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Expressions of Nucleoside Diphosphate Kinase (nm23) in Tumor Tissues are Related with Metastasis and Length of Survival of Patients with Hepatocellular Carcinoma[1]

RUN AN[#,†], JIE MENG[#,*], QI SHI[†], XIAO-XIA DAI[#], JING-HONG CHEN[#], YAN-JUN LEI[#,†], BING SHAN[†], CHEN GAO[†], YONG-LIE CHU[#], AND XIAO-PING DONG[†,2]

[#]*School of Medicine, Xi'an Jiao-Tong University, Xi'an 710061, Shanxi, China;* [†]*State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Ying-Xin Rd 100, Beijing 100052, China;*
[*]*The First People's Hospital of Changzhou 213003, Jiangsu, China*

Objective To evaluate the relationship of expressions of nucleoside diphosphate kinase (nm23) and proliferating cell nuclear antigen (PCNA), as well as apoptosis, with the prognosis of HCC patients by analyzing their pathological and clinical data. **Methods** The expressions of nm23 and PCNA were analyzed by immunohistochemistry and the apoptotic phenomena were detected by TUNEL technique in the liver samples from 43 HCC tissues, 39 para-neoplastic tissues, and 10 normal tissues. The mean apoptosis index (AI) and proliferative index (PI) in individual sample were calculated. **Results** As shown by the detection, 32.6% of carcinomas had negative nm23 signal in tumor tissues, whereas all para-neoplastic and normal tissues had positive nm23. The AI in nm23 positive HCC was significantly higher than that in nm23 negative one, with statistical difference (P<0.05). Furthermore, the expressions of nm23, and the values of AI and PI were contrastively analyzed with some main pathological and clinical data of HCC. It revealed that HCC with extrahepatic metastasis showed remarkable correlation with the negative nm23 (P=0.013) and higher PI values of HCC (P=0.015). The disease-free survival in HCC patients with negative nm23 expression was significantly poorer than that in patients with positive nm23 expression. **Conclusion** These data suggest that expressions of nm23 protein in tumor tissues are correlated with occurrences of metastasis and length of survival of the HCC patients, which may be an indicator for their prognosis.

Key words: Hepatocellular carcinoma; nm23; PCNA; Apoptosis; Prognosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with a high prevalence in Asia and Europe[1]. HCC, as one of the malignant tumors with the highest incidence in China, is characterized with frequent metastasis at an early stage and high rate of recurrence after operations[2-3]. Owing to the high rate of recurrence and the low rate of survival of HCC patients, it is a matter of urgency to develop useful diagnostic and prognostic markers. A wealth of studies have been dedicated to look for such biomarkers and a series of oncogenes and proteins, i.e. MMP9, VEGF, and AFP have been described to be associated with the prognosis of HCC [4-7]. However, accurate prediction of prognosis in HCC patients remains out of the question so far.

The onset and development of HCC are generally considered as the consequence of a multi-stepped procedure, which may involve activation of oncogenes and inactivation of antioncogenes. Nucleoside diphosphate kinase gene (NDPK, nm23) is an antitumor metastasis gene that is firstly reported in murine melanoma with close relationship with metastasis[8] and is currently under intense investigation. Recently, lower or negative expression of nm23 has been reported to be related with either intrahepatic metastasis or poor prognosis of HCC[9]. However, a comprehensive analysis of this molecular marker in relation to the recurrence and survival of HCC patients has virtually not been reported.

Proliferating cell nuclear antigen (PCNA) is an auxiliary factor in DNA polymerase, expressed in the nuclei, particularly at the late G_1 and S phases. Information about cell kinetics may be an useful

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[2]Correspondence should be addressed to Xiao-Ping DONG, Tel: 86-10-83534616, Fax: 86-10-63532053, E-mail: dongxp238@sina.com
Biographical note of the first author: Run AN, female, born in 1978, Ph. D., majoring in immunology.

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adjunct to the understanding of tumor behavior, and evaluation of cellular proliferation can be used to predict the clinical course of malignant neoplasms[10]. A previous report has indicated that proliferative activity in noncancerous hepatic tissue may reflect tumor recurrence or the development of tumor in new regions[11]. Apoptosis, one type of programmed cell death, is critical for embryo development and for homeostasis of normal cells and plays an important role in the pathogenesis of a wide variety of diseases including cancer[12]. In the liver, apoptosis plays a prominent part in the pathogenesis of toxic liver injury due to a variety of agents, viral hepatitis and HCC[13].

