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ALTERACIONES EPIGENÉTICAS EN LA METILACIÓN DEL PROMOTOR DEL GEN MGMT COMO FACTOR PREDICTIVO EN PACIENTES CON DIAGNÓSTICO DE ASTROCITOMA

TESIS QUE PARA OPTAR POR EL GRADO DE MAESTRÍA EN CIENCIAS MÉDICAS, ODONTOLÓGICAS Y DE LA SALUD.

PRESENTA

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ABREVIATURAS

ADN: Ácido desoxirribonucleico.
SNC: Sistema Nervioso Central
NCCN: National Cancer Comprenhensive Network
KPS: Karnofsky Performance Scale
RT: Radioterapia
QT: Quimioterapia
FDA: Food and Drug Administration
IRM: Imagen por Resonancia Magnética
CCTCG: Crisis Convulsivas Tónico-Clónico Generalizadas.

I. ANTECEDENTES

De acuerdo a la Organización Mundial de Salud (OMS) el cáncer es la primera causa de mortalidad en países desarrollados. En México representan el 2% del total de neoplasias[1]. De acuerdo a proyecciones calculadas por el GLOBOCAN, se prevé que para el 2030 el número total de casos se incremente en un 165% (Figura 1) [2]. Dentro de los tumores del Sistema Nervioso Central, el gliobastoma representa la neoplasia primaria más común [1].

Actualmente el tratamiento consiste en cirugía, radioterapia y quimioterapia. Múltiples estudios han ya mostrado el que el grado de resección tumoral es un factor pronóstico importante para los pacientes, teniendo un impacto significativo en la sobrevida cuando se comparan resección macroscópica total contra resección parcial [3] [4-6].

La terapia adyuvante consiste en radioterapia fraccionada a dosis de 60 Gy (en 28-30 fracciones de 1.8-2.0 Gy cada una) [7] [8] [9] mas quimioterapia con temozolamida (Figura 2). Si bien las guías marcan a la temozolamida como el agente de elección, la situación socieconómica de muchos países, incluyendo el nuestro no permite el uso rutinario de la misma, teniendo que echar mano de agentes alternos como carboplatino/vincristina.

El carboplatino es un agente alquilante, cuyo efecto se lleva a cabo por tres mecanismos diferentes 1) incorporación de grupos alquilo al DNA, 2) formación de enlaces cruzados en el DNA, e 3) inducción de “mispairing” de nucleótidos [10].

La vincristina es un agente alcaloide, el cual se une a la cadena beta de la tubulina, del huso mitótico, causando cristalización de los microtúbulos y arresto mitótico o muerte celular, como otros alcaloides, también actúan mediante otros mecanismos 1) metabolismo del glutatión, 2) actividad del transporte dependiente de calmodulina-Ca^{2++} y ATPasa, 3) respiración celular [11].

Durante los últimos 10 años las nuevas técnicas y conocimientos en biología molecular han permitido establecer marcadores moleculares que no sólo clasifican de forma mas precisas las diferentes estirpes gliales, sino que han servido como marcadores pronósticos y predictivos, incluyéndose ya su uso en la nueva clasificación de la OMS [12].

Definición de biomarcador.

En 1998 el National Institutes of Health Biomarkers Definitions Working Group definió biomarcador como “característica capaz de medirse objetivamente y que indica la presencia de un proceso biológico, patogénico o la respuesta farmacológica a una intervención terapéutica” [13]

La Organización Mundial de la Salud, en conjunto con la Organización de las Naciones Unidas lo definen como cualquier sustancia, estructura o proceso que pueda ser medido en el cuerpo o sus productos y que influencie o prediga la incidencia del resultado de una enfermedad [13]

Los marcadores pronósticos marcan una diferencia en el resultado que es independiente del tratamiento recibido, por ejemplo la edad, mientras que los predictivos, como su nombre indica, predicen una respuesta y por lo tanto diferencias en la sobrevida relacionadas con el tratamiento [14]. En tumores como los astrocitomas de alto grado, el uso de múltiples terapias hace necesario conocer que biomarcadores predictivos pueden ser de utilidad en la práctica clínica.

Metil- guanina metil- transferasa (*MGMT*)

El gen metil- guanina metil- transferasa (*MGMT*) se encuentra en 10q26, codifica para la proteína O6- metil guanina metil transferasa de 207 aminoácidos y pesa 21646 Daltons. Esta enzima constitutiva actúa como mecanismo de reparación del ADN al trasferir de forma irreversible un grupo metilo de la posición O6 de nucleótido de guanina del ADN, a un residuo de cisteína de la propia proteína MGMT [15]

Este mecanimso de acción previene la muerte celular inducida por agentes alquilantes, como la temozolamida y el carboplatino, al remover aductos alquilantes de la posición O6 y O4 de la timidina. Uno de los mecanismos de silenciamiento más importantes del gen es la metilación del promotor, produciendo que MGMT no se exprese y disminuyendo así la actividad de reparación [15]. A nivel clínico, esto implica que, aquellas células tumorales con poca expresión de MGMT son quimiosensibles, mientras que aquellas con alta expresión serán quimioresistentes. Sin embargo, esto no siempre es una regla, ya que existen casos que presentan baja expresión de la proteína y aún así son quimioresistentes [16].

Si bien se conoce ampliamente el papel de la metilación del promotor de *MGMT* como factor pronóstico y predictivo en cuanto al tratamiento con temozolamida, sin embargo son pocos los estudios que evalúan su papel como biomarcador en relación a otras terapias, los cuales se observan en la Tabla 1.

Tabla 1. Implicaciones clínicas en glioblastoma y la metilación del promotor de *MGMT*

Estudio	Tratamiento	Pacientes	Sobrevida libre de progresión (meses)	Sobrevida general (meses)
Esteller et al (2000)[17]	RT, cisplatino y carmustina	49 pacientes con astrocitoma anaplásico o glioblastoma, edad 38-70	21 metilado vs 8 no metilado	30 metilado vs 21 no metilado

Heigi et al (2005) [18]	RT vs RT y temozolamida	573 pacientes con glioblastoma, KPS mayor a 70	5.9 vs 10.3 metilado 4.4 vs 5.3 no metilado	15.3 vs 21.7 metilado 11.8 vs 12.7 no metilado
Herrlinger et al (2006) [19]	RT y temozolamida seguido de TMZ o lamustina	31 pacientes con glioblastoma. Edad 18-70 años	19 metilado vs 6 no metilado	34.3 metilado vs 12.5 no metilado
Weller et al (2009) [20]	RT vs RT y TMZ seguido de TMZ	301 con glioblastoma, mayores de 18 años	7.1 vs 11.4 metilado 7.1 vs no metilado	9.9 vs 24.1 metilado 8.8 vs 12.9 no metilado
Gilbert et al (2013) (21)	RT + TMZ seguida de TMZ (en 5/28 dias) vs RT + TMZ seguido de TMZ (en 21/28 días)	833 pacientes. KPS mayor a 60, mayores de 18 años.	8.8 vs 11.7 metilado 7.1 vs 8.2 no metilado	23.5 vs 21.9 metilado 16.6 vs 15.4 no metilado
Abreviaturas: RT, radioterapia; TMZ, temozolamida; KPS, Karnofsky Performance Status.				

Panorama en el Instituto Nacional de Neurología y Neurocirugía

El Instituto Nacional de Neurología y Neurocirugía se considera uno de los centros de referencia más importantes de latinoamerica. En él se realizan un total de mas de 2000 cirugías al año. Datos de nuestro grupo de investigación revelan que la edad al diagnóstico es de 45.7 años, hasta 10 años menor en comparación con lo reportado en la literatura. En cuanto al diagnóstico se obtuvo 6.5% pacientes con Grado I, 12.3% Grado II, 23.2% Grado III, Grado IV 58%. Respecto al tratamiento cabe señalar que solo el 40% de los pacientes reciben quimioterapia y, de estos, solo 10 pacientes recibieron temozolamida, esto se debe a la falta de recursos económicos [22]. El restante reciben esquemas alternos a base de carboplatino y vincristina.

JUSTIFICACIÓN.

Si bien se conoce la participación de *MGMT* en cuanto a su valor como biomarcador predictivo en la respuesta a temozolamida, no se ha evaluado la utilidad de dicho biomarcador con respecto a terapias de uso rutinario en nuestro medio, como son carboplatino y vincristina. La evaluación de la metilación del promotor de *MGMT* es por lo tanto una necesidad para poder individualizar el tratamiento de nuestros pacientes y de esta forma también optimizar recursos.

PLANTEAMIENTO DEL PROBLEMA

A pesar de la amplia gama de protocolos sobre el tratamiento del glioblastoma, el pronóstico continúa siendo malo, con una sobrevida promedio de 15 meses. En nuestro medio se ha implementado recientemente el uso de biomarcadores moleculares que pudieran ser predictivos y pronósticos, sin embargo los protocolos actuales no describen la relación de estos con el uso de quimioterapias que son usadas en países con población socieconómica

con nivel bajo. Es por lo tanto, una necesidad evaluar si dichos marcadores pueden ser aplicables a nuestra población.

PREGUNTA DE INVESTIGACIÓN

¿ La metilación del promotor de *MGMT* es un biomarcador predictivo en pacientes con astrocitoma de alto grado que reciben terapia con carboplatino/vincristina?

HIPÓTESIS.

La metilación del promotor del gen MGMT se correlaciona de manera positiva con la sobrevida en pacientes con glioblastoma que reciban carboplatino/vincristina.

OBJETIVOS.

Objetivo General.

a) Determinar si la metilación del promotor de *MGMT* es un biomarcador predictivo en pacientes con astrocitoma de alto grado que reciben tratamiento con carboplatino/vincristina.

Objetivos particulares.

a) Evaluar el estado de metilación del promotor de *MGMT* en pacientes con astrocitomas de alto grado.

b) Determinar si factores clínicos con edad, género, IK, porcentaje de resección, déficit inicial son útiles como biomarcadores pronósticos.

c) Correlacionar y analizar dichos factores clínicos con el estado de metilación del promotor.

METODOLOGÍA.

A) **Diseño:** cohorte prospectiva.

B) **Población:** Pacientes con diagnóstico de glioblastoma tratados en el Instituto Nacional de Neurología y Neurocirugía, mayores de 18 años y ambos sexos, de los cuales se obtuvo tejido neoplásico y contaban con consentimiento informado. En el periodo comprendido del 1 de marzo del 2015 al 31 de diciembre del 2015.

C) **Lugar de realización:** Instituto Nacional de Neurología y Neurocirugía.

D) **Captación de muestras:** se obtuvo muestras de tejido fresco de pacientes con diagnóstico de glioblastoma en el periodo mencionado.

E) **Evaluación del estado de metilación del DNA del gen *MGMT:*** Se utilizó el método de conversión del DNA por bisulfito de sodio, para posteriormente realizar una PCR sensible a metilación (MS-PCR).

- **Criterios de inclusión casos:**
    a. Pacientes diagnosticados con glioblastoma por dos patólogos certificados, mayores de 18 años y ambos sexos.
    b. Contar con biopsia de tejido tumoral fresco.
    c. Contar con consentimiento informado.
- **Criterios de NO inclusión casos:**
    d. Pacientes diagnosticados con metástasis a cerebro.
    e. Pacientes con diagnóstico de oligodendriomas y ependimomas.
    f. Pacientes con tumores de sistema nervioso central no gliomatosos.
    g. Haber recibido tratamiento antineoplásico antes de la toma de la biopsia.
- **Criterios de eliminación.**
    a. Muestras con autolisis o necrosis extendida.
    b. Pérdida total o parcial de la muestra.
    c. Degradación del ADN.

F) Análisis estadístico. Se analizará la información con el programa de análisis de datos Statistical Package for Social Sciences SPSS V 21.0. Se utilizaran graficas de Kaplan-Meier para el análisis de la supervivencia y regresión logística para el análisis de la asociación de las variables clínicas con las variables moleculares.

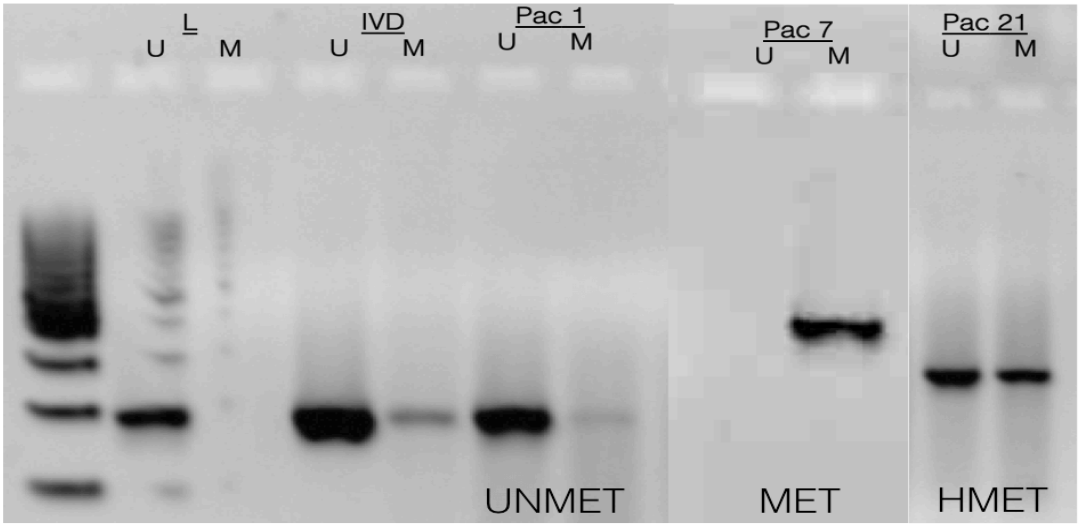
ESTADO DE METILACIÓN DEL PROMOTOR DE MGMT

PCR sensible a metilación (MS-PCR)

Se realizó la modificación de DNA con bisulfito de sodio para análisis de metilación (Kit ab117126 – Bisulfite Conversion Kit –Whole Cell) y PCR sensible a metilación.

