

## RESEARCH ARTICLE

# Prognostic Role of Nucleophosmin in Colorectal Carcinomas

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### Abstract

**Aim:** Recent research suggests that nucleophosmin (NPM) may be a prognostic marker in colorectal carcinomas (CRC). We here tested its use to predict the survival of CRC patients. **Methods:** We investigated NPM expression by immunohistochemistry in histologically normal to malignant colorectal tissues and evaluated its association with clinicopathological variables. Overall and disease-free survival after tumor removal were calculated by the Kaplan-Meier method, and differences in survival curves were analyzed by the log-rank test. The Cox proportional hazards model was used for multivariate analysis of prognostic factors. **Results:** NPM expression was found significantly upregulated in CRC compared to adjacent colorectal tissue, villous adenoma, tubular adenoma and normal colorectal mucosa ( $p < 0.05$  for all). NPM expression was statistically linked to cancer embolus, lymph node metastasis, differentiation grade, and recurrence of CRC. Overall and disease-free survival of NPM-negative CRC patients tended to be better than those for patients with NPM-positive lesions (log-rank statistic,  $p < 0.05$  for all). Multivariate analysis indicated NPM expression as an independent prognostic indicator for CRC patients ( $p < 0.05$ ). **Conclusion:** Our results suggest that NPM expression can predict the survival of CRC patients. Prognosis of CRC is determined by not only many known prognostic factors but also by NPM expression.

**Keywords:** Nucleophosmin - prognosis - colorectal carcinomas

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### Introduction

Colorectal carcinomas (CRC) is the most common one of malignant tumors worldwide and is the common cause of cancer-related deaths (Xu et al., 2010). Prognosis of CRC remains unsatisfactory even after surgical resection. Considerable interest has been generated in identifying factors that influence the prognosis of CRC patients. The most widely studied prognostic factors are related to the pathological characteristics of the neoplasm, including tumor size, differentiation grade, stage, vascular invasion and metastasis. In recent years several molecular markers predicting the survival period of CRC patients have been reported, such as Nucleophosmin (NPM) (Liu et al., 2012; Wong et al., 2013), Ki67 (Guzińska-Ustymowicz et al., 2008; Guzińska-Ustymowicz et al., 2009) and PCNA (Guzińska-Ustymowicz et al., 2008; Guzińska-Ustymowicz et al., 2009).

NPM is a ubiquitously expressed multifunctional nucleolar phosphoprotein. NPM protein has been reported to be over-expressed in rapidly proliferating tumor cells because NPM expression increases rapidly in early G1 phase during mitosis (Feuerstein et al., 1998), thus heavily implicating its possible association with prognosis of cancer patients. Future studies found

that the difference of NPM expression was associated with prognosis and also indicated that NPM may be an important prognosis marker in some solid tumors (Tsui et al., 2008; Kikuta et al., 2009; Coutinho-Camillo et al., 2010). A recent study showed NPM overexpression in CRC and the differential expression of NPM1 along the normal colon-adenoma-carcinoma progression (Wong et al., 2013). Another recent study investigating the potential role of NPM in the regulation of cell migration and invasiveness uncovered a positive association of NPM expression with CRC progression and also reported that NPM overexpression was associated with poor survival rate in CRC patients, however, the prognostic factor was not adjusted using multivariate Cox regression analysis with clinicopathological factors (Liu et al., 2012). Moreover, few studies investigated an association of NPM expression with disease-free survival in CRC patients. We wonder whether NPM may be identified as a useful marker predicting the survival of CRC patients in daily practice.

In this study we investigate NPM expression by immunohistochemistry in histologically normal to malignant colorectal tissues and also evaluate its association with clinicopathological variables and patient survival in CRC patients.

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**Table 1. NPM Expression in Normal to Malignant Colorectal Tissues**

	NPM Expression (n %)	
	(+)	(-)
Colorectal carcinomas	104 (64.6%)	57 (35.4%)
Adjacent colorectal tissue	74 (46.0%)	87 (54.0%)
Colorectal HGIN	8 (61.5%)	5 (38.5%)
Villous adenoma	4 (12.1%)	29 (87.9%)
Tubular adenoma	1 (2.4%)	41 (97.6%)
Normal colorectal tissue	0 (0%)	44 (100%)

