OPEN

Increased PA2G4 Expression Is an Unfavorable Factor in Nasopharyngeal Carcinoma

Yan Xu, MD, PhD, Hongbing Cai, MD, PhD, Wei Tu, MBBS, Lingying Ding, MBBS, and Rongcheng Luo, MD, PhD

Abstract: PA2G4 plays a dual role in tumors. However, the correlation of its expression with clinical feature and prognosis has never been reported in nasopharyngeal carcinoma (NPC). Using immunohistochemical staining, we examined PA2G4 protein level in clinicopathologically characterized 201 NPC cases (138 male and 63 female) with age ranging from 21 to 83 years and 45 nasopharyngeal (NP) tissues. Statistical methods were used to assess the difference in PA2G4 expression and its relationship with clinical parameters and prognosis in NPC. Immunohistochemical analysis showed that the protein expression of PA2G4 examined in NPC tissues was higher than that in the nasopharyngeal tissues ($P = 0.005$). In addition, high levels of PA2G4 protein were positively correlated with tumor size (T classification) ($P < 0.001$), the status of lymph node metastasis (N classification) ($P < 0.001$), distant metastasis ($P = 0.029$), and clinical stage ($P < 0.001$) of NPC patients. Patients with higher PA2G4 expression had a significantly shorter overall survival time than did patients with low PA2G4 expression. Stratified analysis indicated that high expression of PA2G4 showed the inversed survival time in clinical stages III-IV, but not stages I-II. Finally, multivariate analysis suggested that the level of PA2G4 expression was an independent prognostic indicator ($P < 0.001$) for the survival of patients with NPC. Elevated protein expression of PA2G4 was significantly shown, which plays an unfavorable outcome for NPC patient survival.

Key Words: nasopharyngeal carcinoma, PA2G4, prognosis, immunohistochemistry

(*Appl Immunohistochem Mol Morphol* 2021;29:513–518)

Received for publication June 27, 2020; accepted December 29, 2020.

From the Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangdong, P.R. China.

Y.X. and R.L.: contributed toward planning, analysis, discussing, and drafted the initial manuscript. Y.X., H.C., and W.T.: contributed to immunohistochemistry images selection, analysis, and plotting. L.D. and R.L.: provided the tissue specimens, interpreted and supervised the data. All authors read and approved the final manuscript.

Supported by Guangdong Natural Science Foundation (2016A030313724), Project of Administration of Guangdong of China (20171173). Project of Department of Education of Guangdong Provincial (2020KTSCX062).

The authors declare no conflict of interest.

Reprints: Rongcheng Luo, MD, PhD, Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong 510310, P.R. China (e-mail: luorc02@vip.163.com).

Nasopharyngeal carcinoma (NPC) is one of the most common malignant diseases in the south Chinese and other south-east Asians. Unfortunately, most NPC patients tend to present a more advanced stage of disease when first diagnosed due to its deep location and vague symptoms. Therefore, it is of great interest to search valuable factors for early diagnosis, prognosis prediction, and novel therapeutic strategies.

NPC is one of the most common tumors in southern China. EBV infection, environmental carcinogens, and genetic susceptibility are reported to be closely related to NPC pathogenesis, which induces abnormal expression of a few of genes and signals, such as *FOXO1*,[1] *EBV-BART-22*,[2] *CDKs*,[3] *NKG2D*,[4] *PDCD4*,[5] *VPS33B*[6] and thus promotes the occurrence and development of NPC.

PA2G4/EBP1 encodes proliferation-associated protein 2G4/ErbB3-binding protein 1. In previous studies, it plays a complex role in tumor, either oncogene or tumor suppressor gene in tumors.[7–15] However, PA2G4 expression with clinical feature and prognosis has never been reported in NPC.