Como control negativo para el estado de metilación se utilizó ADN de linfocitos de sangre periférica. Para el control positivo se realizó metilación in vitro de ADN de linfocitos con CpG Methylase (M. SssI).

Figura 2. Ejemplos de estados de metilación.



Gel de agarosa al 2%. L: linfocito, IVD: in vitro metilated DNA, U: no metilado, M: metilado, UNMET: paciente 1 que muestra promotor no metilado, MET: paciente 7 con clara metilación del promotor, HMET: paciente 21 en estado de hemimetilación.

RESULTADOS.

Se obtuvieron un total de 45 muestras de tejido neoplásico con confirmación histológica de glioblastoma. El estado de metilación del promotor de *MGMT* se evalúo en las 45 muestras obtenidas, sin embargo en 10 casos no se observó amplificación, por lo que fueron excluidas del análisis.

Características demográficas.

El porcentaje de hombres fue de 54.3% (19 pacientes) y 45.7% mujeres (16 pacientes). En cuanto al estado socioeconómico, el 62.9% de los pacientes se encontraban en nivel 1, clasificado como bajo y sólo 2.9% alto. La edad al diagnóstico fue de 53.3 años. 57.1% tenían algún antecedente de importancia (hipertensión y diabetes mellitus) (Tabla 2).

Tabla 2. Características demográficas.

Variable	Valor (%)
Género	
Hombre	54.3
Mujer	45.7
Nivel socioeconómico	
1	62.9
2	25.7
3	5.7
4	2.9
5	2.9
Edad de presentación	53.31 (años)
Alcoholismo	
Si	8.6
No	91.4
Tabaquismo	
Si	28.6
No	71.4
Antecedentes patológicos	
Si	57.1
No	42.9

Características clínicas.

Las localizaciones mas comunes fueron temporal 42.9%, frontal 31.5%, parietal 17.2%, occipital 2.9% y otros 5.4%.

En cuanto al síntoma de presentación este fue cefalea en el 31.4.3%, seguido de alteraciones en funciones mentales 22.9%, crisis tónico clónico generalizadas (20%), alteraciones motoras (20%), alteraciones en sensibilidad (2.9%) y otros (2.9%).

Tratamiento y estado de metilación.

Se logró resección parcial en 40% y total en 60%. El Karnofsky postoperatorio fue mayor a 70 en 85.7% y menor a 70 en 14.3%.

Sólo 8.6% de los pacientes presentaron alguna complicación postquirúrgica (2 casos con infección sitio quirúrgico y 1 paciente con hematoma lecho quirúrgico).

Los datos sobre terapia adyuvante y el estado de metilación se resumen en las Tabla 3-4. Es importante recalcar que casi el 50% de los pacientes no recibieron radioterapia ni quimioterapia, siendo la principal causa la falta de recursos económicos.

Tabla 3. Terapia adyuvante y estado de metilación

Variable	Valor (%)
Radioterapia	
Si	54.3
No	45.7
Quimioterapia	
Carbo/Vincristina	40 (n=14)
Temozolamida	17.1 (n=6)
No recibió	42.9 (n=15)
Estado de metilación	
Metilado	11.4 (n=4)
No metilado	62.9 (n=22)
Hemimetilado	25.7 (n=9)

Tabla 4. Relación entre estado de metilación y quimioterapia

Tipo de quimioterapia	Estado de metilación		
	Metilado	No metilado	Hemimetilado
Carbo/Vincristina	2	6	6
Temozolamida	0	5	1
No recibió	2	11	2

Sobrevida.

El tiempo de seguimiento fue 12 meses, con una sobrevida promedio de 8 meses.

Se realizaron curvas de Kaplan Meier (figuras 3-7) para estado de metilación, género, radioterapia, quimioterapia, localización y síntoma de presentación inicial. Sólo la quimioterapia y radioterapia resultaron estadísticamente significativos. Se realizo el análisis de supervivencia comparando los estados de metilación en los pacientes que recibieron carboplatino y vincristina y se agruparon a los pacientes hemimetilados dentro de los metilados y no metilados. Si bien en ninguno de los tres casos se mostró significancia estadística, al agrupar los pacientes hemimetilados en metilados, se observo que el grupo metilados presenta una mortalidad mas temprana que los no metilados (figuras 8-10)

