The Role and Therapeutic Potential of Key Molecules in the Developmental Pathways of Meningiomas

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Für meine Eltern.

CONTENT

PUBLICATIONS AND PRESENTATIONS

1. PUBLICATIONS

Pfister, C., Ritz, R., Pfrommer, H., Bornemann, A., Tatagiba, M. S., and Roser, F. Are there attacking points in the eicosanoid cascade for chemotherapeutic options in benign meningiomas? *Neurosurgical Focus* **23**(4):E8. (2007)

Pfister, C., Ritz, R., Endemann, E., Schittenhelm, J., Bornemann, A., Tatagiba, M. S., and Roser, F. Evidence of ubiquitously in vivo and in vitro expression of pro-apoptotic Smac/DIABLO protein in meningioma cell lines. *Oncology Reports* **21**(5):1181-8. (2009)

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Pfister, C., Pfrommer, H., Tatagiba, M. S., and Roser, F. Detection and quantification of farnesol-induced apoptosis in difficult primary cell cultures by TaqMan protein assay. *Apoptosis*, doi:10.1007/s10495-012-0796-0 (2013)

2. BOOK CHAPTER

Roser, F., **Pfister, C.** Proliferation in Meningiomas. In: Ramina R., Aguiar PHP., Tatagiba M. (Hrsg.) Samii's Essentials in Neurosurgery. 2. Auflage 2013, Springer-Verlag, Heidelberg

3. ORAL PRESENTATIONS

Pfister, C., Tatagiba, M. S., and Roser, F. "Selection of suitable reference genes for quantitative real-time polymerase chain reaction in human meningiomas and arachnoidea."

62nd Annual Meeting of the German Society of Neurosurgery; Hamburg, Germany 2011

4. POSTER PRESENTATIONS

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6th International Congress on Meningiomas, AANS. Boston, USA 2008

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Pfister, C., and Roser, F. Evaluation of TaqMan® Protein Assays in meningiomas.

6th Brain Tumor Meeting; Berlin, Germany 2011

Pfister, C., Pfrommer, H., Tatagiba, M. S., and Roser, F. Vascular endothelial growth factor signals through platelet-derived growth factor receptor β not vascular endothelial growth factor receptor in meningiomas *in vitro.*

10th Meeting of the European Association of NeuroOncology; Marseille, France, 2012

1 ZUSAMMENFASSUNG

Meningeome gehören zu den häufigsten intrakraniellen Tumoren. Deren primäre Therapie ist die mikrochirurgische Resektion. Trotz radikaler Exstirpation neigen auch benigne Meningeome zur Bildung von Rezidiven (10-30%), deren weitere Behandlung auf Grund eines Mangels an chemotherapeutischen Therapien deutlich eingeschränkt ist. Da Meningeome außerhalb der Blut-Hirn-Schranke wachsen, ist die Applikation von Medikamenten problemlos möglich. Allerdings müssen bei der Entwicklung einer chemotherapeutischen Behandlung von benignen Tumoren neben der Wirksamkeit des Medikaments auch mögliche Nebenwirkungen mit in Betracht gezogen werden, da diese den Patienten nicht unverhältnismäßig belasten sollten.

Die Grundlage für sämtliche im Folgenden beschriebenen Arbeiten war die Kultivierung und Archivierung von über 600 histologisch bestätigten Meningeomen aus frischen OP-Präparaten.

Der Ausgangspunkt für den ersten Teil der vorliegenden Doktorarbeit war eine Veröffentlichung aus dem Jahr 2005 von Ragel *et al.* In dieser Studie konnte zum einen immunhistochemisch eine ubiquitäre Expression von Cyclooxygenase-2 (COX-2) in Meningeomen nachgewiesen werden und zum anderen eine Dosis-abhängige Wachstumshemmung *in vitro* durch Celecoxib demonstriert werden[1]. Celecoxib ist ein COX-2-Inhibitor, der bei der Behandlung von Arthrose und Polyarthritis eingesetzt wird. Ein bereits zugelassener Wirkstoff, dessen Dosierung und Nebenwirkungen beim Menschen bereits bekannt sind, wäre für die Behandlung von benignen Meningeomen ideal. Da die Datenlage zu diesem Thema in der Literatur bei Meningeomen begrenzt bzw. hauptsächlich auf immunhistochemischen Färbungen beschränkt war, wurde die RNA- und Proteinexpression von COX-1, COX-2, sowie von 5-Lipoxygenase (5-LO) und Prostaglandinrezeptor E4 (PTGER4) mit Hilfe von immunhistochemischen Färbungen *in vivo*, immunzytologischen Färbungen, Western Blot und PCR *in vitro* untersucht. 5-LO ist ebenso wie COX-2 ein Arachidonsäurederivat, welches in verschiedenen Tumorarten überexprimiert wird. PTGER4 wurde bis dato nicht nachgewiesen. Das Substrat von PTGER4 ist Prostaglandin E2 (PGE_2), ein COX-2-Derivat. Mehrere ältere Studien konnten eine Überexpression von PGE_2 in Meningeomen im Vergleich zum normalen Gehirn nachweisen. Die 124