To elucidate the action of PA2G4 in the pathogenesis of NPC, in the current study we investigate the correlation of PA2G4 protein expression with clinicopathologic features, including the survival of patients. The data showed that the protein expression level of PA2G4 was higher in NPC tissues than those in noncancerous nasopharyngeal (NP) tissues. Furthermore, the relatively higher protein expression of PA2G4 was associated with NPC progression and poor prognosis. Our results demonstrated that elevated PA2G4 protein level as an independent prognostic factor plays an unfavorable prognostic factor for NPC patient's survival.

MATERIALS AND METHODS

Microarray Data Analysis

In previous study, Fang et al[16] had reported to use microarray to examine the differential expression genes in NPC tissues or NPC cells compared with NP tissues. In this investigation, we reanalyzed the differential genes of NPC compared with NP based on the data provided by Fang et al.[16]

Sample Collection

Two hundred one NPC specimens and 45 noncancerous nasopharynx paraffin-embedded specimens were obtained

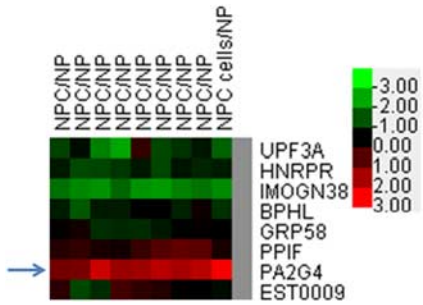

FIGURE 1. PA2G4 messenger RNA is upregulated in nasopharyngeal carcinoma (NPC) tissues or NPC cells compared with nasopharyngeal tissues based on the microarray data analysis.

from Nanfang Hospital, Guangzhou, China. In these cases, there were 138 male and 63 female with age ranging from 21 to 83 years. For the use of these clinical materials for investigation purposes, prior consents from the patients and approval from the Ethics Committees of Nanfang Hospital were obtained. All specimens had proved pathologic diagnosis and were staged according to NPC staging system of the WHO in 2009.

Immunohistochemistry

Immunohistochemical staining was performed according to the previous descriptions.[17,18] NPC and NP specimen paraffin sections were deparaffinized in 100% xylene and rehydrated in descending ethanol series according to standard protocols. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 minutes at 100°C. Endogenous peroxidase activity and nonspecific antigen were blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with mouse PA2G4 antibody (1:100) (Proteintech, Wuhan, China) for 1 hour at 37°C. After washing, the sections were incubated with biotin-labeled rabbit anti-goat antibody for 10 minutes at room temperature, and subsequently were incubated using streptavidin-conjugated horseradish peroxidase (HRP) (Maixin Inc, China). Sections