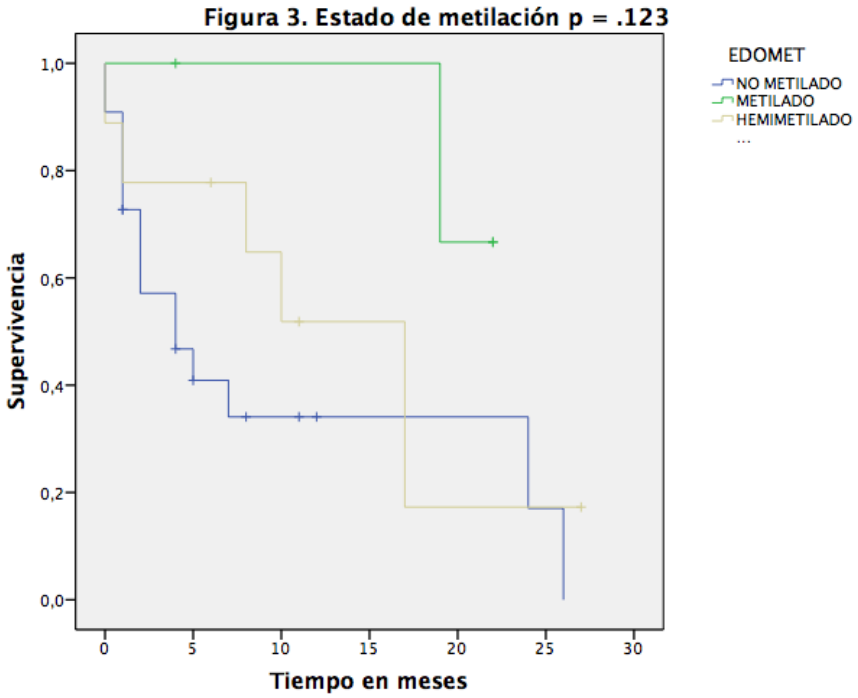


Figura 4: Quimioterapia p= .045

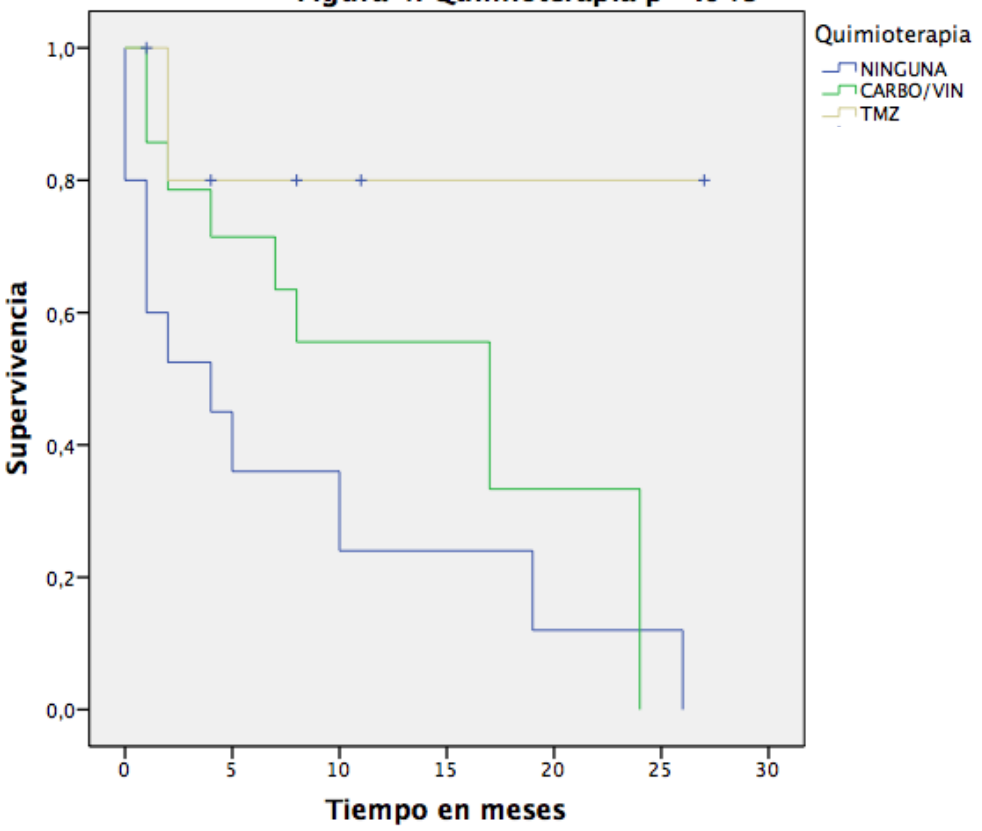


Figura 5: Radioterapia p0 .05

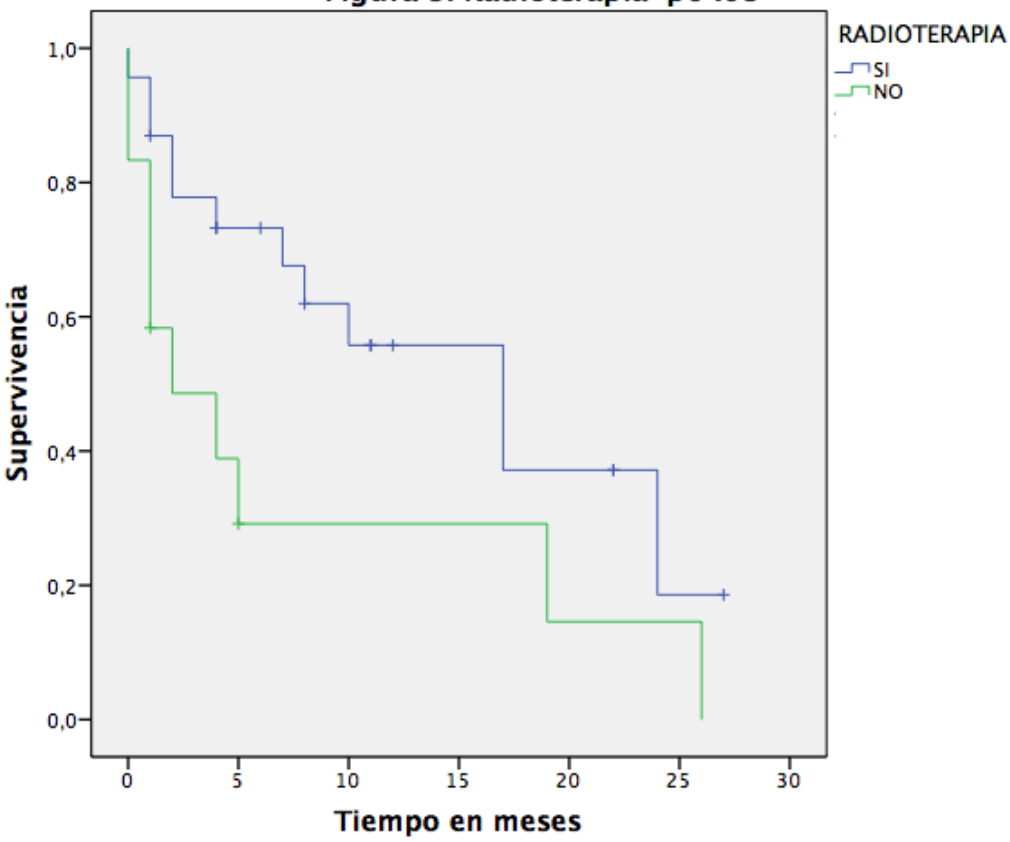


Figura 6: Localización p=.258

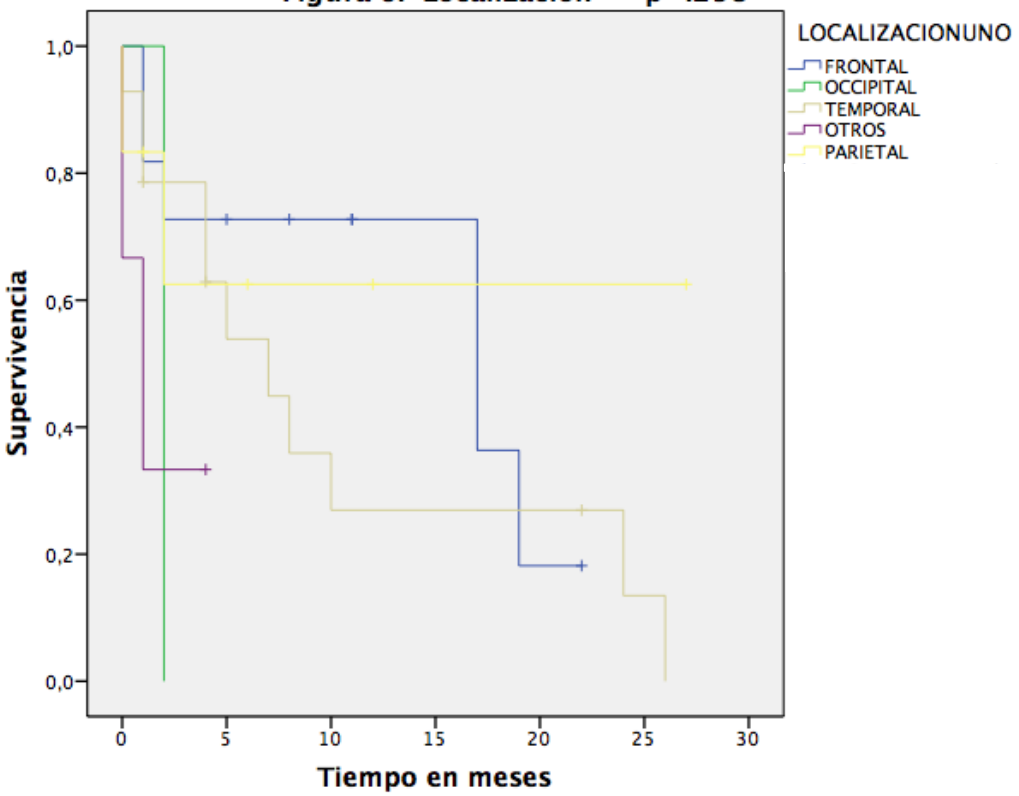


Figura 7: Síntoma de presentación inicial p=.456

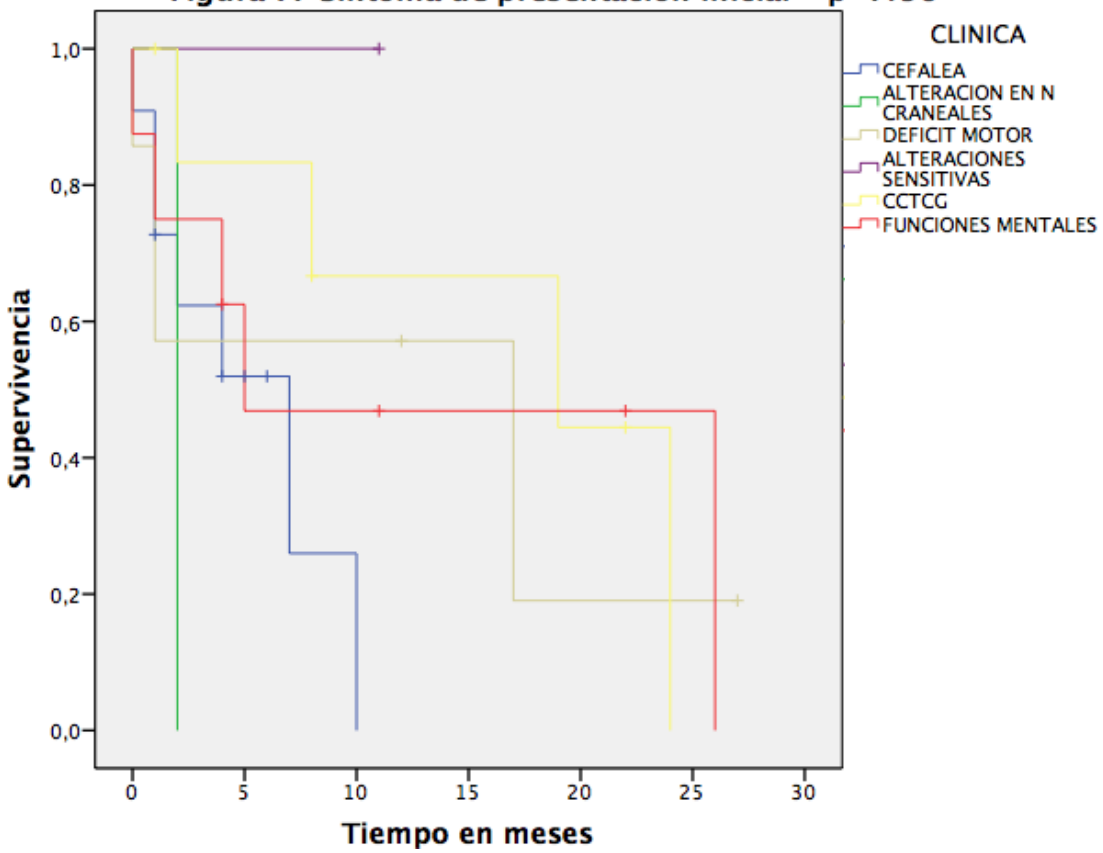


Figura 8. Sobrevida en pacientes con carboplatino/vincristina con relación al estado de metilación (p=.286)

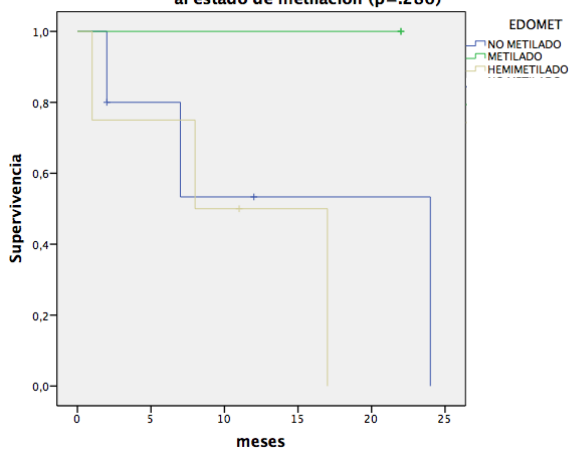


Figura 9. Sobrevida grupo hemimetilado=no metilado (p=.146)

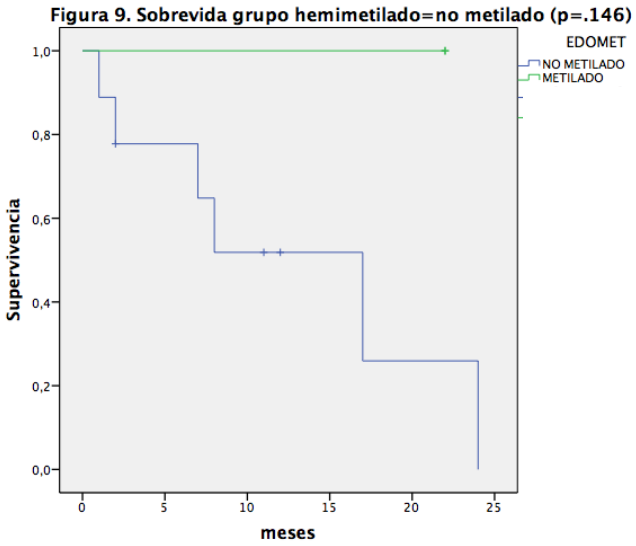
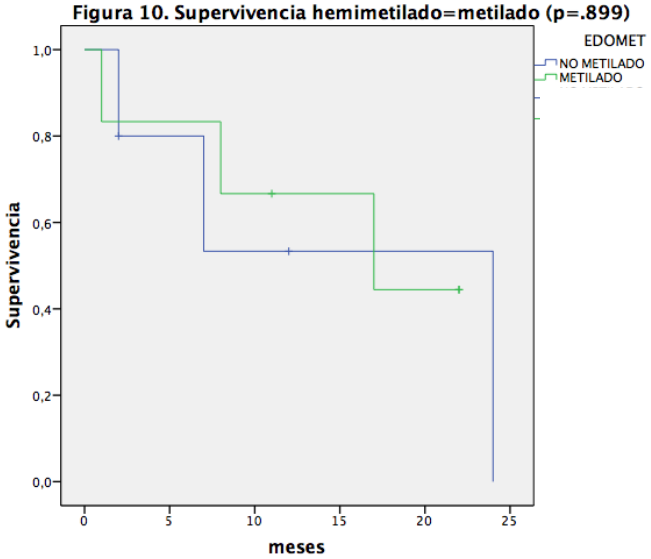


Figura 10. Supervivencia hemimetilado=metilado (p=.899)



DISCUSIÓN.

En nuestro estudio, la edad media de presentación fue de 53.3 años, lo que continúa siendo 10 años menor a la edad de presentación reportada a nivel mundial y es consistente con los resultados obtenidos en nuestro trabajo previo [22]. Esto indica que en nuestra población esta neoplasia se presenta a edades mas tempranas, lo cual representa una mayor carga social y económica para nuestro sistema de salud, ya que se trata de población económicamente activa. Si bien ninguno de los antecedentes personales mostró significancia estadística, es de notar que el 57% de la población presentaba diabetes y/o hipertensión arterial, lo cual podría impactar de manera directa en su respuesta postoperatoria y en su evolución. La localización y el síntoma de presentación inicial no mostraron significancia, sin embargo esto pudo ser debido al tamaño de muestra pequeño, ya que cuando se explora mayor número de pacientes el déficit motor como signo inicial si es estadísticamente significativo.

A pesar de lograr una resección total (mayor al 90%) en 60% de los pacientes, el impacto en la sobrevida se ve disminuido ya que el tratamiento adyuvante solo pudo ser otorgado en menos del 50% de los casos. 42% no recibió quimioterapia y 45.7% no recibió radioterapia. Lo anterior debido a que los pacientes no contaban con los recursos económicos para poder costear dichos tratamientos, siendo esto una de las grandes limitaciones de nuestro estudio.

Sin embargo, ambas variables mostraron significancia con respecto a la sobrevida total y en el caso de quimioterapia, esto se mantiene independientemente de la quimioterapia recibida. Lo anterior respalda la importancia de la terapia adyuvante en el tratamiento del glioblastoma y la necesidad de contar con esquemas alternos que sean aplicables en países en desarrollo, ya que sólo 6 pacientes pudieron recibir temozolamida, debido al costo total de la misma y el resto de los casos fueron tratados con carboplatina/vincristina. Se analizó si el estado de metilación del promotor de *MGMT* fue un factor predictivo en dicho esquema. No se encontró significancia estadística. Al realizar el análisis por subgrupos, colocando a los pacientes hemimetilados en la categoría de metilado y no metilado tampoco se obtuvo significancia, sin embargo se observa una tendencia a una menor sobrevida si se analizan a los pacientes hemimetilados como metilados. Esto puede ser debido a que el tamaño de muestra es muy pequeño, siendo solo 14 pacientes los que recibieron este esquema.

CONCLUSIÓN

Este estudio es el primero en evaluar el estado de metilación del promotor de MGMT en población mexicana con glioblastoma que recibe tratamiento con carboplatino y vincristina. Si bien el tamaño de la muestra es una limitante importante, que nuestro estudio puede abrir campo para evaluar biomarcadores en nuestra población y probar esquemas de quimioterapia mas accesibles en países con economías emergentes.

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Clinical prognostic factors in adults with astrocytoma: Historic cohort

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ABSTRACT

Objective: To explore the clinical prognostic factors for adults affected with astrocytoma.
Patients and methods: Using a historic cohort, we selected 155 clinical files from patients with astrocytoma using simple randomization. The main outcome variable was overall survival time. To identify clinical prognostic factors, we used bivariate analysis, Kaplan Meier, the log rank test and the Cox regression models. The number of lost years lived with disability (DALY) based on prevalence, was calculated.
Results: The mean age at diagnosis was 45.7 years. Analysis according to tumour stage, including grades II, III and IV, also showed a younger age of presentation. Kaplan-Meier survival estimates showed that tumour grade, Karnofsky status (KPS) ≥70, resection type, chemotherapy, radiotherapy, alcohol consumption, familial history of cancer and clinical presentation were significantly associated with survival time. Using a proportional hazard model, age, grade IV, resection, chemotherapy + radiotherapy and KPS were identified as prognostic factors.The amount of life lost due to premature death in this population was 28 years.
Conclusion: In our study, astrocytoma was diagnosed in young adults. The overall survival was 15 months, 9% (n = 14) of patients presented a survival of 2 years, and 3% of patients survived 3 years. On average the number of years lost due to premature death and disability was 28.53 years.
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1. Introduction

Cancer prognoses have significantly improved over the past 40 years. For instance, in 1975, the 5-year overall survival (OS) rates for breast and prostate cancer were 75% and 68%; currently, these rates are 90% and nearly 100%, respectively [1]. However,, the 5- year OS rate has increased by only 13% (22–35%) for central nervous system (CNS) tumours [1].

Malignant CNS tumours disproportionately contribute to cancer mortality, especially for high-risk age groups. In the USA, such tumours are the second and fifth leading causes of cancer mortality in men and woman, respectively, aged 20–39 years [2]. In Mexico brain tumour represent 2.8% of the mortality due to neoplasms and exhibited an incidence of 3.5 per 100,000 habitants in 2014 [3].