Meningeomproben wurden mit normalen zerebralen Cortex und Dura mater *in vivo* (immunhistologische Färbungen) und *in vitro* (immunzytologische Färbungen, Western Blot und PCR) verglichen[2]. Sowohl COX-1, COX-2, 5-LO als auch PTGER4 wiesen eine ubiquitär hohe RNA- und Proteinexpression in allen Meningeomen auf. Allerdings konnte keine erhöhte Expression im Vergleich zu zerebralem Cortex bzw. Dura nachgewiesen werden. Außerdem konnte im Gegensatz zu früheren Studien keine signifikante Änderung der COX-2-Expression mit steigender Malignität festgestellt werden, weshalb weder COX-2 noch einer der anderen untersuchten Eicosanoidderivate als prognostischer Marker bei Meningeomen verwendet werden können. Neben Primärzellkulturen wurde auch die maligne, immortale Meningeomzelllinie IOMM-Lee untersucht. Ragel *et al.* hatte die Dosis-abhängige Wachstumshemmung durch Celecoxib an dieser Zelllinie nachgewiesen. COX-2 konnte im Western Blot in IOMM-Lee-Zellen nachgewiesen werden, aber der RNA-Nachweis mit Hilfe einer PCR lieferte mehrfach unklare Ergebnisse. Deshalb wurde die COX-2 RNA-Expression in der IOMM-Lee Zelllinie erneut untersucht, nachdem ein RealTime-PCR Gerät zur Verfügung stand. Die Verwendung eines hochspezifischen Taqman-Primerpaares für COX-2 bewies, dass IOMM-Lee auch nach 40 PCR-Zyklen keine messbare COX-2 RNA-Expression aufweist. Die Untersuchung der COX-2 RNA-Expression in Meningeomprimärzellkulturen, normalem Gehirn und Dura bestätigten die Ergebnisse der PCR. Dies warf die Frage auf, wie ein selektiver COX-2-Inhibitor wie Celecoxib das Wachstum von COX-2-negativen Zellen hemmt. Außerdem hemmt Celecoxib das Wachstum von Meningeomzellen erst im unphysiologischen Bereich. Aufgrund dieser Ergebnisse wurde COX-2 nicht weiter als Ansatzpunkt für eine chemotherapeutische Behandlung in Betracht gezogen.

Im zweiten Teil der Doktorarbeit wurde der Einfluss von apoptotischen Proteinen in der Evolution von Meningeomen untersucht. Veränderungen von Mechanismen in der Apoptose spielen eine große Rolle in der Tumorpathogenese. Bisher wurden in Meningeomen immunhistochemisch Caspase-3 und das anti-apoptotische Survivin nachgewiesen. Bei beiden Proteinen wiesen die Ergebnisse auf einen Zusammenhang zwischen erhöhter Expression und Malignität bzw. Rezidivwahrscheinlichkeit hin. Aufgrund der dürftigen Datenlage wurde die RNA- und Proteinexpression von Caspase-3, Caspase-9, Survivin und dem pro-apoptotischen Smac/DIABLO mit Hilfe von immunhistologischen bzw. -zytologischen Färbungen, Western Blot und PCR an 100 Meningeomen und deren

