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Original Article

Nuclear division cycle 80 complex is associated with malignancy and predicts poor survival of hepatocellular carcinoma

Xiaowei Chen*, Wenwen Li*, Lushan Xiao, Li Liu

*Hepatology Unit and Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong, P. R. China. *Equal contributors.*

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Abstract: The NDC80 (nuclear division cycle 80) complex takes part in chromosome segregation by forming an outer kinetochore and providing a platform for the interaction between chromosomes and microtubules, thus impacting the progression of mitosis and the cell cycle. The clinical significance of its components, NDC80, nuf2, spc24, and spc25, were widely explored in various malignancies respectively, yet seldom were they studied from the perspective of a complex. This paper explores the clinical importance of the NDC80 kinetochore complex components in terms of their expression level, prognostic value, and therapeutic potential in HCC (hepatocellular carcinoma) patients. With the data from several paired HCC samples from Nanfang Hospital, HCC patients from the TCGA database and other cases from GSE89377, we analyzed the expression levels of the NDC80 complex components, NDC80/nuf2/spc24/spc25, along with the survival data as well as other clinical features using statistical methods and GSEA. The study found that a high expression of NDC80 complex predicts poor survival, and these components have the potential to be used as therapeutic targets.

Keywords: Hepatocellular carcinoma, nuclear division cycle 80 complex, overexpression, prognosis

Introduction

The NDC80 kinetochore complex forms the outer kinetochore to interact with microtubules, therefore assuring proper chromosome segregation during the progression of the cell cycle [1]. The complex has four components: NDC80 (Hec1, Highly Expressed in Cancer Protein), nuf2 (CDCA1), spc24 (Spindle Pole Body Component 24 Homolog) and spc25 (Spindle Pole Body Component 25 Homolog). Aberrant expression or additional configuration [2] of these components got the attention of scholars due to the importance of the cell cycle in cell proliferation. It is well-documented that misregulated NDC80, nuf2, spc24, and spc25 contribute to the unchained proliferation, invasion, and restrained apoptosis [3-6], their expression thus linking extensively to poor prognosis in various tumors [7, 8]. Moreover, siRNA targeting of the four components prohibited cell proliferation and increased apoptosis in gastric and colorectal cancers [9, 10], and an RNA interfering screen also revealed the therapeutic potential of targeting NDC80 and nuf2 [10, 11]. The tumorigenicity of spc24 has been confirmed, and the knockdown of spc24 represses tumor growth and invasion but increased cell apoptosis in anaplastic thyroid cancer [12]. Apart from malignancies, NDC80 has also been reported to be involved in autoimmune diseases like type 1 diabetes [13], and the diagnostic importance of spc25 has been shown in Alzheimer's disease [14]. Thus, efforts exploring the functions and mechanisms of NDC80's components are of great significance.

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer [15] and contributes to more than 500,000 cancer-related deaths worldwide every year [16]. Because of the cancer's highly proliferative and invasive features, most HCC patients merely receive an unsatisfactory curative effect from their hepatectomies, TACE or other therapies [17, 18]. Several studies have reported the significance of the

NDC80 kinetochore complex in the development and regression of HCC [3, 19-21], while further studies are still required for comprehensive understanding of their clinical relevance and therapeutic value. NDC80 components assemble the outer kinetochore, which mediates the interaction between chromosomes and microtubules. Thus, the four components, NDC80, nuf2, spc24, and spc25 should be a structural and functional macrocosm, yet they seldom are analyzed collectively [22].

Hence, we aimed to elucidate the clinical meaning of the expression of the NDC80 complex and discuss its molecular mechanisms to offer a strategy for exploiting their possible therapeutic implications.

Material and methods

Patients and tissues

The experimental design of the current study has been reviewed and approved by the Ethics Committee of Nangfang Hospital, Southern Medical University (Guangzhou, China). Patients enrolled in the study did not receive any anticancer treatments before surgery, and the specimens were collected between January and December 2015. Informed consents were provided by all patients eligible for the collection of HCC and adjacent non-tumor liver specimens.

Data source

Data from the 354 HCC patients were obtained from the TCGA dataset to compare their different expressions of NDC80, nuf2, spc24, and spc25. The data comprise follow-up information and genome-wide expression profiles of these patients for phenotype investigation. Patients with available data on their NDC80/nuf2/spc24/spc25 expression and clinical information were included in the current study. The expression profiles of the tumor tissues from 354 patients and 50 adjacent liver tissues were obtained. Taking NDC80 as an example, the 354 patients were divided into two groups, the NDC80 high group and the NDC80 low group, according to NDC80 expression level with the median expression level of NDC80 as the cutoff. Patients with NDC80 expression below the median were classified as the low NDC80 group and the rest as the high NDC80 group, with 177 cases in each group.

The GSE89377 dataset (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE89377) was downloaded from the GEO website, and the cohort contains 108 cases in total, including 13 healthy people, 9 patients with low-grade chronic hepatitis, 12 with high-grade chronic hepatitis, 12 with cirrhosis, 11 with low-graded dysplastic nodules, 11 with high-graded dysplastic nodules, 5 with early HCC, 9 with Stage I HCC, 12 with Stage II HCC and 14 with Stage III HCC. The analysis of the data was performed based on the platform GPL6947 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL6947).

The gene set enrichment analysis (GSEA)

The gene set enrichment analysis (GSEA) was used to screen gene sets or pathways that are associated with the expression of the interested genes, NDC80, nuf2, spc24, and spc25 in the TCGA dataset. Taking NDC80 as example, the patients were divided into 4 groups with the quantiles of the expression levels of NDC80 and the top and bottom quarter cases were included into the GSEA input as the groups NDC80_high and NDC80_low. The enrichment results were generated by GSEA software through analysis, annotation and interpretation. The gene sets with the most members showing a positive relation to the NDC80 expression were termed associated with NDC80. The significance threshold was set at $P < 0.05$.

