Original Article
The prognostic impact of RAP2A expression in patients with early and locoregionally advanced nasopharyngeal carcinoma in an endemic area

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Abstract: Background: By data mining from published transcriptomic databases, we identified *RAP2A* as a significantly upregulated gene in nasopharyngeal carcinoma (NPC) tissues. *RAP2A*, a member of the RAS oncogene family, is involved in the process of GTP binding and GTPase activity. The aim of this study was to evaluate the expression of RAP2A and its prognostic impact in patients with early and locoregionally advanced NPC. Methods: RAP2A immunohistochemistry was performed for 124 NPC patients who were receiving standard treatment and had no initial distal metastasis. We also performed Western blotting to evaluate the endogenous protein expression of RAP2A in NPC cells and non-neoplastic mucosal cells. The result of RAP2A expression was further correlated with clinicopathological variables, disease-specific survival (DSS), distant metastasis-free survival (DMeFS), and local recurrence-free survival (LRFS). Results: High expression of RAP2A was significantly associated with advanced primary tumor status (P = 0.024) and advanced TNM stage (P = 0.006). In univariate analysis, high expression of RAP2A served as a significant prognostic factor for inferior DSS (P < 0.0001), DMeFS (P < 0.0001), and LRFS (P < 0.0001). In multivariate analysis, RAP2A overexpression still independently predicted worse DSS (hazard ratio [HR] = 2.976, P < 0.001), DMeFS (HR = 4.233, P < 0.001), and LRFS (HR = 4.156, P < 0.001). Moreover, Both HONE1 and TW01 NPC cells, but not non-neoplastic DOK cells demonstrated significantly increased RAP2A expression. Conclusion: Overexpression of RAP2A is associated with advanced disease status and may therefore be an important prognosticator for poor outcomes in NPC, as well as a potential therapeutic target to aid in developing effective treatment modalities.

Keywords: RAP2A, nasopharyngeal carcinoma, NPC, GTPase

Introduction

There is a marked geographic variation in the incidence of nasopharyngeal carcinoma (NPC). It is rare in the United States and Western Europe, but is endemic in southern China and Taiwan. This tumor occurs more commonly in males, with the male: female ratio approaching 2-3:1 [1]. The geographic variation of NPC incidence suggests a multifactorial etiology, including Epstein-Barr virus (EBV) infection, genetic predisposition, and environmental factors such as smoking and high intake of preserved foods [2, 3]. In the endemic area, approximate 99% of cases are classified as non-keratinizing or undifferentiated carcinoma, showing strong association with EBV [4]. Radiotherapy is the mainstay of first-line local treatment for early stage NPC. For more advanced disease, concurrent cisplatin-based chemoradiotherapy improves local control and decreases the rate of distant metastasis [5]. Despite recent ad-

vances in the treatment of NPC, a substantial proportion of patients develop a certain degree of resistance to chemoradiotherapy. Thus, it would be of great value to search for potential biomarkers that might predict the response to treatment, and to further clarify the underlying molecular mechanisms.

Several molecular pathways have been found to be involved in the pathogenesis of NPC, including pathways mediating cell proliferation, angiogenesis, apoptosis, migration and invasion [6]. The GTPase activity plays an important role in the regulation of signal transduction, cell division, protein biosynthesis, protein translocation and vesicle transportation. GTPases are a large family of hydrolase enzymes that function as hydrolyzing guanosine triphosphate [7]. A substantial number of these G proteins are involved in cell cycling, and some of them are known proto-oncogenes. For example, the RAS superfamily of G proteins is a key regulator of normal cell growth and malignant transformation because RAS is a common downstream target of numerous growth factors [8]. To search for potential transcripts involved in the regulation of cell cycling, we performed data mining on published expression profiles in the Gene Expression Omnibus databases (GSE-12452 and GSE34573) focusing on genes associated with GTPase activity [9, 10]. We identified *RAS-related protein 2A (RAP2A)* as the most significant gene that was differentially expressed between the high-stage and low-stage tumors. It was also significantly upregulated in the tumor tissue when compared with normal tissue. The RAP proteins belong to the RAS family of small G proteins and consist of five members, including RAP1A, RAP1B, RAP2A, RAP2B and RAP2C. Although their structures are quite similar, they clearly are involved in different signaling pathways and possess unique biological functions [11, 12]. Recent studies have suggested that RAP-mediated signaling pathways have certain influence on the invasiveness and metastasis of some human cancers, such as prostate cancer and leukemia [13, 14]. However, little is known about the role of RAP proteins in NPC.