Kontrollgewebe untersucht[3]. Caspase-3 war in Meningeomen ubiquitär hoch exprimiert ohne signifikante Veränderungen mit steigender Malignität. Caspase-9 wies ebenfalls eine omnipräsente Expression auf, allerdings auf einem niedrigeren Niveau als Caspase-3. Als Erste konnte diese Studie eine ubiquitär hohe Expression von Smac/DIABLO in Meningeomen nachweisen. Smac/DIABLO zeigte keine veränderte Expression in Meningeomen sowohl im Vergleich mit normalem Gehirn und Dura mater als auch mit steigender Malignität. Gleichzeitig wiesen die Meningeome eine niedrige, omnipräsente Expression von Survivin auf, ohne eine signifikante Erhöhung der Expression in malignen Tumoren bzw. Rezidiven. Außerdem konnte keine Veränderung der Survivin-Expression im Vergleich mit normalem Gehirn und Dura mater nachgewiesen werden. Diese Ergebnisse weisen auf ein ähnliches apoptotisches Verhalten in Meningeomen wie im normalen Gehirn hin. Zudem fehlt eine in vielen Tumoren auftretende pathologische Überexpression von Survivin. Die RNA-Expression von Survivin wurde im Nachhinein mit Hilfe der Real-Time PCR genauer analysiert.

Bei der Real-Time PCR handelt es sich um eine deutlich sensitivere Methode als die klassische PCR zur relativen bzw. absoluten Quantifizierung von RNA. Zur relativen Quantifizierung wird ein endogenes Kontrollgen benötigt, welches im verwendeten Versuchsaufbau eine möglichst geringe Variation der Expression in Zellen und Geweben aufweist. Da es sich bei Meningeomen um eine heterogene Tumorart handelt und die Ergebnisse mit der Expression in Arachnoidea, Dura mater und Gehirn verglichen werden sollten, war es besonders wichtig, ein stabiles endogenes Referenzgen zu ermitteln. Dazu wurden aus 32 Genen, welche häufig als endogene Kontrollen verwendet werden, mit Hilfe von mehreren Algorithmen das stabilste Referenzgen (RPL37A) für Meningeome und deren Kontrollgewebe ermittelt[4]. Dieses Ergebnis war die Voraussetzung für sämtliche weiteren Real-Time PCR Versuche, um belastbare Daten im Bezug auf signifikante RNA-Expressionslevel zu erhalten.

Um die RNA-Expression von Survivin in Meningeomen im Vergleich zu Normalgewebe genauer zu bestimmen, wurde zusätzlich eine höhere Anzahl von Dura-Präparaten, Arachnoidea sowie „Human Brain Reference RNA“ der Firma Applied Biosystems eingesetzt. „Human Brain Reference RNA“ ist aus einem Pool von mehreren Donatoren und verschiedenen Gehirnregionen zusammengesetzt und dient als hochwertige Referenz für

Mikroarrayanalysen. Die Daten zeigten eine signifikant erhöhte Expression von Survivin in Meningeomen verglichen zu Arachnoidea und „Human Brain Reference“, aber nicht im Vergleich zur Dura, unabhängig von Rezidivwahrscheinlichkeit und Malignität. Diese Unterschiede zeigen, dass Ergebnisse in hohem Maße sowohl von den verwendeten Methoden als auch von den Vergleichskontrollen abhängen. Das ideale Vergleichsgewebe für Meningeome ist die Arachnoidea, aus deren Zellen Meningeome entstehen. Allerdings sind Arachnoidea-Präparate sehr schwer zu bekommen. Die Arachnoidea umhüllt das Gehirn wie ein feines Spinnennetz. Die OP-Präparate sind sehr klein, so dass nur geringe Mengen RNA isolieren werden können. Daher war nur ein RNA-Vergleich zwischen Arachnoidea und Meningeomen möglich. Um belastbarere Daten zu erhalten wurde als zusätzliches Vergleichsmaterial Dura mater verwendet, welche zusammen mit der Arachnoidea und der Pia mater die zerebralen Meningen bilden. Desweiteren wurde normales Gehirn als Vergleichsmaterial verwendet. Da dieses an Meningeome angrenzt, können signifikant veränderte Expressionsprofile zwischen Gehirn und Tumor Hinweise für einen möglichen Therapieansatzpunkt geben.

