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Research Article

Hsp70-2 is Highly Expressed in Nasopharyngeal Carcinoma and Involved in Nasopharyngeal Carcinoma Invasiveness

Guizhi Tang^{1,2}, Guodong Chen², Jianhua Xiao^{1#} and Faqing Tang^{3*#}

¹Institution of Pathogenic Biology, Medical College, University of South China, Hengyang, Hunan, P.R. China ²Center for Medical Genetics, School of Life Sciences, Central South University, Changsha, Hunan, P.R. China ³Department of Clinical Laboratory, The Affiliated Cancer Hospital of Xiangya Medical School, Central South University, Changsha, Hunan, P.R. China [#]Contributed equally

ARTICLE INFO

Article history: Received: 24 February, 2020 Accepted: 25 March, 2020 Published: 30 March, 2020

Keywords: Nasopharyngeal carcinoma N, N'-dinitrosopiperazine HSP70-2 invasion

ABSTRACT

Nasopharyngeal carcinoma (NPC) has a highly metastatic character, and its metastasis is closely related to carcinogens. N, N'-Dinitrosopiperazine (DNP) is a specific carcinogen for NPC. Our previous studies have shown that DNP enhances NPC invasion through up-regulating the heat shock protein 70-2 (HSP70-2), but the mechanisms underlying its regulation in NPC metastasis are unclear. In this study, we found that HSP70-2 expression in NPC tissue samples was significantly higher than that in the normal nasopharyngeal tissues. Moreover, HSP70-2 in metastatic cancer tissues was higher than that in primary cancer tissues. Importantly, NPC cell line, 5-8F cells with high metastatic potential had a significantly higher HSP70-2 expression and HSP70-2 ATPase activity than that in 6-10B cells with low metastatic potential. Strikingly, we further found that the NPC specific carcinogen, DNP induced HSP70-2 expression and increased HSP70-2 ATPase activity, displaying a dose dependence and time dependence. And DNP also enhances the interaction between HSP70-2 atPase. These results suggest that HSP70-2 participates in NPC invasiveness, and DNP-inducing HSP70-2 ATPase activity may be a novel mechanism of NPC metastasis.

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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy in south China and southeast Asia, which is closely related to Epstein Barr Virus (EBV) infection, chemical carcinogen, N, N'-dinitrosopiperazine (DNP) and genetic susceptibility [1]. NPC is a highly malignant neoplasm derived from nasopharyngeal epithelium with high local metastasis and early distant metastasis. In the early stage of the disease, cancer cells spread to surrounding tissues and causes blood and lymphatic metastasis, which eventually lead to the death of NPC patients [2]. Although radiation therapy and adjuvant chemotherapy have greatly improved the cure rate of NPC, the overall 5-year average survival rate remains at around 70%. About 30-40% of the patients were diagnosed with advanced NPC, and a significant proportion of NPC patients developed distant metastasis and recurrence within 4 years after treatment. So far, the molecular mechanism of NPC metastasis has not been fully elucidated. Therefore, it is urgent to further study the pathogenesis of NPC and find the mechanism underlying the high metastasis. Previous studies have shown that DNP induces rat nasopharyngeal carcinogenesis, which enhances the expression of heat shock protein 70 (HSP70), and the rats with high DNP concentrations exhibit high metastasis [3]. This implies that HSP70 is involved in NPC metastasis.

Heat shock proteins (HSPs) are a kind of stress protein family with conserved structure, extensive existence and multiple functions. HSP family is composed of HSP70-2, HSP70t, HSC70, GRP75, GRP78 (HSP70-5), and HSP70-4 [4]. The expression of HSPs can be induced under the conditions of thermal stimulation, hypoxia and ultraviolet irradiation [1]. HSP70 family members mainly include HSP70-1, HSP70-2, HSP70-hom and HSP70B, its molecular weight is about 70Kd, which is the most abundant HSP family in cells [2]. HSP70 acts as a molecular chaperone, regulates protein anabolism and facilitates

^{*}Correspondence to: Prof. Faqing Tang, M.D., Ph.D., Department of Clinical Laboratory, The Affiliated Cancer Hospital of Xiangya Medical School, Central South University, Changsha, Hunan, P.R. China; E-mail: tangfaqing33@hotmail.com

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proper folding of unfolded, misfolded, and refolded proteins [5]. In addition, it also plays an important role in hormone receptor regulation, gene product metabolism, cell cycle regulation and tumor cell growth regulation [6]. High expression of HSP70 has been reported in many tumor cells, such as breast cancer, pancreatic cancer, prostate cancer, bladder cancer, ovarian cancer and nasopharyngeal cancer [7].