RNA extraction, cDNA synthesis and RT-qPCR analysis

The total RNA of HCC and non-tumor adjacent liver tissues was extracted using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. cDNA synthesis was performed using the PrimeScrip™ RT Reagent Kit strictly according to the protocol offered by Takara Biotechnology Co., Ltd., Dalian, China. First, genomic DNA were removed using the DNA Eraser and the corresponding buffer and minimal volume of the sample were subjected to a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Inc.) to measure the quality

and concentration of total RNA. The RNA was then transcribed into cDNA using a Primer mix RT kit (Takara Biotechnology Co., Ltd.) in a 20 μl reaction volume with 1 μg RNA. Subsequently, amplification reactions were performed using a SYBR Green PCR kit from Takara Biotechnology Co., Ltd., using the following primers: NDC80 sense, 5'-ATCAAGG ACCCGAGACCACT-3', and anti-sense, 5'-GTGCAAAAGGATACCCAAGGT-3'; nuf2 sense, 5'-GAAGGAAGCCTGCAGACAGA-3', and anti-sense, 5'-GCAAGACTTCAGGCTTTGGA-3'; spc24 sense, 5'-CTGCGAGAGATCCTCACCAT-3', and anti-sense, 5'-TTGTGACTGTCGTGTCCTCG-3'; spc25 sense, 5'-TACGGACACCTCCTGTCAGA-3', and anti-sense, 5'-GGGCACTATCTGACACTTCAT-3'; GAPDH sense, 5'-GACAGTCAGCCGCATCTTCT-3', and anti-sense, 5'-AAATGAGCCCCAGCCTTCTC. GAPDH was amplified as an internal control. Gene-specific amplification was performed using a LightCycler® 480 Instrument II (Roche Diagnostics, Basel, Switzerland). The specific conditions for the qPCR reaction were as follows: Preliminary denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 20 seconds. A melting curve analysis of the PCR products was used to assess the specificity of amplification. Fold changes were calculated using the relative quantification ($2^{-\Delta\Delta Cq}$) method.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software (IBM, Chicago, IL, USA). The results were reported as the mean ±standard deviation (SD). Student's t-test for independent samples was used to compare the two groups; for example, the different expression levels of NDC80, nuf2, spc24, and spc25 in the HCC samples and in the normal liver tissues. Student's t-test for paired samples was applied to compare the expression level of NDC80, nuf2, spc24, and spc25 in HCC and in the adjacent non-tumor liver tissues. A log-rank based survival analysis was applied in the comparison of overall survival (OS) or disease-free (DFS) time between the high and low NDC80/nuf2/spc24/spc25 groups. A Cox proportional hazardous model was used for univariate and multivariate analysis in evaluating the prognostic significance of NDC80/nuf2/spc24/spc25 expression and other factors. All figures were generated by Graphpad Prism.

