NUBPL, a novel metastasis-related gene, promotes colorectal carcinoma cell motility by inducing epithelial–mesenchymal transition

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Colorectal cancer (CRC) is the second leading cause of cancer death worldwide(1) and over 90% of these deaths result from metastasis.(2) However, effective treatments for patients with metastatic CRC are still unavailable. Hence, an improved understanding of the molecular mechanisms underlying metastasis may enhance therapeutic strategies or prevent early metastasis.

Double minute chromosomes (DMs) are considered to be associated with tumor metastasis or malignant phenotype. They are extrachromosomal circular DNA ranging from hundreds of kilobases to megabases in size. Nucleotide binding protein-like (NUBPL) was found amplified in human CRC cell line NCI-H508, which is known to contain many DMs. Therefore, NUBPL might play a role in the development of CRC. NUBPL, also known as IND1 or huInd1, is an assembly factor for human mitochondrial complex I, which is a large ~1000-kDa mitochondrial inner membrane enzyme composed of 45 protein subunits.(3,4) The expression of NUBPL is closely correlated with complex I protein and activity levels. However, the relationship between NUBPL and tumorigenesis has not been described.

One of the major mechanisms of cancer cell metastasis includes epithelial–mesenchymal transition (EMT), which allows cells to leave the site of the primary tumor, invade surrounding tissues, disseminate through the lymphatic or hematogenous systems, and migrate to distant organs.(6,7) During the process of EMT, epithelial cells undergo a loss of polarity and cell–cell contact, and convert into mesenchymal cells with increased motility. Biochemically, the expression of epithelial markers such as E-cadherin and α-catenin are repressed and the expression of mesenchymal markers such as N-cadherin and vimentin are upregulated.(8) Reduction of E-cadherin is a well-established hallmark of EMT, and is also correlated with poor clinical prognosis in many kinds of cancers.(9–11)

In the present study, the expression of NUBPL was compared between CRC tissues and paired adjacent non-tumor colorectal tissues. Additionally, the role of NUBPL in human...
Cancer cell lines was investigated using various assays. Furthermore, the mechanism of NUBPL in tumor metastasis was also addressed.

Materials and Methods

**Cell lines and cell culture.** Colorectal cancer cell lines SW620 and HT29 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Colorectal cancer cell lines SW480, NCI-H716, T84, COLO320HSR, SK-CCr1, and NCI-H508 were purchased from ATCC (Manassas, VA, USA). COLO320HSR, NCI-H508, and NCI-H716 cells were maintained in RPMI-1640 medium, and SW480 and SW620 cells in Leibovitz’s L-15 medium. SK-Cr1 cells in minimal essential medium, T84 cells in a 1:1 mixture of DMEM and HT-29 cells in DMEM containing high glucose. All cells were supplemented with 10% FBS.

**Materials and Methods.**

**Western blot analysis.** Colorectal cancer cells were dissolved in RIPA (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 40 μg total protein was separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 10% blocking solution (Roche, Basel-Stadt, Switzerland) and incubated with primary antibodies overnight at 4°C. Then cells were treated with secondary antibody for 1 h at room temperature. The bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The following primary antibodies were used: NUBPL (SAB1408017, Sigma-Aldrich), E-cadherin, α-catenin, Vimentin, fibronectin, α-smooth muscle actin (α-SMA) (all from Proteintech, Rosemont, IL, USA), ERK, phospho-ERK (both from Cell Signaling Technology, Danvers, MA, USA), and GAPDH (Kangchen Bio-tech, Shanghai, China).

**Quantitative RT-PCR.** Total RNA was extracted from CRC cells using the High Pure RNA Isolation Kit (Roche) according to the manufacturer’s instructions. The synthesis of cDNA from total RNA was carried out with the PrimeScript RT Reagent Kit Perfect Real Time (Takara, Dalian, China). Reverse transcription–PCR for quantification was undertaken with LightCycler 480 SYBR Green I Master (Roche). The specific primer sets used in this study were obtained from Outdo Biotech (Shanghai, China). This CRC tissue microarray contains 75 pairs of human CRC and their matched normal tissues. The mRNA expression of NUBPL was found elevated in CRC compared with normal colorectal tissues, as analyzed in three databases (Fig. 1a). Additionally, the expression pattern of NUBPL was studied by immunohistochemistry in 75 pairs of CRC tissues and adjacent normal tissues. The statistical analysis was carried out using Student’s t-test. The 2-test was used to determine the correlation between NUBPL expression and clinopathologic features. Wilcoxon’s signed-rank test was used to analyze the expression differences between CRC and adjacent normal tissues. The statistical analysis was carried out using spss for Windows, version 19.0 (IBM, Armonk, NY, USA) and P < 0.05 was considered significant.