HSP70 may participate in the conformational regulation of protooncogenes or tumor suppressor genes, affect cell cycle and disrupt cell proliferation by regulating the binding of kinases and apoptosis-related proteins in apoptotic pathways, and affect the occurrence and development of tumors [8]. Studies have shown that high expression of HSP70-2 can promote the proliferation, infiltration and metastasis of various tumor cells such as cervical cancer, breast cancer, liver cancer and bladder urothelial carcinoma [9, 10]. The bladder cancer with high metastasis has high expression of HSP70-2. HSP70-2 inhibits the activity of JNK and inhibits the phosphorylation of 14-3-3 zeta. Bax cannot be separated from 14-3-3 zeta [11]. It has been reported that DNP increases HSP70-2 expression and enhances the motility and invasion of NPC cells, and HSP70-2 may participate in NPC metastasis [12]. In this study, we detected HSP70-2 expression in NPC tissues, and focussed on NPC metastasis tissues, and probed the mechanism of HSP70-2 mediating NPC metastasis.

Materials and Methods

I NPC Patients and Tumor Samples

This study was conducted on paraffin-embedded NPC samples obtained from 84 patients with NPC who were histologically and clinically diagnosed in Chenzhou No.1 People's Hospital (Hunan, China) from April 2009 to March 2018. Prior written patient consent and the approval of the Institutional Research Ethics Committee of Chenzhou No.1 People's Hospital were obtained. 84 NPC patients included 65 patients with carcinoma in situ and 29 patients with distant metastasis. The area of normal paracancer tissue from samples obtained from 40 patients with NPC was used as a control.

II Reagents and Antibodies

The antibody against HSP70-2 was purchased from Abcam China (Shangshai, China). Antibody against β -actin and normal mouse IgG were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Secondary antibodies used in these experiments were horseradish peroxidase-linked anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G, and purchased from Santa Cruz Biotechnology, Inc. DNP was donated by the Cancer Research Institute of Central South University. Its boiling point, melting point, and other chemical characteristics were all determined to be within an acceptable range [3].

III Cell Culture and DNP Treatment

Human NPC cell lines 5-8F and 6-10B were purchased from the Cancer Research Institute of Sun Yatsen University (Guangzhou, China). 5-8F cell line has high metastatic ability, while 6-10B is only slightly metastatic cell line [13]. Cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L L-glutamine, 100 mg/ml penicillin, and 100 IU/ml streptomycin (Invitrogen, Carlsbad, CA), and were maintained in an incubator at 37°C and 5% CO₂. For DNP treatment, DNP crystals were dissolved in dimethyl sulfoxide (DMSO), and the appropriate amounts of DNP stock solution were added to the cultured cells to achieve the indicated concentrations. The cells were then incubated for the specified amount of time. To investigate the dose-course dependency of DNP treatment, cells were treated with 2 or 4 mM DNP for 24 h. For time-course assays, cells were treated with 4 mM DNP for 12 and 24 h [12].

IV ATPase Capture

NPC biopsy tissues were taken at 2 mg and washed with cold PBS for 3 times. NPC tissues were frozen with liquid nitrogen and ground into powder. The sample powder was added to the cell lysis buffer [1×phosphate buffer saline (PBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and freshly added 100 µg/ml phenylmethanesulfonyl fluoride, 10 µg/ml aprotinin, and 1 mM sodium orthovanadate], and was fully mixed. The lysis fluid was collected and centrifuged at 10000 rpm to collect the supernatant. ATPase was captured using Abcam immune capture beads (Abcam China). Specifically, the ATP Synthase Immunocapture Kit (ab109715) provided by Abcam China was used for reference, and then the anti-HSP70-2 antibody was used for Western-blotting detection.

V Western Blot

Western blot was performed as previously described [12]. Cells were seeded into a 60-mm plate at 4×10^5 cells per plate and harvested after 48 h of culture. The cells were lysed in the lysis buffer. The cell lysates obtained were centrifuged, and protein concentration of the clarified lysates was measured using Easy II Protein Quantitative Kit (BCA). 40 µg of the supernatant protein was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The protein membrane was blocked with 5% non-fat milk, incubated with the indicated antibody, and then incubated with an appropriate peroxidase conjugated secondary antibody. The signal was developed using 4-chloro-1-napthol/3,3-o-diaminobenzidine, and the relative photographic density was quantified by a gel documentation and analysis system.