Astrocytomas are the most common and lethal CNS tumours, and the majority of these tumours are classified as grade IV (glioblastoma) (4.37 per 100,000 individuals) [2]. The OS for astrocytomas is 15 months despite the best treatment available, and only approximately 2% of all glioblastoma patients survive longer than 36 months [4].

The prognosis for astrocytomas can be predicted using specific clinical factors, which allow neurosurgeons and neuro-oncologists

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to define the best treatment for each patient. Certain patient features, such as age, gender, performance status and tumour localization, have been studied as potential prognostic factors [5]. However, few studies have reported demographic and clinical characteristics and prognostic factors in adult patients in Mexico [6–8].

The aim of this study was to explore the clinical and sociodemographic variables that affect OS in adult Mexican astrocytoma patients.

2. Patients and methods

2.1. Design

Study design. For this historical cohort, we used data from the National Institute of Neurology and Neurosurgery of Mexico (NINN) (Instituto Nacional de Neurología y Neurocirugía of México) from 2008 to 2014. The NINN is a national referral centre that receives patients from all geographic areas of the country. Clinical data were collected from pathological astrocytoma diagnoses until the time of death or last appointment.

Setting. The data featured 1100 patients diagnosed with a tumour of the central nervous system (CNS). Studies have reported that 33% of tumours treated at the NINN are gliomas [6]. The data show a confidence level of 95%, precision of 5% and proportion of at least 12% of surviving patients at two years of follow-up [9]. Three hundred records, were selected by simple randomization: from these, 155 patients who met the criteria for eligibility were included in the data analyses. The total CNS tumours and approximately 43% of all gliomas treated at the NINN during 7 years.

Eligibility criteria. Diagnoses of astrocytoma were confirmed by a certified pathologist; all individuals were naive to treatment and elected to receive diagnostic, therapeutic or surgical care. Other gliomas, tumours with components of other gliomas and metastases were excluded. All patients included were adults, more than 18 years old).

2.2. Variables

All variables were collected from clinical files. The main outcome variable was overall survival, which was defined as the time between diagnosis and the time of death or last appointment. The time of diagnosis was considered the date of surgery. The time of death was taken from the death certificate or active surveillance. Additionally, demographic and clinical data were collected and analysed; these variables were defined and used as follows. The tumour grade was determined by a certificated pathologist, who is an expert in neuro-diagnosis based on the World Health Organization (WHO) criteria [10]. The degree of resection was based on computed tomography (CT) or magnetic resonance imaging (MRI) results. The familial history of cancer was determined from the clinical file. Alcohol consumption, smoking history and comorbidities were self-reported in the clinical file. The Karnofsky status (KPS) was dichotomized as $\geq$70 and <70 based on work from Ewelt et al., who retrospectively analysed a large cohort of elderly glioblastoma multiform (GBM) patients and found that a preoperative KPS score ~70 was associated with significant survival benefits [11]. Socio-economic status was studied and classified by a social worker; personal and familial income, expenses, occupation, type of dwelling and health status were considered (a score of 1–2 represents low class, 3–4 represents medium class, and 5–6 represents high class). Body mass index (BMI) was classified based on the WHO parameters. Clinical presentation was considered the main characteristic that led the patient to seek treatment.

The number of potential years of healthy life lost to premature death was calculated by subtracting the actual age at the time of death from the of life expectancy reported for Mexico (women, 77 years and men, 72 years). The number of disability-adjusted life years (DALYs) was based on prevalence. It was obtained by multiplying the expected duration of disability (until remission or death) by a disability weight reflecting the average severity of the disease compared to individuals with perfect health and those who had died. We used specific disability weights for age and, treatment for form as of sequelae included in the Global Burden of Disease study.

Table 1
Demographical and clinical characteristics.

n = 155	n (%)
Demografic variables	
Age*	45.7 ± 15.1
Sex	
Female	68 (46)
Male	87 (56)
Socio-economic status	
Low class	121 (78)
Medium class	30 (19.4)
High class	4 (16.7)
Marital status	
Single	45 (29)
Common low marriage	26 (16.7)
Marriage	60 (38.7)
Divorce	14 (9.1)
Widower	10 (6.5)
Clinical variables	
BMI	
Malnutrition	2 (1.3)
Normal	70 (45.2)
Overweight	60 (38.7)
Obese	23 (14.8)
Morbility	
Previews neoplasia	9 (5.8)
Diabetes Mellitus 2	11 (7)
Hypertension	20 (13)
History of smoking	55 (35.5)
History of alcoholism	23 (14.8)

* = years.

2.3. Statistical analysis

The demographic and clinical characteristics of the subjects were summarized and reported. The bivariate analysis was generated using independent-sample Chi square or student's *t*-tests according to the type of variable, with comparisons between patients who were living and deceased. The effect of each measured factor on time to death was identified using Kaplan Meier curves Cox, regression models, and log rank tests and were used to identify differences in survival function between subgroups. A value of $p \leq 0.05$ was considered statistically significant. All analyses were performed using SPSS v21 and STATA 12.

2.4. Ethics

The NINN Ethical Committee on human experimentation approved the use of the human subject information included in this study. Internal codes were used to preserve patient confidentiality.

3. Results

3.1. Demographical characteristics

In total, 155 patients with astrocytomas were included in the analysis. The demographic data are shown in Table 1. The mean age at diagnosis was 45.7 ± 15.1 years, and a familial history of

cancer was present in 26.5% of the patients (n = 41). The remaining variables are described in Table 1.

3.2. Tumour characteristics

Ten patients (6.5%) were classified as grade I, 19 were classified as grade II (12.3%), 36 were classified as grade III (23.2%), and 90 were classified as grade IV (58%).

The mean tumour volume was 16.77 ± 11.79 cm. The mean age of patients with grade I tumours was 26; the grade II patient mean age was 39; the grade III patient mean age was 42; and the grade IV patient mean age was 49 years. The tumour localizations were 72 (46%) in the right hemisphere and 72 (46%) in the left hemisphere; the remaining 8% reached both hemispheres. By area, 52 (34%) tumours were found at the frontal lobe, 37 (24%) tumours were found at the temporal lobe, 32 (21%) tumours were found at the parietal lobe, 7 (4%) tumours were found at the occipital lobe, and the remaining 27 (17%) tumours were found in other areas, such as the thalamus, intraventricular area, insular area, brainstem and corpus callosum.

3.3. Clinical presentation and treatment

The main clinical presentation was headaches in 48 (31%) patients followed by motor deficit in 35 (23%) patients, generalized seizures in 35 (21%) patients, changes in mental function in 18 patients (12%) and focal seizures in 13 patients (8%); the remaining 6 (5%) patients mainly presented with other symptoms.

The first surgery consisted of total resection in 55 (35%) patients, a partial/incomplete surgery in 79 (51%) patients and stereotactic biopsy in 21 (14%) patients. After surgery, only 9% of patients presented a complication (oedema, haemorrhage or hydrocephaly).

The mean of number of surgeries was 1.24 with a range of 1–4 surgeries. After the surgery, the KPS status was ≥70 in 130 (84%) patients and <70 in 15 (16%) patients.

Fifty-six patients (87%) received radiotherapy; of these, 97% received standard focal brain radiotherapy (60 Gy in 28–30 cycles), and 3% received hypo-fractionated radiotherapy. Only 40% of the patients received chemotherapy. Temozolamide was offered to all patients, but only 10 could afford this treatment. The remainder received other available drugs based on the clinical experience at the NINN (carboplatin, chloroquine, vincristine, cisplatin, and carmustine) [12–15].

A comparison of the prognostic variables between the living and deceased groups is shown Table 2. The variables that differed between groups included age, tumour grade, KPS, resection type and chemotherapy. Alcohol intake also exhibited a positive association with death; for the group of patients who indicated that they frequently drank alcohol, 17% were living, while 83% were deceased.

3.4. Status and survival time

The mean follow-up period was 17.3 months (1–109 months). At the time the article was written, 29% of the patients were living with the disease, 4% [6] were living without the disease, 61% [94] had succumbed to the disease, 1% [2] has succumbed to another cause, and 5% [8] were lost to follow-up. The OS rate was 15 months; 9% (n = 14) of patients presented a survival of 2 years, and 3% presented a survival of 3 years (Fig. 1).

The Kaplan-Meier survival estimates showed that tumour grade, KPS status >70, resection type, chemotherapy, radiotherapy, alcohol consumption, familial history of cancer and clinical presentation were significantly associated with survival time (Figs. 2 and 3). Age, BMI, gender, tumour volume, socioeconomic and civil status, smoking status, comorbidities, familial history of cancer, and number of surgeries were not associated with OS.

Regarding the proportional hazard ratio the variables age, grade, resection type and KPS were significant, but in the multivariable model the significance was lost for the variables alcoholism, family history of cancer and clinical presentation. This loss could be due the sample size or could that that these variables are related to the time of death but not mortality (Table 3). No significant differences were found when only GBM was included in the model (data not shown).

Table 2
Differences between alive and dead patients.

Variable	Alive patients n (%) 51	Dead patients n (%) 96	p
Age	41+- 14.57	48 ± 14.8	0.0034*
Grade			
I	5 (9.8)	3 (3.15)	0.0000*
II	13 (25.45)	5 (5.21)	
III	16 (31.37)	17 (17.71)	
IV	17 (33.33)	71 (73.96)	
Gender			
Male	25 (49.03)	56 (58.33)	0.280
Female	26 (50.98)	40 (41.67)	
Localization			
Frontal	20 (39.22)	26 (27.08)	0.236
Parietal	13 (25.49)	18 (18.75)	
Occipital	2 (3.92)	5 (5.21)	
Temporal	11 (21.57)	26 (27.08)	
other	5 (9.8)	21 (21.88)	
KPS + = 70	10 (19.61)	43 (44.79)	0.002*
KPS–70	41 (80.39)	53 (55.21)	
Resection			
Biopsy	1 (1.96)	19 (19.79)	0.000*
Partial	21 (41.18)	54 (56.25)	
Total	29 (56.86)	23 (23.96)	
QT			
YES	30 (58.82)	65 (67.71)	0.002*
NO	21 (41.18)	31 (32.29)	
RT			
YES	34 (66.67)	52 (54.17)	0.288
NO	17 (33.33)	44 (45.83)	
Alcohol intake	4 (7.84)	19 (19.79)	0.058
	47 (92.16)	77 (80.21)	
Smoke			
yes	16 (31.37)	16 (31.37)	0.460
No	35 (68.63)	35 (68.63)	
Familiar with cancer			
yes	18 (35.25%)	20 (64.71)	0.057
No	33 (64.71)	76 (79.17)	
Clinical presentation	17 (33.33)	29 (30.21)	0.235
Headache			
Deficit motor	7 (13.73)	26 (27.08)	
Generalized convulsive crisis	14 (27.45)	17 (17.71)	
Other	13 (25.49)	24 (25)	

* = Statistical significance.

Table 3
Proportional hazards model.

	HR	[95% Conf. Interval]	P
Age	1.04	1.02 1.05	0.000
Grade I	Reference		
Grade II	0.5	0.09 13.87	0.38
Grade III	0.95	0.57 48.32	0.94
Grade IV	5.05	1.46 17.50	0.01
Biopsy	Reference		
Partial resection	0.46	0.26 0.64	0.000
Resection total	0.30	0.16 0.58	0.000
Chemotherapy + Radiotherapy	0.3	0.22 0.54	0.000
KPS < = 70	Reference		
KPS > 70	0.4	0.26 0.64	0.000

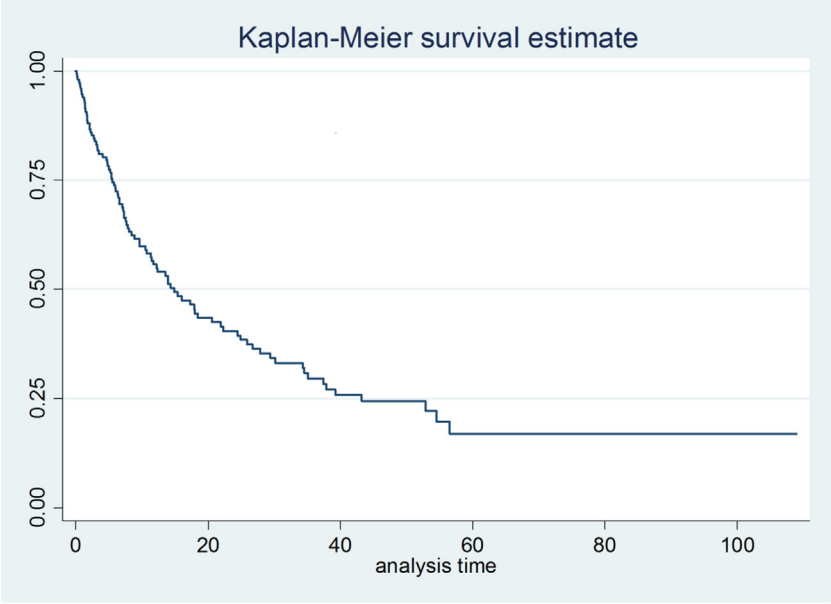

Fig. 1. Kaplan-Meier survival time. n = 155, the overall survival time was 15 months.