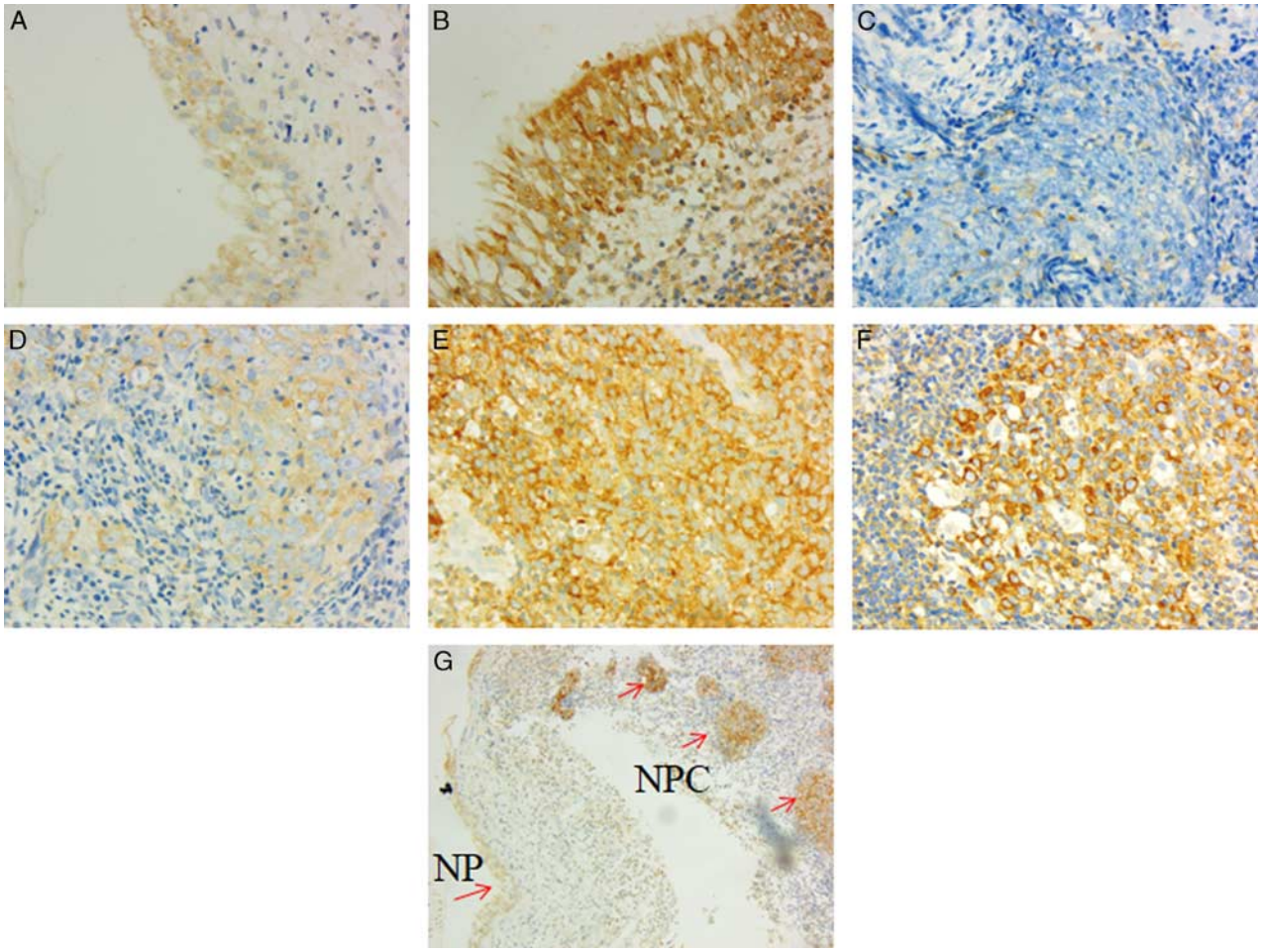

FIGURE 2. PA2G4 expression in NPC and NP tissues. Specific PA2G4 protein expression was observed in the cytoplasm of the malignant tumor cells and full thickness nasopharyngeal mucosa epithelium, including cilia. A, Low expression of PA2G4 in NP tissues (×400). B, High expression of PA2G4 in NP tissues (×400). C and D, Low expression of PA2G4 in NPC tissues (×400). E and F, High expression of PA2G4 in NPC tissues (×400). G, Low expression of PA2G4 in NP tissue and high expression in NPC tissues in the same section (×100). NP indicates nasopharyngeal; NPC, nasopharyngeal carcinoma. full color online

were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum, and analyzed using a bright field microscope.

Evaluation of Staining

The immunohistochemically stained tissue sections were reviewed and scored separately by 2 pathologists blinded to the clinical parameters. The staining score was previously described according to the sum of staining intensity and the percentage of positive staining areas (0 to 7).[19] For statistical analysis, a final staining scores of 0 to 5 and 6 to 7 were, respectively, considered to be low and high expression.

Statistical Analyses

All statistical analyses were carried out using SPSS 20.0 software. Data were shown as mean ± SD. The χ^2 test was explored to analyze the association between the levels of PA2G4 expression and clinicopathologic characteristics. Survival curves were plotted by the Kaplan-Meier assay and compared using the log-rank test. The significances of various variables in survival were analyzed using multivariate Cox proportional hazards model. A *P*-value of <0.05 was considered to be statistically significant.