VI ATPase Activity Assay

The treated cells were washed 3 times with cold phosphate buffer (PBS) at logarithmic growth stage (5×10^5) and 200 µl lysis buffer was added to lyse the cells. The lysate was collected by scraping and was broken by ultrasound. The supernatant of cell lysate was collected by centrifugation. BAC protein assay was used to determine the protein concentration in the supernatant. 200 mg protein of the supernatant was used to be immunoprecipitated with the beads with HSP70-2 antibody, and thoroughly incorporated at 4°C to precipitate overnight. The bead complex was centrifuged to collect the beads, the beads were eluted, and centrifuged to collect the supernatant. ATPase activity assay kit was used to detect ATPase activity in the supernatant according to the kit instructions. The standard curve of ATPase activity was established. The supernatant 10 µl, buffer 10 µl and ATP 10 µl were added into reaction solution 30 µl for reaction, and ATPase activity were determined.

VII Immunohistochemical Analysis

The tissue sections of the NPC samples were stained with HSP70-2 antibody (dilution 1:1000) as described previously [6]. The stained tissue sections were evaluated independently by two pathologists who were blinded to the clinical features and clinical outcome. Each case was scored based on intensity and percentage of the positive cells. At least 10 high-power fields were chosen randomly, and > 1000 cells were counted for each section. The intensity of HSP70-2 staining was scored as 0 (no signal), 1+ (weak), 2+ (moderate), and 3+ (marked). Percentage scores were assigned as 0, negative; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The sum (extension + intensity) was used as the total score [14].

VIII Statistical Analysis

The statistical significance of differences was analyzed using t test and chi-square test when indicated. p-values less than 0.05 were considered significant.

IX Ethics Statement

The present study protocol was approved by the ethical committee at Chenzhou No.1 People's Hospital, China.

Results

I HSP70-2 is Highly Expressed in Patients with Nasopharyngeal Carcinoma

To investigate the relationship between NPC and HSP70-2 expression, we detected the HSP70-2 expression in the NPC tissues of 84 cases (65 cases of primary and 29 cases of lymph metastasis) and the nasopharyngeal tissues of 40 cases. The results showed that the HSP70-2 expression was significantly high in the cancerous tissues when compared with the nasopharyngeal tissues (Figure 1, Table 1) (P < 0.001). HPS70-2 expression in the primary tissues and lymph metastatic tissues were also analyzed. The data showed that HSP70-2 levels in the metastatic tissues were significantly higher than those in the primary tissues (Table 2) (P < 0.01). These results suggest that HSP70-2 is involved in NPC development.

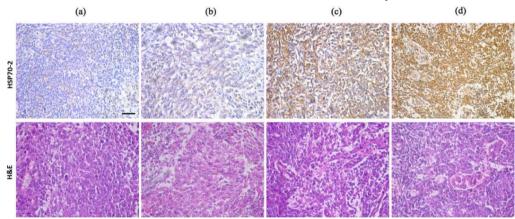


Figure 1: Immunohistochemical staining of HSP70-2 in NPC tissues. Positive staining was observed as a brown color. (a) negative (-), (b) weakly positive (+), (c) moderately positive (2+), (d) strongly positive (3+) staining of HSP70-2. Original magnification, \times 400. Scale bar, 5 μ m.

Table 1: HSP70-2 expression in NPC and nasopharyngeal tissues.

		HSP70-2 expression						
	Ν	(%)	+(%)	++ (%)	+++ (%)	PR(%)		
NPC	80	7(0.87)	13(0.16)	39(0.48)	21(0.26)	91		
Control	40	28(0.70)	12(0.30)	0(0)	0(0)	30		
P-value						< 0.001		

Table 2: HSP70-2 expression in NPC primary and metastatic tissues.

		HSP70-2 expression					
	n	- (%)	+(%)	++(%)	+++(%)	SPR(%)	
preinvasive carcinoma	59	7(0.12)	12(0.20)	30(0.51)	10(0.17)	16.9	
metastatic carcinoma	21	0(0)	1(30%)	9(0)	11(0)	52.3	
<i>P</i> -value						< 0.01	

- negative; + weak; ++ moderate; +++ strong staining. SPR, strong positive rate.