HGIN: high-grade intraepithelial neoplasia

## Materials and Methods

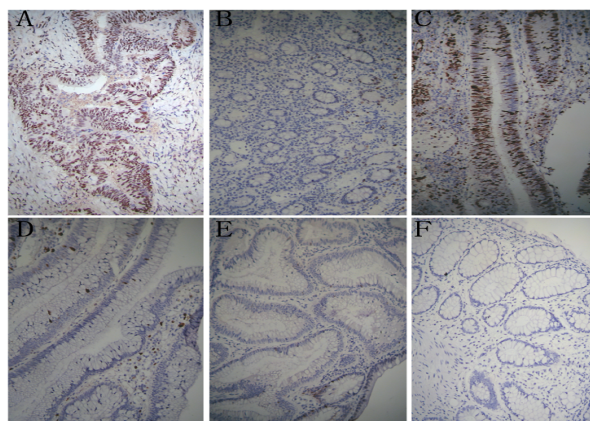
### Clinical samples

161 well-documented surgically matching pairs of CRC and the corresponding adjacent tissue samples were obtained in the files of Dongguan Shilong People's Hospital (an academic teaching hospital with 1000 beds and over 1.5 million outpatients each year) from 2002 to 2010. The characteristics of the patients and their tumors were collected through review of medical records and pathologic reports. The patients were followed postoperatively for a mean of 49.24 months (range, 23-81 months). In addition, we also collected the other colorectal tissue samples including 13 cases of colorectal high-grade intraepithelial neoplasia (HGIN), 33 cases of villous adenoma, 42 cases of tubular adenoma and 44 cases of normal colorectal mucosa. The diagnostic criteria of colorectal tumor were based on the WHO classification. All of the tissues were sectioned for immunohistochemistry of NPM. The collection of the human specimens in the study was approved by the Independent Ethics Committee of Shilong People's Hospital.

### Immunohistochemistry

Tissue samples were processed according to routine procedures. In brief, each paraffin-embedded tissue section (4  $\mu$ m in thickness) was deparaffinized, hydrated, and incubated in 3% H<sub>2</sub>O<sub>2</sub> and microwaved to block endogenous peroxidase activity. After appropriate antigen retrieval, the primary antibodies (NPM, mouse monoclonal antibody, Clone No. NA24, Santa Cruz Biotechnology, CA, USA) and then secondary antibody (PV6000 Kit, ZhongShan Co., China) were used. Subsequently, the sections were stained with 3'3'-diaminobenzidine, counterstained with hematoxylin and mounted. Positive controls comprised sections of known NPM-positive CRC. Negative controls by omitting the primary antibody showed weak background staining in all cases.

For the tissue evaluation of NPM, each slide was scored based on the percentage of positively stained malignant nuclei. Every stained nucleus was considered positive, irrespective of intensity. In agreement with previous studies, the cut-off of NPM positivity was >50% positive tumor cells with nuclear staining (Wong et al., 2013). Staining results were evaluated by two independent observers blinded to clinicopathological data. Regarding the cases with discordant evaluation, two pathologists performed a consensus adjudication review using a multiheaded microscope.



**Figure 1. NPM Expression in Normal to Malignant Colorectal Tissues.** A and C: Very high levels of NPM is expressed throughout the nucleus in colorectal carcinomas and colorectal high-grade intraepithelial neoplasia glands, respectively. B, D, E and F: NPM shows low/negative expression in tissues adjacent to tumor, villous adenoma, tubular adenoma, normal colorectal mucosa, respectively (All fields, 200  $\times$ )

### Statistical analysis

Pearson's chi-square test was used to determine the difference of NPM expression in histologically normal to malignant colorectal tissues, also to determine the relationship between the clinical parameters of gender, age, tumor position, tumor size, depth of invasion, cancer embolus, lymph node metastasis, pathologic grade, histologic type, tumor occurrence and NPM expression. Overall and disease-free survival after tumor removal were calculated by the Kaplan-Meier method, and difference in survival curves was analyzed by the log-rank test. The Cox proportional hazards model was used for multivariate analysis of prognostic factors. Statistical significance is initially set at  $P < 0.05$ . All statistical analysis was performed using the SPSS 16.0 software (Los Angeles, CA).

## Results

### NPM expression in normal to malignant colorectal tissues

NPM expression in normal to malignant colorectal tissues was showed (Table 1) (Figure 1). NPM expression was found significantly upregulated in CRC compared to adjacent colorectal tissue, villous adenoma, tubular adenoma and normal colorectal mucosa, respectively ( $p < 0.05$  for all). However, NPM expression in the CRC tissue was the same as that in colorectal HGIN ( $p > 0.05$ ). NPM expression in HGIN was higher than that in villous adenoma, tubular adenoma and normal colorectal mucosa, respectively ( $p < 0.05$  for all). There is a clear trend of progressive increase in positive rates for NPM expression from normal to malignant colorectal tissues.