TABLE 2. Correlation Between the Clinicopathologic Characteristics and Expression of PA2G4 Protein in NPC

		PA2G4 (%)		
Characteristics	**n**	**High Expression**	**Low Expression**	***P***
Sex				
Male	138	72	66	
Female	63	29	34	0.450
Age (y)				
≥50	99	48	51	
<50	102	53	49	0.673
Smoking				
Yes	32	17	15	
No	169	84	85	0.847
T classification				
T_1-T_2	155	60	85	
T_3-T_4	56	41	15	<0.001
N classification				
N_0-N_1	107	41	66	
N_2-N_3	94	60	34	<0.001
Distant metastasis				
Yes	15	12	3	
No	186	89	97	0.029
TNM clinical stage				
I-II	75	19	56	
III-IV	126	82	44	<0.001

RESULTS

On the basis of the analysis of microarray data, we observed that PA2G4 level was significantly increased in pooled NPC cells and 8 NPC pooled tissues compared with NP tissues (Fig. 1).

Then we measured the expression levels and subcellular localization of PA2G4 protein in 201 paraffin-embedded NPC specimens and 45 noncancerous NP samples using immunohistochemical staining assay. Specific PA2G4 protein expression was observed in the cytoplasm of the malignant tumor cells and full thickness NP mucosa epithelium, including cilia (Figs. 2A–G). Furthermore, we observed that in 50.2% (101/201) of NPC samples, PA2G4 protein level was elevated. In comparison, 26.7% (12/45) of noncancerous NP samples had increased PA2G4 protein level, significantly lower than that in the NPC samples ($P=0.005$) (Table 1).

We next analyzed the relationships between clinicopathologic characteristics and PA2G4 expression levels in individuals with NPC (Table 2). Although we did not find a significant association of PA2G4 expression levels with patient's age, sex, and smoking, we observed that the expression level of PA2G4 was positively correlated with tumor size (T classification) ($P<0.001$), the status of lymph node metastasis (N classification) (N0-N1 vs. N2-N3) ($P<0.001$) and status of distant metastasis (M classification) ($P=0.029$) and clinical stage (I-II vs. III-IV) ($P<0.001$) in 201 NPC patients (Table 2).

To investigate the prognostic value of PA2G4 expression for NPC, we further assessed the association between the levels of PA2G4 expression and patients' survival using Kaplan-Meier analysis with the log-rank test. In 201 NPC cases with prognosis information, we observed that the level of PA2G4 protein expression was significantly correlated with the overall survival of NPC patients (Fig. 3A). Patients with high level of PA2G4 expression had poorer survival than those with lower level of PA2G4 expression ($P=0.001$). We also observed that higher tumor PA2G4 protein expression was associated with a shorter survival time for patients in clinical stage III-IV (Fig. 3C) ($P=0.010$), but not I-II ($P=0.821$) (Fig. 3B).

Univariate assay showed that PA2G4 expression, T, N, M classification, and clinical stage were correlated significantly with patient survival ($P<0.001$, $P<0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively). A multivariate analysis of PA2G4 protein expression levels adjusted for T, N, M classification, and clinical stage showed that the level of PA2G4 expression was an independent prognostic factor for NPC ($P<0.001$; Table 3).

TABLE 1. PA2G4 Was Highly Expressed in NPC Tissues Compared With NP Tissues

	Protein (n)		
Expression Level	**NPC**	**NP**	***P***
High expression	101	12	
Low expression	100	33	0.005
Total	201	45	

NP tissues indicates noncancerous nasopharyngeal tissues; NPC tissues, nasopharyngeal carcinoma tissues.

