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High expression of nucleophosmin is closely related to the grade and invasion of colorectal cancer

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This study explores the differential protein expression in the colorectal cancer (CRC) patients to validate a new biomarker for tumor progression. CRC tissues and their adjacent non-cancerous tissues were analyzed by two-dimensional LC/MS/MS. Nucleophosmin 1 (NPM1) was selected and confirmed its differential expression by Western blot. Immunohistological staining of NPM1 in tissues was performed to validate its correlation with clinicopathologic parameters of CRC patients. There were 39 candidates with significant difference between cancerous tissues and their adjacent non-cancerous tissues, which included 19 increased proteins and 20 decreased proteins in CRC samples. Especially, NPM1 was correlated with poor differentiation, and lymph node metastasis according to the analysis of patients' clinicopathologic parameters. Increased expression of NPM1 can be as a critical biomarker for clinical diagnosis of tumor progression of CRC patients.

Keywords: Colorectal cancer, NPM1, Two-dimensional LC/MS/MS

Colorectal cancer (CRC) is the fourth-leading cause of cancer-related deaths globally[1]. Its prognosis largely depends on the stage of CRC, with 5-year overall survival (OS) ranging from close to 90% for early-stage cancer to little over 10% for advanced-stage cancer even in the well-developed countries[2]. That is, patients with early-stage CRC can obtain benefits from surgical resection combined with systemic chemotherapy[3]. However, the 5 year OS of patients with advanced CRC is only 60%, which might be due to only partial patients can respond to the chemotherapeutic agent 5-fluorouracil (5-FU), irinotecan or oxaliplatin, and are easily prone to recurrence[4].

So far, some molecular mechanisms can partially elucidate the phenomena of recurrence and poor prognosis of CRC patients. For example, the genetic variants in some biological pathways involved in drug transportation or metabolism, repair of DNA damage, and cell cycle modulation, which might affect the efficacy of oxaliplatin treatment in patients with advanced CRC[5]. In addition, serum level of miR-760 with higher diagnostic power and miR-92a with higher prognostic power for the discrimination between CRC stages in Egyptian patients have also been proven[6]. However, these prognostic biomarkers are still not enough for identifying advanced-stage CRC patients with high risk of recurrence after surgical resection. Although the advancements in the molecular diagnosis and targeted therapy of CRC, identification of new diagnostic biomarkers continues to be a challenge[7].

Nucleophosmin (NPM), also known as nucleolar phosphoprotein B23 or numatrin, is a protein encoded by the NPM1 gene in humans[8]. It is located in the nucleolus, and can be translocated to the nucleoplasm in response to serum starvation or treatment with anticancer drugs. NPM1 plays an essential role in cell growth and proliferation by regulating cell cycle progression and centrosome duplication[9,10]. NPM has multiple functions, including genomic stability and DNA repair, centrosome duplication during cell cycle, and inhibition of caspase-activated DNase. Other studies also demonstrated that NPM1 can regulate the activity of several tumor suppressors, including P53 and Rb *via* direct binding[11]. It is also involved in transcriptional activation through interaction with transcription factors, such as c-Myc and NF-κB[12].

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NPM1 has also been proven to be associated with several cancers, including prostate, liver, gastric, colon, pancreas, glioma and glioblastoma, astrocytoma and others[13,14].

In the present study, we analyzed the expression values of candidates from the CRC and the adjacent non-cancerous tissues by using two-dimensional LC/MS/MS. NPM1 protein was selected to measure its increased expression in CRC patients by using Western blot and immunohistological staining, and then further analyzed with the clinicopathologic parameters of patients by using statistics.