In the present study, the expression of nm23 and PCNA, as well as the apoptosis in HCC tissues, were determined by immunohistochemical staining. The state of nm23, the value of proliferative index (PI) and apoptosis index (AI) in each of HCC, para-neoplastic and normal liver tissues were separately counted. Combined with the pathological and clinical data of HCC patients, the biological significances of nm23, AI, and PI in tumorigenesis, especially in the prognosis, were evaluated. The nm23-negative tissues were observed in a portion of HCC, but not in normal livers. Expressions of nm23 protein in tumor tissues were statistically correlated with occurrences of metastasis and survival of the HCC patients.

PATIENTS AND METHODS

Patients and Samples

A total of 43 HCC samples and 10 normal liver tissues were obtained from surgical resections in the First Affiliated Hospital of Xi'an Jiaotong University from 2000 to 2004. Among the HCC patients, 35 were men and 8 were women, with the mean age of 48.7 years (ranging from 29 to 77 years). All patients were histopathologically diagnosed as hepatocellular carcinoma by pathologists. Among these patients, 35 were classed as at grade Ⅰ-Ⅱ, 8 at grade Ⅲ according to Edmondson grading; 12 were classed at stage Ⅰand Ⅱ, 31 at stage Ⅲ and Ⅳ according to the Pittsburgh modified TNM criteria of International Union Against Cancer (UICC)[14]. Sixteen patients' tumor sizes were smaller than 50 mm in diameter and other 27' were larger than that. Eighteen cases had tumor envelope invasion and the other 25 cases had otherwise. Thirteen cases saw local cancer embolus or extrahepatic metastasis and the remaining 30 cases failed to do so. Twenty three cases were of nodular type and 20 were of mass type. All patients underwent surgical operations to remove the tumor tissues. ICF forms were signed by the patients involved in the study.

Immunuhistochemical Assays of the Expressions of nm23 and PCNA

Specimens of surgical resection were fixed in 10% formalin and embedded in paraffin. Thin sections (4 μm) were deparaffinized twice with xylene and rehydrated in a series of ethanol solutions (100%, 90%, and 80%). Immunohistochemical analyses of the expressions of nm23 and PCNA were performed according to the routine processes. Briefly, 4-μm sections of tumor or normal tissues were mounted on poly-D-lysine coated slides. The sections were deparaffinized in xylene (20 min) and rehydrated through serial baths of alcohol to water. Antigen retrieval was performed with pressure cooking using 1 000 mL of 0.01 mol/L sodium citrate buffer, pH 6.0. After treatment with 0.3% H_2O_2 for 15 min to remove endogenous peroxidase activity, nonspecific blockage was carried out with commercially ultrablock nonspecific blocking agent (Labvision Co.) for 10 min. The sections were incubated with 1:150 diluted nm23 antibody (nm23/NDPKinase, Neomarkers) at room temperature for 2 hours or with 1:50 diluted PCNA monoclonal antibody (PC-10, Dako) at room temperature for 1 h. The slides were rinsed in phosphate-buffered saline solution and incubated with a biotinylated secondary antibody (Ultravision-Labcision Co., Fremont, CA, USA). After washing with phosphate buffered saline (PBS), the slides were incubated with an avidin-biotin-preoxidase complex (Ultra-streptavidin/HRP, Labvision Co.) for 30 min according to the manufacturer's instruction. As a chromogen, 3-3′-diamino-benzene tetrahydrochloride was used with hydrogen peroxide. The slides were finally counterstained with hematoxylin.

TUNEL Staining

TUNEL (TdT-mediated dUTP nick end labeling) assays were performed according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, after having been deparaffinized and rehydrated, the sections were incubated with TUNEL reaction mixture in the volume of 50 mL at 37 ℃ for 60 min. Then, the slides were counterstained in 0.5 mL of propidium iodide (PI) solution at 37 ℃ for 40 min and developed in BCIP/NBT for 15-30 min.

Calculation of the Index of Apoptosis (AI) and the Index of Proliferation (PI)

PCNA positive cells with brown grains and TUNER-positive stained cells with blue and purple

signals in nuclei were counted under the microscope (×400). PI or AI indexes (%) were calculated by dividing the numbers of the positive cells with that of the total cells.

Statistic Analysis

Statistic analysis were performed using SPSS (Statistical Package for the Social Sciences, Chicago, IL) 11.5 for Microsoft Windows. The relationships between the expression levels of nm23 or PCNA and the tumor size or metastasis progression were analyzed by the χ^2 test or Fisher's exact test. The index of apoptosis (AI) and the index of proliferation (PI) were analyzed by the *t* test. The symptom-free interval and overall survival were calculated according to the Kaplan-Meier method. *P* values less than 0.05 were considered statistically significant.