To the best of our knowledge, the expression of RAP2A has never been studied in a large cohort of patients with NPC, particularly in the endemic area. This study aimed to evaluate the expres-

sion of RAP2A in patients with early and locoregionally advanced NPC. We specifically wished to elucidate the prognostic significance of RAP2A expression and its association with various clinicopathological factors.

Materials and methods

Analysis of published transcriptomic datasets

To identify genes critical in the pathogenesis of NPC, we reappraised the transcriptome dataset deposited in the Gene Expression Omnibus (GSE12452), identifying NPC tissues (n = 31) versus non-neoplastic nasopharyngeal mucosal epithelial tissues (n = 10) enriched by laser capture microdissection for cells of interest [10]. The raw CEL files of the Affymetrix HUMAN Genome U133 Plus 2.0 microarray platform were imported into Nexus Expression 3 software (BioDiscovery) to analyze all probe sets without pre-selection or filtering. We performed supervised comparative analysis and functional profiling to identify significant genes that are differentially expressed, with special attention to pathways related to GTPase activity (GO: 0003924), as previous described [15-17]. Those with $P < 0.01$ and $\log_2$-transformed expression fold change > 0.1 when comparing tumor versus non-tumor and high-stage (III-IV) versus low-stage (I-II) tumors were chosen for further analysis. The significance of selected candidates was further validated in another independent transcriptome dataset of NPC (GSE34573) [9].

Patients and tumor specimens

The institutional review boards approved procurement of formalin-fixed NPC tissue for this study (IRB102-03-001, 103-02-013). Available paraffin-embedded tissue blocks were retrieved from 146 consecutive NPC patients who underwent biopsy between January 1993 and December 2002 in the Chi Mei Medical Center. Of these, 10 patients were diagnosed with systemic disease, and 12 who had not completed a standard course of therapy and/or loss of follow-up were excluded. The remaining 124 patients formed the basis of this retrospective study. All these patients were free of distant metastasis at initial diagnosis. Two pathologists (T.J.C & H.L.H) reappraised the histological subtypes according to the current WHO classification [4]. The tumor staging was reap-

praised by the 7th *American Joint of Cancer Committee (AJCC)* system [18].

Immunohistochemical staining and assessment of RAP2A expression

Tissue sections were cut from paraffin-embedded blocks at 3-μm thickness. Slides were deparaffinized with xylene, rehydrated with ethanol, and heated by microwave treatment for retrieval of antigen epitopes in a 10 mM citrate buffer (pH 6) for 7 min. Endogenous peroxidase was quenched by 3% H_2O_2 treatment. Slides were washed with Tris buffered saline for 15 min and then incubated with a primary polyclonal antibody targeting RAP2A (1:250; Novus Biologicals, Cat No. NBP2-24574, LLC, CO) for one hour. Primary antibodies were detected using the ChemMate EnVision kit (DAKO, K5001, Carpinteria, CA). The slides were incubated with the secondary antibody for 30 minutes, developed with 3,3-diaminobenzidine for 5 minutes, and then counterstained with hematoxylin. Following the manufacture's recommendations, human lung tissue with respiratory epithelium was used as a positive control. Rabbit serum IgG was used to replace primary antibody as a negative control. Without prior knowledge of clinical and follow-up information, two pathologists (T.J.C & H.L.H) scored the RAP2A immunoexpression using a multiheaded microscope to reach a consensus on the H-score. In brief, we calculated it using the following equation: H score = $\sum Pi$ (i+1), where i is the intensity (ranging from 0 to 3), and Pi is the percentage of stained tumor cells varying from 0% to 100%. Tumors with H-scores greater than the median of all cases were classified as having high expression.