Materials and Methods

Patients

The study was performed in accordance with the Helsinki Declaration and was also approved by Ethics Committee at China-Japan Union Hospital of Jilin University. Patients with CRC had been given verbal explanations and had signed informed consent before surgery began. Forty patients in the study were clinically or pathologically diagnosed as stage III colorectal adenocarcinoma, who had not received any therapy before the surgery began (Table 1). Specimens from the cancerous tissues and their adjacent non-cancerous

Table 1 — Clinicopathologic characteristics and stages of CRC patients

No.	Gender	Age	Differentiation	TNM stage
1	Male	68	Moderately	T1N0M0
2	Female	48	Poorly Differentiation	T1N1M0
3	Male	40	Poorly Differentiation	T1N2aM0
4	Male	68	Moderately	T1N2bM1a
5	Male	46	Well	T2N0M0
6	Female	71	Well	T2N0M0
7	Female	50	Poorly Differentiation	T2N1M0
8	Male	58	Poorly Differentiation	T2N1M0
9	Male	52	Poorly Differentiation	T2N1M0
10	Male	44	Poorly Differentiation	T2N1M0
11	Female	54	Well	T3N0M0
12	Female	49	Moderately	T3N0M0
13	Male	46	Poorly Differentiation	T3N0M0
14	Male	54	Poorly Differentiation	T3N1M0
15	Male	49	Poorly Differentiation	T3N1M0
16	Male	43	Poorly Differentiation	T3N1M0
17	Male	36	Moderately	T3N1M1a
18	Male	37	Poorly Differentiation	T3N2bM0
19	Female	51	Moderately	T4aN0M0
20	Male	53	Moderately	T4aN0M0
21	Female	52	Poorly Differentiation	T4aN0M0
22	Male	61	Moderately	T4aN0M0
23	Male	74	Moderately	T4aN0M0
24	Female	60	Poorly Differentiation	T4aN1aM0
25	Male	52	Poorly Differentiation	T4aN1M0
26	Male	59	Poorly Differentiation	T4aN1M0
27	Male	65	Poorly Differentiation	T4aN1M0
28	Female	46	Poorly Differentiation	T4aN1M1a
29	Female	54	Poorly Differentiation	T4aN1M1a
30	Male	56	Moderately	T4bN0M0
31	Female	54	Poorly Differentiation	T4bN0M0
32	Male	50	Moderately	T4bN0M0
33	Male	54	Moderately	T4bN1M0
34	Female	59	Poorly Differentiation	T4bN1M0
35	Male	48	Poorly Differentiation	T4bN2bM0
36	Male	48	Poorly Differentiation	T4bN2bM1a
37	Male	54	Poorly Differentiation	T4N1M0
38	Male	51	Poorly Differentiation	T4N1M0
39	Male	47	Poorly Differentiation	T4N1M0
40	Male	50	Poorly Differentiation	T4N1M0

tissues (10 cm away from the cancerous tissues) were collected to freeze in a −80°C refrigerator immediately after resection, then stored in liquid nitrogen.

Reagents

N,N,N',N'-Tetramethylethylenediamine, monoclonal antibody against NPM1, Sodium Dodecyl Sulfate (SDS), absolute alcohol, RCDC Protein Assay Kit were purchased from Bio-Rad (Richmond, CA). DAB staining kit, bromophenol blue, citric acid antigen retrieval agent, Coomas bright blue, Xylene, mineral oil, modified hematoxylin, and PMSF were obtained from Sigma-Aldrich (St. Louis, MO). Iodoacetamide (IAM), goat anti-rabbit IgG, 3% H_2O_2, DTT, TPCK-trypsin were from Promega (Madison WI). Dehydration machine (Tissue-Tek VIP Sakura) and Agilent 1200 nanoflow LC system were from Agilent Technologies (Wilmington, DE). Embedding machine (Tissue-TekVIP TEC) and LTQ Orbitrap XL MS/MS spectrometer were from Thermo Fisher (San Jose, CA). Tissue slicer (LEICA SM 2000) was from Leica Microsystem (Nussloch, Germany).

Two-dimensional LC/MS/MS

The frozen samples were digested followed by strong cation exchange-RPLC/MS/MS as briefly described following. The frozen samples were treated with DTT and alkylated with IAM before digestion with trypsin. The separations of peptides were achieved using an Agilent 1200 nanoflow LC system (Agilent Technologies) by using strong cation exchange column (150 mm × 0.32 mm) coupled with reverse-phase column (150 mm × 0.17 mm). Fifty μg of peptides in Buffer A (0.1% FA) were operated by using a mobile phase with ammonium acetate, which concentrations were linearly increased from 0 mM to 1 M (0 mM, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, and 1 M). The reverse-phase column was operated in 30% Buffer B (0.1% formic acid and 99% acetonitrile) with 2 μL/min flow rate for 3 h. Peptide analysis was carried out using a Thermo LTQ Orbitrap XL MS/MS spectrometer (San Jose, CA, USA) with data-dependent MS/MS scan over the m/z range 400-2000 by 35% normalized collision energy. Thermo Bioworks 3.3.1 SP1 with SEQUEST was used to search against the database ipi.human.v3.05, and results were filtered using standard values for Xcorr and ΔCN.