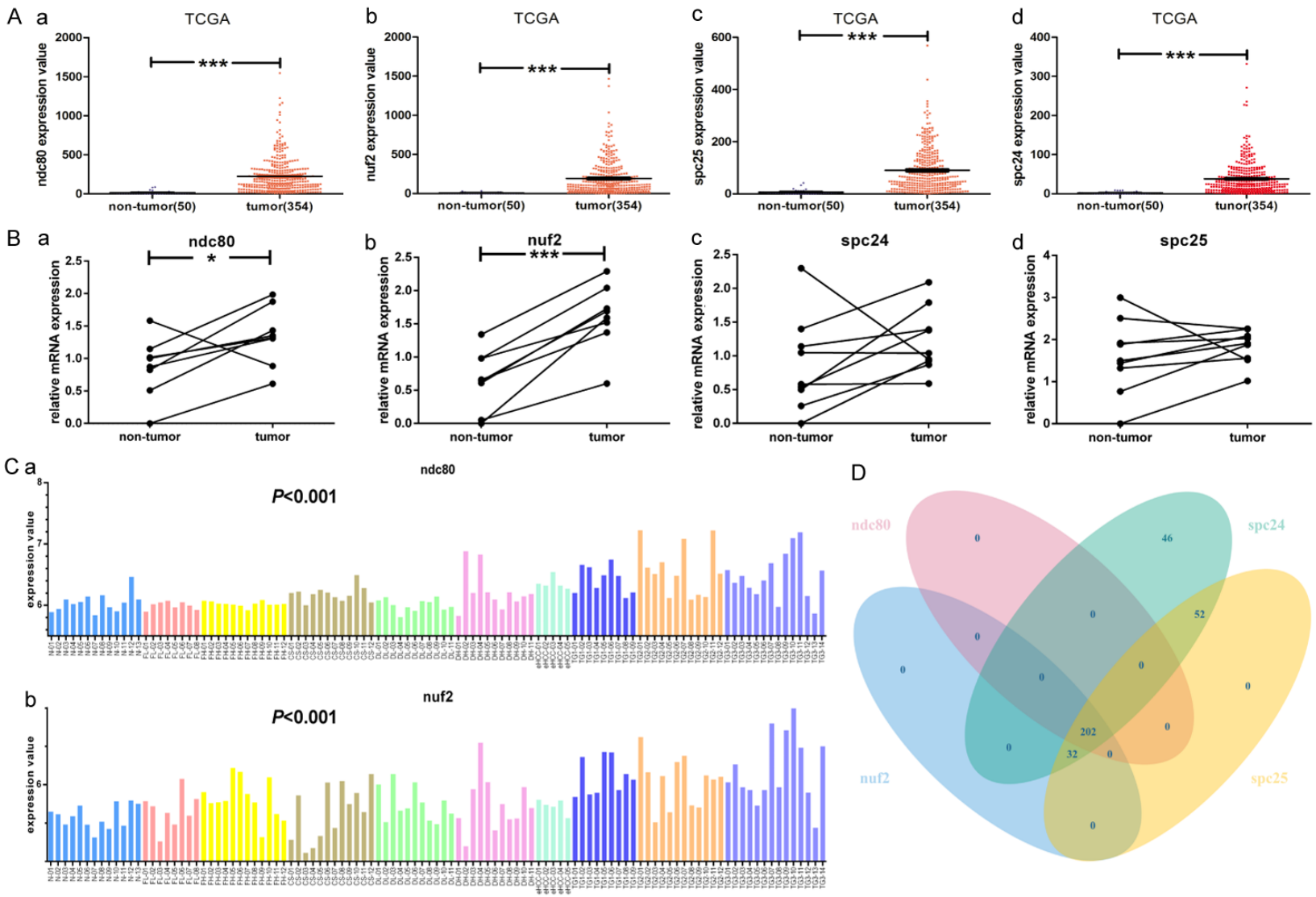
Results

Components of NDC80 complex are overexpressed in HCC

The NDC80 complex is composed of four components: NDC80, nuf2, spc24 and spc25. In order to determine the expression levels of these components in HCC, we analyzed the data of the HCC patients obtained from The Cancer Genome Atlas (TCGA) database and found that all of them were notably overexpressed in HCC as compared with non-tumor hepatic tissues ($P < 0.001$, **Figure 1A**). Also, we detected the expression of these components in HCC and in the adjacent normal liver tissues and found notable overexpressions of NDC80 and nuf2 in the tumor lesions while spc24 and spc25 showed little difference between the tumor and non-tumor tissues, probably due to the sample size of the detection (**Figure 1B**). Further, the gradual upregulation of NDC80, nuf2, spc24 and spc25 was manifested in a GEO dataset (GSE89377) (**Figure 1C**) and an increased expression of these components between tumor and non-tumor groups was also found with significance. Next, we examined the co-expression of these components in the TCGA cohort by crossing the high expression groups of each gene, and their intersections were manifested in a Venn diagram (**Figure 1D**). In the total cohort of 354 HCC patients from the TCGA database, 202 patients showed simultaneously high expression of the four components, yet hardly any were overexpressed individually. Interestingly, spc24 co-overexpressed with nuf2 and spc25 but not NDC80. The NDC80 components possess a different affinity to each other and accordingly form two subcomplexes, NDC80-nuf2 and spc24-spc25 [23]. The protruding upregulation of spc24 indicated another configuration of these components or an additional function of spc24 which required further investigation.

Upregulation of NDC80, nuf2, spc24 and spc25 was correlated with malignant phenotypes of HCC

Next, the relationship of the NDC80 complex expression and the clinicopathological features was investigated. Firstly, the upregulation of the four components was more commonly seen in patients with higher pathological degrees (*P*


A
a
TCGA
ndc80 expression value
***
non-tumor(50)
tumor(354)
b
TCGA
nuf2 expression value
***
non-tumor(50)
tumor(354)
c
TCGA
spc25 expression value
***
non-tumor(50)
tumor(354)
d
TCGA
spc24 expression value
***
non-tumor(50)
tunor(354)
B
a
ndc80
*
relative mRNA expression
non-tumor
tumor
b
nuf2
***
relative mRNA expression
non-tumor
tumor
c
spc24
relative mRNA expression
non-tumor
tumor
d
spc25
relative mRNA expression
non-tumor
tumor
C a
ndc80
P<0.001
expression value
b
nuf2
P<0.001
expression value
D
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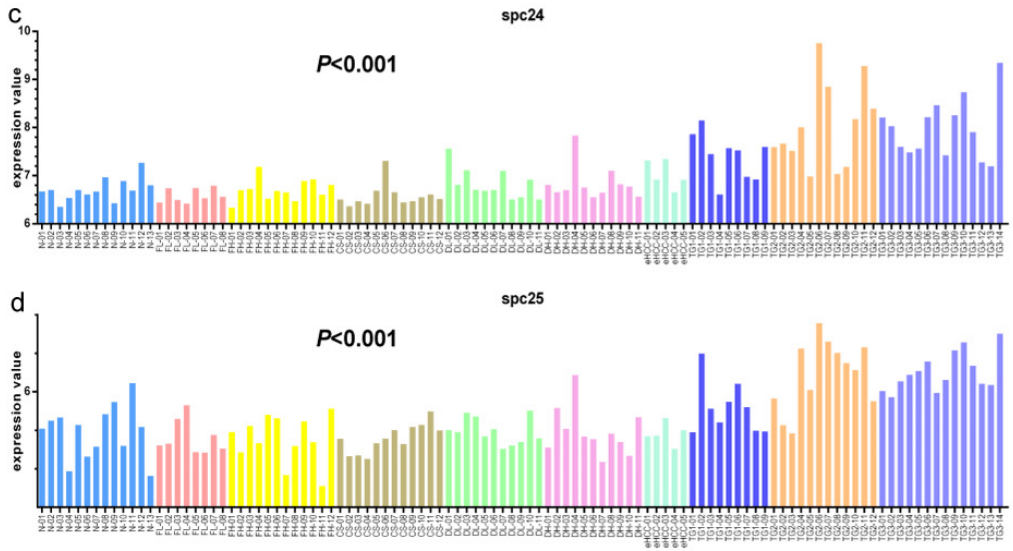


