Progesterone Receptor Together with PKC α Expression as Prognostic Factors for Astrocytomas Malignancy

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Introduction: Astrocytomas are the most common and aggressive primary brain tumors, and they are classified according to the degree of malignancy on a scale of I to IV, in which grade I is the least malignant and grade IV the highest. Many factors are related to astrocytomas progression as progesterone receptor (PR), whose transcriptional activity could be regulated by phosphorylation by protein kinase C alpha (PKC α) at the residue Ser400. Our aim was to investigate if PR phosphorylation together with PKC α expression could be used as a prognostic factor for astrocytomas malignancy.

Methods: By immunofluorescence, we detected the content of PKC α , PR and its phosphorylation at Ser400 in 46 biopsies from Mexican patients with different astrocytoma malignancy grades; by bioinformatic tools using TCGA data, we evaluated the expression of PR and PKC α mRNA according to astrocytoma malignancy grades. For all statistical analyses, significance was $p < 0.05$.

Results: We detected a positive correlation between the tumor grade and the content of PKC α , PR and its phosphorylation at Ser400, as well as the intracellular colocalization of these proteins. Interestingly, using an in silico assay, we found that the PR and PKC α expression at mRNA level has an inverse ratio with astrocytomas tumor grade.

Discussion: These results indicate that PR and its phosphorylation at Ser400 site, as well as PKC α and their colocalization, could be considered as possible malignancy biomarkers for astrocytomas grades I–IV.

Keywords: astrocytoma, glioblastoma, progesterone receptor, protein kinase C alpha, biomarker

Introduction

Astrocytomas are the most common primary brain tumors which represent about 76% of all gliomas¹ and can be found in any part of the brain, especially in the brain cortex, thalamus and basal ganglia.² The World Health Organization (WHO) proposed a scale from I to IV for their classification, according to the degree of malignancy. Grade I exhibits the lower degree and grade IV or glioblastoma (GBM) exhibits more advanced features of malignancy, including vascular proliferation, mitosis and necrosis, typically associated with rapid disease evolution and with poor prognosis.³ Epidemiological data report that GBM occurs in a greater proportion in men than in women (3:2).² The average survival of patients with astrocytoma depends on the histological grade, 7 years for patients with astrocytoma grade II, 3 years for grade III, and 12–15 months for patients with glioblastoma,

Table I Clinic and Pathological Characteristics from 46 Astrocytic Tumor Samples

MR	Age	HAG	Sex
101	17	I-II	M
102	18	I-II	F
103	26	I-II	F
104	22	I	F
105	40	I	F
206	25	II	M
207	34	II	F
208	56	II	M
209	35	II	M
310	30	III	M
311	73	III	M
312	68	III	M
313	66	III	M
314	48	III	F
315	55	III	M
316	38	III	F
317	65	III	F
318	48	III	F
319	66	III	M
320	27	III	F
321	32	III	F
422	16	IV	F
423	23	IV	M
424	50	IV	M
425	55	IV	F
426	27	IV	M
427	48	IV	M
428	43	IV	F
429	74	IV	F
430	27	IV	M
431	39	IV	M
432	66	IV	M
433	16	IV	M
444	41	IV	M
445	52	IV	F
446	29	IV	M
447	44	IV	M
448	70	IV	F
449	48	IV	F
450	56	IV	M
451	61	IV	F
452	46	IV	F
453	79	IV	M
454	56	IV	M
455	21	IV	F
456	49	IV	F
057	34	NT	F
058	18	NT	F
059	26	NT	F
060	20	NT	F

(Continued)

Table I (Continued).