Die Grundlage für den dritten Teil der Doktorarbeit waren Vorversuche, bei denen die Effektivität von verschiedenen Apoptose-induzierenden Wirkstoffen (Cycloheximid, Pac1, Cyclophosphamid, Dexamethason, Gambogic Acid, Epicatechin Gallat und Farnesol) an Meningeomprimärzellkulturen getestet wurde. Dabei zeigten Farnesol und Gambogic Acid die stärkste apoptotische Wirkung auf Meningeomzellen. Beides sind pflanzliche Stoffe, die *in vitro* eine deutlich stärkere apoptotische Wirkung auf Tumorzellen als auf normale Zellen aufweisen. Gambogic Acid ist nicht nur ein potenter, krebshemmender Wirkstoff, sondern weist auch eine anti-angiogenetische Wirkung auf. Mit Hilfe von quantitativer Real-Time PCR und der neuen hochsensitiven Methode Taqman® Protein Assay wurden vaskulärer endothelialer Wachstumsfaktor A (VEGFA), VEGF-Rezeptor 2 (KDR), thrombozytärer Wachstumsfaktor B (PDGFB) und PDGF-Rezeptor β (PDGFRβ) in Meningeomen *in vitro* und *in vivo* quantifiziert. Zudem wurde der Effekt von VEGFA, PDGFB und Tyrosinkinase-Inhibitoren wie Gambogic Acid auf die Zellproliferation und die Tyrosin-Phosphorylierung von PDGFRβ in Meningeomprimärzellkulturen untersucht[5]. In der Studie konnte demonstriert werden, dass Meningeome *in vitro* und *in vivo* wenig bzw. kein KDR exprimieren, sondern stattdessen PDGFRβ überexprimieren. Desweiteren zeigten

Meningeome eine niedrige PDGFB-Expression. Meningeomprimärzellkulturen, die mit exogenem VEGF stimuliert wurden, wiesen eine deutlich erhöhte Proliferation auf. Wurde PDGFRβ vor der Gabe von exogenem VEGFA gehemmt, hob sich dieser proliferierende Stimulus auf. Außerdem konnte belegt werden, dass VEGFA vergleichbar wie PDGFB konzentrations-abhängig die Tyrosin-Phosphorylierung von PDGFRβ induziert. Basierend auf diesen Ergebnissen wurde die Wirkung verschiedener Tyrosinrezeptor-Inhibitoren verglichen. Sunitinib, ein bereits zugelassenes Chemotherapeutikum, welches vorzugsweise KDR aber auch PDGFRβ hemmt, beeinträchtigte die Migration von Meningeomen in gleicher Weise wie Tandutinib, das eine hohe Aktivität gegenüber PDGFRβ aufweist aber eine sehr geringe Aktivität gegenüber KDR. Zudem konnte gezeigt werden, dass Gambogic Acid ebenso die Zellmigration von Meningeomen beeinträchtigt und dabei die VEGFA-induzierte PDGFRβ-Tyrosinphosphorylierung unterdrückt. Damit ist es gelungen zu beweisen, dass erstens VEGFA in Meningeomen die Zellmigration hauptsächlich durch PDGFRβ und nicht durch KDR reguliert und dass zweitens Gambogic Acid ein potenter Inhibitor des Meningeomwachstums *in vitro* ist.

Um die apoptotische Wirkung von Farnesol auf Meningeome *in vitro* genauer zu untersuchen, musste zuerst eine neue Methode zur Proteinquantifizierung als Apoptosenachweis etabliert werden. Für einen sicheren Apoptosenachweis ist es sinnvoll, zwei unterschiedliche Methoden zu verwenden, die zudem an verschiedenen Punkten im Ablauf der Apoptose messen. Die verwendeten Methoden sind stark vom Ausgangsmaterial abhängig. Die Verwendung von benignen Meningeomprimärzellkulturen weist mehrere Probleme für den Apoptosenachweis auf: 1. Versuche lassen sich nur in einem engen zeitlichen Rahmen reproduzieren, da Meningeomprimärzellkulturen ihren Phänotyp bei langanhaltender Kultivierung verändern; 2. Es steht nur eine begrenzte Zellzahl zur Verfügung, da Meningeomprimärzellkulturen eine sehr niedrige Proliferationsrate aufweisen und nach wenigen Passagen in Seneszenz gehen. Als eindeutigen apoptotischen Nachweis wurden folgende Methoden angewandt: die immunhistochemische Färbung von einzelsträngiger DNA (ssDNA), Western Blot-Nachweis von geschnittener PARP1 und die Quantifizierung von aktiver Caspase-3 mit Hilfe von Taqman® Protein Assay. Während das Auftreten aktiver Caspase-3 und geschnittener PARP1 Kriterien früher Apoptose darstellen, kennzeichnet die Fragmentierung von DNA das Spätstadium der Apoptose. Der