RESULTS

Immunohistochemical Staining of nm23 in HCC, Para-neoplastic and Normal Liver Tissues

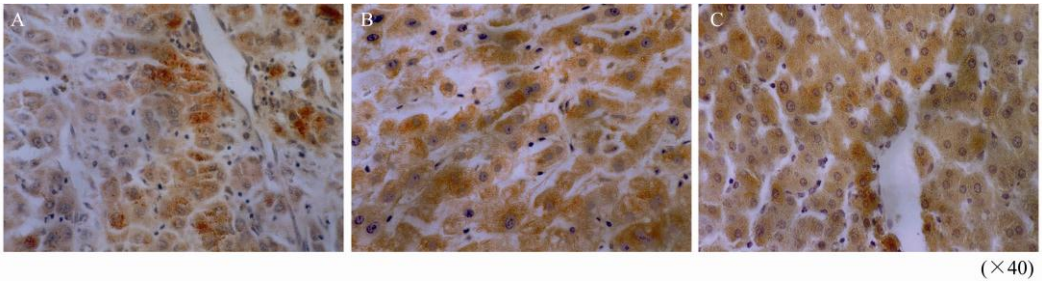
Totally 43 HCC tissues, 39 para-neoplastic liver tissues and 10 normal liver tissues were immunohistochemically stained for the evaluation of the presences and expressions of cellular protein nm23. All tested normal and para-neoplastic liver tissues showed strongly stained signals in hepatocytes, mostly in cytoplasm (Fig. 1A and B). However, among the 43 tumor tissues, 29 (67.4%) showed positive staining comparable to normal tissues, whereas 14 (32.6%) carcinomas revealed a significantly weak signals or loss of positive signal in tumor tissues (Fig. 1C). Analyses of the positive stained numbers among the three groups showed a statistical difference (Table 1, $P<0.05$).

Immunohistochemical Staining of PCNA and TUNEL Staining in HCC and Para-neoplastic Liver Tissues

To address the possible difference in cellular proliferation and apoptosis between normal and carcinoma tissues, 43 HCC tissues and 39 para-neoplastic liver tissues were screened by PCNA-specific immunohistochemical staining and TUNEL staining. Although PCNA positive cells with brown grains mostly in nucleoli were observed in both malignant and para-neoplastic tissues, careful analyses revealed more positive-stained nucleoli with denser colour in HCC tissues, forming big circularity or ellipse in the area of nucleolus (Fig. 2A), whereas notably fewer positive nucleoli were morphologically observed in the normal tissues as compared to those from para-neoplastic liver tissues (Fig. 2B). The average indexes of proliferation (PI) of HCC and para-neoplastic tissues were 30.47% and 27.47%, respectively, showing a statistical difference (Table 2, $P=0.038$). TUNEL-positive stained cells were also detected in both groups that the nuclei of cells were blue and purple, but more apoptostic cells with granule in nucleoli were observed in tumor tissues (Fig. 2C) than in para-neoplastic tissues (2D). The average indexes of apoptosis (AI) of HCC and para-neoplastic tissues were 8.61% and 6.93%, respectively, revealing a statistical difference (Table 2, $P=0.028$).

Analyzing the Relationships of the States of nm23 and the Values of AI/PI with Pathological and Clinical Characteristics of HCC

To identify the possible influence of expression of nm23 in HCC on the values of AI and PI, the average values of AI and PI in HCC were separately calculated. Table 3 showed that the values of AI in the groups of nm23-positive and nm23-negative HCC were 9.17% and 7.43%, respectively, with a statistical difference ($P=0.020$), while PI in these 2 groups was 30.72% and 29.93%, respectively, without a statistical difference ($P=0.575$, which indicated thatnm23-negative HCC tissues might have comparable proliferating capacity, but lower apoptostic tendency.



(×40)

FIG. 1. Immunochemistry staining of nm23 protein in liver tissues. The sections were incubated with 1:150 diluted nm23 antibody and subsequently with a biotinylated secondary antibody. After reaction with an avidin-biotin-preoxidase complex, the slides were finally counterstained with hematoxylin. Nm23 positive cells were stained as brown and nucleoli were stained as blue. A: HCC tissue. B: Para-neoplastic liver tissues. C: Normal liver tissues.

TABLE 1

Assays of nm23 Staining in the Liver Cells from HCC, Para-neoplastic and Normal Liver Tissues

Tissues	Numbers	nm23 Staining		Positive Rate
		Positive	Negative	
HCC Tissues	43	29	14	67.4%[a]
Para-neoplastic Liver Tissues	39	39	0	100%
Normal Liver Tissues	10	10	0	100%

Note. [a]P<0.01 HCC vs. Para-neoplastic and Normal Liver Tissues.