DISCUSSION

The PA2G4 gene belongs to a member of the described PA2G4 family.[20] The mouse p38-2G4 gene is the prototype of this family, which was isolated by generating monoclonal antibodies against DNA binding proteins. The sequence of the human PA2G4 cDNA encodes a 394 amino acid protein and is ~45 kDa molecular weight. It

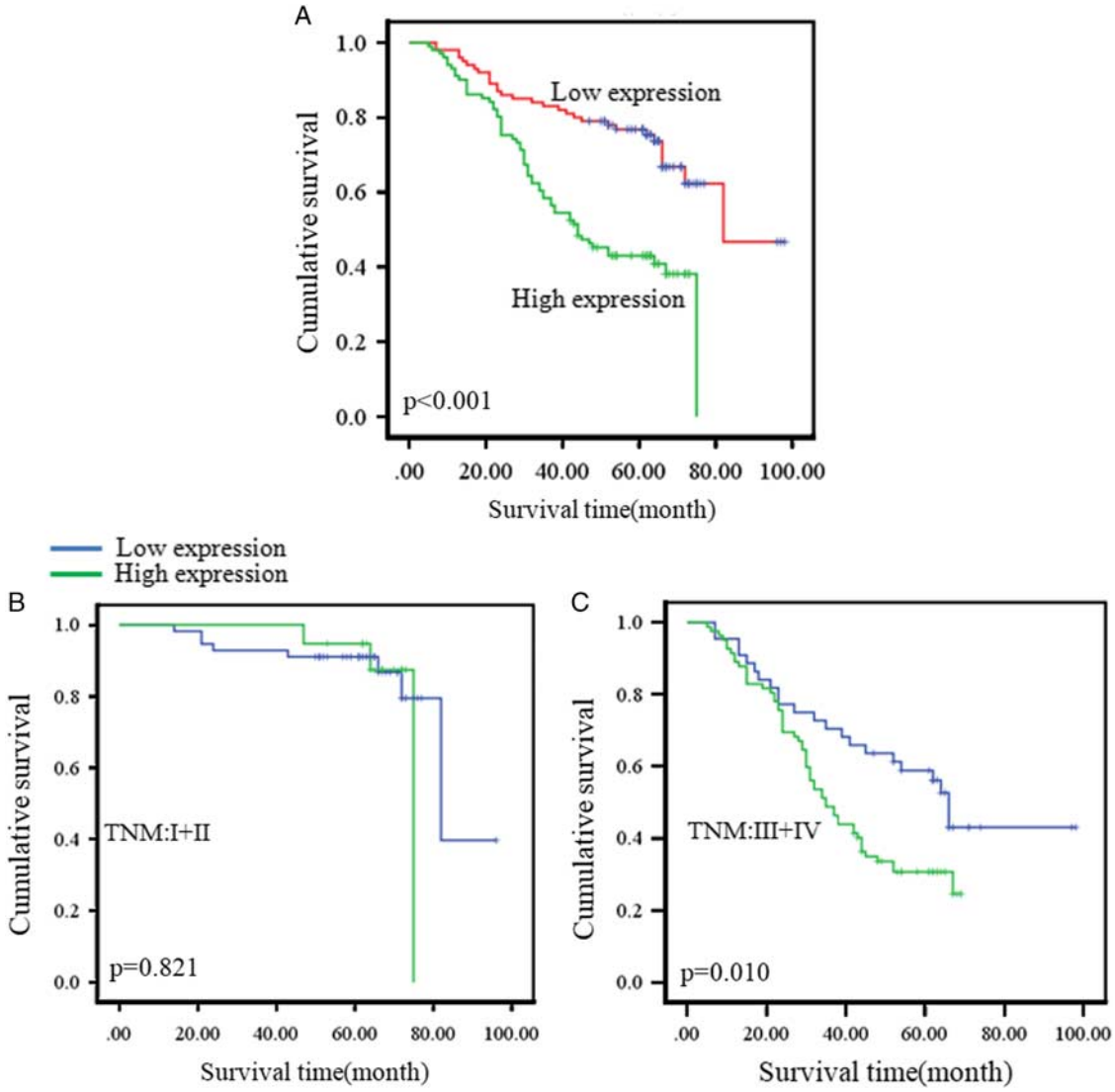

FIGURE 3. Overexpression of PA2G4 promotes an unfavorable outcome for NPC patient survival. A, Overexpression of PA2G4 shortens the overall survival time of NPC patients. B, Overexpression of PA2G4 is not associated with the overall survival time of NPC patients in clinical stage I+II. C, Overexpression of PA2G4 leads to a poor prognosis for NPC patients in clinical stage III+IV. NPC indicates nasopharyngeal carcinoma. full color online

has a longer N-terminal region than mouse p38-2G4 protein.[21] In previous studies, PA2G4 might play a role in an ERBB3-regulated signal transduction pathway. It was shown to be interacted with ERBB3[22,23] and promoted or suppressed the tumor pathogenesis. Furthermore, PA2G4 seems to be involved in growth regulation and acts a corepressor of the androgen receptor[24] and is modulated by the ERBB3 ligand neuregulin-1/heregulin (HRG).[22] However, the role of PA2G4 was still unclear in NPC.

PA2G4 has reported to be overexpressed in some tumors including salivary adenoid cystic carcinoma, brain tumor, pancreatic ductal adenocarcinoma, acute myelogenous leukemic cells, cervical cancer, oral cancer.[7–11] However, inversed data showed its downregulated expression in breast cancer, bladder cancer, prostate cancer,[12–15] which hinted a dual role in tumors. In previous study, Xiao et al[25] found that PA2G4 was upregulated in NPC compared with NP and serum samples based on proteomics assay. Here, we firstly analyzed the microarray data provided by Prof Fang and observed that PA2G4 was obviously increased in NPC tissues or