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RESEARCH

Nuclear Protein Phosphatase 1 α (PP1A) Expression is Associated with Poor Prognosis in p53 Expressing Glioblastomas

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Abstract
Background Protein phosphatase 1 α (PP1A) is an enzyme intimately associated with cell cycle, the over expression of which has been demonstrated in glioblastoma (GBM). Further, the nuclear expression of PP1A has been shown to be highly specific to GBM. In addition, PP1A has been shown to be a connecting molecule in the p53 containing GBM sub network. In view of these, we evaluated the prognostic relevance of PP1A.
Methods GBM tissues were examined for protein expression of PP1A by immunohistochemistry (IHC). Nuclear expression of PP1A was scored in all tumor tissue samples. Survival analyses were performed by Cox-Regression and Kaplan-Meier survival analysis with Log Rank tests. IDH1, ATRX and p53 IHC and stratification of all GBM cases were performed and subgroup specific evaluation of nuclear PP1A correlation with overall and progression free survival was performed.
Results PP1A protein expression showed no correlation with prognosis in all cases of GBM or on stratification based on IDH1 or ATRX expression. However on p53 stratification nuclear PP1A expression emerged as strong independent predictor of poor overall survival only in p53 positive GBMs both in univariate and multivariate analysis.
Conclusions While PP1A expression uniquely associates with poor prognosis only in p53 expressing GBMs, there is a notable absence of such correlation in p53 negative GBMs; thus skewing the overall relation of this molecule with prognosis in GBM. PP1A emerging as a strong prognostic marker in p53 expressing GBMs, enables us to foresee this molecule as a potential therapeutic target.

Keywords Glioblastoma · Prognosis · TP53 · Protein phosphatase 1 α · PPP1CA

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Introduction

Glioblastoma (GBM) has been well documented as the most aggressive primary neoplasm of the brain [1]. The dismal prognosis due to this disease has fostered several high throughput studies ranging from gene expression analysis to proteomics and epigenomics. Handling such large data arising from these involves use of computational biology techniques. One such previous study from our group employing bioinformatics methods, explored GBM specific protein interaction networks and had identified Protein Phosphatase 1 α (PP1A) as a novel connecting molecule between cell cycle associated genes [2].

PP1A, also referred to as Protein Phosphatase 1 Catalytic subunit Alpha isoenzyme (PPP1CA), is a catalytic subunit of the enzyme Protein Phosphatase 1 (PP1), plays an integral role in signal transduction pathway, and acts as a key molecule in cell division [3, 4]. While in normal scenario PP1A has been described to bring about apoptosis [5] and cellular senescence [6], contributing to tumor suppression [6], its tissue/tumor specific role is still not described.

In GBMs, PP1A has been demonstrated by us to be overexpressed both at mRNA and protein levels, the nuclear expression of PP1A was shown to be highly specific for GBMs [2]. The identification of PP1A was predominantly as a connecting molecule in the p53 containing subnetwork of GBMs.

While on one hand PP1A contributes to tumor suppression [6], literature also suggested that PP1 could inhibit p53, a key regulator of cellular outcome [7]. Further, oncogene induced senescence was shown to be dependent on the activity of both p53 and PP1A [8]. These studies suggest that there is a complex interplay of these molecules in determining cellular outcome and thereby deciding the fate at the tissue level. These have lead to an intriguing possibility of a biological interplay between p53 and PP1A in determining the patient outcomes in various cancers.

In the current study, we have evaluated the expression pattern of IDH1, ATRX, p53 and PP1A expression in GBMs and assessed their prognostic value in a large prospective cohort of uniformly treated adult patients with newly diagnosed GBM.

Materials and Methods

Patient and Tissue Samples

GBM (n=136) tumor tissue samples were obtained following institutional ethical clearance and informed patient consent, from patients who underwent surgery at the two clinical centres (National Institute of Mental Health and Neurosciences and Sri Sathya Sai Institute of Higher Medical Sciences, Bangalore, India) between 2006 and 2009. GBM samples were selected from a clinical cohort of adult patients who were newly diagnosed with GBM, and had undergone maximal safe resection of the tumor and had a post operative Karnofsky's Performance Score (KPS) ≥70. Following histopathological confirmation of the diagnosis, the tumor samples were further characterised and noted to consist of 17 cases (12.5 %) positive for mutant IDH1 staining, and 15/132 cases (11.4 %) showing absence of ATRX expression as per the evaluation and assessment criteria detailed elsewhere [9, 10]. The patient cohort received standard adjuvant therapy which included radiotherapy (total dose=59.4 Gy), along with concomitant chemotherapy with temozolomide (100 mg/day, daily for 45 days) and cyclical chemotherapy with temozolomide (150 mg/ sq. m body surface area for 5 days every 28 days). The patients were regularly followed up clinically and with radiological MR imaging.