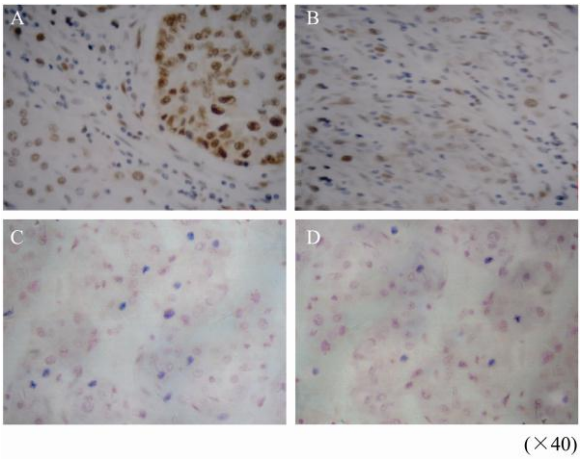


FIG. 2. Assays of PCNA expression and apoptosis in liver tissues. Immunochemistry staining of PCNA in HCC (A) and para-neoplastic (B) liver tissues. The sections were incubated with 1:50 diluted PCNA monoclonal antibody and subsequently with a biotinylated secondary antibody. After reacted with an avidin-biotin-preoxidase complex, the slides were finally counterstained with hematoxylin. Nm23 positive cells were stained as brown and nucleoli were stained as blue. TUNEL staining for evaluating apoptosis in HCC (C) and para-neoplastic (D) liver tissues. The sections were incubated with TUNEL reaction mixture and counterstained with PI solution. TUNEL-positive nucleoli were stained as blue and purple.

TABLE 2

AI and PI in HCC and Para-neoplastic Liver Tissues ($\bar{x} \pm s$)

	Tumor Tissues	Para-neoplastic Liver Tissues	P
AI (%)	8.61±2.34	7.46±2.28	0.028
PI (%)	30.48±4.30	28.41±4.51	0.038

TABLE 3

The Relationship between the Presence of nm23 and the Values of AI/PI in HCC

nm23 Expression	Numbers	AI (%)	P	PI (%)	P
Positive	29	9.17±2.09	0.020	30.72±4.52	0.575
Negative	14	7.43±2.47		29.93±3.89	

To figure out the possible relationships between the laboratory data and main pathological and clinical characteristics of HCC, the numbers of nm23 positive or negative HCC and the average values of AI and PI were re-counted based on several essential indexes, e.g. tumor grade and stage, tumor size, envelope invasion and metastasis. Table 4 summarized the data with statistic analyses. Tumor grade (I-II or III), tumor size and envelope invasion did not show any significant correlation with the states of nm23 or the values of AI or PI. The values of AI in HCC at stage I-II (7.33%) were lower than those at stage III-IV (9.10%), showing a statistical difference (P=0.025), while the states of nm23 and the values of PI did not reveal correlation between various stages of HCC. Interestingly, HCC with extrahepatic metastasis showed remarkable correlation with the negative nm23 (P=0.013) and higher PI values HCC (P=0.015), indicating that the HCC tissues with negative expression of nm23 and relatively higher proliferating activities had higher possibility of metastasis.

To analyze the correlations of the state of nm23 with the values of AI and PI, as well as other pathological and clinical characteristics of HCC, Kaplan-Meier method was employed. Among the 43 patients in the present study, the median overall survival was 14 months. The survival was significantly affected by some clinical factors, such as extrahepatic metastasis, clinical staging, and local tumor envelop invasion (Table 5). Moreover, the symptom-free survival (12.18 months) in the patients with negative nm23 expression was shorter than that (19.00 months) in the patients with positive nm23 expression, with a statistical difference (P=0.046). In contrast, the values of AI and PI failed to show significant influence on the survival of HCC patients (Table 5).

DISCUSSIONS

HCC is one of the most common malignant tumors with poor prognosis. Studies on the possible genetic alterations during HCC development has identified a high genomic heterogeneity[15-17]. Scores of candidates have been proposed to be correlative with the prognosis of HCC, regardless of radical hepatectomy[18-22]. However, the reliability of these biomarkers in practice remains controversial. In this study, we have addressed close correlations of the negative nm23 expression and higher PI value with the occurrence of metastasis, and negative nm23 expression with shorter disease-free survival in HCC patients.