### Correlation between NPM expression and clinicopathologic parameters in colorectal carcinomas

We further investigated the differences between NPM expression in CRC tissue on the basis of different clinical parameters, including the gender, age, tumor position, tumor size, depth of invasion, cancer embolus, lymph

**Table 2. Correlation Between NPM Expression and Clinicopathologic Parameters in Colorectal Carcinomas**

	NPM expression		P value
	(+)	(-)	
Age at diagnosis			
<50	59	37	NS
≥50	45	20	
Gender			
Male	58	32	NS
Female	46	25	
Position			
Ascending colon	27	13	NS
Transverse colon	21	13	
Descending colon	19	6	
Sigmoid colon	37	25	
Size (cm)			
<5	47	31	NS
≥5	57	26	
Infiltration of serosa			
Absent	47	27	NS
Present	57	30	
Cancer embolus			
Absent	80	52	0.024
Present	24	5	
Lymph node metastasis			
Absent	51	38	0.031
Present	53	19	
Differentiation grade			
Well	8	11	0.029
Moderate	78	42	
Poor	18	4	
Histologic type			
Non-mucinous	95	54	NS
Mucinous	9	3	
Recurrence			
Absent	58	42	0.025
Present	46	15	

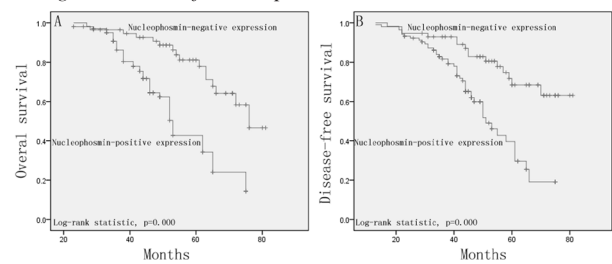
NS, not significant

node metastasis, differentiation grade, histologic type and tumor occurrence (Table 2). We observed a statistical correlation between NPM expression and cancer embolus, lymph node metastasis, differentiation grade, tumor recurrence in CRC tissue ( $p < 0.05$  for all). NPM-positive expression on tumor cells was more likely to be the presence of cancer embolus, positive lymph node, poor differentiation grade and tumor recurrence. No statistical significance was found between NPM expression and the other clinicopathologic parameters.

**Table 3. Significant Prognostic Factors of (a) Overall Survival and (b) Disease-free Survival by Multivariate Analysis**

Factor	Coefficient	HR	CI 95%	P value
(a) Overall survival				
NPM expression (positive vs. negative)	0.652	1.919	1.056-3.488	0.032
Recurrence (present vs. absent)	3.166	23.719	8.522-66.017	0.000
(b) Disease-free survival				
NPM expression (positive vs. negative)	0.955	2.598	1.409-4.791	0.002
Cancer embolus (present vs. absent)	1.539	4.659	2.720-7.980	0.000
Positive lymph nodes (present vs. absent)	0.64	1.896	1.131-3.179	0.015

HR, Hazard ratio; CI, confidence interval



**Figure 2. Relationship between Overall and Disease-free Survival and NPM Expression in Colorectal Carcinomas Patients.** A: The overall survival of colorectal carcinomas patients according to the intensity of NPM expression. B: The disease-free survival of colorectal carcinomas patients according to the intensity of NPM expression

*Relationship between survival and NPM expression in colorectal carcinomas patients*

We observed a statistical correlation between NPM expression and overall survival in CRC patients. The 3-year and 5-year overall survival of patients were 86.1% [95% confidence interval (CI) 82.5% to 89.7%] and 40.0% (95% CI 33.0% to 47.0%) with NPM-positive expression respectively, compared with 94.6% (95% CI 91.6% to 97.6%) and 77.9% (95% CI 71.5% to 84.3%) with NPM-negative expression respectively. The survival curve for patients with NPM-negative on tumor cells tended to be better than that for patients with NPM-positive (log-rank statistic,  $p < 0.05$ ; Figure 2A). The Cox multivariate regression analysis with clinicopathological factors was used to assess the independent prognostic factors for overall survival and the following were shown to be independent prognostic factors (Table 3a). The intensity of NPM staining was identified as an independent prognostic factor in CRC patients.