The overall survival was defined as the duration between surgery and death of the patient due to disease. The progression free survival was defined as the duration between surgery and the earliest onset of clinically detectable recurrence or radiological progression of tumor measured as an increase in the volume of the tumor by at least 25 % in comparison with the prior imaging study [11]. Failure to follow up due to death or clinical deterioration was also considered as disease progression in line with the previous studies [12, 13].

Immunohistochemistry (IHC)

Formalin fixed paraffin embedded tumor tissue samples were collected on silane-coated slides, and the protein expression of PP1A and p53 was assessed by IHC. Following deparaffinization of the tissue samples, antigen retrieval was done by heat treatment at 850 W in citrate buffer. After the initial processing steps, sections were incubated overnight with primary antibody. The primary antibody for PP1A (Cell Signaling Technologies) was used at a dilution of 1:25, ATRX (Rabbit polyclonal, Sigma Lifesciences) at 1:200, IDH1 R132H (Mouse#H09 clone, Dianova) at 1:50 and p53 (Mouse Monoclonal DO-7; Biogenex, USA) at 1:200. This was followed by incubation with secondary antibody (QD440-XAK, Biogenex). 3,3′-Diaminobenzidine (Sigma-Aldrich) was used as the chromogenic substrate. GBM tumors that showed PP1A and p53 overexpression by IHC respectively in our previous studies [2, 14] served as positive controls. A negative control (slide in which the primary antibody is omitted) was included with each batch of staining.

The nuclear expression pattern of p53 in GBM tissues is well documented. Tumor tissues with 20 % or more cells demonstrating strong staining were labelled as p53 expressing (p53 positive) GBMs. Tumor tissues were thus categorised into p53 positive GBM and p53 negative GBM. PP1A demonstrated both cytoplasmic and nuclear staining, however, only the strong nuclear staining was considered for further analysis, in view of our previous demonstration of high specificity of nuclear expression of PP1A in GBM [2]. In each slide >1000 cells were counted and the percentage of cells with strong nuclear staining was depicted as the labelling index (LI).

Statistical Analysis

Survival Analysis

The clinical parameters like the extent of surgical resection and post operative KPS were standardized as part of inclusion criteria; hence the only clinical variable included for analyses was patient's age. SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL) was used for analysis. A P-value of <0.05 was considered significant.

For correlation of PP1A protein expression with overall survival and progression free survival, Cox Regression analysis was employed. The prognostic significance of PP1A was then assessed following p53 stratification of all GBM cases. Multivariate analysis was performed using Cox regression

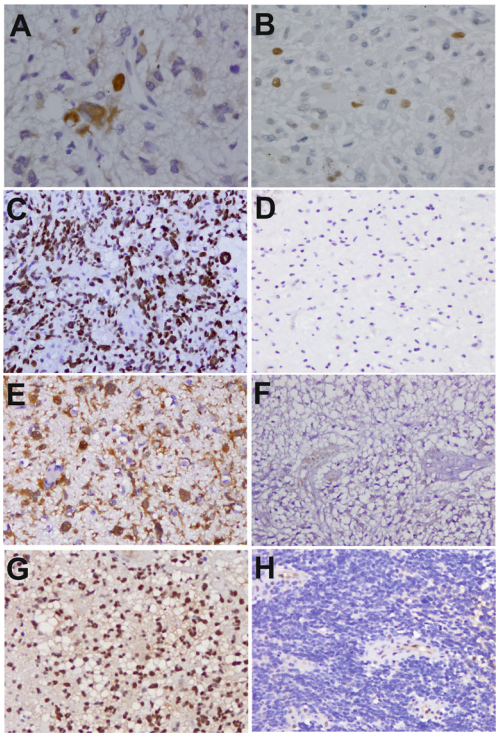

Fig. 1 Demonstrates the staining pattern for various markers in different glioblastoma tumor tissues. Tumor tissue in **a** shows both cytoplasmic and nuclear expression of PP1A while **b** shows nuclear expression of the PP1A (magnifications ×320). **c** and **d** show tumor tissue staining positive and negative for p53. **e** and **f** positive and negative for IDH1R132H, **g** and **h** positive and negative staining patterns of ATRX (magnifications ×160)

models. For purpose of clinical utility, the PP1A protein expression was dichotomised into positive tumors and negative tumors with a median cut off value of labelling index at 10 %. Log Rank tests for significance were performed and the Kaplan Meier curves were generated. Results were reported using the *P*-value and the estimated hazard ratio (HR) with their 95 % confidence intervals.