TABLE 4

Relationship of the States of nm23 and the Values of AI/PI with Main Clinical and Pathological Features of HCC

Factors	Numbers	nm23 Expression		*P*	AI (%)	*P*	PI (%)	*P*
		negative	positive					
Grade								
I-II	35	10	25	0.404	8.89±2.46	0.100	30.63±4.24	0.608
III	8	4	4		7.38±1.19		29.75±4.74	
Stage								
Ⅰ-Ⅱ	12	3	9	0. 720	7.33±2.35	0.025	29.25±3.72	0.253
Ⅲ-Ⅳ	31	11	20		9.10±2.18		30.94±4.46	
Tumor Size								
≤50mm	16	5	11	0.888	8.00±2.07	0.196	29.56±4.70	0.294
>50mm	27	9	18		8.96±2.46		31.00±4.03	
Cancer Embolus or Extrahepatic Metastasis								
yes	13	8	5	0.013	8.92±1.93	0.563	32.85±3.93	0.015
no	30	6	24		8.47±2.52		29.41±4.08	
Envelope Invasion								
yes	18	8	10	0.158	8.89±2.72	0.506	31.78±3.75	0.089
no	25	6	19		8.40±2.06		29.52±4.48	

TABLE 5

Correlations of Main Biological, Pathological and Clinical Characteristics with the Length of Survival of HCC Patients

Factors		Length of Survival	S.E.	95%CI	*P*
Gender	Male	17.09	1.78	(13.59, 20.58)	0.849
	Female	17.67	4.00	(9.83, 25.51)	
Age	≤45	20.07	2.31	(15.55, 24.59)	0.227
	>45	15.01	2.01	(11.07, 18.95)	
Type	Nodular	16.81	1.75	(12.75, 19.61)	0.408
	Large Mass	18.32	2.76	(12.91, 23.73)	
Tumor Size	≤50mm	19.42	2.52	(14.48, 24.36)	0.168
	>50mm	15.51	1.95	(11.69, 19.32)	
Grade	Well-Differentiated	16.91	1.79	(13.39, 20.43)	0.675
	Poorly Differentiated	18.72	4.02	(10.85, 26.59)	
Clinical Stage	Early	24.42	3.16	(18.21, 30.62)	0.003
	Advanced	13.76	1.32	(11.17, 16.34)	
Extrahepatic Metastasis	No	19.70	1.97	(15.84, 23.56)	0.003
	Yes	10.96	1.67	(7.68, 14.24)	
Envelop invasion	No	20.98	1.98	(17.11, 24.86)	0.004
	Yes	11.22	1.73	(7.84, 14.61)	
nm23 expression	Negative	12.18	2.67	(6.95, 17.41)	0.046
	Positive	19.00	1.68	(15.70, 22.29)	
AI	<AI Mean	16.54	2.19	(12.24, 20.84)	0.799
	> AI Mean	16.30	2.00	(12.38, 20.23)	
PI	< PI mean	20.07	1.94	(16.27, 23.87)	0.083
	> PI mean	13.27	2.13	(9.09, 17.45)	

Note. S.E.: standard error; CI: confidence interval.

Nm23 is a new class of metastasis suppressor gene that is originally identified by differential hybridization between low- and high-metastatic murine melanoma cell lines, in which reduced nm23 mRNA levels has been proposed to be correlated with increased metastatic potential[23]. Subsequently, the relationship between nm23 and metastatic potential has been investigated in human malignant tumors. An inverse correlation between expression level of nm23 and metastatic potential of tumor has been demonstrated in carcinomas of the stomach, ovary, liver, breast, bladder, and esophagus[24-28]. The association of nm23 protein level with the prognosis of HCC patients has also been described elsewhere[29-30]. Moreover, Nanashima and his co-workers have observed that the disease-free survival in HCC patients after hepatic resection with a low nm23 expression in the tumor sample is significantly shorter than that in those with high nm23 expression[31]. In another study 63% of patients with a low tendency of recurrence show high expression of nm23-H1, whereas only 21% with a high tendency of recurrence reveal high nm23 expression[32]. All our data have strongly indicated that nm23 may act as biomarker for estimating metastasis and prognosis of HCC.

nm23-H1 protein has been identified as the Granzyme A (GzmA)-activated DNase responsible

for a caspase-independent apoptosis pathway elicited by cytotoxic T lymphocytes (CTL) in some virus-infected or tumor cells. It is therefore suggested that nm23-H1 expression levels in caspase-resistant transformed cells can be relevant to successful immune-surveillance, which may possibly be involved in the prevention of primary tumors development[33]. Previous reports have indicated that proliferative activity in noncancerous hepatic tissue may reflect tumor recurrence or the development of tumor in new regions. Less apoptostic cells and more proliferative cells in nm23 negative HCC tissues in this study outline the participation of nm23-H1 in the apoptostic process, which plays a potential role in the early phases of tumorigenesis.

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