Treatment and follow-up

Of these 124 patients with NPC, 114 were monitored regularly after radiotherapy until death or their last appointment, with the mean follow-up duration being 67.0 months (range, 3-141). Based on the published protocols, all 124 cases received a complete course of radiotherapy with the daily fractionation of 180 cGy to 200 cGy, with five fractions weekly, to achieve a total dose of no less than 7,000 cGy [19]. As a rule, at least three cycles of cisplatin-based chemotherapy was performed in those with stage II-IV disease. However, there was one patient with stage II and four with stage IV disease who received radiotherapy alone due to their poor general condition. The method of radiotherapy was generally uniform within this period. However, among those treated in the earlier phase of this cohort, seven patients were not available to obtain instantaneous image evaluation after therapy as a baseline to evaluate treatment response. In total, there were 110 complete and 7 partial responders in accordance with the previously reported methodology adapted from WHO criteria [20].

EBER in situ hybridization

In situ hybridization for the EBV-encoded mRNA (EBER-ISH) was performed in an autostainer (Bond MAX, Vision BioSystems Ltd, Mount Waverley, Australia) using a polymer-based detection system (Bond Polymer Refine Detection, Vision BioSystems Ltd) with an EBV specific probe (Bond ISH EBER Probe) and 3,3'-Diaminobenzidine as chromogen.

Cell lines and culture conditions

Dysplastic oral keratinocyte (DOK) [21], NPC-derived HONE1 [22] and TW01 [23], cell lines were maintained in Dulbecco's modified Eagle's medium/Hams-F12 (Invitrogen, Carlsbad, California, USA), supplemented with 10% fetal bovine serum (Biological Industry, Kibbutz Beit Haemek, Israel) at a 37°C, 5% CO_2 incubator.

Western blot assay

To evaluate the endogenous RAP2A expression levels, equal amounts of total protein (25 μg) extracted from the cell lines were separated on 4%~12% gradient sodium dodecylsulfate polyacrylamide gel electrophoresis gel NuPAGE (Invitrogen, Carlsbad, CA), and then transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% skim milk in TBST buffer at room temperature for 1 h, the membranes were then probed with antibodies at 4°C overnight against RAP2A (ZA003, 1:1000, Invitrogen, Paisley, Scotland), and GAPDH as a loading control (6C5, 1:10,000, Millipore, Beverly, MA). The membranes were then incubated with the secondary antibody at room temperature for 1.5 h, and detected with enhanced chemiluminescence reagents (Amersham Biosciences).

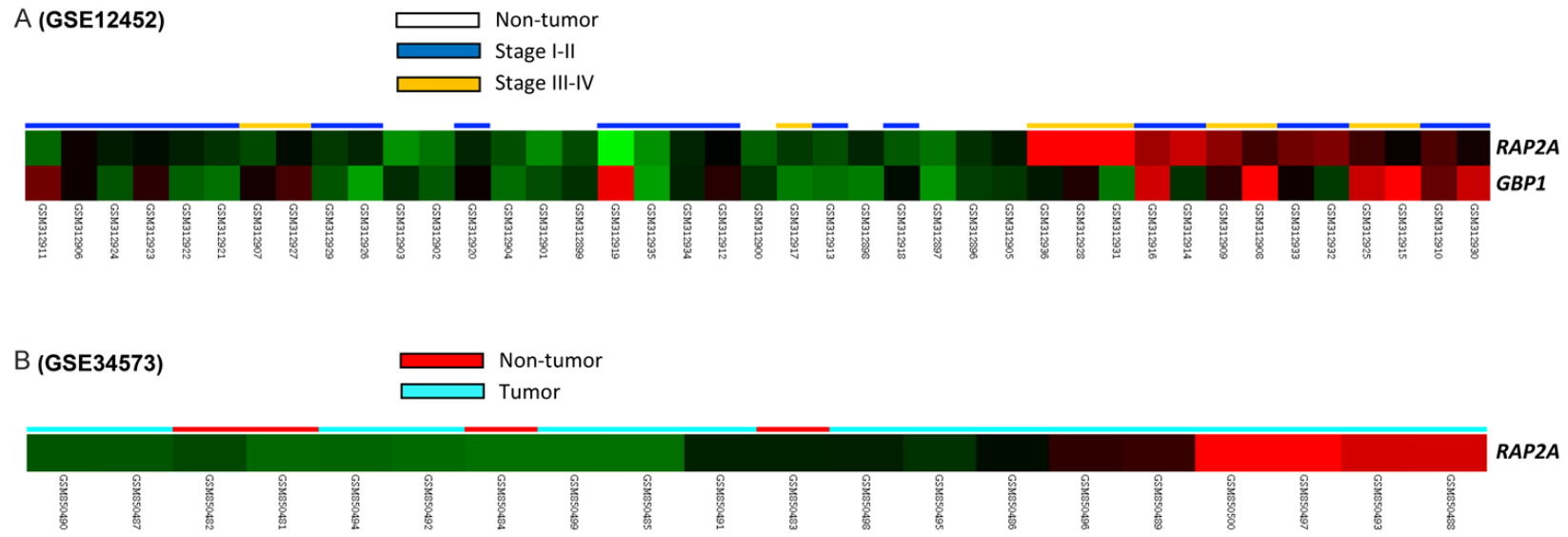# RAP2A overexpression in nasopharyngeal carcinomas