The SEQUEST criteria was performed as follows: mass tolerance = 3 Da; 2 leakage points; a modification of +57 Da on cysteine residue; FPR=1%, Δ Cn ≥0.19; Xcorr = 2.2 for +1 charged peptides; Xcorr = 2.5 for +2 charged peptides; Xcorr = 2.9 for +3 charged peptides.

Western blot analysis

Protein (20 μg) from each tissue sample was denatured and separated by 10% SDS-polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA). The membranes were blocked in 5% skim milk at 4°C for overnight, and then incubated with the anti-NPM1 antibody (dilution 1:200 in 5% BSA-PBS) for 2 h at RT. After washing 3 times in 1X PBST, the membranes were subsequently incubated with HRP-conjugated anti-IgG secondary antibody (dilution, 1:10000) and then washed 3 times in 1X PBST. The proteins were visualized by using an enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The intensity of the images was quantified by using ImageJ software (National Institute of Health, USA). An anti-β-actin mouse monoclonal antibody (dilution 1:1000 in 5% BSA-PBS) was used to normalize the protein loading.

Immunohistological staining

Paraffin-embedded tissue samples were heated in 60℃ incubator for 20 min, followed by being dewaxed in xylene for 5 min twice, rehydrated with graded alcohol solutions, and put in distilled water for 5 min twice. Antigen retrieval was conducted by autoclaving the slides for 10 min in 0.01 mol/L sodium citrate buffer, pH 6.0. After cooling down, the slides were washed with 1X PBS for 5 min. Endogenous peroxidase was blocked by incubation in fetal calf serum for 10 min, and followed by washing in PBS for 3 min 3 times. The tissue sections were then incubated at RT for 60 min with antibody against NPM1 (dilution, 1:200), and followed by washing in PBS for 5 min 3 times. The antigen-antibody complex was then detected with a biotinylated goat anti-rabbit antibody at RT for 10 min, and washed in PBS for 3 min 3 times. The slides were then incubated with streptoavidin-horseradish peroxidase for 10 min, washed in PBS for 3 min 3 times, and then visualized by staining with 50 μL diaminobenzidine (DAB) for 5 min for color detection. The tissue sections were then counterstained with hematoxylin for 2-3 min, and dehydrated with graded alcohol solutions for mounting. The samples were observed and photographed with an optical microscope. Tumor grade was evaluated according to the grading system established by the World Health Organization in 2004.

Statistical analysis

Raw files from two-dimensional LC/MS/MS were analyzed by using SEQUEST algorithm. Statistical analysis was conducted with SPSS version 11.0, and reported as mean ± standard deviation (SD). The *P*-value less than 0.01 (0.05/5=0.01) was considered to be statistical significance according to Bonferroni correction.

Results

Differential expression of 39 proteins detected by two-dimensional LC/MS/MS

In the present study, the number of spectra of the two-dimensional LC/MS/MS was used to evaluate the abundance of proteins. The criteria of the significant differences between two groups was defined as follows. First, the difference in the number of protein spectra of the two samples should larger than 72; Second, the ratio of the numbers of protein spectra in two groups should larger than 1. There were 39 proteins with significant difference between cancerous tissues and their adjacent non-cancerous tissues according to the above criteria (Table 2). Among them, 19 proteins were increased, however, 20 proteins were decreased in CRC samples (Table 2). These targets might be the highly promising sensitive and specific tumor markers of CRC, and need to be validated clinically in the future. The identification of NPM1 with two-dimensional LC/MS/MS was shown in (Table 3 and Fig. 1).