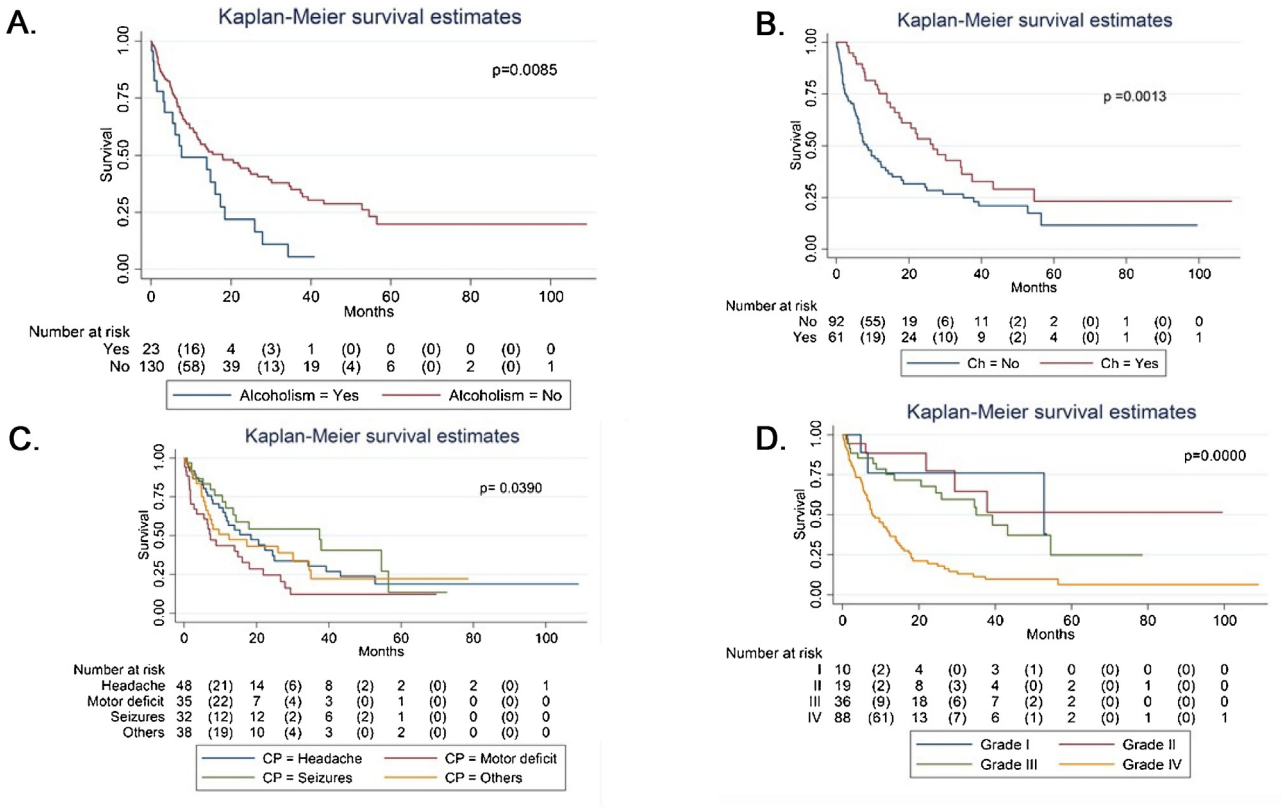

Fig. 2. Kaplan-Meir survival estimates (A) alcohol consumption (B) chemotherapy (C) CP: clinical presentation (D) grade.

The average the number of DALYs was 28.53 years. Based on this data, we assume that by 2020 there will be 33,799 prevalent cases, resulting in a total loss of 964.262 years.

4. Discussion

In the study sample, the average age of death was of 46 years which indicates the challenge that this disease could represent for the Mexican Health System; these data also indicate that, in our population, this disease presents among a younger population that

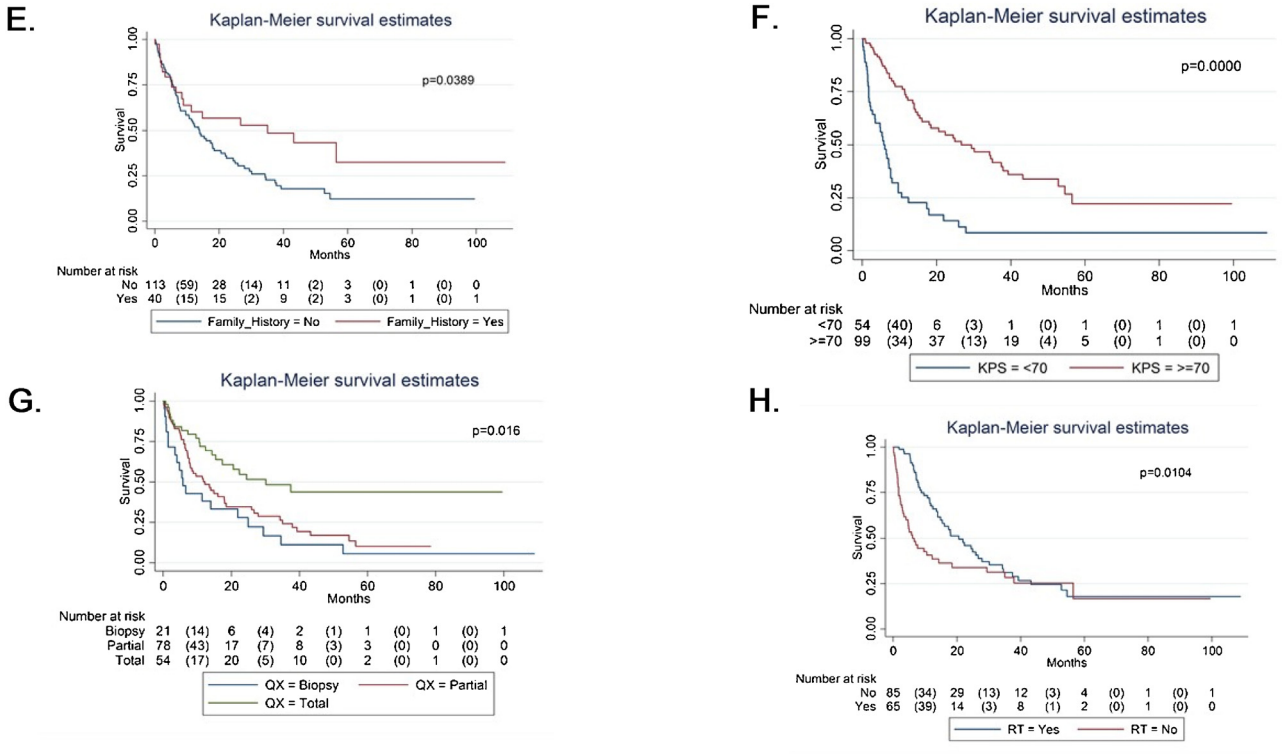

Fig. 3. Kaplan-Meier survival estimates (E) family history of cancer. (F) Karnofsky status (G) resection type (H) radiotherapy.

is still economically productive. Therefore, this disease represents an economic and social burden for the entire family [5].

The mean age at diagnosis in our study was 45.7 years, which is 15 years younger than that reported elsewhere. Indeed, age is a consistent prognostic factor for patients with astrocytoma [5,16–19]. In multiple series, patient age is the single most important prognostic factor, and the median age at diagnosis is ~65 years. Among elderly patients, the median survival is markedly reduced to only 4–5 months in GBM patients. The shorter survival among elderly patients may be due to less favourable tumour biology, less aggressive care, treatment toxicity due to lower physiological reserves, and comorbidities that may also shorten the lifespan. For GBM, the best predictor is patient age; patients aged ≥50 years exhibit a shorter survival than patients <50 years old. In GBM patients aged >50 years with favourable factors (KPS > 70), complete surgical resection, preserved neurocognitive function, and the ability to perform active work), the median survival is 11.1 months with a 2-year survival rate of 15% [20–22]. We hypothesized that survival time may be influenced by the mean age at diagnosis because our population was diagnosed at a younger age. The explanation for this young age at presentation could involve many factors; it could be due the demographical transition of the population, in which the younger population is growing in proportion to the older population [23]. Environmental exposure could also be another cause, but this is much more difficult to prove due to the geographical diversity of our population. Another explanation could be genetics factors such as germinal mutation *TP53* or Lynch syndrome genes [18,24–26]. Multidisciplinary investigations are required in which epidemiologists, clinical and basic researchers, work together to understand this phenomenon.

Only 5.8% (n = 9) of patients showed a positive history for a previous neoplasm, but up to 41 patients (26.5%) showed a family history of cancer. A familial history of cancer has been suggested to be a protective factor. One explanation for this finding is that having a family member with cancer could lead to patient awareness of the symptoms. Another explanation is that certain hereditary syndromes are related to astrocytoma, such as Lynch syndrome, for which the general prognosis of colon cancer is better than in patients without MMR mutations [27]. In this cohort, the incidence of a familial history of cancer should be considered with caution because it was indirectly collected from the file and, therefore, may have been underestimated. Another limitation is that no molecular data were included in our analysis.

Alcohol consumption was statistically associated with a poor prognosis in the Kaplan Meier analysis. Alcohol is a known neurotoxic and carcinogenic agent; indeed, it is classified by the IARC as a class 1 carcinogen, and its chronic use is a known risk factor for oral cavity, pharynx, larynx, oesophagus, liver, colorectal and breast cancer [28]. As a risk factor for astrocytoma, alcohol has been widely studied with contradictory results. One of the most significant studies featured a cohort of 41,514 patients, in which the participants' history of alcohol consumption was statistically associated with a risk of GBM in a dose-response relationship [29]. In our study, alcohol consumption was determined from the patient's clinical file and was defined through the patient self-reporting as a heavy drinker. When this variable was analysed in the multivariable model, an effect was not observed, likely because only 23 patients were heavy drinkers. Thus, more studies designed to analyze this comorbidity should be performed; it may be especially interesting to determine whether modifying alcohol consumption improves astrocytoma patient outcomes. Only a few studies have addressed the role of alcohol consumption and prognosis in astrocitoma patients [30]; to our knowledge, ours is the first study to identify a significant association. This finding is important because alcohol is a modifiable factor that can be control by the patient.

Among the risk factors that show statistical significance in early mortality, one that deserves special attention is the presence of motor deficit. The corticospinal tract begins in the V layer of the cerebral cortex and passes though the corona radiata, internal capsule, cerebral peduncle, pons, and medulla oblongata, where it

crosses to form the lateral corticospinal tract in the spine. The anatomical dispositions of the corticospinal fibres and the association fibres of the motor system act as an effective dissemination route for tumour cells and render them more infiltrative with a greater capacity for an invasion. The final effect of these features is early recurrence and progression, which yield a worse prognosis.

In these patients, the KPS post-op score is 70 or less due to motor deficit, which is associated with poor survival. Moreover, the time that these patients spend in bed could contribute to the development of conditions such as pneumonia, urinary tract infections, and skin lesions as well as other comorbidities.

The main limitation of our study was the economic status of the studied population; 78% of the population was classified as low income. This fact has an important impact on our data because not all patients received post-surgery MRI analysis. Further, not all patient received radiotherapy; only 10 patients received the standard treatment, which included radiotherapy and temozolamide. This characteristic of our population is a limiting factor for comparisons with the international standards, but it may be an advantage for the generalizability of this study because these data may be applied to other developing countries. Further, this limitation provides a window of opportunity for developing research in populations that do not meet international standards. Additional studies are needed to determine the best options for the patients who cannot afford the Stupp protocol.

This study was performed in a National Health Institution that specializes in neurology and neurosurgery. Patients who reside in all areas of the country, although most are from the centre region, are referred to this institution for diagnosis and treatment. It is important to mention that only 13 neurological hospitals in Mexico are recognized by the Mexican board of Neurosurgeons (including 8 in Mexico City). The NINN is the biggest, and one of the 3 health centres which depends from the National Ministry of Health [31]. The localization of the NINN could be a source of potential bias because patients with no resources for traveling or with a low KPS might not be included in our cohort. However, as mentioned, we classified 78% of our population as low income, and data from the general population (INEGI) described 59% of the population as low income. The KPS status of our patients was similar to that in the international literature [16]. So our data represents the socioeconomic status of Mexican population.

5. Conclusion

In our cohort, the main age at diagnosis was 20 years younger than that of other populations [2,5,32], and the loss of this working-age group likely has a significant impact on society. Possible explanations for this phenomenon should be addressed in futures studies. Most prognostic factors associated with OS in our study are consistent with previous reports in the literature, including age, tumour grade, resection type, radiotherapy, chemotherapy and KPS [33]. A family history of cancer was also a good prognostic factor in our study, which should reinforce physician awareness of performing a complete family history for all patients. Interestingly, alcohol consumption was a poor prognostic factor; thus, future studies should focus on this variable because it is a modifiable factor. Motor deficit was also identified as a poor prognostic factor. The OS rate was 15 months, with 9% of patients showing 2-year survival, and 3% of patients showing long-term survival (more than 3 years).

Conflicts of interest

None.