Figure 1. Analysis of gene expression from a published transcriptome dataset of NPC (GSE12452) focusing on pathways associated with GTPase activity (GO:0003924) demonstrated *RAP2A* and *GBP1* were the significant genes that were differentially expressed in the tumor versus non-tumor, as well as high-stage (III-IV) versus low-stage (I-II) tumors (A). Of these two, *RAP2A* showed the greatest $\log_2$-transformed expression fold ($\log_2$ ratio = 0.9717 and 1.0288, respectively). Furthermore, in the validation dataset (GSE34573), only *RAP2A* ($\log_2$ ratio = 1.4248, P = 0.0078) but not *GBP1* was significantly upregulated when comparing tumorous versus non-tumorous tissues (B). In the heatmap, upregulation and downregulation of genes are illustrated as a spectrum of brightness of red and green, respectively, with those unaltered coded as black.

Table 1. Summary of two significantly and differentially expressed genes related to GTPase activity (GO:0003924) and associated with both initiation and progression of NPC in the transcriptome of nasopharyngeal carcinoma (GSE12452)

Probe	Comparing tumor to non-tumor		Comparing high to low stage		Gene symbol	Gene name	Molecular function
	Comparison $\log_2$ ratio	Comparison *P*-value	Comparison $\log_2$ ratio	Comparison *P*-value			
225585_at	0.9717	0.0008	1.0288	0.0006	*RAP2A*	RAP2A; member of RAS oncogene family	GTP binding, GTPase activity, nucleotide binding
231578_at	0.5398	< 0.0001	0.3883	0.0024	*GBP1*	Guanylate binding protein 1; interferon-inducible; 67 kDa	GTP binding, GTPase activity, nucleotide binding

Table 2. Associations between RAP2A expression and other important clinicopathological variables

Parameters	Category	RAP2A Exp.		P-value
		Low	High	
Gender	Male	45	50	0.289
	Female	17	12	
Age (years)	< 60 years	49	49	1.000
	≥ 60 years	13	13	
Primary tumor (T)	T1-T2	46	34	0.024*
	T3-T4	16	28	
Nodal status (N)	N0-N1	33	23	0.071
	N2-N3	29	39	
Stage	I-II	26	12	0.006*
	III-IV	36	50	
Histological type	Keratinizing	2	3	0.094
	Non-keratinizing	33	21	
	Undifferentiated	27	38	

*Statistically significant.

Statistical analysis

Statistical analysis was performed using the SPSS 14 software package. The associations between RAP2A expression status and various clincopathological parameters were evaluated by Chi-square test. The endpoints analyzed were disease-specific survival (DSS), distant metastasis-free survival (DMeFS), and local recurrence-free survival (LRFS), calculated from the start date of radiotherapy to the date the event developed. We used the Kaplan-Meier method to plot survival curves and performed log-rank tests to evaluate prognostic significance. Multivariate analysis was performed using the Cox proportional hazards model. For all analyses, two-sided tests of significance were used with the P value < 0.05 considered significant.