immunhistochemische Nachweis von ssDNA wurde dem wesentlich häufiger verwendeten TUNEL-Test vorgezogen, da der TUNEL-Test kein spezifischer Apoptosenachweis ist. Taqman® Protein Assay ist eine erst kürzlich entwickelte hochsensitive Methode zur Proteinquantifizierung, welche Antikörper mit Hilfe des „Proximity Ligation Assays“ mit der quantitativen Real-Time PCR verbindet. Die Studie stellt die Entwicklung, Verifizierung und Verwendbarkeit eines Taqman® Protein Assays dar, der aktive Caspase-3 in Meningeom-primärzellkulturen nachweist[6]. Die immunzytologische Färbung von ssDNA wies die Induktion von Apoptose in Meningeomen nach einer 24-stündigen Behandlung mit 2 µM Farnesol nach. Außerdem konnte gezeigt werden, dass die mit dem Taqman® Protein Assay quantifizierte Expression von aktiver Caspase-3 mit der mit Hilfe eines Western Blots nachgewiesenen geschnittenen PARP1-Expression korrelierte. Dabei wies der Taqman® Protein Assay eine signifikant höhere Sensitivität auf als ein klassischer Western Blot. Zudem konnte gezeigt werden, dass Farnesol in sehr niedrigen Dosen unterschiedlich auf Meningeome wirkt. Während die eine Gruppe Meningeome einen geringen Effekt auf die Zellviabilität bei einer 24-stündigen Behandlung mit 0,4 µM und 0,8 µM Farnesol aufwies, zeigte die andere Gruppe bereits eine signifikante Reduktion um 70% der lebensfähigen Zellen. Nach einer 24-stündigen Behandlung mit 1,2 µM Farnesol war die Zellviabilität bei beiden Gruppen um mehr als 90% reduziert. Dieser Effekt zeigte sich auch beim Nachweis von aktiver Caspase-3 mit dem Taqman® Protein Assay. In Gruppe 1 stieg die Expression von aktiver Caspase-3 erst nach 2-stündiger Gabe von 2 µM Farnesol abrupt an, während sich in Gruppe 2 der aktive Caspase-3 Level mit steigender Farnesol-Konzentration erhöhte. Damit konnte mit Hilfe des Taqman® Protein Assay für aktive Caspase-3 demonstriert werden, dass aktive Caspase-3 bereits in sehr geringen Konzentrationen in Meningeomen zuverlässig detektiert und quantifiziert werden kann.

Die hohe Sensitivität von Meningeomzellen auf Gambogic Acid und Farnesol zeigt, dass diese beiden Wirkstoffe prädestiniert sind als potentielle chemotherapeutische Therapie bei Meningeomen.

2 SUMMARY

Meningiomas are the most common intracranial brain tumors. The primary therapy is microsurgical resection. Benign meningiomas tend to develop recurrences despite radical extirpation (10-30%). Their further treatment is significantly restricted due to a lack of chemotherapeutical therapies. Since meningiomas reside outside the blood-brain barrier drugs can administered without problems. However, in the development of a chemotherapeutical treatment of benign tumors not only the efficacy of a drug has to be taken into consideration but also possible side effects, so the patient is not disproportionately strained.

The basis for all experiments detailed below was the cultivation and archiving of over 600 histological confirmed meningiomas from fresh surgical specimens.