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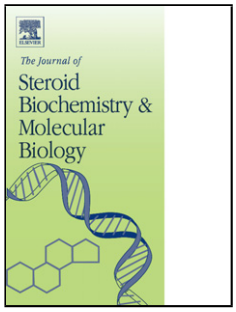

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The interplay between intracellular progesterone receptor and PKC plays a key role in migration and invasion of human glioblastoma cells

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Highlights:

- PR participates in migration and invasion of glioblastoma cells via PKC.
- There is a cross-talk between PKC and PR in human glioblastoma progression.
- The activation of PKCα and PKCδ induces PR transcriptional activity.

Abstract

Intracellular progesterone receptors (PRs) and protein kinases C (PKCs) are known regulators of cancer cell proliferation and metastasis. Both PRs and PKCs are found overexpressed in grade IV human astrocytomas, also known as glioblastomas, which are the most frequent and aggressive brain tumors. In the present study, we investigated whether PR activation by PKC induces the migration and invasion of glioblastoma derived cell lines and if PKCα and δ isoforms are involved in PR activation. We observed that PKC activation with tetradecanoylphorbol acetate (TPA) increases the migration and invasion capacity of two glioblastoma derived human cell lines (U251 MG and U87) and that the treatment with the PR receptor antagonist RU486 blocks these processes. Interestingly, the pharmacological inhibition of the isoenzymes PKCα and PKCδ also resulted in a blocked PR transcriptional activity. Also, TPA-dependent PR activation increases the expression of progesterone-induced blocking factor (PIBF), a known PR target gene. These results hint to an existing cross-talk between PKCs and PRs in regulating the infiltration process of human glioblastomas.

Key words: glioblastoma, progesterone receptor, PKCα, migration, invasion, PIBF.

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1. Introduction

The most frequent and aggressive human brain tumors are astrocytomas, which are glial cell derived tumors (gliomas) with high malignant potential. They originate anywhere in the brain but are mainly located in the cerebral cortex, appearing more frequently in adults between 50 and 70 years old [1]. Astrocytomas are classified by WHO according to their histopathological and molecular features into four grades (I-IV), where grade IV, also known as glioblastoma (GBM), represents the maximal evolution stage and is one of the most lethal human malignancies [1]. The median survival in GBM patients is 12-15 months [2,3], and only 3-5% of the patients survives for more than 3 years [4].

Progesterone (P4) plays different physiological roles in the organism [5–7]; however, it has also an important participation in pathological processes, *e.g*. cancer. In the human breast cancer cell line T47-D, this hormone is able to induce cell cycle progression by promoting the expression of cyclins [8,9], proliferation associated genes (c-myc, c-fos) [10], and growth factors (EGF, VEGF, TGF-α) [11] as well as their receptors (EGFR) [10,11].

Some of the effects exerted by P4 on cell proliferation, differentiation, and cell death are mediated by the progesterone receptor (PR), located in the cytoplasm and nucleus of target cells [12]. In humans, two PR isoforms with different functions and regulation have been characterized: PR-A (94 kDa) and PR-B (116 kDa) [13]. When bound to DNA, PR interacts with components of the basal transcriptional machinery, recruiting co-regulator proteins thus modulating gene transcription [14].

PR is expressed in human astrocytomas [15–17] and its expression is directly related to tumor progression, suggesting that astrocytomas overexpressing PR have a high rate of cell proliferation and infiltration [17]. It has been reported in *in vitro* and *in vivo* experiments that the treatment with P4 increases the number of cells of a grade III human astrocytoma derived cell line (U373), and this effect was blocked with RU486 (PR antagonist) [18,19]. Moreover, two additional studies report an increased growth rate in glioblastoma tumors during pregnancy (where P4 levels are considerably elevated) [20,21]; Besides, a case study reported that

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the life of a 43-year-old man with glioblastoma was extended after treatment with RU486 [22], suggesting PR participation in tumor growth.

PR activation not only depends on the direct binding of its own ligand but also on post-translational modifications such as phosphorylation. There is evidence that different cell signaling pathways promote PR phosphorylation and hence regulate its transcriptional activity [23]. Until now, fifteen phosphorylation sites have been identified for the human PR [24–28].

Phosphorylation of PR through various signaling pathways involves different kinases: casein kinase II (CKII) [27], cyclin-dependent kinase 2 (CDK2) [24,26], mitogen activated protein kinases 44 and 42 (MAPK 44/42) [25], glycogen synthase kinase-3β (GSK3β) [28], and recently, we have reported PKCα and PKCδ [29].

PKCs are a family of proteins that consists of 12 isoforms. They are divided into three subfamilies based on the second messenger they require for activation: conventional, novel, and atypical [30]. The conventional PKCs include α, ßI, βII, and γ isoforms and need Ca^{2+}, diacylglycerol (DAG) and phospholipids such as phosphatidylcholine for activation. The novel PKCs comprise δ, ε, η, and θ isoforms, which require DAG, but not Ca^{2+} for their activation. Finally, the atypical PKCs include ζ, ι and λ isoforms and require neither Ca^{2+} nor DAG for activation [30].

PKCs activate a wide range of proteins such as enzymes, cell cycle regulators, transcription factors, structural proteins, etc. Therefore, the PKC signal transduction pathway plays a crucial role in regulating metabolism, cell proliferation, differentiation, survival, migration, and angiogenesis. The relevance of PKC has been further studied in pathological processes such as cancer. In gliomas, PKCα, PKCδ, and PKCε have been associated with invasive and apoptotic processes along with an increase in oncogenes expression [31–33] .

In a previous study, we identified a putative residue (Ser400) in PR that can be phosphorylated by PKCα and PKCδ in grades III and IV human astrocytoma derived cell lines [29]. The phosphorylation of Ser400 induced PR transcriptional activity and its subsequent degradation by the 26S proteasome [29]. The obtained

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data demonstrate a key role of PKCs in PR regulation and function in human astrocytoma cells; however, the relevance of PR and PKC interplay in cell migration and invasion of human astrocytomas has not yet been addressed. Therefore, the present study aims to clarify whether PR activation by PKC induces the migration and invasion of glioblastoma derived cell lines and if PKCα and δ isoforms are involved in the activation of the receptor.

2. Materials and Methods

2.1 Cell Culture and Treatments

Human glioblastoma derived cell lines U251 MG and U87 (ATCC, VA, USA) were grown in 10 cm dishes and maintained in DMEM medium (In vitro, S.A., D.F., MEX), supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids (GIBCO, NY, USA) at 37°C, 5% CO_2. 24 h before treatment, phenol red and hormone-free DMEM medium (In Vitro, S.A., MEX) supplemented with 10% charcoal-stripped FBS, 1 mM pyruvate, 1 mM antibiotics and 0.1 mM non-essential amino acids (Biowest, USA) was added to the cultures. Tetradecanoyl phorbol acetate (TPA 1 µM Sigma-Aldrich, MO, USA) was used to activate classical and novel PKCs. PKCα and PKCδ inhibitors: Gö 6976 (1 µM) and Rottlerin (1 µM) (Sigma-Aldrich, MO, USA), respectively, were added 30 min before TPA treatment. P4 and RU486 were purchased from Sigma-Aldrich (MO, USA). RU486 (1 µM) was added to the culture media at the same time as P4 (10 nM) or TPA (1 µM).

2.2 Migration Assay

Migration assays were performed as described by Piña-Medina et al., 2016 [34]. Briefly, U251 MG and U87 cells were grown to confluence and scratched with a pipette tip to make a thin “wound”. The medium was aspirated to remove cell debris and phenol red and FBS-free DMEM was added. Cell proliferation inhibitor Ara-C (10 µM) was added 1 h before treatment. Cell migration was monitored at 0, 24 and 48 h after treatment using an inverted microscope (Bx43; Olympus, PA, USA). The number of cells migrating into the “wound” was counted using the

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ImageJ 1.45S software (National Institutes of Health USA). The images were captured with an Infinity1-2C camera at a 20X magnification.

2.3 Invasion Assay

Invasion experiments were performed as described by Piña-Medina et al., 2016 [34]. Briefly, Transwell chambers (Corning, NY) were covered with 2 mg/mL of Matrigel supplemented with the DNA synthesis inhibitor AraC (Sigma Aldrich, St. Louis, MO) and left at room temperature for gelation. 300,000 U251 MG and U87 cells suspended in 1.5 mL serum-free medium and treatments were added to the upper chambers. The lower chambers were filled with 2 mL DMEM with 10% FBS. The chambers were incubated at 37°C, 5% CO_2 for 24 hours. After incubation, the Matrigel with the non-invading cells was removed from the upper surface of the transwell membrane. The invading cells were fixed in 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 20 min. Then, the inserts were observed under an inverted microscope (Olympus CKX41) and the images of 5 randomly selected fields were captured with an Infinity1-2C camera at 40X magnification.

2.4 Luciferase Activity Assay

For transient transfections, $5x10^4$ U251 MG cells were seeded in hormone-free medium (phenol red free DMEM with 1% charcoal-stripped FBS) into 24-well plates. 24 h later, cells were transfected with 200 ng of Mouse Mammary Tumor Virus-luciferase (MMTV-Luc, with two PRE in its promoter) reporter construct and 20 ng of pCytomegalovirus-β-galactosidase (pCMV-β-gal) expression vector as an internal control using Lipofectamine 2000 (Invitrogen, Life Technologies, CA, USA) according to the manufacturer´s protocol. After 48 h, cells were treated with vehicle (V, DMSO 10%); P4 (10 nM); TPA (1 µM); P4+Gö (PKCα inhibitor 1 µM); P4+Rott (PKC δ inhibitor 1 µM); TPA+Gö and TPA+Rott for 24 h. In all cases, cells were harvested in luciferase lysis buffer (100 mM potassium phosphate buffer pH 7.8 containing 1% Triton X-100 and 1 mM DTT). Relative light units (RLU) from firefly luciferase activity in cell lysates were measured using a Monolight 3010

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luminometer (BD Biosciences, NJ, USA) and were normalized to the level of β-galactosidase activity measured in the same sample after incubation with Galacton-Star substrate (Tropix Inc., MA, USA) for 60 min. Transfection data are shown as the ratio of luciferase RLU/β-galactosidase RLU [29]. The results represent at least three independent experiments.

2.5 RT-PCR

Total RNA was isolated from U251 MG cells 12 h after treatment with either V (DMSO 10%), P4 (10 nM), TPA (1 μM), TPA+RU486 (1 μM) or RU486, with the single-step method based on guanidine isothiocyanate-phenol-chloroform extraction according to the TRIzol reagent manufacturer's protocol (Invitrogen, CA, USA). RNA was quantified using the spectrophotometer Nanodrop-2000 (Thermo Scientific, MA, USA). The cDNA was synthesized with the SuperScript II reverse transcriptase (Invitrogen, CA, USA) using 3 μg of total RNA and oligo $(dT)_{12-18}$ primers (Sigma–Aldrich, MO, USA) according to its protocol. 3 μL of RT reaction were subjected to PCR in order to amplify a gene fragment of a PR regulated gene known as progesterone-induced blocking factor (PIBF) that increases glioblastoma cell proliferation [35], and the internal control 18S ribosomal RNA. The sequences of the specific primers for PIBF amplification were 5′-GACAGAGCCAATTCGCTATTAAACCAGACTCAACAGC -3′ for the sense primer and 5′-GCTGAGTACACGATTAAGCTGAATTTTGTTTTCCATCAG -3′ for the antisense primer; and those primers amplifying the 18S were 5′-CGCGGTTCTATTTTGTTGGT-3′ for the sense primer and 5′-AGTCGGCATCGTTTATGGTC-3′ for the antisense (Sigma–Aldrich, MO, USA). The 25 μL PCR reaction included: 3 μL of previously synthesized cDNA, 2.5 μL 10X buffer PCR, 1.25 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 μM of each primer, and 2.5 units of Taq DNA polymerase. Negative controls without RNA and with non-retrotranscribed RNA were included in all the experiments. After the initial denaturation step at 94°C for 5 min, PCR reaction was performed for 30 cycles. The cycle profile for PIBF and 18S amplification was: 30 s at 94°C, 30 s at 68°C and 30 s at 72°C. A final extension cycle was performed at 72°C for 5 min. The

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number of performed cycles was within the exponential phase of the amplification process [35]. The PCR products were resolved on a 2% agarose gel and stained with GelRed™ (Biotium, HAY, CA, USA) and the image was captured under a UV transilluminator. The intensity of PIBF and 18S bands was quantified by densitometry using the ImageJ software (National Institute of Health, WA, USA). Gene expression levels were normalized to those of 18S.