Results

RAP2A was the only differentially upregulated gene related to GTPase activity

From the dataset of 31 NPC cases in the public transcriptome (GSE12452), we focused on 501 probes covering 203 genes related to GTPase activity. Of these genes, only *RAP2A* and *GBP1* met the selection criteria. Of these two, *RAP2A* showed the greatest log_2-transformed expression fold when comparing tumor versus non-tumor (log_2 ratio = 0.9717) and high-stage (III-IV) versus low-stage (I-II) tumors (log_2 ratio =

1.0288). Moreover, in the validation dataset (GSE34573), only *RAP2A* (log_2 ratio = 1.4248, P = 0.0078) but not *GBP1* was significantly upregulated when comparing tumorous versus non-tumorous lesions. Accordingly, we selected *RAP2A* for further validation (**Figure 1**, **Table 1**).

Immunohistochemical expression of RAP2A and its association with clinicopathological variables

As shown in **Table 2**, these 124 cases of NPC included 95 males and 29 females with a mean age of 48.6 years (range, 20-83). Seven cases were classified as stage I, 31 as Stage II, 46 as Stage III, and 40 as Stage IV, respectively. Except for one keratinizing NPC, all tumors enrolled were positive for EBER expression. RAP2A immunoexpression was low in the non-neoplastic nasopharyngeal mucosal epithelia without and with squamous metaplasia (**Figure 2A**, **2B**). It was detectable in all tumor samples with a wide range of expression (H-scores from 160 to 390, **Figure 2C**, **2D**) and was significantly associated with primary tumor status (pT, P = 0.024) and TNM stage (P = 0.006). It was not related to other clincopathological variables, including histological type.

Prognostic significance of RAP2A expression

The mean follow-up period was 67.0 months (range, 3-141). Of the three survival endpoints analyzed (**Table 3**), T3-4 status, N2-3 status, and AJCC III-IV stage at presentation were all significantly predictive for worse outcomes in univariate analysis. Notably, high RAP2A expression in patients with NPC was also associated with a more aggressive clinical course, with significantly shorter DSS (P < 0.0001, **Figure 3A**), DMeFS (P < 0.0001, **Figure 3B**), and LRFS (P < 0.0001, **Figure 3C**). In multivariate comparison, high expression of RAP2A still remained prognostically independent for DSS (P < 0.001, hazard ratio = 2.976), DMeFS (P < 0.001, hazard ratio = 4.233), and LRFS (P < 0.001, hazard ratio = 4.156), together with AJCC III-IV stages (**Table 4**).