Results

Immunohistochemistry (IHC):

p53 expression was localized to the nucleus (Fig. 1c) and 45.5 % (62/136) of the cases were p53 positive, while 54.4 % (74/136) were negative for p53 expression (Fig. 1d).

PP1A immuno-staining was noted to show both cytoplasmic and nuclear pattern (Fig. 1a, b), and as previously described only the nuclear expression was considered for further analysis. Of the total 136 tumor tissue samples studied, nuclear PP1A expression (LI) ranged from 0 to 35 % (Median±SD =10±9 %). With the median LI of 10 % as a cut off, we observed that p53 expressing GBMs consisted of 34 PP1A positive and 40 negative cases. The p53 negative GBMs also showed a similar pattern with 29 PP1A positive and 33 negative cases. We also noted PP1A immuno-positivity in 8/17 and 55/119 IDH1 positive and negative tumors, and 57/117 and 4/15 ATRX positive and negative tumors respectively in this cohort.

Table 1 summarises the number of cases in each subgroup.

Survival Analysis

The present cohort had a maximum follow up of 85 months, median progression free survival of 9 months and median overall survival of 14 months. On univariate analysis, we noted that patient age was associated with poor overall survival (HR: 1.026; p=0.002; CI 1.010 to 1.043) and early disease progression (HR: 1.022; p=0.007; CI 1.006 to 1.039).

Nuclear PP1A showed no correlation with overall survival or progression free survival in present cohort. We then stratified the patients based on the IDH1, ATRX and p53 expression status and performed subgroup analyses. While PP1A showed no association with prognosis in subgroups based on IDH1 or ATRX expression; p53 based stratification revealed some unique findings in terms of survival which are depicted in Table 2. In patients with GBM lacking p53 protein expression, nuclear PP1A showed no correlation with overall survival or progression free survival. Interestingly, in p53 positive GBMs, nuclear PP1A demonstrated, statistically significant association with poor overall survival (p=0.004) as well as progression free survival (p=0.039) on univariate analysis. Further in p53 expressing GBMs, on multivariate survival analysis, nuclear PP1A emerged as the strongest predictor (p=0.016) of poor overall survival while age lost significance (Table 3).

The Kaplan-Meier curves generated to evaluate the effect of nuclear PP1A expression have been depicted in Fig. 2. The study revealed that in the entire cohort of GBM, nuclear PP1A expression lacks correlation with overall and progression free

Table 1 Depicts the distribution of all cases of GBM based on their p53, IDH1, ATRX, and nuclear PP1A expression patterns

nPP1A	P53 expression			IDH1 expression			ATRX expression		
	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total
Negative	33	40	73	64	9	73	11	60	71
Positive	29	34	63	55	8	63	4	57	61
Total	62	74	136	119	17	136	15	117	132

Table 2 Survival analysis depicting effect of the patient's age and nuclear PP1A expression in all cases of GBM and following stratification into subgroups

Univariate cox regression analysis						
		Variable	Overall survival		Progression free survival	
			Significance	Hazard (95 % CI)	Significance	Hazard (95 % CI)
All GBMs		Patient age	0.002*	1.026 (1.010–1.043)	0.007*	1.020 (1.006–1.036)
		PP1A - LI[a]	0.377	1.009 (0.989–1.030)	0.290	1.010 (0.991–1.029)
Following stratification into subgroups						
Based on IDH1 status	IDH1 negative GBMs	PP1A - LI[a]	0.531	1.007 (0.985–1.029)	0.478	1.007 (0.987–1.028)
	IDH1 positive GBMs	PP1A - LI[a]	0.461	1.022 (0.964–1.085)	0.213	1.038 (0.979–1.100)
Based on ATRX status	ATRX negative GBMs	PP1A - LI[a]	0.081	1.067 (0.992–1.149)	0.096	1.059 (0.990–1.133)
	ATRX positive GBMs	PP1A - LI[a]	0.787	0.997 (0.975–1.019)	0.936	0.999 (0.979–1.020)
Based on p53 status	p53 negative GBMs	PP1A - LI[a]	0.391	0.988 (0.961–1.016)	0.968	1.000 (0.976–1.024)
	p53 positive GBMs	PP1A - LI[a]	0.004*	1.051 (1.016–1.087)	0.039*	1.035 (1.002–1.069)

[a] PP1A – LI represents the labelling index (percentage of nuclei staining strongly positive for PP1A protein). While age is strongly associated with poor survival, PP1A expression is strongly associated with poor prognosis only in the p53 expressing GBM subgroup (*$p<0.05$)

survival respectively (Fig. 2a and d). Interestingly, following p53 stratification, we noted that nuclear PP1A expression was not associated with prognosis in p53 negative GBMs (Fig. 2b and e). In p53 expressing GBMs however PP1A positive GBM had a poor overall median survival of 14 months as opposed to 21 months in the PP1A negative cases (Fig. 2c) ($p=0.001$). The progression free survival in the p53 expressing subgroup (Fig. 2f) demonstrated that the PP1A positive cases had median progression free survival of only 9 months as against 14 months in the PP1A negative GBMs ($p=0.003$).