2.6 Western blot

$1x10^6$ U251 MG cells were grown in 10 cm dishes and maintained as described in the "Cell culture and Treatments" section. 24 h before treatment, the medium was changed for phenol red free DMEM and cells were incubated at 37°C under a 5% CO_2 atmosphere. Four experiments were independently performed. Cells were treated with vehicle (V), DMSO 10%; PKCα inhibitor Gö 6976 (Gö 1 µM) or PKCδ inhibitor Rottlerin (Rott 1µM) for 24 h. After treatment, cells were homogenized in RIPA lysis buffer with protease inhibitor cocktail (1 mM EDTA, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM PMSF). Proteins were obtained by centrifugation at 12500 rpm, at 4°C for 15 min and quantified using the spectrophotometer NanoDrop-2000 (Thermo Scientific, MA, USA). Approximately 70 µg of total extracted protein were resolved in an 8% SDS-PAGE at 20 mA. Colored markers (Bio-Rad, CA, USA) were included as a molecular weight standard. Gels were transferred to nitrocellulose membranes (Millipore, MA, USA) for 7 h (35 mA, at room temperature in semi-dry conditions). Membranes were blocked with 3% non-fat dry milk and 1% bovine serum albumin at room temperature for 2 h. Membranes were incubated with the following antibodies: PR isoforms (3.3 µg/mL) (ab58565, Abcam, Cambridge, ENG), PKCα or PKCδ (0.66 µg/mL; sc-8393, sc-213, respectively; Santa Cruz Biotechnology, TX, USA) at 4°C, overnight. Blots were then incubated with anti-mouse or anti-rabbit secondary antibodies (1:5000 or 1:7500, respectively) conjugated to horseradish peroxidase (Santa Cruz Biotechnology, TX, USA) for 45 min at room temperature. In order to correct for differences in the amount of total protein loaded in each lane, protein content was normalized to that of α-tubulin. Blots were stripped with glycine (0.1 M,

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pH 2.5, 0.5% SDS) at 4°C overnight and at room temperature for 30 min, and reprobed with 0.2 µg/mL of mouse anti-α-tubulin monoclonal antibody (sc-5286, Santa Cruz Biotechnology, TX, USA) at 4°C overnight. Blots were incubated with a 1:3000 dilution of goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, TX, USA) at room temperature for 1 h [29]. Chemiluminescent signals were detected exposing the membranes to Kodak Biomax Light Film (Sigma-Aldrich, MO, USA) using Western blotting substrate (Thermo Scientific, MA, USA). The antigen–antibody complex was detected as the area under a peak corresponding to a band density (the area is given in inches with a default scale of 72 pixels/inch) in a semiquantitative way using the ImageJ 1.45S software (National Institutes of Health, USA). In order to minimize inter-assay variations, all Western blots were carried out in parallel.

2.7 Statistical Analysis

All data were analyzed and plotted using the GraphPad Prism 5.0 software for Windows XP (GraphPad Software, CA, USA). Statistical analysis between comparable groups was performed using a one-way ANOVA with a Bonferroni post-test. For post-test, a value of $P<0.05$ was considered statistically significant as stated in figure legends.

3. Results

3.1 TPA-induced cell migration and invasion is decreased by PR blockade

In the present study, we explored the involvement of PR in cell migration and invasion induced by PKC activation. TPA treatment induced a 10-fold increment in the number of U251 MG migrating cells ($p<0.001$ for 24 and 48 h) and a 2-fold increase in U87 cells ($p<0.001$ for 24 and 48 h) when compared with control cells (10% DMSO vehicle) (Fig. 1A and B). Interestingly, the PR antagonist RU486 diminished TPA effect by 55% ($p<0.001$ for 24 and 48 h) in U251 cells and 20% ($p<0.01$ and $p<0.05$ for 24 and 48 h, respectively) in U87 cells (Fig. 1B). The PR antagonist RU486 did not exert any significant effect on cell migration when compared with control cells (Fig. 1B). Concerning the invasion assays, PKC

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activation increased the number of invasive cells in both U251 and U87 cell lines as compared with vehicle-treated cells 24 hours after treatment (Fig. 2). The presence of cell clusters after TPA treatment, unobserved with other treatments, is noteworthy.In fact, managing the counting of single cells within these cell clusters became a difficult task. As in the case of migration, RU486 blocked the invading effect of TPA (Fig. 2). Treatment with RU486 alone neither increased nor modified the number of invading cells as compared with the vehicle in both cell lines (Fig. 2). These results suggest that the PKC/PR activation pathway regulates the processes of cell migration and invasion of glioblastoma cells.

3.2 PKCs α and δ induce PR transcriptional activity

To further elucidate the role of PKC in PR activation, we explored whether pharmacological inhibition of α and δ isoenzymes, which we previously reported that participate in PR phosphorylation [29], can modify PR transcriptional activity when induced by TPA. To achieve this, U251 MG cells were transfected with the MMTV-Luc reporter plasmid carrying two progesterone response elements (PRE) in its promoter, and then treated with TPA, P4 and/or PKCα (Gö 6976 1 μM) or PKCδ (Rottlerin 1 μM) inhibitors. The addition of P4 or TPA induced a 2 fold increase in PR transcriptional activity ($p<0.001$) when compared with vehicle (Fig. 3A); as hypothesized, the treatment with PKCα inhibitor reduced 33% luciferase activity induced by TPA ($p<0.05$) (Fig. 3A) whereas PKCδ inhibitor completely blocked PR transcriptional activity ($p<0.001$) induced by P4 or TPA ($p<0.001$).

We also evaluated PR, PKCα, and PKCδ content after treatment with either Gö 6976 or Rottlerin in order to fully determine if the observed effects in PR activity were due to PKC activity block rather than to modifications in their respective protein expression. The content of PR, PKCα or PKCδ was not modified after 24 h of treatment with PKC inhibitors. These results suggest that changes in PR transcriptional activity are due to the pharmacological inhibition of PKCα and δ isoforms and not to changes in PKCs or PR protein content (Fig. 3B).

3.3 **PR activation by PKC increases PIBF expression**

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In order to evaluate the effect of TPA over a PR target gene, we analyzed the expression of PIBF, a protein known to participate in the growth of astrocytoma cells [29]. We treated cells with TPA for 12 h and evaluated PIBF mRNA expression (Fig. 4). We observed that TPA treatment increased PIBF expression in ~50% ($p<0.05$) when compared with vehicle, and this effect was even higher than that observed with P4 (Fig. 4). Interestingly, the use of the PR antagonist RU486 blocked TPA-dependent PIBF expression. RU486 alone did not exert any effect over PIBF expression (Fig. 4).

4. Discussion

In this study, we observed that TPA activation of PKC resulted in the increased migration and invasion capacity of two human glioblastoma derived cell lines (U251MG and U87) and that the treatment of these cells with the PR receptor antagonist RU486 blocked these processes. We also observed that the pharmacological inhibition of the PKCα and PKCδ enzymes blocked PR transcriptional activity induced by TPA. We also found evidence that TPA-dependent PR activation increases the expression of progesterone-induced blocking factor (PIBF), a known PR target gene involved in cell proliferation.

Several types of cancer, including GBM, represent a great challenge when designing an effective therapeutic strategy, mainly due to their high proliferation and infiltration capabilities. In particular, GBM tumor infiltration, a process in which both migration and invasion are involved, often allows tumors to prevail despite surgical resection, chemotherapy, and radiation. In this study, we observed that the activation of PKCs by TPA induces the migration and invasion of U251MG and U87 cells, which are derived from human GBM; these processes should occur through PR since RU486 treatment reduced the number of migrating and invasive cells. Another study, performed by Piña-Medina *et al*., reported that P4 induces cell invasion and migration through PR in D54 and U251 MG cells, both derived from human GBM [34]. In a cell line derived from a human astrocytoma grade III, a 2-fold increase in cell proliferation after PKC activation with TPA was observed; the treatment with the PR antagonist RU486 reduced this effect thus suggesting that

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PR participates in the proliferation of cancer cells induced by PKC activation [29]. It must be highlighted that the increased number of migrating cells in response to TPA was due to changes in cell motility rather than to an increased cell proliferation rate, because of the treatment of these cells with the DNA synthesis inhibitor AraC. Taken together, these results suggest that the activation of PKCs is crucial to mediate PR activation that is in turn involved in regulating the migration and invasion processes.

It has been demonstrated that PR activation mechanisms, either through its own ligand or by an alternative pathway such as PKC, induces cell proliferation in astrocytomas. The cell lines U373 and D54, derived from human astrocytoma grades III and IV, respectively, show an increased proliferation rate after treatment with P4, whereas the co-administration of RU486 significantly blocked this effects in both cell lines [18]. Moreover, PKC activation also increased U373 cell proliferation which was regulated by PR [29].

It has also been reported that high doses of P4 (80 µM) exert an anti-metastatic effect on grade IV human GBM cells lines U87 and U118 MG; at this concentration, the hormone reduces cell proliferation and its combination with temozolomide decrease migration [36]. These results correlate with the effects observed after the inhibition of the EGFR/PI3K/Akt/mTOR pathway, and revealed a dual effect of P4 that is dose-dependent: at low concentrations (nM) this hormone induces proliferation and migration whereas at higher ones (µM) these processes become impaired. This dual effect is probably due to its interaction with different cell signaling pathways.

PR involvement in tumor invasiveness has been previously proposed in ES-2 and NIH-OVCAR-3 cell lines, which are derived from ovary carcinomas. Lima *et al*., 2016 reported that P4 increases mRNA expression and protein content of two proteases of the "A disintegrin and metalloproteinase with thrombospondin motifs" protein family (ADAMTS-1 and ADAMTS-4); these proteins are involved in collagen processing, cleavage of the proteoglycan matrix, and angiogenesis. The effect of P4 over ADAMTS-1 and 4 expression was reversed by RU486 treatment, indicating that P4 acts directly through its intracellular receptor to increase the

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expression of these proteases in ovarian cancer cell lines, thus favoring the invasion process [37]. PIBF is a PR target gene that is overexpressed in stomach and uterus solid tumors and in cells derived from different types of cancer i.e. ovary, cervical, breast, lymphoma, leukemia [38–41], and astrocytoma [35,42]. PIBF is known to be involved in astrocytoma cell proliferation [35,42] and it has been shown that P4 induces PIBF expression through PR activation in a cell line derived from a grade III astrocytoma (U373 MG) [35]. In this study, we show that in U251 MG cells PKCs α and δ activation increases PR transcriptional activity that correlates with an increased PIBF expression. These results suggest that the PKC/PR pathway should induce the expression of genes involved in cell proliferation.

PR activation by P4 (10 nM) increases the expression of three genes involved in cell proliferation and metastasis e.g. epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and cyclin D1, such effects were blocked by RU486 [43], suggesting that P4 effects on EGFR, VEGF, and cyclin D1 expression are mediated by the classical PR.

In vivo models also suggest an important role for PR in glioblastoma growth. A previous report showed that in nude mice bearing xenografts of U87 cells an elevated dose of the PR antagonist RU486 (0.5 mg/day, during 4 days) was highly effective in reducing tumor volume when compared with lower doses (0.1 mg/day) [44]. Another study reported that, in xenografts of U373 MG cells implanted in the motor cortex of adult male rats, P4 induced tumor infiltration and growth that were blocked by RU486 [45]. In patients, a strong PR nuclear immunopositivity has been detected in glioblastomas compared to lower grade astrocytomas [46,47]. PR gene expression was observed in 83 and 100% of biopsies from human astrocytomas grades III and IV, respectively [15].

During the last trimester of pregnancy, P4 levels reach and maintain an 8-fold increase above the basal levels. In a study of 18 patients with grade II or III gliomas, 8 (44%) confirmed tumor progression during the progression of pregnancy or [48]; also in a case report, the glioblastoma of a 36-year-old pregnant female increased its size during pregnancy [21]. These data suggest a subtle but

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important role for P4 during the progression of this type of tumors. Besides, a 43-year-old male with a GBM exclusively treated with RU486 showed definite palliative effects for several weeks after treatment, and his life was extended beyond pre-treatment predictors [22], suggesting the participation of PR in tumor growth. It should be taken into consideration that the male:female ratio of glioblastoma patients in the USA is 1.26 [49] and 1.4 in México [50], implying that other factors besides P4 are involved in tumor growth.

The phosphorylation of PR after 5 minutes of treatment with TPA enhances its transcriptional activity [29]. In this study, we observed that after the pharmacological inhibition of PKCα and PKCδ, the TPA-induced transcriptional activity of PR was blocked in 33% and 100%, respectively. Surprisingly, the treatment with Rottlerin also completely inhibited PR transcriptional activity induced by P4. These results suggest that PR activation by PKCα is important for its ligand-independent transcriptional activity while PKCδ is important for both its ligand-dependent and –independent transcriptional activity. PR can be activated in a ligand-dependent or –independent manner, and these differences lead to the regulation of specific target genes [51]. In fact, studies concerning the phosphorylation of human PR by different kinases have shown the functional role for site-specific phosphorylation, including hormone response sensitivity, DNA binding, stability, subcellular localization, dimerization, and protein–protein interactions that altogether modulate the expression of target genes. In breast cancer cells, phosphorylation of the Ser294 residue in PR is hormone-dependent, increasing ligand-dependent PR transcriptional activity [25]. Other studies revealed that CDK2 and PKCs α and δ phosphorylate PR at Ser400 in the absence of progestins, thus inducing a ligand-independent activation of PR [29,52].

It must be highlighted that Gö 6976 and Rottlerin are not only specific inhibitors of PKCα and PKCδ, since Gö6976 inhibits PKC βI and βII isoforms with less affinity, while Rottlerin inhibits other kinases such CaM-kinase III [53]. Since U251 MG cells also express PKC isoenzymes βI and βII [54] and there are no current studies concerning CaM-kinase III expression in this cell line, we cannot discard the participation of these kinases in PR activation.

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It has been reported that P4 partly induces the growth of astrocytomas by interacting with PR, however, other studies revealed that the membrane progesterone receptors mPRα and mPRβ are expressed in U251 MG and U87 cells [55]. Therefore, it should be taken into consideration that mPRs could also mediate P4 action in glioblastomas in addition to the effects of intracellular PR.