RAP2A was more abundant in NPC cells than DOK1 cells

Western blot assay demonstrated a distinct band at 43 kDa detected in both NPC cell lines, which confirmed RAP2A protein expression. In
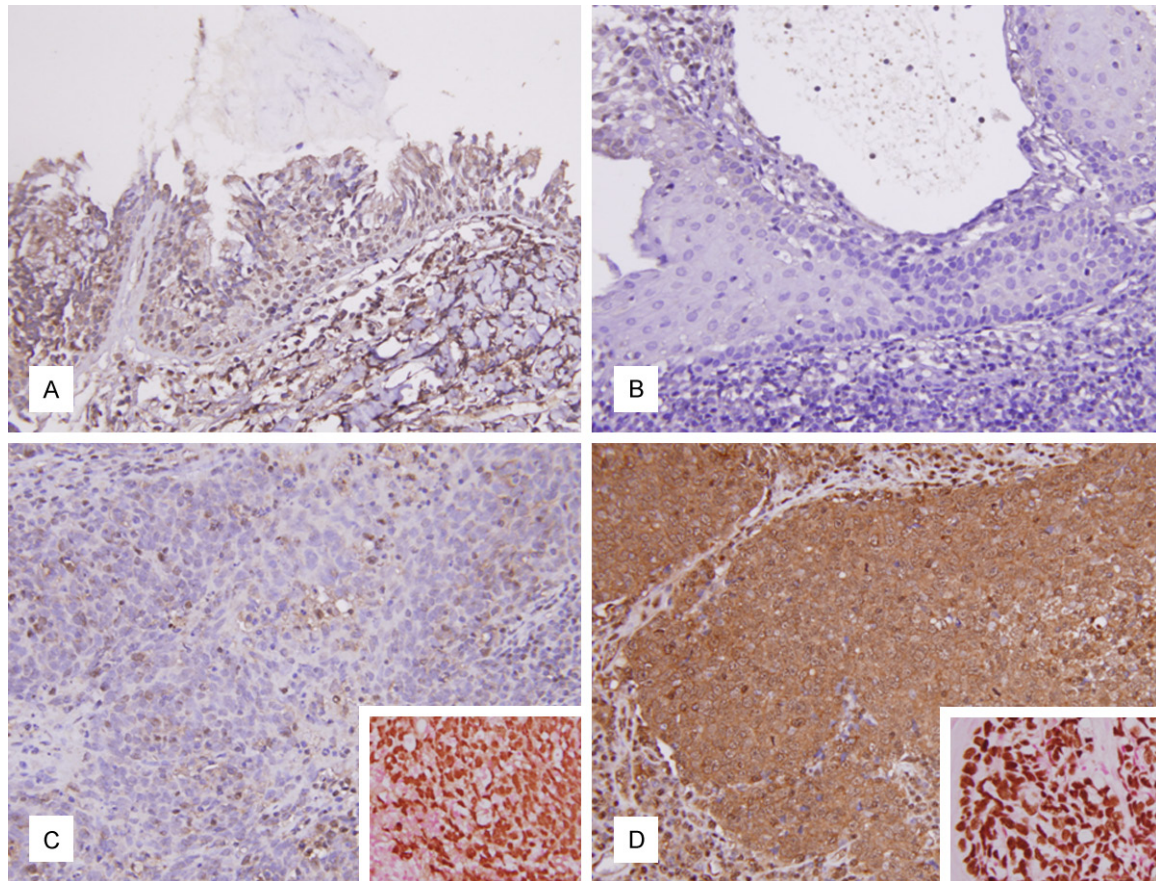
Figure 2. Immunohistochemical study of RAP2A in NPC. The immunoexpression of RAP2A was low in the non-neoplastic nasopharyngeal mucosal epithelia without (A) and with squamous metaplasia (B). Representative cases of NPC showed weak (C) and strong (D) cytoplasmic immunostains for RAP2A, respectively. Both cases are positive for EBER (inlets of C and D).

Table 3. Univariate log-rank analyses

Parameters	Category	No. of case	DSS		DMeFS		LRFS	
			No. of event	*P*-value	No. of event	*P*-value	No. of event	*P*-value
Gender	Male	95	45	0.7870	38	0.6128	30	0.3240
	Female	29	14		11		7	
Age (years)	< 60 years	98	48	0.8600	42	0.3091	29	0.8206
	≥ 60 years	26	11		7		8	
Primary tumor (T)	T1-T2	80	32	0.0289*	25	0.0085*	19	0.0180*
	T3-T4	44	27		24		18	
Nodal status (N)	N0-N1	56	18	0.0008*	17	0.0132*	12	0.0160*
	N2-N3	68	41		32		25	
Stage	I-II	38	10	0.0020*	9	0.0072*	5	0.0026*
	III-IV	86	49		40		32	
Histological type	Keratinizing/Non-keratinizing	47	20	0.1980	17	0.2753	15	0.9521
	Undifferentiated	77	39		32		22	
RAP2A	Low Exp. (H-score < median)	62	16	< 0.0001*	11	< 0.0001*	8	< 0.0001*
	High Exp. (H-score ≥ median)	62	43		38		29	