Discussion

The protein phosphatase (PPP) family of proteins consist of many subfamilies including PP1, PP2A etc. [3]. In recent times, the role played by these molecules in GBM biology is being uncovered. PP2A has been demonstrated to be associated with dormancy in GBM stem cells [15] and its' inhibition was shown to enhance the effect of cancer chemotherapy [16]. Similarly, Anisomycin a drug which could down-regulate PP2A has also been shown to induce glioma cell death [17].

PP1A however, in all its complexity, acting as a molecule associated with both pro-apoptotic and pro-tumorigenic roles [6–8], has come to light only recently in GBM, with our previous demonstration of PP1A as a novel connecting molecule in GBMs in the p53 subnetwork [2]. Further the recent literature on specificity of nuclear PP1A expression in GBMs [2] and its potential as a targetable molecule [18–20] made it imperative for us to understand the prognostic relevance of this molecule in GBM.

In the current study which addresses the above issue, we have demonstrated that while nuclear PP1A expression does not correlate with survival in all cases of GBM, a strong prognostic relevance of this molecule is noted in p53 expressing GBMs, both in terms of overall survival and progression free survival. The lack of survival correlation of PP1A in the p53 negative GBMs points to a possible subgroup specific role even within GBM tumors. In the present study, we also demonstrate how the relation of a molecule with survival can be

Table 3 Multivariate Cox regression analysis model to study effect of patient's age and nuclear PP1A expression on survival in p53 positive GBMs

Multivariate Cox regression analysis—p53 positive GBMs				
Variable	Overall survival		Progression free survival	
	Significance	Hazard (95 % CI)	Significance	Hazard (95 % CI)
Patient age	0.070	1.020 (0.998–1.041)	0.043*	1.021 (1.001–1.042)
PP1A - LI[a]	0.016*	1.044 (1.008–1.081)	0.075	1.031 (0.997–1.066)

[a] PP1A - LI represents the labelling index (percentage of nuclei staining strongly positive for PP1A protein). PP1A shows a strong association with poor overall survival (*$p<0.05$) on a multivariate analysis while age loses significance. In terms of Progression free survival, patient age continued to demonstrate a strong association while PP1A lost significance