MR	Age	HAG	Sex
061	49	NT	F
062	18	NT	F

Note: (-) information was not available**Abbreviations:** MR, medical reference; HAG, histological astrocytoma grade; NT, non-Tumor.

even with the standard treatment (radiotherapy + temozolomide).⁴ The National Institute of Neurology and Neurosurgery (INNN) reports that in Mexico, 9% of all brain tumors are GBM,⁵ which present an incidence of 3.5 per 100,000 habitants⁶ and, particularly for the Mexican population, the average of diagnosis is 49 years old.⁶

Some factors support the development of astrocytomas, for example, the activated pathways of NFκB, PI3K/AKT and the expression of progesterone receptor (PR).^{7,8} Regarding PR, immunohistochemical analysis revealed higher detection of this receptor in GBM compared with lower-grade malignant tumors,⁹ furthermore, blocking this receptor with RU486 antagonist reduces proliferation, migration, and invasion of GBM derived cell lines.¹⁰ In *in vivo* models, the size of tumors resulting from heterotopic and orthotopic xenograft of murine glioma cells (a tumor-like glioblastoma) decreased by 50% after treatment with RU486.^{11–13}

PR receptor belongs to the family of the nuclear transcriptional regulators that can be activated by ligand or without it^{14,15}; PR presents post-translational modifications, such as phosphorylation, sumoylation, acetylation and ubiquitylation.¹⁶ The state of phosphorylation of the PR affects its transcriptional activity, cellular localization, specificity and replacement rate.¹⁷ At the moment, for the human PR, 15 phosphorylation sites have been recognized.^{18–21} The residue Ser400 can be basally phosphorylated and in response to mitogens, and this phosphorylation is necessary for the regulation of ligand-independent activity.²² Our group has identified that the protein kinase C (PKC) is able to phosphorylate PR in this residue, in two cell lines derived from GBM.^{23,24}

The protein kinases C (PKC) are a family of isoenzymes that have the activity of Ser/Thr kinases. These proteins participate in many signaling pathways and are implicated in diverse cellular responses.²⁵ The over-expression or hyper-activation of PKC is a characteristic

Table 2 Clinical Pathological Features of the Samples from Data Bases TCGA and GTEX

Clinical Pathological Features	Non-Tumor	G II	GIII	GIV
n	283	258	271	166
Mean age	-	39.9	45.5	59.4
Survival (months)	-	37.6	28.3	14.2

Note: (-) information was not available.

Abbreviation: G, astrocytoma grade.

of GBM²⁶; some studies show that the activity of PKC is increased in cell lines derived from gliomas, and the PKC inhibitors reduce their proliferation significantly.²⁷ Isoforms PKC α and PKC δ are expressed in U373 cells (cell line derived from astrocytoma grade III) and its activation induces proliferation, migration and invasiveness,²³ interestingly through an interaction between PKC α and PR, which does not exist with the isoform δ .²⁸

In this work, we analyzed 46 biopsies from Mexican patients with diagnosis of astrocytoma (9 for grades I and II, 12 for grade III and 25 for grade IV). These biopsies showed a positive correlation between the tumor grade and the content of PKC α , PR and its phosphorylation at Ser400, as well as the colocalization of these proteins. An *in silico* assay on Xena browser using the data base of TCGA and GTEX was performed; interestingly, we found that PR and PKC α expression at mRNA level has an inverse ratio with astrocytomas tumor grade.

Materials and Methods

Samples

Pathology slides from 46 astrocytic tumors were obtained from patients (22 females and 24 males). The slides were obtained from the National Institute of Neurology and Neurosurgery Manuel Velasco Suárez (in accordance with the protocol N° 67/12 approved by the Institutional Review Board: Comité Científico y de Bioética del Instituto Nacional de Neurología y Neurocirugía) and

Figure 1 PR, PKC α localization, and their merge increase according to the astrocytoma grade. Representative sections of astrocytic tumors from (NT) non-tumor tissue, (G) astrocytomas grade, which were immunostained vs PR and PKC α . Blue: nucleus, green: PKC α , red: PR and orange: merge among the three channels (original magnification, 600 $\times$).

were classified according to the WHO histopathological classification (low grade: 9 samples, grade III: 12 samples, and 25 samples for GBM) (Table 1). For this work, six pathology slides of brain epileptic foci (women) were obtained, which were used as non-tumoral tissue.