*Statistically significant; DSS, disease-specific survival; DMeFS, distal metastasis-free Survival; LRFS, local recurrence-free survival.
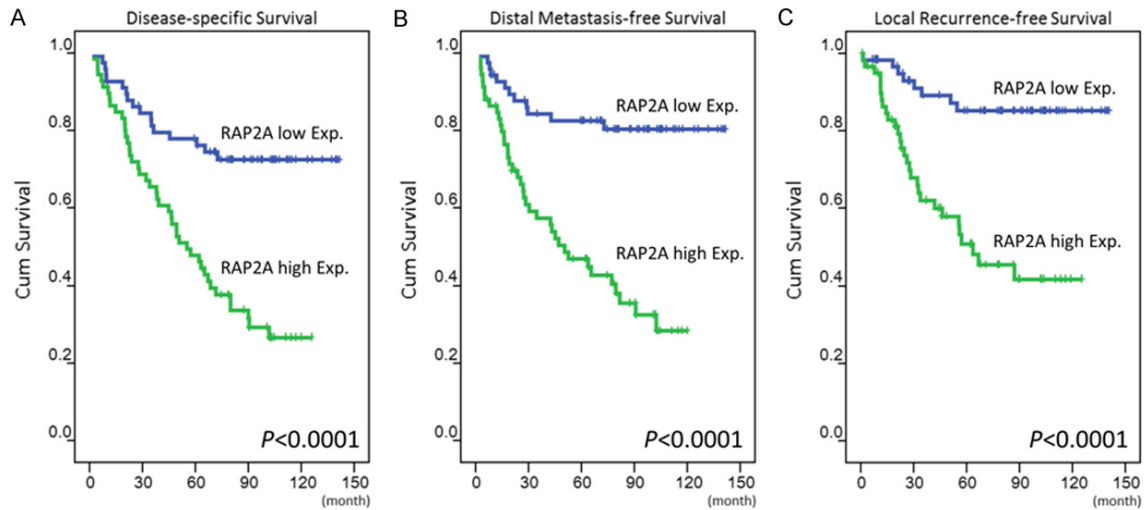
Figure 3. Kaplan-Meier survival analysis revealed high expression of RAP2A predicted worse disease-specific survival (A), distant metastasis-free survival (B), and local recurrence-free survival (C).

Table 4. Multivariate survival analyses

Parameter	Category	DSS			DMeFS			LRFS		
		H.R	95% CI	P-value	H.R	95% CI	P-value	H.R	95% CI	P-value
Stage	I-II	1	-	0.029*	1	-	0.087	1		0.035*
	III-IV	2.168	1.084-4.334		1.903	0.912-3.971		2.803	1.077-7.299	
RAP2A Exp.	Low Exp.	1	-	< 0.001*	1	-	< 0.001*	1	-	< 0.001*
	High Exp.	2.976	1.657-5.345		4.233	2.135-8.392		4.156	1.874-9.221	

*Statistically significant; DSS, disease-specific survival; DMeFS, distal metastasis-free Survival; LRFS, local recurrence-free survival.
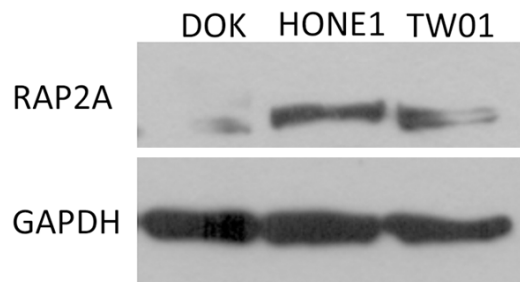

Figure 4. Western blot analyses in detecting RAP2A expression in NPC cells. Higher expression of RAP2A protein was found in HONE1 and TW01 cells, but not in non-tumorigenic DOK cell.

contrast, we found that RAP2A protein expression was barely detected in non-tumorigenic DOK cells. (**Figure 4**), suggesting that RAP2A plays a role in neoplastic transformation.

Discussion

RAP proteins are small molecular weight GTPases that belong to the RAS family. RAP

GTPases mediate a wide variety of biological processes such as cell adhesion, migration, differentiation, proliferation and survival [11, 24]. RAP proteins, like other GTPases, function as switches to control a signal transduction on and off, through an active GTP-bound and inactive GDP-bound conformation, respectively. The switch function of RAP is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which are impinged by various extracellular and intracellular stimuli [25]. Upstream signals can be transduced via binding with lipids or proteins, the posttranslational modifications of GEFs and GAPs, and second messengers, such as cAMP. These distinct upstream signals regulate individual RAP-GEFs and RAP-GAPs and control their activity, cellular distribution or stability. More importantly, the temporospatial distribution of RAP-GEFs or RAP-GAPs plays a crucial role in regulating RAP activity at various times and places in the tissue, and subsequently interacts with diverse downstream effectors to control distinct biological functions [11].