Increased expression of NPM1 in the CRC tissues

The differential expression of NPM1 in the two groups was confirmed by Western blot analysis. The

Table 2 — Expressions of differential proteins in CRC and adjacent non-cancerous tissues

	Proteins
Up-regulation in CRC (19)	KRT18, ATP5B, NPM1, TPM4, FGA, HSPA5, MUC2, IGHA1, SERPINH1, ENO1, TPM3, CKAP4, IGHA2, POSTN, COL6A3, FGG, PKM2, HIST1H2AE, MYH10
Down-regulation in CRC(20)	SYNM, MYH11, PKM2, MYL9, KRT10, TPM1, FLNC, DES, H2AFX, CSRP1, CAP1, CNN1, MYL6, ACTA2, TUBB2A, HSPB1, TLN1, ACTA1, FLNA, CKB

Table 3 — Identification of NPM1 by LC/MS/MS

IPI number	Protein Name	Sequence	Charge	Parent Ion Mass	M+H+ Deviation	XCorr	DeltaCn
IPI:IPI00549248	NPM1 Isoform 1 of Nucleophosmin	R.TVSLGAGAKDELHIVEAEAMNYEG SPIKVTLATLK.M	3	3658.1175	0.0205	7.023	0.637
IPI:IPI00549248	NPM1 Isoform 1 of Nucleophosmin	R.MTDQEAIQDLWQWR.K	2	1821.0065	0.1451	3.387	0.326
IPI:IPI00549248	NPM1 Isoform 1 of Nucleophosmin	K.MSVQPTVSLGGFEITPPVVLR.L	2	2228.6401	0.7745	5.03	0.627
IPI:IPI00549248	NPM1 Isoform 1 of Nucleophosmin	K.ADKDYHFKVDNDENEHQLSLR.T	3	2574.7039	0.022	5.364	0.273

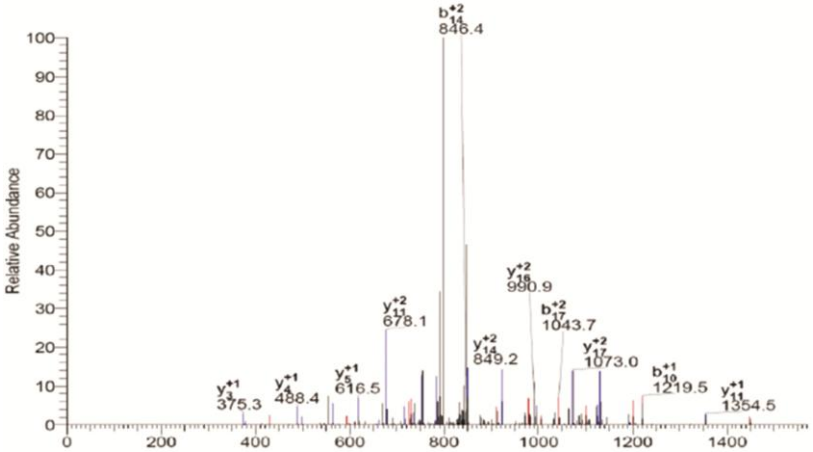

Fig. 1 — Peptide sequence of NPM1 from the results of LC/MS/MS. The peptide sequence "ADKDYHFKVDNDENEHQLSLR" was shown to identify NPM1 by using two-dimensional LC/MS/MS

expression of NPM1 was higher in the cancerous tissues than in the non-cancerous tissues (Fig. 2A & B). The results were consistent with those from two-dimensional LC/MS/MS analysis.

In addition, high expression of NPM1 in cancerous tissues was also observed by using immune histological staining. It indicated that NPM1 was located predominantly at the cytoplasm (Fig. 2C & D).