NPC cells compared with NP tissues. Further, we used immunohistochemistry staining to examine the expression of PA2G4 in NPC and NP tissues. The data showed that PA2G4 protein expression was significantly upregulated in NPC. This data supported Xiao and colleagues' data, which suggested that elevated expression of PA2G4 promoted the pathogenesis of NPC.

In previous documents, overexpression of PA2G4 was reported to correlate with the clinical progression of PA2G4 in tumors.[7,10] In this study, we observed that

TABLE 3. Summary of Univariate and Multivariate Cox Regression Analysis of Overall Survival Duration

	Univariate Analysis			Multivariate Analysis		
Parameter	***P***	**HR**	**95% CI**	***P***	**HR**	**95% CI**
Sex						
Male vs. female	0.324	1.265	0.792-2.020			
Age (y)						
≥50 vs. <50	0.384	1.203	0.794-1.823			
Smoking						
Yes vs. no	0.936	1.024	0.578-1.814			
T classification						
T_1-T_2 vs. T_3-T_4	0.000	3.303	2.154-5.064	0.011	1.949	1.165-3.261
N classification						
N_0-N1 vs. N_2-N_3	0.000	3.350	2.156-5.207	0.032	1.871	1.056-3.315
M classification						
M_0 vs. M_1	0.000	5.402	2.973-9.817	0.001	2.908	1.564-5.408
Clinical stage						
I-II vs. III-IV	0.000	7.030	3.707-13.334	0.026	2.657	1.123-6.285
PA2G4 level						
High expression vs. low expression	0.000	2.741	1.751-4.291	0.000	0.239	0.125-0.458

CI indicates confidence interval; HR, hazard ratio.

although overexpressed PA2G4 was not associated with sex, age, and smoking, it positively correlated with T classification (tumor size), N classification (lymph node metastasis), M classification (distant metastasis), clinical stages of NPC patients. These data were similar to Mei et al's report,[10] which suggested that overexpressed PA2G4 significantly accelerated the pathogenesis of NPC and played an unfavorable role for NPC prognosis. However, the relationship between PA2G4 expression and the survival of NPC patients was still to be determined.