**Results.**

**Overexpression of NUBPL is identified in CRC tissues.** To explore the relationship between NUBPL and CRC, we investigated the expression levels of NUBPL in CRC tissues and normal tissues. The mRNA expression of NUBPL was found elevated in CRC compared with normal colorectal tissues, as analyzed in three databases (n = 197) from Oncomine (GSE20916, GSE5206, and GSE9348) (Fig. 1a). Additionally, the expression pattern of NUBPL was studied by immunohistochemistry in 75 pairs of CRC tissues and adjacent non-tumor tissues. The protein expression level of NUBPL was significantly higher in CRC tissues compared with their non-tumor counterparts (P < 0.05; Fig. 1b), suggesting that NUBPL was frequently overexpressed in CRC.
NUBPL expression was detected in metastatic lymph node of CRC patients by immunohistochemical staining. High expression level of NUBPL was observed in metastatic lymph nodes (Fig. 1c). These results indicate that NUBPL may play a vital role in CRC metastasis.

Expression of NUBPL in CRC cell lines. The expression of NUBPL in eight colorectal adenocarcinoma cell lines (SW620, SW480, NCI-H716, NCI-H508, T84, HT29, COLO320HSR, and SK-CO-1) was examined by quantitative RT-PCR (qRT-PCR). Results showed that SW620 and SW480 exhibited the lowest NUBPL expression among these cell lines (Fig. 2a). Therefore, they were used for the subsequent overexpression experiments with control plasmids and pEGFP-C1-NUBPL. The expression of NUBPL in SW620 and SW480 cells was upregulated after transfection as detected by Western blot analysis (Fig. 2b). Then, the NUBPL gene was further silenced by three targeted siRNAs (siNUBPL-1, siNUBPL-2, and siNUBPL-3) in SW480-NUBPL cells. Quantitative RT-PCR (Fig. 2c) and Western blot analysis (Fig. 2d) results suggested that the expression levels of NUBPL in both mRNA and protein could be effectively silenced by siRNAs (siNUBPL-1, siNUBPL-2, and siNUBPL-3).

Migration and invasion of CRC cells enhanced by NUBPL. As our clinical data revealed that NUBPL overexpression was correlated with lymph node metastasis, we hypothesized that NUBPL increases cell motility. In order to determine whether ectopic expression of NUBPL could alter CRC cell migration and invasion, we undertook a series of biological experiments using SW480 and SW620 cells. The wound-healing assay showed that NUBPL-transfected cells obtained a remarkably faster healing rate of the scratched “wound” compared with control cells ($P < 0.05$; Fig. 3a). Cell migration assay also showed that NUBPL could significantly increase cell motility compared with control cells ($P < 0.05$; Fig. 3b). In addition, Transwell invasion assay further revealed that NUBPL overexpression was able to promote cell invasion significantly ($P < 0.05$; Fig. 3c).

To determine whether the above functional changes were actually caused by NUBPL, we then knocked down the NUBPL expression in SW480-NUBPL cells. As expected, both cell migratory (Fig. 3d) and invasive (Fig. 3e) abilities were significantly suppressed in NUBPL knockdown cells compared with the controls ($P < 0.05$). Collectively, these results suggest that NUBPL overexpression can lead to the aggressive phenotype of CRC cells.
NUBPL promotes CRC cell motility

Table 1. Correlation between nucleotide binding protein-like (NUBPL) expression and clinicopathologic features of colorectal carcinoma

| Variable                        | All cases | NUBPL expression | P-value |
|---------------------------------|-----------|------------------|---------|
|                                 |           | High             | Low     |         |
| Gender                          |           |                  |         |         |
| Female                          | 32        | 8                | 24      | 0.129†  |
| Male                            | 43        | 18               | 25      |         |
| Age, years                      |           |                  |         |         |
| <60                             | 23        | 9                | 14      | 0.589†  |
| ≥60                             | 52        | 17               | 35      |         |
| Lymph node metastasis          |           |                  |         |         |
| –                               | 39        | 9                | 30      | 0.028†**|
| +                               | 36        | 17               | 19      |         |
| Distant metastasis             |           |                  |         |         |
| –                               | 66        | 21               | 45      | 0.303‡  |
| +                               | 9         | 5                | 4       |         |
| Differentiation                 |           |                  |         |         |
| 1–2                            | 62        | 22               | 40      | 0.997‡  |
| 3                               | 13        | 4                | 9       |         |
| Staging                         |           |                  |         |         |
| I–II                           | 36        | 8                | 28      | 0.030†**|
| III–IV                         | 39        | 18               | 21      |         |

†Pearson’s χ²-test. †Yates correction χ²-test. *P < 0.05.