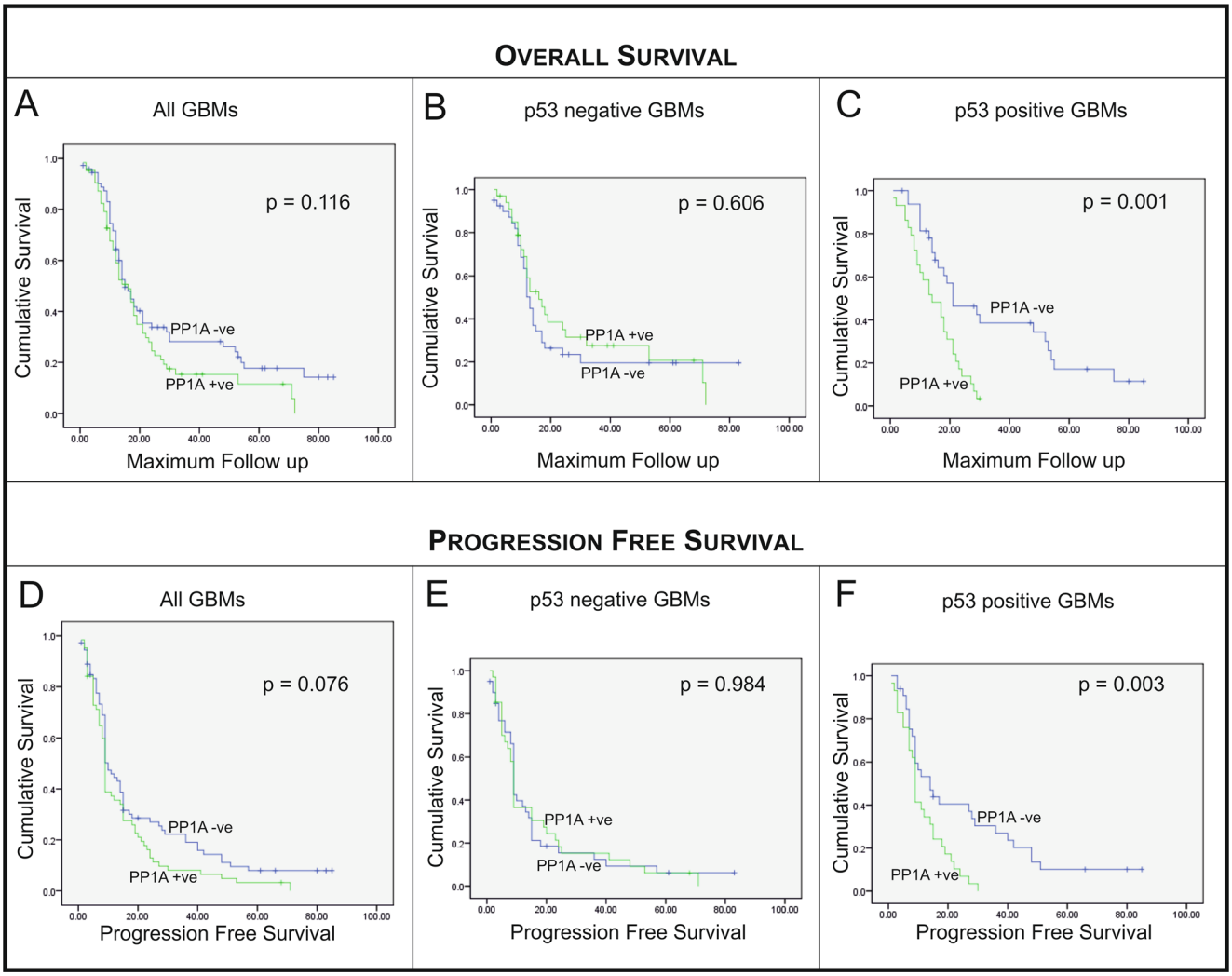

Fig. 2 Depicts the Kaplan-Meier survival curves generated to evaluate the effect of nuclear PP1A expression. **a**–**c** represent the overall survival patterns while **d**–**f** depict the progression free survival pattern. **a** and **d** demonstrate that in the entire cohort of GBM, PP1A expression lacks survival correlation. **b** and **e** also demonstrate lack of survival correlation of PP1A expression in p53 negative GBMs. The p53 expressing GBMs are depicted in **c** and **f** where PP1A expression demonstrates a statistically strong association with poor overall survival and progression free survival respectively

skewed due to underlying tumor heterogeneity. Similarly various molecules such as follistatin-like 1 (FSTL1) in GBM [21], c-erbB2 in breast cancers [22, 23], p-glycoprotein in osteosarcoma [24], Myc in B cell lymphoma [25], emerge as strong predictors of poor prognosis upon co-expression with p53. The prognostic association of PP1A, emerging only upon p53 stratification but not in the IDH1/ATRX based subgroups of GBM, further point towards the biology of p53 positivity and its pathway playing a key interaction as opposed to the developmental origin of GBM.

In gliomas p53 immunopositivity has been shown as a moderately sensitive and highly specific marker to predict TP53 mutations [26], enabling p53 IHC to be employed as a surrogate to identify p53 mutation in routine practice. In normal scenario, p53 protein has a short half-life, remains undetectable on IHC but plays a key role acting as a regulator of cell cycle. P53 has also been shown to bring about cellular senescence acting in conjunction with PP1A [8]. Just as p53 'the guardian of the genome', turns pro-tumorigenic following mutation in cancers, PP1A which has been described to prevent oncogenic transformation [27], has also been shown to be overexpressed in oral cancer [28] and in GBM [2], however its role in cancer biology is yet unknown.

Despite the lack of literature on the biological role of PP1A in cancers, our study paves way for further studying the biological role of PP1A in GBM as well as the functional relation between p53 and PP1A. A context dependent functional role of PP1A in GBM can also be foreseen from the current study. Further biological experiments could investigate the role of PP1A as a potential therapeutic target to be exploited in the clinical setting.

Conclusion

The identification of therapeutic targets in GBM revolves around the demonstration of novel molecules expressed specifically in GBM and with a prognostic relevance. In view of our previous demonstration of GBM specific expression of nuclear PP1A and the current demonstration of PP1A to be strongly associated with worse prognosis in p53 expressing GBM, we propose PP1A could be a potential target in p53 expressing GBM. Further studies on its contribution to glioma pathogenesis are currently desirable to biologically establish its role as a therapeutic target.

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