In prior documents, expression of PA2G4 in tumor cells has been indicated to be a favorable or unfavorable prognostic factor depending on tumor types. Hu et al[26] found that decreased expression of PA2G4/EBP1 is a favorable factor in hepatocellular carcinoma. Interestingly, opposite results were reported in salivary adenoid cystic carcinoma, Sun et al[7] showed that the higher expression of PA2G4 caused an unfavorable outcome.

In the present study, we provide the proof to demonstrate that elevated PA2G4 protein expression was inversely correlated with patient's overall survival prognosis in NPC. The patients with higher expression of PA2G4 protein had shorter survival time. Stratified analysis further indicated that although overexpressed PA2G4 was not related to survival prognosis in clinical stage I and II, it indicated the worse survival prognosis level in clinical stage III and IV. The data indicated that the overexpression of PA2G4 could be used as a prognostic indicator for mid-advanced NPC, but not mid-early NPC.

Finally, we analyzed the possibility of PA2G4 as independent prognostic factor. On the basis of multivariate analyses, elevated expression of PA2G4 protein was a significantly independent predictor of poor prognosis for NPC patients. These data further revealed the significance of overexpressed PA2G4 in NPC pathogenesis.

In summary, this study confirmed that the expression level of PA2G4 was significantly upregulated in NPC and correlated with the malignant progression of NPC. Furthermore, our data demonstrated that overexpression of PA2G4 was an unfavorable prognostic factor for NPC. Finally, PA2G4 expression level is an independent prognosis factor predicting NPC pathogenesis.

ACKNOWLEDGMENT

The authors thank Prof WeiYi Fang, PhD (Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China) for providing the microarray data.

REFERENCES

1. Li Y, Liu X, Lin X, et al. Chemical compound cinobufotalin potently induces FOXO1-stimulated cisplatin sensitivity by antagonizing its binding partner MYH9. *Signal Transduct Target Ther*. 2019;4:48.
2. Liu Y, Jiang Q, Liu X, et al. Cinobufotalin powerfully reversed EBV-miR-BART22-induced cisplatin resistance via stimulating MAP2K4 to antagonize non-muscle myosin heavy chain IIA/glycogen synthase 3β/β-catenin signaling pathway. *EBioMedicine*. 2019;48:386–404.
3. Syn N, Lim PL, Kong LR, et al. Pan-CDK inhibition augments cisplatin lethality in nasopharyngeal carcinoma cell lines and xenograft models. *Signal Transduct Target Ther*. 2018;3:9.
4. Huang Y, Chen X, Guo K, et al. Sunitinib induces NK-κB-dependent NKG2D ligand expression in nasopharyngeal carcinoma and hepatoma cells. *J Immunother*. 2017;40:164–174.
5. Zhen Y, Fang W, Zhao M, et al. miR-374a-CCND1-pPI3K/AKT-c-JUN feedback loop modulated by PDCD4 suppresses cell growth, metastasis, and sensitizes nasopharyngeal carcinoma to cisplatin. *Oncogene*. 2017;36:275–285.
6. Liang Z, Liu Z, Cheng C, et al. VPS33B interacts with NESG1 to modulate EGFR/PI3K/AKT/c-Myc/P53/miR-133a-3p signaling and induce 5-fluorouracil sensitivity in nasopharyngeal carcinoma. *Cell Death Dis*. 2019;10:305.
7. Sun J, Luo Y, Tian Z, et al. Expression of ERBB3 binding protein 1 (EBP1) in salivary adenoid cystic carcinoma and its clinicopathological relevance. *BMC Cancer*. 2012;12:499.
8. Kim CK, Nguyen TLX, Joo KM, et al. Negative regulation of p53 by the long isoform of ErbB3 binding protein Ebp1 in brain tumors. *Cancer Res*. 2010;70:9730–9741.
9. Nguyen LXT, Zhu L, Lee Y, et al. Expression and role of the ErbB3 binding protein 1 in acute myelogenous leukemic cells. *Clin Cancer Res*. 2016;22:3320–3327.