GBM are considered highly heterogeneous tumors, and despite sharing similar molecular profiles, extensive variations in gene expression and treatment response have been reported [56]. Glioma stem cells are present within these tumors and could, therefore, spawn cell sub-populations with different sensitivity to drugs, as well as different proliferation and infiltration capabilities [57]. This heterogeneity drives the need for a further in-depth study into the diverse mechanisms by which this kind of tumors proliferate, one of such being the PKC/PR signaling pathway, which has the potential to be turned into a pharmacological target for controlling glioblastoma growth.

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Figure Legends

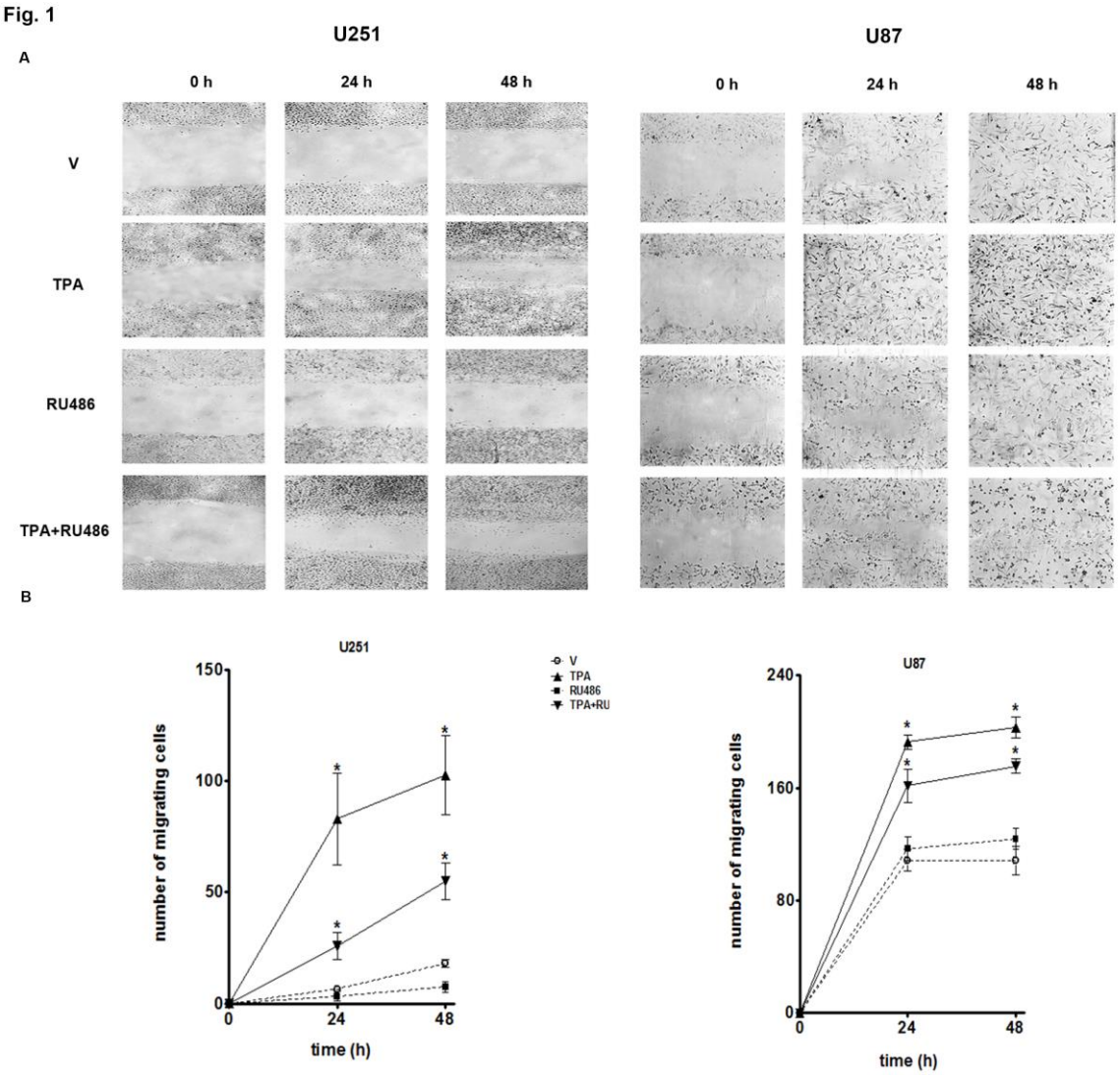

FIGURE 1. PKC activation with TPA increases the migration capability of U251 MG and U87 cells through PR activation. U251 MG and U87 cells were incubated with vehicle (V, DMSO 10%), TPA (1 µM), TPA+RU486 (1 µM) or RU486 during 0, 24 and 48 h and subjected to a migration assay. (A) Representative photographs of 3 scratch-wound assays in U251 MG and U87 cell lines are shown.

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(B) Graphs represent the number of U251 MG and U87 migrating cells into the wound over time. Results are expressed as the mean ± S.E.M. (n=3), %CV range in U251 cells= 9.6-14.8, %CV range in U87 cells = 7.2-14.6, ANOVA $p<0.0001$, Bonferroni post-test $*p<0.05$.

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Fig. 2

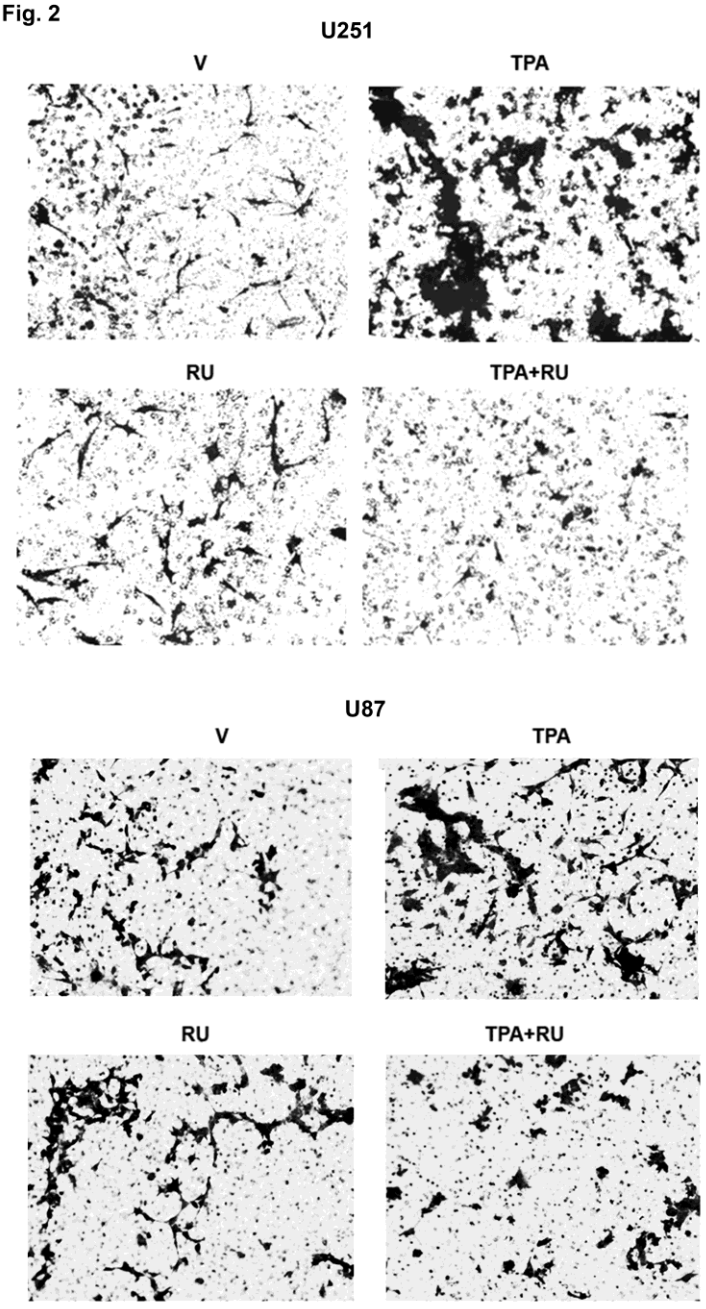

FIGURE 2. PKC activation with TPA induces U251 MG and U87 cell invasion through PR activation. U251 MG and U87 cells were incubated with vehicle (V, DMSO 10%), TPA (1 µM), RU486 (1 µM) or TPA+RU486 during 24 h and subjected to a transwell invasion assay. Cells invading the matrigel after each treatment were stained and photographed. Representative photographs of 3 independent transwell invasion assays for U251 MG and U87 cell lines are shown.

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Fig. 3

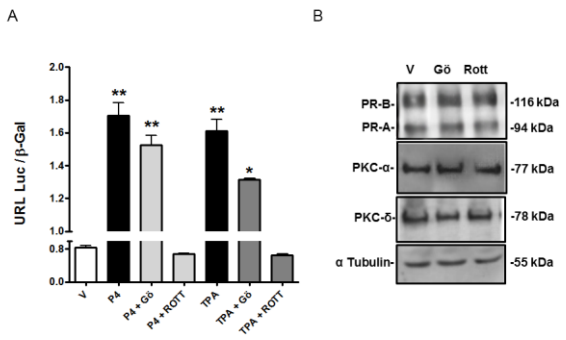

FIGURE 3. PKCα and PKCδ regulate PR transcriptional activity. (A) U251 cells were transfected with an MMTV-Luciferase reporter plasmid with two PREs in its promoter region. After 48 h of transfection, cells were treated with vehicle (V, DMSO 10%); P4 (10 nM); P4+Gö (PKCα inhibitor 1 μM); P4+Rott (PKC δ inhibitor 1 μM); TPA (1 μM); TPA+Gö and TPA+Rott for 24 h. Relative luciferase light units (RLU) were normalized to β-galactosidase activity. Graphs represent the mean ± S.E.M. (n=3), %CV range= 1.0-13.2 ANOVA p<0.0001, Bonferroni post-test **p*<0.01 *vs.* all groups; ***P*<0.01 *vs.* V, P4+Rott, and TPA+Rott. (B) Western blot showing the effect of PKC inhibitors on the protein content of PR isoforms, PKCα and PKCδ. U251 MG cells were treated with vehicle (V); PKCα inhibitor Gö 6976 (Gö 1μM) or PKCδ inhibitor Rottlerin (Rott 1μM) during 24 h. After treatment, cells were lysed to obtain total protein, and 70 μg were used to evaluate PR-B, PR-A, PKCα and PKCδ content. A representative image of 2 independent experiments is shown, α-Tubulin was used as a loading control.

FIGURE 4. PKC activation regulates PIBF expression in U251MG cells. PIBF mRNA expression was assessed by RT-PCR, U251 MG cells were treated with V,

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P4 (10 nM), TPA (1 µM) , TPA+RU486 (1 µM) or RU486 during 12 h. PIBF, and the internal control 18S, were amplified and resolved on a 2% agarose gel, stained with gel red and detected with UV light. A representative assay of 3 independent experiments is shown. The graph represents the densitometric analysis of PIBF expression with respect to its control gene. Results are expressed as the mean ± S.E.M. (n=3), %CV range=2.8-11.7, ANOVA $p<0.0001$, Bonferroni post-test $*p<0.05$ compared with V and RU; $**P<0.05$ compared with V, TPA+RU, and RU.

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Table1 Required description of antibodies

Information of the antibody					
Name of the antibody	**Manufacturer, catalogue#, batch #**	**Protein target**	**Antigen sequence**	**Species raised, monoclonal/polyclonal**	**Dilution used**
Anti-Progesterone Receptor antibody [N559]	Abcam Catalogue # ab58565 Batch # GR39794-3	Human progesterone receptor (A and B isoforms)	Amino-terminal domain aa. 551-564 (ASQSPQTSFESLPQ) of synthetic peptide to human progesterone receptor (PR)	Mouse monoclonal IgG1	3.3 µg/mL
PKC α Antibody (H-7)	Santa Cruz Biotechnology Catalogue # sc-8393 Batch # G0512	PKC δ of mouse, rat and human origin	amino acids 645-672 at the C-terminus of PKC α of human origin	Mouse monoclonal IgG1	0.66 µg/mL

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PKC δ Antibody(C-17)	Santa Cruz Biotechnology Catalogue # sc-213 Batch # D2210	PKC δ of mouse, rat and human origin	C-terminus of PKC δ of rat origin	Rabbit polyclonal IgG	0.66 µg/mL
α Tubulin Antibody (B-7)	Santa Cruz Biotechnology Catalogue # sc-5286 Batch # B2715	α Tubulin of human origin	amino acids 149-448 of α Tubulin of human origin	Mouse monoclonal IgG_{2a}	0.2 µg/ml
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology Catalogue # sc-2004 Batch # F0515	Affinity purified secondary antibody raised in goat against rabbit IgG and conjugated to HRP	Rabbit IgG	Rabbit polyclonal	1.3 ng/mL
Goat anti-mouse	Santa Cruz Biotechnology Catalogue # sc-2005 Batch # F1515	Affinity purified secondary antibody raised in goat against mouse IgG and conjugated to HRP	Mouse IgG	Mouse polyclonal	1.3 ng/mL