Immunofluorescence

The slides were dewaxed in a dry bath at 60°C for 50 minutes; after that, they were passed for the following gradients: xylol, xylol:ethanol (1:1), ethanol (100%), ethanol (96%) and ethanol (90%), for 5 minutes each. For the identification of antigens, the epitopes were exposed to a solution of sodium citrate (10 mM, pH 6.0), boiling for an hour. The tissue was blocked in 5% normal goat serum/TBS-Tween 0.01% (blocking buffer) overnight at 4°C; the sections were washed and then incubated with the primary antibodies: rabbit anti-PR (2 µg/mL) (sc-7208, Santa Cruz Biotechnology, Dallas, TX) and mouse anti-PKCα (2 µg/mL) (sc-8393, Santa Cruz Biotechnology, Dallas, TX) and rabbit anti-PR pSer400 (1.3 µg/mL) (ab60954, Abcam) at 4°C overnight. The antibodies were removed and the sections were washed three times with TBS-Tween 0.1% for 5 minutes and then incubated with secondary antibodies at room temperature for 1 hour: Alexa Fluor 488 anti-mouse (1:1000) (A11001, Life Technologies, Carlsbad, CA) and Alexa Fluor 594 anti-rabbit (2 drops/mL according with the guide) (R37117, Molecular Probes by Life Technologies, Carlsbad, CA). Nuclei were stained with Hoechst 3342 (Thermo Scientific, Waltham, MA). Sections were covered from light, washed, mounted with Fluoro Care Anti-Fade Mountant (Biocare Medical, Concord, CA) and visualized in an Olympus Bx43 fluorescence microscope. The images were analyzed using the program Image-Pro Plus 7.0 Media Cybernetics (Rockville, MD). Twenty-five fields were taken for each sample; subsequently, the fluorescence intensity for each field was evaluated, considered as a proportional measure of the density of positive cells. Finally, the values were averaged to have a unique value for each patient. An immunofluorescence control was done using samples and secondary antibody, non-signal was detected. Two persons determined fluorescence intensity for each sample independently and blinded (no one knew the malignancy grade of samples).

Results Classification

In order to define three intervals for the fluorescence intensity as low, medium, or high for PR or PKCα based on the average of 25 fields per sample, we used the formula

Figure 2 Percentage of positive cells for PR, PKCα and their merge. Percentage of positive cells for PR (A) for PKCα (B) and their colocalization PR/PKCα (C). For each sample, 25 fields were taken. Results are expressed as the mean ± SD. Dunnet post-test determined the statistical difference (* $p \leq 0.05$ vs NT and & $p \leq 0.05$ vs all groups).

Abbreviations: NT, non-tumor; G, astrocytomas grade.

modified from Villegas-Pineda et al.²⁹ $I = (H-L)/3$, where I is the intensity, H is the highest intensity value of all samples and L is the lowest intensity value of non-tumoral tissue. With this formula, we can define the intervals as follows:

Figure 3 Spearman correlation between tumor grade and % of positive cells. A Spearman correlation coefficient was calculated to measure the strength of association between tumor grade and % of positive cells for PR (A), PKC α (B) and their merge (C).

- Low intensity for the interval $[L, f]$,
- Medium intensity for the interval $(f, s]$,
- High intensity for the interval $(s, H]$,

Table 3 Fluorescence Intensity from Each Protein and Their Classification in Intervals

Protein	HAG	% of Samples	FI
PR	I–II	66.66 22.22 11.11	Low Medium High
	III	25 66.66 8.33	Low Medium High
	IV	8.0 20.0 72.0	Low Medium High
PKC α	I–II	70.0 20.0 10.0	Low Medium High
	III	7.69 61.54 30.77	Low Medium High
	IV	8.0 28.0 64.0	Low Medium High

Note: Bold values indicate the highest percentage of samples ubicated in certain fluorescence values.

Abbreviations: HAG, histological astrocytoma grade; FI, fluorescence intensity. Bold values indicate the highest percentage of samples ubicated in certain fluorescence values.

where $f = L + I$ and $s = f + I$. Each sample was categorized in one of these intervals.

