with that in adjacent non-neoplastic mucosa, similar to the results of a previous study (14).

In gastric and colorectal carcinomas, the genomic region 17q21 is frequently associated with microsatellite instability and loss-of-heterozygosity. The nm23 gene is located in this area. This region contains several putative tumor suppressor genes, including the Spn gene. Spn downregulation is associated with a poorer survival in patients with advanced stages of colorectal carcinoma (15), and downregulation of nm23-H1 contributes to tumorigenesis in lung cancer (16). Also, Qin *et al* (17) support the potential utility of targeting Nm23-H1 as a therapeutic approach for the treatment of Kaposi's sarcoma (17).

Data from a previous study has suggested that part of the antimetastatic function of Nm23-H1 lies in the pathways that it interrupts via binding and inactivation of proteins (18). In human hepatoma and colon carcinoma cells, nm23-H1 was implicated as a metastasis suppressor, in maintaining adherens junctions and limiting the invasive potential. It appears that nm23 is crucial for inhibiting invasive migration, cell-cell adhesion and cell migration (13). Enhanced expression of nm23-H1 protein in Chinese patients with digestive system cancer inhibited cancer metastasis (19). However, the mechanistic basis of the metastasis suppressor function of nm23 and its regulated expression remains to be elucidated.

Disorders of gene structure or gene expression can cause abnormalities in cell growth and differentiation, thereby causing cancer and having a malicious biological behavior. Generally, these are mechanisms that lead to gene silencing, mutation, genomic deletion and promoter methylation. Mutation screening is always performed by PCR-SSCP. At present, this technique has been used for detecting the known mutation hot-spots, screening of unknown mutations

and analyzing of the gene mutation and cancer suppressing gene (20). The present study assessed 63 specimens of Chinese human colon cancer tissue via PCR-SSCP. The results indicated that the nm23 gene mutation does not serve the dominant role in the metastasis of colon cancer.

A previous study demonstrated that treatment with 5-aza-2-deoxycytidine reduces the expression of Nm23-H1, which causes hypermethylation in the nm23-H1 promoter (17,21). Protein arginine methyltransferase 5 (PRMT5) methylates histones H3 at the nm23 promoter region and inhibits methylation of H3K9. PRMT5 regulates cell growth and proliferation by modulating the expression of nm23 (22). As with the previous studies, it is not possible to determine the mechanism of nm23 responsible for the regulation of cell growth and proliferation involved in tumor suppression. The mechanisms by which nm23-H1 exerts its metastasis-suppressor functions involve multiple pathways, which remain to be elucidated (23,24).

Although colon cancer metastasis may be due to other genes or due to the methylation of the control portion of nm23 gene, the mechanism of the nm23 as a suppressor gene is more complicated than previously hypothesized. Further research is required to fully understand the mechanism and to identify its role in the cancer process.

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