10. Mei Y, Zhang P, Zuo H, et al. Ebp1 activates podoplanin expression and contributes to oral tumorigenesis. *Oncogene*. 2014;33:3839–3850.
11. Liu L, Xu DY, Yang SS, et al. Ebp1 protein expression in cervical cancer tissue and its significance. *Genet Mol Res*. 2015;14:5496–5500.
12. Zhang Y, Akinmade D, Hamburger AW. Inhibition of heregulin mediated MCF-7 breast cancer cell growth by the ErbB3 binding Protein EBP1. *Cancer Lett*. 2008;265:298–306.
13. He H, Ling X, Zhu J, et al. Down-regulation of the ErbB3 binding protein 1 in human bladder cancer promotes tumor progression and cell proliferation. *Mol Biol Rep*. 2013;40:3799–3805.
14. Zhang Y, Linn DE, Liu Z, et al. EBP1, an ErbB3-binding protein, is decreased in prostate cancer and implicated in hormone resistance. *Mol Cancer Therap*. 2008;7:3176–3186.
15. Zhang Y, Ali TZ, Zhou H, et al. ErbB3 binding protein 1 represses metastasis-promoting gene anterior gradient protein 2 in prostate cancer. *Cancer Res*. 2010;70:240–248.
16. Fang W, Li X, Jiang Q, et al. Transcriptional patterns, biomarkers and pathways characterizing nasopharyngeal carcinoma of Southern China. *J Transl Med*. 2008;6:32.
17. Ma R, Wang S, Qin S, et al. Preparation and characterization of monoclonal antibody against glypican-3. *Hybridoma*. 2012;31: 455–461.
18. Lin X, Li A, Li Y, et al. Silencing MYH9 blocks HBx-induced GSK3β ubiquitination and degradation to inhibit tumor stemness in hepatocellular carcinoma. *Signal Transduct Target Ther*. 2020; 5:1–12.
19. Liu Z, Li X, He X, et al. Decreased expression of updated NESG1 in nasopharyngeal carcinoma: its potential role and preliminarily functional mechanism. *Int J Cancer*. 2011;128:2562–2571.
20. Lamartine J, Seri M, Cinti R, et al. Molecular cloning and mapping of a human cDNA (PA2G4) that encodes a protein highly homologous to the mouse cell cycle protein P38-2G4. *Cytogenet Genome Res*. 1997;78:31–35.
21. Yoo JY, Wang XW, Rishi AK, et al. Interaction of the PA2G4 (EBP1) protein with ErbB-3 and regulation of this binding by heregulin. *Br J Cancer*. 2000;82:683–690.
22. Zhang Y, Hamburger AW. Heregulin regulates the ability of the ErbB3-binding protein Ebp1 to bind E2F promoter elements and repress E2F-mediated transcription. *J Biol Chem*. 2004;279:26126–26133.
23. Zhang Y, Akinmade D, Hamburger AW. The ErbB3 binding protein Ebp1 interacts with Sin3A to repress E2F1 and AR-mediated transcription. *Nucleic Acids Res*. 2005;33:6024–6033.
24. Zhang Y, Wang XW, Jelovac D, et al. The ErbB3-binding protein Ebp1 suppresses androgen receptor-mediated gene transcription and tumorigenesis of prostate cancer cells. *Proc Natl Acad Sci USA*. 2005;102:9890–9895.
25. Xiao Z, Chen Y, Yi B, et al. Identification of nasopharyngeal carcinoma antigens that induce humoral immune response by proteomic analysis. *Proteomics Clin Appl*. 2007;1:688–698.
26. Hu B, Xiong Y, Ni R, et al. The downregulation of ErbB3 binding protein 1 (EBP1) is associated with poor prognosis and enhanced cell proliferation in hepatocellular carcinoma. *Mol Cellular Biochem*. 2014;396:175–185.