Figure 1. Comparison of NDC80/nuf2/spc24/spc25 expression between HCC samples and normal liver tissues from TCGA. A. In TCGA cohort, (a) NDC80, (b) nuf2, (c) spc24 and (d) spc25 were upregulated in HCC (n=354) than non-tumor liver tissues (n=50) as determined by Student's *t*-test. **P* < 0.05 and ****P* < 0.001. B. The expression of (a) NDC80 (n=8), (b) nuf2 (n=8), (c) spc24 (n=9), and (d) spc25 (n=9) in HCC and adjacent non-tumor tissues were compared by Student's' t-test for paired samples. C. The expression of (a) NDC80, (b) nuf2, (c) spc24, and (d) spc25 in GSE89377 cohort (n=108). N-normal (n=13), FL-chronic hepatitis with low grade (n=8), FH- chronic hepatitis with high grade (n=12), CS-cirrhosis (n=12), DL-dysplastic nodules with low grade (n=11), DH-dysplastic nodules with high grade (n=11), eHCC-early HCC (n=5), TG1/2/3-HCC (n=9, 12, 14). D. Venn diagram exhibiting the number of TCGA HCC patients where a specific gene was overexpressed or given genes were upregulated at the same time. Among 354 cases from the TCGA database, 22 patients showed simultaneously low expression of the four NDC80 components. **P* < 0.05 and ***P* < 0.01.

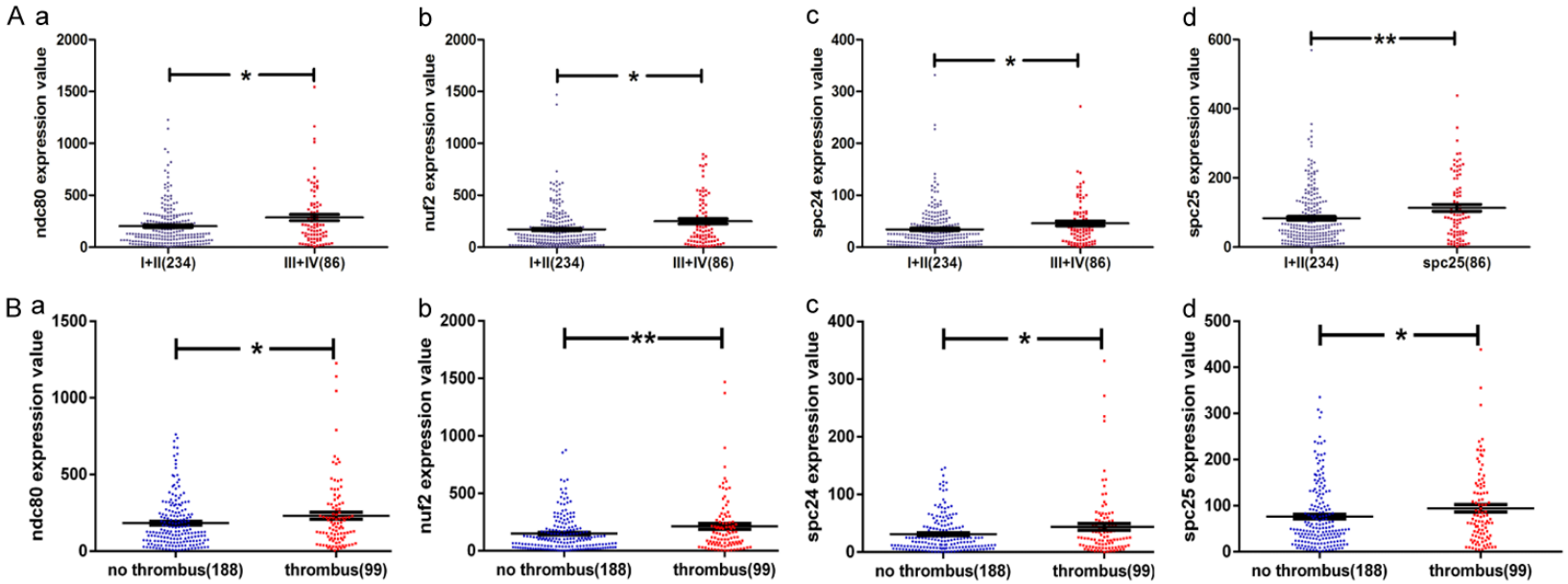
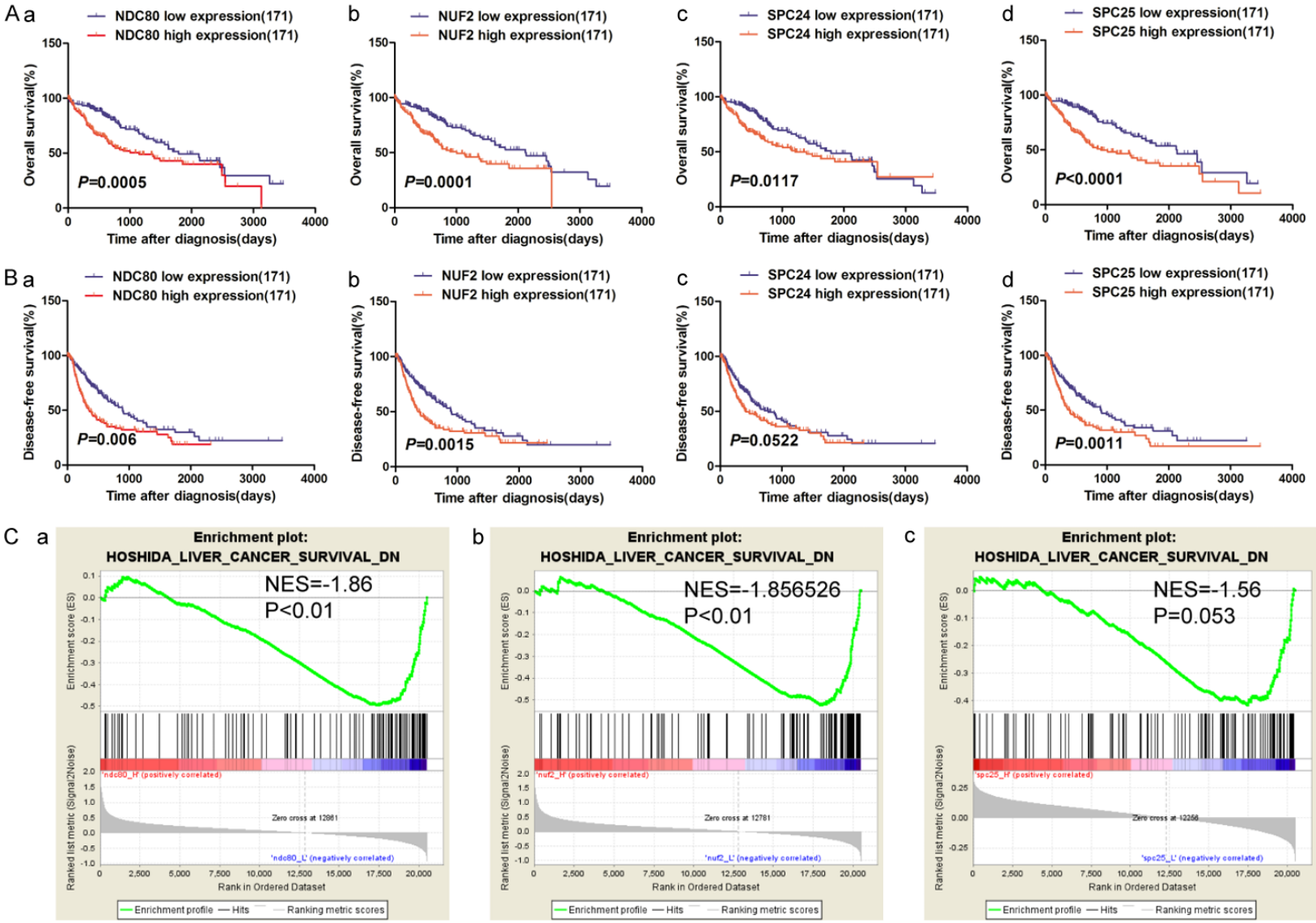