PGR and PRKCA Genes Evaluation

For the determination of *PGR* and *PRKCA* mRNA we used the platform of the California University, Santa Cruz: UCSC Xena (XenaBrowser.net) and the database from TCGA and GTEX for tumor tissue and non-tumor tissue, respectively. For the non-tumor tissue a total of 283 samples from brain cortex of GTEX database were used. For the tumor tissue, we used the TCGA database and obtained 258 samples for grade II (GII), 271 for grade III (GIII) and 166 for grade IV (GIV or GBM). The levels of mRNA were obtained and plotted on Graph Pad Prism 5.0. For this analysis, we used *EGFR* (Supplementary Figure 4) as positive control expression for GBM. The data are summarized in Table 2.

Spearman Correlations

To obtain the correlation between the degree of expression of *PGR* and *PRKCA*, the gene expression database called

“TCGA Glioblastoma (GBM)” were used, the samples that did not have data for both genes were eliminated; 172 samples were used and the Spearman coefficient was determined using the GraphPad Prism 8.0.2 program.

Survival Curves

To evaluate the relationship between gene expression of *PGR* and *PRKCA* with patients survival, Kaplan–Meier type curves were performed using the XenaBrowser.net platform, the database “TCGA low grade astrocytoma and glioblastoma,” we obtained the following data: for *PGR*: grade II: 257 samples, grade III: 270 samples, grade IV: 172 samples, for *PRKCA*: Grade II: 270 samples, Grade III: 270 samples, and Grade IV: 172 samples.

Statistical Analysis

The results were expressed as the mean $\pm$ SD for % of positive cells and for their overlap. Statistical analysis between groups was performed with an ANOVA followed by a Dunnet’s post-test. A value $P < 0.05$ was considered

statistically significant as stated in figure legends. All these analyses were performed in Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA).

Results

PR, PKC α Localization and Their Merge Increase According to the Tumor Grade

Immunofluorescence assays were performed to evaluate the presence of PR, PKC α , and their colocalization in astrocytoma biopsies (Figure 1). We observed that the number of PR and PKC α positive cells increased in accordance with the tumor malignancy grade: the percentage for PR positive cells in the non-tumor tissue (NT) was 6.2%, GI–II 16.1%, GIII 26.7% and GIV (GBM) 51.6% (Figure 2A). For PKC α , the data were as follows: NT 9.2%, GI–II 22.5%, GIII 31.1% and GIV 41.5% (Figure 2B), and for colocalization: 5.9% for NT, 12.6% for GI–II, 20.8% for GIII and 43.9% for GIV (Figure 2C).

To know if a correlation exists between the tumor grade and the PR/PKC α detection, we performed a Spearman

Figure 4 PR pSer400, PKC α detection and their colocalization increased according to the tumor grade. Representative sections of astrocytic tumors from (NT) non-tumor tissue, (G) astrocytomas grade, which were immunostained for PR pSer400 and PKC α . Blue: nucleus, green: PKC α , red: PR pSer400 and orange: merge between three channels (original magnification, 600 $\times$).

Figure 5 Percentage of positive cells pSer400 PR, PKCα and their merge. Percentage of positive cells for pSer400 PR (A), PKCα (B) and cells overlap pSer400 PR/PKCα (C). For each sample, 25 fields were taken. Results are expressed as the mean $\pm$ SD. Dunnet post-test determined the statistical difference ($^{\&}$ $p \leq 0.05$ vs all groups). (NT) non-tumor, (G) astrocytomas grade. Correlation between the colocalization of pSer400 PR/PKCα and astrocytoma grade (D). Spearman correlation coefficient was calculated to measure the strength of association between PR/PKCα colocalization and tumor malignancy.

Abbreviations: NT, non-tumor tissue; G, astrocytomas grade.

Correlation. We found a positive statistical correlation between PR (Figure 3A), PKCα (Figure 3B), and their colocalization with tumor malignancy grade (Figure 3C).

To determine if some features as sex or age could influence PR and PKCα detection, the samples were separated according to the sex in two groups (F: feminine and M: masculine), but no significant differences were found between sexes (Supplementary Figures 1a-c). No correlation was observed between astrocytomas grades and the age of patients according to detection of PR, PKCα and PR/PKCα colocalization (Supplementary Figures 2a-c).