II DNP Increases HSP70-2 Expression and ATPase Activity

The above data showed that HSP70-2 is highly expressed in NPC and associated with NPC metastasis. 5-8F and 6-10B cells were used to confirm the relationship of HSP70-2 and NPC metastasis. 5-8F cells with high metastasis had a high HSP70-2 expression, and 6-10B cells with low metastasis had a low HSP70-2 expression (Figure 2). Our previous works showed that DNP induces HSP70-2 expression, while HSP70-2 ATPase activity plays an important role in HSP70-2 function. In the next step, we probed whether HSP70-2 ATPase activation participates in NPC development. At first, 5-8F and 6-10B cells were used to compare HSP70-2 ATPase activity. The results showed that ATPase activity in 5-8F cells was significantly higher than that in 6-10B cells (Figure 3A, p < 0.01). To further probe the mechanism of increasing HSP70-2 ATPase activity, DNP, a specific carcinogen for NPC was used to investigate whether it increases the HSP70-2 ATPase activity. After 6-10B cells being treated with DNP, HSP70-2 ATPase activity dramatically increased (Figure 3A, p < 0.05). This suggests that DNP induces an increase in HSP70-2 ATPase activity. To further observe the dose- and

time- dependence of DNP-induced HSP70-2 ATPase activity, 6-10B cells were treated with approximately 4, 8 mM DNP or with 4 mM DNP for approximately 60 ~ 85 min, and then the HSP70-2 ATPase activity was examined. The DNP-mediated HSP70-2 ATPase activity is both dose and time-dependent (Figure 3B).

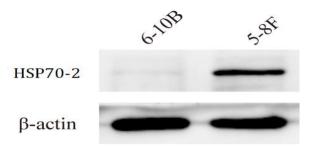


Figure 2: HSP70-2 expression in human NPC cell line. Human NPC cell lines, 5-8F and 6-10B cell lines (5×10^5) were subjected to Westernblotting with HSP70-2 antibody. β -actin served as a loading control.

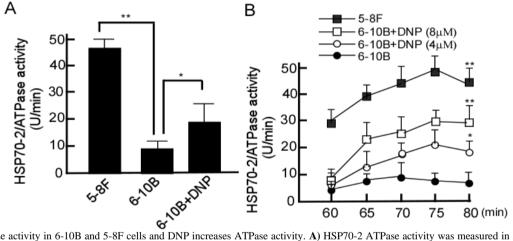


Figure 3: ATPase activity in 6-10B and 5-8F cells and DNP increases ATPase activity. **A)** HSP70-2 ATPase activity was measured in 5-8F, 6-10B cells and 6-10B+DNP using ATPase activity assay. **B)** 6-10B cells were treated with the indicated concentration of DNP for the indicated time and subjected to the ATPase activity assay. *, p < 0.05; **, p < 0.01.

III DNP Promotes the Binding of ATPase and HSP70-2 in NPC Cells

In order to further explore the mechanism of DNP up-regulating HSP70-2 ATPase activity, the interaction of HSP70-2 with ATPase, ATPase was respectively captured in the cell lysates of 6-10B, 5-8F and DNP-treated 6-10B cells by Abcam immunocapture beads, and then HSP70-2 was detected by Western blotting. The results showed that 5-8F cells had a high interaction of HSP70-2 with ATPase, and 6-10B had a relatively low the binding of HSP70-2 and ATPase. And after DNP treatment, the interaction of HSP70-2 with ATPase was significantly increased in 6-10B (Figure 4). These data suggested that DNP significantly increased the binding of HSP70-2 and ATPase.

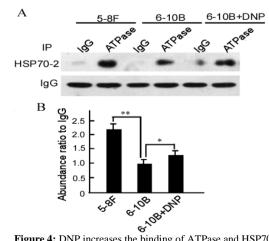


Figure 4: DNP increases the binding of ATPase and HSP70-2 in 6-10B cells. **A)** ATPase was captured from 6-10B, 5-8F cells and 6-10B cells treated with DNP, and then subjected to the Western-blotting with HSP70-2 antibody. IgG served as a loading control. **B)** Abundance ratio to IgG was calculated. *, p < 0.05; **. p < 0.01.