Figure 2. The association between NDC80/nuf2/spc24/spc25 expression and malignant phenotypes. A. Different expression levels of (a) NDC80, (b) nuf2, (c) spc24, and (d) spc24 in patients with lower (I+II, n=234) and higher (III+IV, n=50) pathological degrees. B. Different expression levels of (a) NDC80, (b) nuf2, (c) spc24, and (d) spc24 in HCC patients (n=287) with (n=99) or without (n=188) a thrombus. The total of 354 patients from TCGA were included in the analysis, but the thrombus data were only available for 287 of the patients. *$P < 0.05$ and **$P < 0.01$.


A a
NDC80 low expression(171)
NDC80 high expression(171)
Overall survival(%)
P=0.0005
Time after diagnosis(days)
b
NUF2 low expression(171)
NUF2 high expression(171)
P=0.0001
c
SPC24 low expression(171)
SPC24 high expression(171)
P=0.0117
d
SPC25 low expression(171)
SPC25 high expression(171)
P<0.0001
B a
Disease-free survival(%)
P=0.006
P=0.0015
P=0.0522
P=0.0011
C a
Enrichment plot:
HOSHIDA_LIVER_CANCER_SURVIVAL_DN
NES=-1.86
P<0.01
Enrichment score (ES)
'ndc80_H' (positively correlated)
Zero cross at 12861
'ndc80_L' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
b
NES=-1.856526
P<0.01
'nuf2_H' (positively correlated)
Zero cross at 12781
'nuf2_L' (negatively correlated)
c
NES=-1.56
P=0.053
'spc25_H' (positively correlated)
Zero cross at 12256
'spc25_L' (negatively correlated)

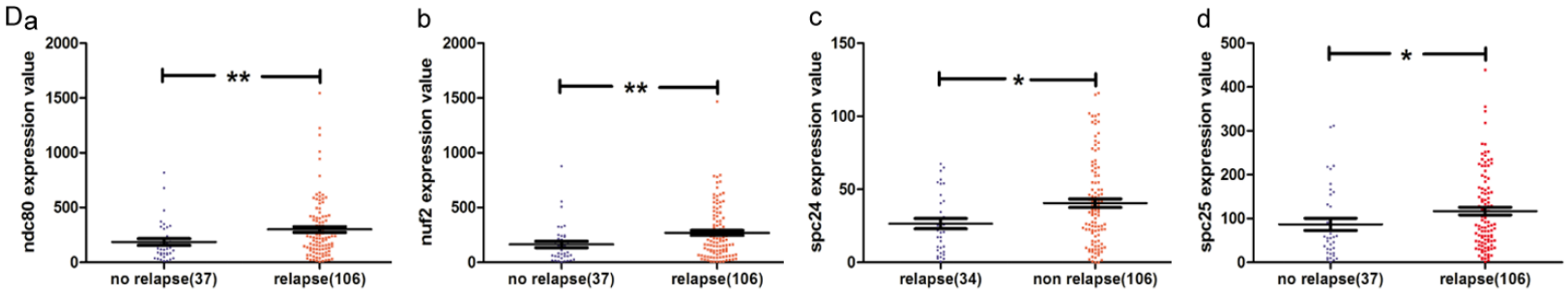


Figure 3. The association between NDC80/nuf2/spc24/spc25 expression and the poor prognosis of HCC patients. A. Shortened overall survival time for patients overexpressing (a) NDC80, (b) nuf2, (c) spc24 or (d) spc25 as compared with patients expressing low NDC80, nuf2, spc24 and spc25. B. Patients overexpressing (a) NDC80, (b) nuf2, (c) spc24 or (d) spc25 showed reduced disease-free time as compared with patients expressing low NDC80, nuf2, spc24 and spc25. C. (a) NDC80, (b) nuf2 and (c) spc25 presented negative associations with a gene set enriched for HCC survival. D. Patients from the TCGA database were grouped based on whether the patient developed a recrudescent HCC lesion or not. Patients found with a recurrence (n=106) showed a higher level of NDC80, nuf2, spc24, and spc25 than those undiscovered (n=37). $*P < 0.05$ and $**P < 0.01$.