Fluorescence Intensity from Each Protein in Immunofluorescence Assays

We sorted the fluorescence intensity for each protein in three intervals (low, medium, and high) according to the formula described in the Materials and Methods section,²⁸ then the samples were classified in accordance with their

intensity (Table 3): 66.6% of the samples from grade I-II showed a low intensity for PR, and 70% for PKCα, in the case of astrocytomas grade III, 66.66% for PR and 61.54% for PKCα of the samples showed a medium intensity, and for grade IV or GBM 72% for PR and 64% for PKCα presented high intensity.

PR pSer400 and PKCα Detection, and Their Colocalization Increased According to the Tumor Grade

For the analysis of phosphorylation, we randomly took six samples for each group. We found that the percentage of positive cells for pSer400 and their merge with PKCα increased according to the tumor grade (Figure 4). The mean of cells positive to PR pSer400 in the NT tissue was 4.2%, 8.4% for GI-II, 17.9% for GIII, and 42.4% for GIV (Figure 5A), for PKCα this mean was 4.1% for NT, 8.0% for GI-II, 20.63% for GIII and 41.3% for GIV (Figure 5B).

Figure 6 Expression of mRNA for PGR and PKCA and its correlation. PGR (A) and PRKCA (B) mRNA expression in normal tissue (NT) and in astrocytomas with different malignancy grade (G). Results are expressed as the mean $\pm$ SD. Dunnet post-test determined the statistical difference (* $p \leq 0.005$ vs NT). Correlation between the expressions of both genes (C). Spearman correlation coefficient was calculated to measure the strength of association between PR and PKC α .

The colocalization of these two proteins provided a mean of positive cells of 3.3% for NT, 6.7% for GI-II, 16.1% for GIII, and 38.9% for GIV (Figure 5C). We performed a Spearman correlation between colocalization of PR pSer400/PKC α with tumor grade, a positive statistical correlation was found (Figure 5D). In Supplementary Figure 3, representative images of PR pSer400 and PKC α colocalization for each patient are shown. These results suggest an astrocytoma grade-dependent correlation with the detection of PKC- α and PR pSer400.

Expression of mRNA for PGR and PRKCA

In silico analysis using TCGA data showed that mRNA levels for both genes are proportionally inverse to the tumor grade (Figure 6A and B). We observed that no correlation exists at mRNA level between PGR and PRKCA expression (Figure 6C), suggesting that the expression of one of these factors does not depend on

the other. For this analysis, we used the gene *EGFR* (Supplementary Figure 4) as a positive control because many reports indicate that the mRNA level of this gene correlates positively with tumor astrocytoma grade.³⁰ From the in silico data, we separated the data according to the patient's sex to determine if a difference exists between mRNA for PGR and PRKCA. No difference was observed between sexes or tumor grade (Supplementary Figures 5a and 5b).

Expression of PGR and PRKCA Correlation with Patient Survival

In order to evaluate the relationship between PGR and PRKCA expression (mRNA) with patient survival, Kaplan-Meier graphs using TCGA data were done (Figure 7). Interestingly, patients with astrocytoma grade III and IV with less expression of both factors have a large survival than those with high expression (Figure 6C-F).

Figure 7 Survival curves. The Kaplan–Meier graphs show survival curves in patients that express *PGR* and *PRKCA* in different grades of astrocytomas: Grade II (**A** and **B**) Grade III (**C** and **D**) and Grade IV (**E** and **F**).

Patients with astrocytoma grade II survival are independent of the expression of these factors (Figure 6A and B). The results suggest that the expression level of both factors in high-grade astrocytomas correlates with a poor prognosis.