Significant association of NPM1 with clinicopathologic characteristics of CRC patients

We further analyzed the correlation of NPM1 expression with clinicopathologic characteristics in CRC patients. The expression of NPM1 was higher in patients with poorly differentiation than in patients with moderately/well differentiation ($P< 0.05$) (Tables 4 and 5). 18 out of 22 CRC patients with lymph node

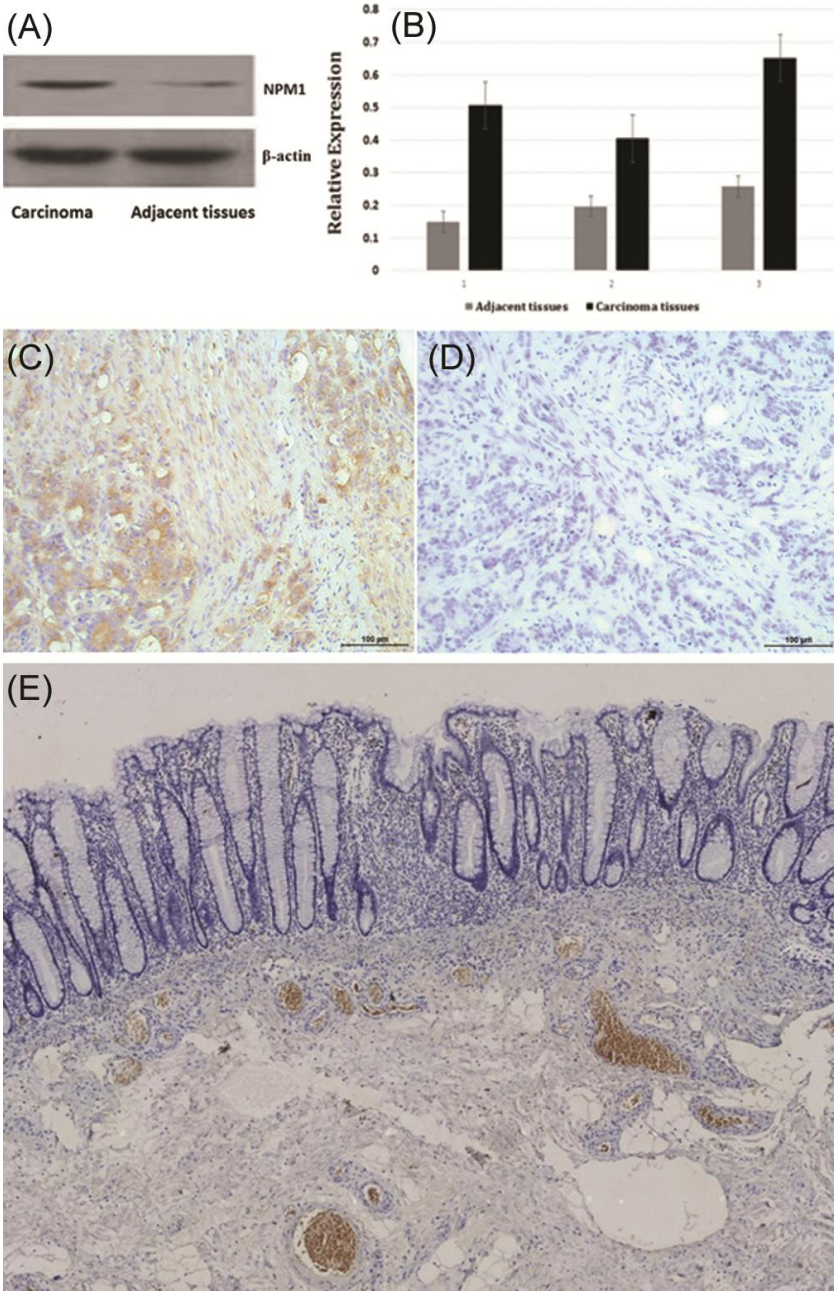

Fig. 2 — Different expression of NPM1 protein in carcinoma tissues and adjacent non-cancerous tissues. (A & B) The expression of NPM1 protein in carcinoma tissues is significantly higher than that in adjacent non-cancerous tissues; (C & D) Comparison with adjacent non-cancerous tissues, NPM1 protein in tumor tissues revealed strong expression; and (E) Normal tissue was shown (× 40)

Table 4 — NPM1 expression in CRC and adjacent non-cancerous tissues

Type	Sample (n)	NPM1 expression		*P*-value
		+	–	
CRC tissue	100	72 (72.0%)	28 (28.0%)	0.008
Adjacent non-cancerous tissue	100	46 (46.0%)	54 (54.0%)	

Table 5 — Correlation of NPM1 expression with clinical-pathologic characteristics of CRC patients