Table 1. Correlation between NDC80, nuf2, spc24 and spc25 expression and HCC clinicopathological features in the TCGA liver cancer cohort

Variates	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
NDC80	1.376	1.111-1.705	1.111-1.705			
Nuf2	1.575	1.268-1.957	1.268-1.957	1.745	1.205-2.528	0.003[a]
Spc24	1.253	1.012-1.550	1.012-1.550			
Spc25	1.347	1.088-1.667	1.088-1.667			
Age	0.953	0.769-1.181	0.769-1.181			
Stage	0.811	0.645-1.019	0.645-1.019			
Micro-invasiveness	1.209	0.946-1.544	0.946-1.544			

[a]Value indicates a statistically significant difference. HR, hazard radio; CI, confidence interval. Univariate analysis and multivariate Cox regression.

< 0.05, **Figure 2A**). Secondly, patients with a microthrombus expressed a higher level of NDC80, nuf2, spc24 and spc25 ($P < 0.05$, **Figure 2B**). While the microthrombus manifests invasion and migration of cancer cells from the tumor lesion to the adjacent vasculature and unaffected tissues, a high pathological degree is more directly linked to poor prognosis. Hence, these data suggested the association of NDC80/nuf2/spc24/spc25 expression with HCC malignancy.

Upregulation of NDC80, nuf2, spc24 and spc25 was correlated with poor prognosis of HCC patients

Given that NDC80, nuf2, spc24 and spc25 were universally upregulated in HCC and their association with unfavorable clinical features, we sought to investigate if the upregulation of these components had any impact on the outcome of HCC patients. According to the TCGA samples, the upregulation of the four components correlated positively with shorter overall survival respectively ($P < 0.05$, **Figure 3A**). Furthermore, NDC80, nuf2, and spc25 upregulation were associated with a poor living standard as shown by shortened disease-free survival ($P < 0.01$, **Figure 3B**). Also, GSEA revealed the association between the expression of the four components and genes relating to liver cancer survival ($P < 0.01$, **Figure 3C**, data not shown for spc24). On the other hand, patients expressing higher levels of the four components were more inclined to the recurrence of liver cancer ($P < 0.05$, **Figure 3D**). A univariate analysis recognized NDC80, nuf2, spc24, and spc25 as hazardous factors for the survival of HCC patients, while a multivariate analysis further confirmed the prognostic value of the expression of nuf2 (**Table 1**).

GSEA revealed the significant roles of NDC80 complex in cell cycle, migration and proliferation

Since the overexpression of the NDC80 complex components was related to the malignant phenotypes and poor prognosis in HCC patients, we thus wondered about the mechanism underlying their clinical significance. Gene set enrichment analysis (GSEA) of HCC patients' data obtained from TCGA demonstrated a strong association between the expression of NDC80 complex components and the cell cycle-related genes (**Figure 4A**). Accordingly, patients with high NDC80 complex expression manifested enrichment for DNA replication-related genes (**Figure 4B**, data not shown for spc25). Moreover, NDC80, nuf2, and spc25 showed a strong correlation with the proliferation gene cluster (**Figure 4C**). Meanwhile, a STRING analysis confirmed the relationship of NDC80 with genes involved in chromatin segregation and mitosis (**Figure 4D**). In another aspect, EMT-related genes were also enriched in these patients (**Figure 4E**), indicating the role of the NDC80 complex in promoting the invasiveness of HCC.

Discussion

The aberrant expression of the NDC80 complex components plays multiple roles in the development and progression of HCC. Regarding their functions in the cell cycle, their overexpression has been reported to promote unlashed mitosis and proliferation. It has been

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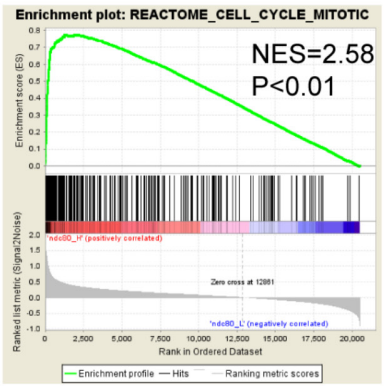

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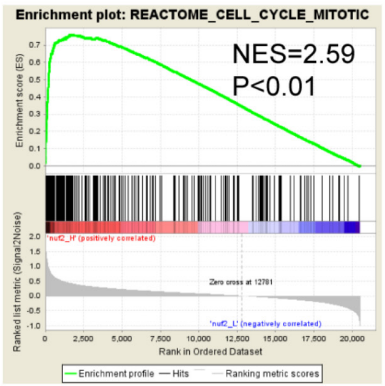

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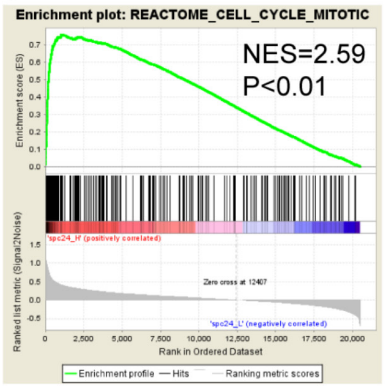

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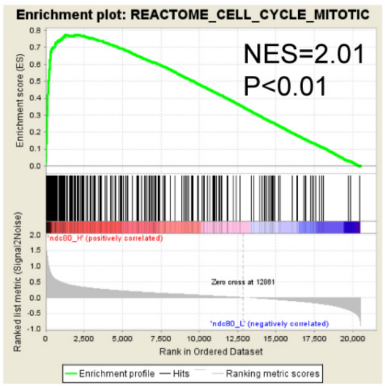