RAP1 and RAP2 are closely related proteins that have structural similarity. Dysregulation of RAP1 has been found in various human cancers or cell lines, such as colon cancer [26], prostate cancer [27], pancreatic cancer [28] and squamous cell carcinoma of the head and neck [29]. In contrast to RAP1, much less is known about the role of RAP2 in carcinogenesis and tumor progression. Although limited data have suggested that RAP2A over-expression stimulated cell growth in androgen-dependent LNCaP human prostate cancer cells [30], the definite biological effect of RAP2A in other cancer tissue remains obscure. In this study, we found that high expression of RAP2A was significantly associated with advanced primary tumor status and advanced TNM stage. Moreover, in survival analysis, high expression of RAP2A also predicted worse DSS, DMeFS and LRFS. These findings indicated that there was a potential relationship between RAP2A expression and an aggressive phenotype in NPC. In support of these findings, as mentioned above, one recent study pointed out an important role of RAP2A expression in androgen hypersensitivity and promoting cell growth in human prostate cancer cells [30]. But interestingly, another study found that over-expression of RAP2A inhibits migration and invasion of glioma cells by down-regulating p-AKT [31]. To account for such diverse findings, we hypothesized that the diverse effect of RAP2A expression in tumor formation, invasion or progression is via different biological signaling pathways in different cell types. With regard to RAP2A expression in NPC, more basic functional researches are needed to clarify the regulatory role of RAP2A in cancer progression, as well as its interaction with other upstream activators or suppressors.

In endemic areas such as Taiwan, the development of NPC is strongly associated with EBV infection. EBV, also called human herpesvirus 4 (HHV-4), usually establishes a life-long latent infection in most adults [32]. EBV has evolved numerous mechanisms, such as via evasion of host immune surveillance, to maintain the survival of infected cells long enough for the virus to establish persistent latent infection [33]. Viral latency is characterized by three distinct processes including viral persistence, restricted viral gene expression that alters cell growth, and retained potential for reactivation to lytic replication [34]. In latent infection, the most abundant EBV RNAs in infected B-cells are small, noncoding and nonpolyadenylated nuclear RNAs, named EBV-encoded small nuclear RNA 1 (EBER1) and EBER2 [35]. In immortalized nasopharyngeal epithelial cells, stable expression of EBER has been found to confer resistance to apoptotic stress by inhibiting phosphorylation double-stranded RNA-dependent protein kinase (PKR) [36]. However, the contribution of the apoptotic-resistant phenotype associated with EBER expression to chemo- or radio-resistance in NPC is still unknown. In our study, all but one case had EBER expression. The only one case with EBER negativity was classified as keratinizing squamous cell carcinoma. Because of the extremely high incidence of EBV-infected NPCs in Taiwan, we were unfortunately unable to assess the prognostic significance of EBER expression in NPC, as well as the association between RAP2A expression and EBER expression.

In conclusion, our data revealed for the first time that high expression of RAP2A is associated with advanced tumor status. More importantly, it acts as a useful marker for predicting early local recurrence and distant metastasis in early and locoregionally advanced NPCs. Identifying the prognostic significance of RAP2A expression in NPC may aid in developing specific signaling inhibitors and providing effective treatment modalities for NPC. Further studies are needed to determine the regulatory role of RAP2A in RAS-related signaling pathways as well as its interaction with upstream GAPs or GEFs.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; RAP2A, RAS-related protein 2A; DSS, disease-specific survival; DMeFS, distant metastasis free survival; LRFS, local recur-

rence-free survival; ISH, in situ hybridization; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; HHV4, human herpesvirus 4; EBER, EBV-encoded small nuclear RNA; PKR, double-stranded RNA-dependent protein kinase

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