A statistical correlation was also observed between NPM expression and disease-free survival in CRC patients. The disease-free survival of patients with NPM-positive expression in 3 and 5 years were 61% (95% CI 77.7% to 85.5%) and 36.3% (95% CI 28.9% to 43.7%) respectively, whereas 89.0% (95%CI 84.7% to 93.3%) and 68.4% (95%CI 61.1% to 75.7%) disease-free survival in 3 and 5 years were observed in patients with NPM-negative expression. Overexpression of NPM on tumor cells was significantly associated with a poor disease-free survival of CRC patients (log-rank statistic,  $p < 0.05$ ; Figure 2B). Using the Cox regression model, we performed multivariate analysis to assess the independent prognostic factors for disease-free survival and the following were shown to be independent prognostic factors (Table

3b). NPM expression was found to be an independent prognostic factor for CRC patients.

## Discussion

In this study our data indicated that NPM expression was found not only significantly upregulated in CRC compared to corresponding colorectal tissue adjacent to the tumor, but also in CRC compared to villous adenoma, tubular adenoma and normal colorectal mucosa. These results show that increased expression of NPM play important roles in carcinogenesis.

NPM is a multifunctional protein involved in a complex network of interactions. The role of NPM in oncogenesis is controversial. On one hand, precious studies reported NPM overexpression in numerous solid tumors, such as gastric cancer (Tanaka et al., 1992), CRC (Nozawa et al., 1996), prostate cancer (Subong et al., 1999), bladder cancer (Tsui et al., 2004), liver cancer (Yun et al., 2007), ovarian cancer (Shields et al., 1997) and thyroid cancer (Pianta et al., 2010). These results including ours show that NPM contributes to cancer pathogenesis as an oncogene. The NPM gene is mutated or rearranged in a number of hematological disorders (Cilloni et al., 2008), also suggesting that NPM functions as a proto-oncogene, but NPM gene mutation has not been detected in solid cancers (Jeong et al., 2007). In addition, NPM knockdown decreases cell viability, induces cellular senescence and growth arrest in CRC cells, also supporting its function as an oncogene (Wong et al., 2013). On the other hand, experiments with cultured NPM-null cells and with mice carrying a single inactivated NPM allele indicate a tumor suppressor function for NPM (Feuerstein et al., 1988). Moreover, tumor suppressive role for NPM is reported in breast cancer (Karhemo et al., 2011) and NPM acts as a haploinsufficient tumor suppressor in the hematopoietic compartment (Sportoletti et al., 2008). NPM functions both as oncogene and tumor suppressor gene. Although NPM appears to contribute to oncogenesis through many mechanisms, its role in tumor development is unclear. Role of NPM mutations in promoting leukemia has been widely studied. NPM mutations result in abnormal transport of NPM into cytoplasm which probably triggers "multiple cellular pathways" and then causes "leukemogenesis" (Shahab et al., 2013). However, a recent study reporting none of NPM mutations in children acute myeloid leukemia (AML) indicates that NPM mutations may not be significantly involved in the pathogenesis of childhood AML (Mukda et al., 2011).

The primary goal of our study is to investigate an association of NPM expression with survival in CRC patients and also to investigate whether NPM could be identified as a potential prognostic marker for CRC patients in daily practice.

Many clinical and histopathological factors that can assist in predicting survival of CRC patients have been studied. These parameters, when used with the TNM system, will serve as useful and specific tools to provide crucial information about prognosis of CRC cancer. Several potential molecular markers for CRC have been also identified. Currently, PCNA and Ki-67 are the most

popular molecular markers investigated as prognostic factors in CRC because of their properties reflecting cancer cells proliferation. Many previous studies have reported the relationships between Ki67 and PCNA expression and the survival of CRC patients and/or between Ki67 and PCNA expression and many known prognostic factors related to the pathological characteristics of the neoplasm, heavily showing their values as useful markers of survival in CRC (Oshima et al., 2005; Evans et al., 2006; Salminen et al., 2005; Choi et al., 1997; Nakae et al., 1998). However, some studies investigating the prognostic values of Ki67 and PCNA using multivariate cox regression analysis show that Ki67 and PCNA are not independent prognostic factors in CRC (Fodor et al 2012; Tanaka et al., 1994). The differential expression of Ki-67 and PCNA seem to be certain practical, rather than reliable and independent, value to predict survival of CRC patients. An ideal molecular markers as prognostic factors for CRC must be biologically meaningful and reliable. Thus, new potential molecular markers as prognostic factors for CRC patients should be searched.