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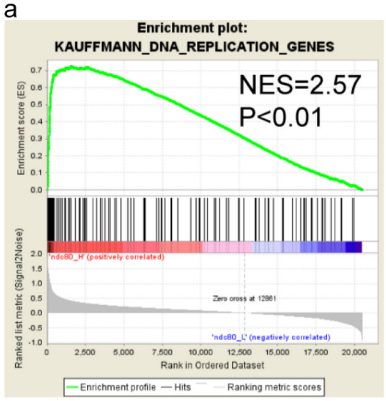

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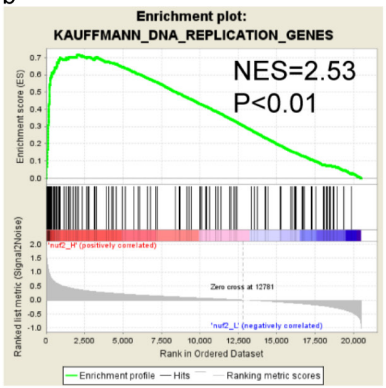

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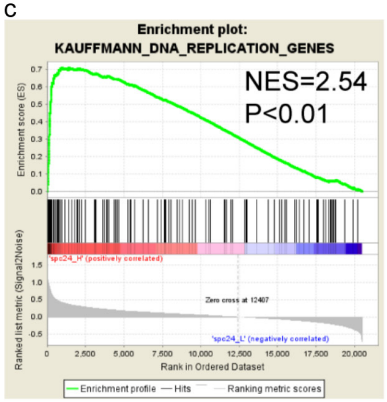

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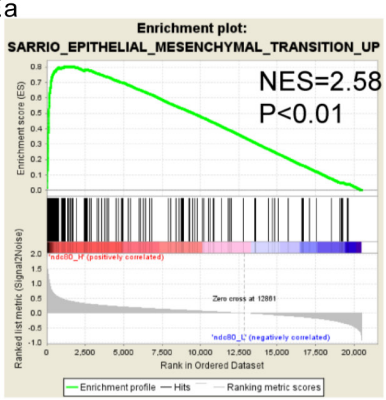

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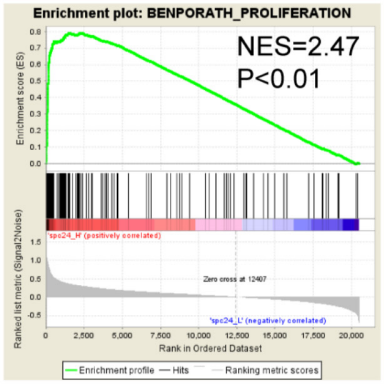

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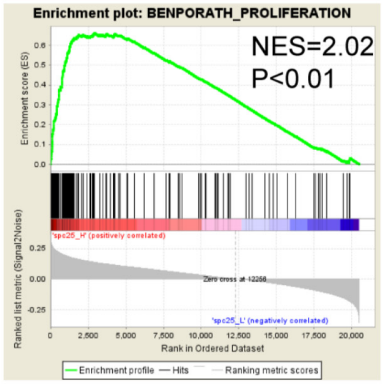

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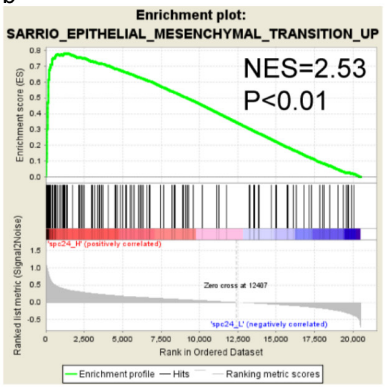