Discussion

In this work, we detected the presence of PR, PKC α , and the colocalization of both proteins in 46 biopsies from Mexican patients with a diagnosis of astrocytomas grades I–IV. Interestingly, we observed that the number of positive cells for total PR and its phosphorylation at the Ser400 site, as well as PKC α and their respective colocalization, positively correlates with the tumor grade. Previously, a positive relationship between the percentage of PR positive cells with respect to tumor grade had been observed in patient biopsies; however, it had not been compared with respect to non-tumor tissue.^{9,31}

The activity of transcription factors is regulated by post-translational modifications such as phosphorylation; PR can be activated by phosphorylation at Ser400 by

PKC α in human GBM cell lines.²⁸ In human glioblastoma cell lines, PKC α can be activated by lysophosphatidic acid receptor 1 (LPA₁) which activates G α_q , which in turn activates PKC α ,²⁴ the activation of this kinase causes an increase in the transcriptional activity of PR²³ that regulates expression of genes involved in proliferation, migration, and invasiveness.^{32,33} In this work, we detected that PR phosphorylated at Ser400 and their colocalization with PKC α increased according to the tumor grade, which suggests that the PKC α induces PR phosphorylation, which afterward could induce receptor transcriptional activity.^{23,28,34} Phosphorylation sites have been proposed as possible tumor biomarkers; the phosphorylation at Ser294 of PR in breast cancer is associated with bad prognostic. This phosphorylation induces stem cell phenotype and increases signaling pathways activated by growth factors; in relation to this, a use of therapies with anti-estrogen activity has been suggested to avoid the recurrence of patients with a high content of pSer294 PR.³⁵

Interestingly, we observed that the expression of *PGR* and *PRKCA* genes decreased in relation to the malignancy grade of

astrocytomas, opposite effect observed at the protein level. The abundance of mRNA and the protein levels are unrelated in many cases³⁶; in lung adenocarcinoma, 29 genes with at least two isoforms, show a different correlation coefficient mRNA/protein, for example, for OP18 (oncoprotein 18), three of the four isoforms showed a statistically significant correlation between the mRNA abundance and the protein, the fourth isoform showed no correlation between protein and mRNA expression.³⁷ On patients with lung adenocarcinoma, some mechanisms that can modify the transcription at mRNA levels have been suggested; the Kozak sequences alterations, the codons polarization, the nonsense lectures, or even these alterations can be attributed to the quantification methods, such as temperature and lifetime of mRNA.³⁶ Differences between the transcriptome and proteome studies from samples of the same patient with GBM have been found; in genes as synapsin 1 (*SYN1*, related to cellular communication), mRNA levels are under-expressed, meanwhile protein is over-expressed.³⁸ In another study, a profile of proteins was performed in eight patients with GBM and compared to their respective non-tumor tissue; afterward, they analyzed the relation between mRNA/protein, they observed that only 2% of total proteins correlated with their respective mRNA.³⁹

Gene mutations could be related with differences between mRNA and protein levels. In chordoid gliomas *PRKCA*^{D463H} transcript is overexpressed compared to *PRKCA*^{WT} mRNA transcripts.⁴⁰ We determined the presence of punctual mutations in the *PGR* and *PRKCA* genes in glioblastoma tissue using the TCGA data bank in the Xena browser platform ([Supplementary Figure 6](#)), we found the presence of mutations that provides changes in protein amino acids at the sites: 709, 813 and 836 for PR and 506 for PKC α , it would be necessary to study if these transcripts are abundant in GBM compares to NT tissue. Interestingly, Kaplan–Meier curves point out that the expression of mRNA of *PGR* and *PRKCA* in different astrocytoma grades correlates with patient survival, indicating the relevance of these factors for this pathology.

Conclusion

Our results indicate that PR and its phosphorylation at Ser400 site, as well as PKC α and their colocalization, could be considered as possible malignancy biomarkers for astrocytomas grades I–IV and that the analysis of mRNA expression in GBM is not enough to determine the role of a gene in tumor malignancy.

Ethical Statement

The pathology slides were obtained from the National Institute of Neurology and Neurosurgery Manuel Velasco Suárez in accordance with the protocol N° 67/12 approved by the Institutional Review Board. All patients signed the informed consent and the guidelines outlined in the Declaration of Helsinki were followed.

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Disclosure

The authors reported no conflicts of interest for this work and declare that they have not known competing financial interests or personal relationships that could have appeared to influence this work.

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