Discussion

Compared with other head and neck malignant tumors, NPC has a specific characteristic of high recurrence and metastasis. In the initial diagnosis, about 5-7% of patients can find tumor metastasis, and the total metastasis rate for NPC is up to one-third. The main metastatic sites are external cervical lymph nodes, followed by liver, lung, bone, etc. [15, 16]. Although NPC is sensitive to radiation and chemotherapy, it often leads to treatment failure, because NPC is prone to in situ recurrence and major distant metastasis [17]. Standard radiotherapy and chemotherapy alone can cure more than 90% of stage I NPC patients, and more than 50% of stage III or IV patients without distant metastasis have recurrence within five years after radiotherapy, while the median survival of patients with distant metastasis is only about one year [18]. Therefore, the recurrence and metastasis of NPC are the main factors affecting the prognosis of patients. So, clarification of the molecular mechanism of NPC carcinogenesis, invasion and metastasis, and finding the molecular targets for NPC therapy have become important topics in the field of NPC research. For such a kind of malignant tumor with high recurrence and metastasis, it is believed that its occurrence is related to chemical carcinogens, virus infection and genetic factors. However, little research has been done on the mechanism of carcinogenesis of chemical carcinogens.

Common chemical carcinogens include DNP, nitrosamines, mycotoxins, aromatic amines, etc. Since Magee and Barnes found that dimethylnitrosamines can induce liver cancer in animals, nitrosamines have become increasingly important carcinogens. At present, more than 300 carcinogenic nitrosamines have been found, and their carcinogenic effects involve more than 30 animals [19]. Among the chemical carcinogens of NPC, nitrosamines are one of the important. Chen et al., having induced NPC in rats with different nitroso compounds, found that the use of ring nitrosamines such as dinitronitrosamine and Nitroso mofulin has high incidence of cancer, and observed that small DNP only induced NPC. Therefore, the nasopharynx is more sensitive to carcinogenic effects [3, 20]. Our previous works have found that HSP70 protein expression was increased in DNP-induced NPC in rats. Subsequently, after DNP was used to induce malignant transformation of HENE cells, we also found the up regulation of HSP70 protein in HENE cells [3].

Our further study found that DNP could increase the invasiveness of nasopharyngeal carcinoma in 6-10B cells by increasing the level of HSP70-2 protein, but DNP did not turn up other proteins in HSP70 family. In this paper, the levels of HSP70-2 in NPC tissues of 84 cases and 40 nasopharyngeal tissues were analyzed by immunohistochemistry, and it was found that HSP70-2 protein in NPC tissues were significantly higher than that in normal nasopharyngeal tissues. Moreover, HSP70-2 expressions in NPC metastatic tissues were higher than that in the primary site. This further demonstrates the important role of HSP70-2 in the invasiveness of NPC. We also preliminarily explored the possible molecular mechanism for HSP70-2 up-regulation and found that DNP may increase HSP70-2 expression, especially increase HSP70-2 ATPase activity. The further finding is DNP strengthening the binding of HSP70-2 and ATPase, eventually increasing HSP70-2 ATPase activity. In conclusion, our study confirmed the important role of HSP70-2 in NPC invasiveness. Thus, HSP70-2 may serve as a biomarker for

nasopharyngeal carcinoma invasiveness, allowing us to detect its potential metastasis at the early stage of tumor metastasis.

Author Contributions

GT and GC conducted the study design. GT and GC carried out the assays and collected the samples. GT and GC drafted the manuscript. FT and JX revised the manuscript.

Acknowledgements

We appreciate the contributions and helpful discussion of various members in Clinical Laboratory of Chenzhou NO.1 People's Hospital.

Funding

This work was supported in part by the National Natural Science Foundation of China (81402265, 81502346), Research Project of Hunan Health Commission (B2019084).

Competing Interests

None.

Ethical Approval

All procedures were consistent with the National Institutes of Health Guide and approved by the institutional board. This study was evaluated and approved by the Ethics Committee of the Affiliated Cancer Hospital of Xiangya Medical School, Central South University.

Consent

The patient's written consent was taken with respect to all procedures. All the patients enrolled in this study approve publication.

Abbreviations

DMSO: dimethyl sulfoxide DNP: N, N'-dinitrosopiperazine EBV: Epstein Barr Virus FBS: fetal bovine serum HSP70-2: Heat shock protein 70 NPC: nasopharyngeal carcinoma PBS: phosphate buffer saline SDS: sodium dodecyl sulfate

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