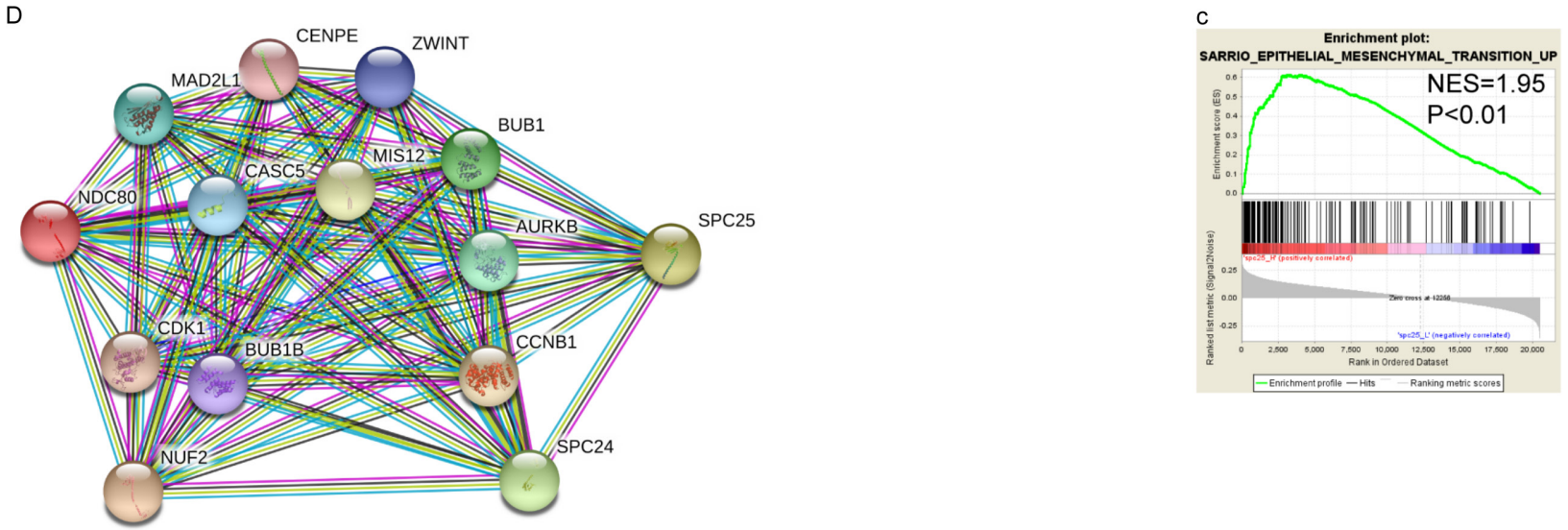

Figure 4. Further exploration into the mechanism of ndc80/nuf2/spc24/spc25 function using GSEA. A. Genes enriched for cell cycle progression presented positive correlation with (a) ndc80, (b) nuf2, (c) spc24 and (d) spc25. B. (a) ndc80, (b) nuf2, and (c) spc24 were found associated with genes enriched for DNA replication. C. Relevance of (a) spc24 and (b) spc25 with genes related to proliferation were found with significance. D. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis marked factors reported with links to ndc80, nuf2, spc24 and spc25. These factors including proteins involved in mitosis (CENPE and MIS12) as well as cell cycle checkpoint (BUB1B, CCNB1, CDK1, AURKB, ZWINT, MAD2L1 and CASC5). E. Genes involved in epithelial-to-mesenchymal transition were highly relevant with (a) ndc80, (b) spc24 and (c) spc25.

previously reported that the overexpression of NDC80 in a mouse model led to the hyperactivation of the mitotic checkpoint *in vitro* and tumorigenesis *in vivo* [24]. Upregulation of NDC80 was also noted in different kinds of neoplastic tissues and exerted a pro-proliferation function in these lesions, including gastric cancer [25], prostate cancer [26], pancreatic cancer [27], breast cancer [28], and so forth. An increased expression of nuf2, spc24, and spc25 was also found to correlate with promoted proliferation or restored stemness of cancer cells as well as the poor survival of patients [29-34]. Meanwhile, targeting NDC80 components produced therapeutic effects in several tumors [25, 26, 35-37]. Further, NDC80 and nuf2 were able to regulate the expression of lncRNA (long non-coding RNA) [26, 30], indicating their potential roles beyond cell mitosis. β-catenin is a classic moonlighting protein that mediates cell adhesion and transcriptional activation in response to Wnt signaling [21]. In light of this, NDC80 and nuf2 might as well function not only as a skeleton protein but also as transcription factors or the like in response to cellular stimuli.

The excessive modules of the kinetochore complex contribute to the instability of chromosomes. Overproduced NDC80 molecules break the equilibrium of microtube-associated protein 1, resulting in improper chromosome segregation and aneuploidy [38]. Chromosomes or their segments incorporated into daughter cells are stagnated in the cytoplasms [39]. Due to failed DNA damage repair, the cGAS-STING pathway could be activated in such cells, ultimately leading to the apoptosis of nearby cells [40]. Therefore, it is reasonable to infer that cells overexpressing NDC80 components should be sensitive to genetically toxic treatments like radiation or genotoxic drugs regarding the high frequency of p53, the main regulator of DNA damage repair in cancer cells. Consistently, TAI-1, a specific inhibitor of NDC80 that disrupts the interaction of NDC80 with Nek2 where chromosome alignment is monitored, showed a stronger anti-tumor potency in the cells with p53 or RB depletion or mutation [41]. A small molecular SM15, another NDC80-specific inhibitor that blocks the correction of the error in kinetochore-microtubule interaction, induces apoptosis by indulging the mitosis catastrophe [42]. However, cells resistant to paclitaxel showed induced NDC80 expression, and NDC80 depletion rescued the sensitivity of ovarian cancer cells to paclitaxel [43], suggesting a switch between sensitive and resistant status mediated by NDC80. Hence, further research into the characteristics of chromosome instability incurred by the mis-regulated NND80 complex is of strategic significance to the clinical prescription. Furthermore, NDC80 inhibitors might also increase cell death through other mechanisms, like the intrinsic apoptosis pathway, namely the mitochondrial-mediated apoptotic pathway, and cellular metabolism-related cell death, like reactive oxygen species (ROS) [42].

The upregulation of NDC80 components involves multiple classical tumorigenesis pathways. NDC80 expression is strictly maintained by the pRb pathway in normal cells, but only partial degradation is observed in transformed malignant cells [44]. A bioinformatics analysis revealed the correlation and possible regulatory relationship between EZH2 and nuf2 [28]. The Wnt/β-catenin pathway, cooperating with PRC1 (Protein regulator of cytokinesis 1), transcriptionally activates spc25 [21]. These antitumor or oncogenic pathways exert different functions on the cell cycle to restrain or boost cell proliferation, and the NDC80 complex stands as the ultimate carrier and a key executor of cell division. Thus, chemicals targeting the NDC80 complex might be promising adjuvant drugs alongside of radiotherapy or other chemotherapies. TAI-1 and another NDC80 inhibitor, INH (N-(4-[2,4-dimethyl-phenyl]-thiazol-2-yl)-benzamide) are able to interfere with the interaction between NDC80 and Nek2 [41, 45]. Aurora kinase A inhibitor structurally disrupts its binding to MYC and subsequently results in cell death [46]. Given that NDC80 is also regulated by aurora kinase B during mitosis [47], an intertwined network monitoring cell mitosis and apoptosis might be underlined.

In all, the present study discussed the components of the NDC80 complex in the context of clinical relevance and molecular function in a comprehensive way. That is, the four components, NDC80, nuf2, spc24 and spc25, were investigated at the same time as a whole. The potential value of NDC80 components, especially NDC80 (Hec1) was highlighted in previous reports and our research.

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Disclosure of conflict of interest

None.

Address correspondence to: Li Liu, Hepatology Unit and Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, 1838 Guangzhou Avenue North Road, Guangzhou 510515, Guangdong, P. R. China. E-mail: liuli.fimmu@gmail.com

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