Parameters		Sample (n)	NPM1		*P*-value
			Positive (%)	Negative (%)	
Gender					0.605
	Male	53	37 (69.8%)	16 (30.2%)	
	Female	47	35 (74.5%)	12 (25.5%)	
Age					0.342
	<55	39	26 (66.7%)	13 (33.3%)	
	≥55	61	46 (75.4%)	15 (24.6%)	
Differentiation					0.001
	Moderately/Well	35	18 (51.4%)	17 (48.6%)	
	Poorly Differentiation	65	54 (83.1%)	11 (16.9%)	
Lymph node metastasis					0.001
	N_x	71	58 (81.7%)	13 (18.3%)	
	N0	29	14 (48.3%)	15 (51.7%)	
Tumor size					0.137
	T_1-T_2	10	5 (50%)	5 (50%)	
	T_3-T_4	90	67 (74.4%)	23 (25.6%)	

metastasis (81.9%) were NPM1 positive, 9 out of 18 CRC patients without lymph node metastasis (50%) were NPM1 negative ($P< 0.05$). The results indicated that NPM1 expression was significantly associated with differentiation and lymph node metastasis, but was not associated with tumor size, gender and age of patients ($P> 0.05$) (Tables 4 and 5).

Discussion

CRC is one of the most common tumors in China, with increasing incidence annually. Its prognosis and the OS are closely related with sensitive and specific diagnosis in the early stage. Some studies reported that patients received the surgery at early-stage of CRC exhibited a higher 5 year OS close to 90%[2]. Therefore, exploring the molecular biomarkers for early diagnosis or recurrence after therapy is crucial to increase CRC patients' survival rates.

By using two-dimensional LC/MS/MS, we found several proteins differentially expressed between CRC and its adjacent non-cancerous tissues (Table 2). Among them, NPM1 was interesting to us to further validate its clinical roles in CRC. In CRC cells, Wong *et al.* proved that NPM1 can affect p53-related aging and growth arrest, indicating that NPM1 promotes tumorigenesis[15]. Liu *et al.* also demonstrated that high expression of NPM1 is associated with distant metastasis and poor survival in CRC patients[16]. In the present study, we further demonstrated that increased NPM1 expression in patients was associated with poorly differentiation and lymph node metastasis (Tables 4 & 5). It indicates that NPM1 can be utilized in the clinical diagnosis for the CRC patients' progression according to the tumor grade. However, its roles in CRC need to be further elucidated.

It was first demonstrated the NPM1 is related to about one-third of anaplastic large-cell non-Hodgkin's lymphomas, and linked to the catalytic domain of anaplastic lymphoma receptor tyrosine kinase (ALK)[17]. In addition, NPM1 overexpression in solid tumors is associated with poor prognosis, including astrocytomas[18], oral squamous cell carcinoma, non-small cells lung cancer (NSCLC), hepatocellular carcinomas (HCC), colon cancer, ovarian cancer, and endometrial carcinoma[19]. Londero *et al.* revealed a link between an overexpression of nuclear NPM1 protein and poor outcomes for women diagnosed with high-grade ovarian serous cancer[20]. Kalra *et al.* elucidated a functional relevance of NPM1, RAD50 and XRCC5 DSB-repair proteins towards ensuring survival and evasion of apoptosis during ovarian

transformation to emphasize their contribution and association with disease progression in high-grade Serous Ovarian Carcinoma[21]. Furthermore, chemotherapeutic sensitivity is poor in lung adenocarcinoma patients with overexpression of c-Src and NPM1 protein, which indicates higher expression of c-Src and NPM1 might be associated with poorly differentiated adenocarcinoma[22].

Taken together, we suggest that NPM1 plays critical roles in the CRC development, migration/ invasion, and progression. It is also interesting to us to explore the cross-talk of NPM1 with other biomarkers or oxidative stress of CRC, which indicates to be critical to tumor differentiation and progression[23].

Conclusion

Based on our findings and the contribution of NPM1 in other cancers and in the genome stability, it might be a promising target to utilize in the diagnosis, prognosis, and even in the therapeutic strategy to CRC patients. Increased expression of NPM1 can be as a critical biomarker for clinical diagnosis of tumor progression of CRC patients.

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