Discussion

Double minute chromosomes are extrachromosomal cytogenetic structures and commonly found to carry oncogenes in various cancers, including epidermal growth factor receptor (13) in gliomas, C-MYC (14) in CRC, and EIF5A2 (15) or RPL22L1 (16) in ovarian cancer. In the report presented here, we identified a novel CRC metastasis-related gene, namely NUBPL, that was also amplified on DMs. It is reported that NUBPL participates in the assembly of mitochondrial complex I (5,17) but its relationship with cancer has not been investigated. So far, the functions of the 45 subunits of complex I are not well understood. Recent studies reported that GRIM-19, one of the subunits of complex I, is not only associated with mitochondrial metabolism, but is also involved in numerous cancers, such as hepatocellular cancer, renal cell carcinoma, and prostate cancer (18–20). This suggests that other subunits may also play a role in cancers.

In our study, the protein expression of NUBPL was first detected in a collection of CRC and adjacent non-tumor tissues. Results from immunohistochemical staining revealed that the level of NUBPL expression was remarkably higher in CRC tissues compared with non-tumor counterparts (P < 0.05). Moreover, analysis of three databases (GSE20916, GSE5206, and GSE9348) from Oncomine indicated that the mRNA expression of NUBPL is considerably higher in CRC compared with normal colorectal tissues (P < 0.05). Importantly, overexpression of NUBPL was significantly correlated with lymph node metastasis (P = 0.028) and advanced clinical stage (P = 0.030). High expression levels of NUBPL were also observed in lymph node metastatic cases. Taken together, these data strongly suggest that NUBPL is frequently overexpressed in CRC tissues and the high expression level of NUBPL may facilitate the metastatic phenotype.

To further understand the biological role of NUBPL in CRC, we studied the function of NUBPL in CRC cell lines, SW480 and SW620, using pEGFP-NUBPL transfection. The underlying the promotion of EMT by NUBPL. The results showed that expression of phosphorylated ERK was increased (Fig. 5a). Further studies showed that ERK inhibitor could decrease N-cadherin and vimentin expression and increase E-cadherin expression in NUBPL-transfected SW480 cells (Fig. 5b). Inhibition of ERK was also found to suppress migratory and invasive abilities of SW480-NUBPL cells (Fig. 5c).
Fig. 3. Effect of nucleotide binding protein-like (NUBPL) overexpression or knockdown on colorectal carcinoma cell migration and invasion. (a) Wound-healing assay detecting the motility of SW480-NUBPL cells. Columns: mean ± SD of triplicate experiments. *P < 0.05, independent Student’s t-test. (b, c) Cell migration (b) and invasion (c) assays investigating the influence of NUBPL overexpression on SW480 and SW620 cells. Representative images of migrated or invaded cells are illustrated in the upper panels (magnification, ×100). Scale bar = 200 μm. Columns: mean ± SD of triplicate experiments. *P < 0.05, independent Student’s t-test. (d, e) Effect of NUBPL knockdown on SW480-NUBPL cell migration (d) and invasion (e) in Transwell assays. Examples of cells migrated through the PET-membrane or Matrigel-coated Transwell are shown in the left panel (magnification, ×100). Scale bar = 200 μm. Columns: mean ± SD of triplicate experiments. siNC, negative control siRNA. *P < 0.05, independent Student’s t-test.
results showed that ectopic expression of NUBPL strongly enhanced cell migration and invasion, and knockdown of NUBPL by siRNA further supported it. These data are consistent with the clinical analysis, suggesting that NUBPL is associated with CRC metastasis. Therefore, NUBPL is not only essential for assembly of complex I, but also vital in tumor progression.

Tumor metastasis is usually induced by EMT. Our data showed that expression of mesenchymal markers (N-cadherin and vimentin) was increased and expression of epithelial marker (E-cadherin) was decreased in CRC cells overexpressing NUBPL. Vimentin is an intermediate filament that is constitutively expressed in mesenchymal cells. Increased vimentin expression can facilitate cell migration and is commonly used as a canonical EMT marker in cancer. E-cadherin is an important mediator of cell-cell adhesions in epithelial tissues. The E-cadherin to N-cadherin switch, which occurs during cancer progression, is used to monitor EMT. The loss of E-cadherin and gain of N-cadherin is considered a key step in the EMT process and is able to enhance metastatic behavior in various epithelial cancers. Thus, NUBPL probably undergoes EMT to achieve higher motility and invasiveness, promoting CRC metastasis.

Accumulating evidence suggests that EMT is one of the pathways mediated by MAPKs, and MAPK–ERK has been considered to be a determinant of EMT. In the present study, we found that NUBPL could enhance the activation of ERK. Furthermore, inhibition of ERK was found to suppress EMT and cell motility in NUBPL-transfected SW480 cells. These results indicate that NUBPL promotes EMT through the activation of ERK.

Collectively, NUBPL has been identified to be a novel metastasis-related gene that plays an essential role in the progression of CRC by promoting EMT through the activation of ERK. These discoveries suggest NUBPL as a novel biomarker for CRC diagnosis, and also provide insights for the...
development of new anticancer therapies for better clinical outcomes for patients with CRC.

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Disclosure Statement
The authors have no conflict of interest.

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