NPM is a nucleolar phosphoprotein constantly shuttling between the nucleolus and cytoplasm (Grisendi et al 2006). It is involved in a complex network of interactions and has multiple functions involved in the ribosomal biosynthesis (Herrera et al., 1995; Savkur et al., 1998), control of centrosome reproduction (Okuda, 2002), cell apoptosis (Liu et al., 1998), molecular chaperones (Okuwaki et al., 2001), environmental stress responses (Kurki et al., 2004), the regulation of the tumor suppressor proteins p53 (Colombo et al., 2002) and p14ARF (Bertwistle et al., 2004), and post-translational modification by acetylation (Swaminathan et al., 2005), sumoylation (Liu et al., 2007), ubiquitinylation (Sato et al., 2004), and phosphorylation (Okuda et al., 2000).

NPM plays an essential role in cell growth and proliferation by regulating cell cycle progression, ribosome biogenesis, and centrosome duplication (Liu et al., 2012). NPM protein is reported to be over-expressed in rapidly proliferating tumor cells (Feuerstein et al., 1998), and also is a marker of the cell kinetic cycle. NPM expression increases rapidly in early G1 phase during mitosis, reaches a peak at the onset of S phase and declines at the end of S phase (Feuerstein et al., 1998). Using a tissue microarray of sections of multiple organs, Yun et al observed robust nuclear expression of NPM in carcinomas that originated from stratified epithelia that were derived from several organs, including the skin, esophagus, cervix, and lung (Yun et al., 2007). In CRC, our findings, similar to a reported observation (Wong et al., 2013), showed an association of NPM upregulation paralleling progression from normal mucosa to overt CRC, likely reflecting this protein's cell proliferative properties. It seems that NPM may be a potential marker of cell proliferation, thus suggesting its possible value regarding prognosis of cancer patients. It is cellular activities of NPM involved in cell growth and proliferation that have sparked interest in evaluating NPM's prognostic value in cancer patients.

NPM expression has been reported to be correlated with the stage of tumor progression (Grisendi et al., 2006). In bladder carcinoma, an immunohistochemical

analysis showed that NPM protein staining is importantly associated with tumor stage, histological grade, and recurrence. Tumors with relatively high NPM levels are poorly differentiated at high stage, and have a high recurrence rate, suggesting a potentially important role of NPM in the development and progression of bladder carcinoma (Tsui et al., 2008). Two recent studies also report that NPM overexpression is associated with tumor progression in CRC (Liu et al., 2012; Wong et al., 2013). Liu et al provide the direct evidence that the expression level of NPM1 is critical for colon cancer cell migration and invasion (Liu et al., 2012). In the current study, our findings show that NPM overexpression is associated with known markers of poor prognosis (cancer embolus, lymph node metastasis, poor differentiation grade and tumor occurrence), suggesting that the differential expression of NPM may be related to prognosis of CRC patients, which are consistent with above-mentioned results.

A positive correlation is found between NPM overexpression and poor five-year survival of CRC patients (Liu et al., 2012). However, the limited number of the patients (n=31) in the above-mentioned study does not allow the authors to make complete statistical adjustment for other potential clinicopathological factors. Accordingly, multivariate analysis is not possible. Similar results are also observed in other solid tumors (Tsui et al., 2008; Kikuta et al., 2009; Coutinho-Camillo et al., 2010). Furthermore, in AML mutated NPM also behaves as a favorable prognostic marker for overall survival or disease-free survival (Shahab et al., 2013). All above-mentioned findings indicate that NPM may be an important prognostic marker for cancer patients. In our study with relatively large number of patients (n=161) recurrence of CRC was associated with NPM overexpression, suggesting that its expression is a useful marker in CRC. Moreover, overall and disease-free survival were significantly worse in patients with NPM overexpression, and the cox multivariate analysis suggested that NPM could be an independent prognostic factor in CRC. However, the mechanism by which NPM overexpression is involved in recurrence and progression of CRC is unclear and requires further investigation.

In summary, our results show that NPM expression is found to be a reliable and independent prognostic factor for CRC patients apart from conventional prognostic factors. Accordingly, prognosis of CRC is determined by not only conventional prognostic factors but also NPM expression. Of course, future studies investigating a larger number of patient sample from diverse hospitals are necessary to continue evaluating NPM's prognostic value in CRC patients. In addition, further in vitro and in vivo studies are also necessary to better understand the mechanisms of NPM regulation and its correlation with proliferation, invasion and metastatic potential in CRC.

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