The starting-point for the first part of this doctoral thesis was a publication in 2005 by Ragel *et al*. In this study a ubiquitous expression of cyclooxygenase-2 (COX-2) in meningiomas was shown immunohistochemically and also growth inhibition in a dose-dependent manner *in vitro* by celecoxib was demonstrated[1]. Celecoxib is a COX-2 inhibitor, which is used in the treatment of arthrosis and polyarthritis. A pre-approved drug with known dosage and side effects would be ideal for the treatment of benign meningiomas. The data for meningioma research on this topic is limited respectively is mainly restricted to immunohistochemical stainings. Therefore RNA and protein expression of COX-1, COX-2, as well as 5-lipoxygenase (5-LO), and prostaglandine receptor E4 (PTGER4) was examined using immunohistological stainings *in vivo*, immunocytological stainings, western blot, and PCR *in vitro*. 5-LO is also an arachidonic acid derivate, which is overexpressed in several tumor types. To date PTGER4 was not described in meningiomas. The substrate of PTGER4 is prostaglandine E2 (PGE_2), a COX-2 derivate. Several previous studies demonstrated an overexpression of PGE_2 compared to normal brain in meningiomas. 124 meningioma specimens were compared with normal cerebral cortex and dura mater *in vivo* (immunohistochemical stainings) and *in vitro* (immunocytological stainings, Western Blot, and PCR)[2]. Both COX-1, COX-2, 5-LO, and PTGER4 displayed a ubiquitous high RNA and protein expression in all examined meningiomas. But the comparison with normal cerebral cortex and dura mater displayed no elevated expression. In addition, significant changes in COX-2 expression with increasing

malignancy could not be established contrary to previous studies. Therefore neither COX-2 nor another of the examined eicosanoid derivates qualifies as prognostic marker for meningiomas. Besides primary cell cultures the malign, immortal meningioma cell line IOMM-Lee was analyzed. Ragel *et al*. demonstrated dose-dependent growth inhibition by celecoxib using this cell line. Despite positive detection of COX-2 protein using Western blot RNA detection using PCR provided indeterminate results. After a Real-Time PCR device was available RNA expression of COX-2 in the IOMM-Lee cell line was re-examined. The application of highly specific Taqman primers for COX-2 showed that IOMM-Lee does not display detectable COX-2 RNA after 40 PCR cycles. The examination of COX-2 RNA expression in meningioma primary cell cultures, normal brain and dura mater confirmed PCR results. These contrary results raised the question how selective COX-2 inhibitors like celecoxib inhibit growth of COX-2 negative cells. In addition, celecoxib inhibits meningioma cell growth only in an unphysiological range. On the basis of these results COX-2 was not taken into consideration as a starting point for a chemotherapeutical therapy.

In the second part of this thesis the influence of apoptotic proteins in the evolution of meningiomas was examined. Changes of apoptotic mechanisms play an important role in tumor pathogenesis. To date expression of caspase-3 and the anti-apoptotic survivin were demonstrated immunohistochemically. Both proteins displayed a connection between elevated expression and malignancy respectively likelihood of recurrence. Due to sparse data RNA and protein expression of caspase-3, caspase-9, survivin, and the pro-apoptotic Smac/DIABLO were analyzed using immunohistochemical respectively immunocytological stainings, Western blot, and PCR in 100 meningiomas and their control tissue[3]. Caspase-3 displayed omnipresent high expression without significant changes with increasing malignancy. Caspase-9 was also expressed ubiquitously but at a lower level than caspase-3. This was the first study detailing the omnipresent high expression of Smac/DIABLO in meningiomas. Smac/DIABLO showed unchanged expression compared to both normal brain respectively dura mater and increasing malignancy. At the same time, meningiomas displayed a ubiquitous low expression of survivin without significant elevated expression in malign or recurrent tumors. Also survivin expression displayed no changes compared to normal brain and dura mater. These results indicate a similar apoptotic behavior of meningiomas compared to normal brain tissue, as well as the lack of a pathological

overexpression of survivin common in many cancer types. RNA expression of survivin was re-examined using Real-Time PCR.

Real-Time PCR is used for relative respectively absolute RNA quantification and has a distinctly higher sensitivity than classic PCR. An endogenous control is needed for relative quantification, which displays a minimal expression variation in cells and tissues. Since meningiomas are a heterogeneous tumor type and results are compared with arachnoidea, dura mater, and brain, it is essential to determine a stable housekeeping gene. Therefore RPL37A was established as the most stable endogenous control for meningiomas and their control tissue from 32 genes, which are commonly used as housekeeping genes, using several algorithms[4]. This result was a requirement for all further Real-Time PCR experiments to obtain reliable data regarding RNA expression level.

A higher number of dura specimens, arachnoidea, plus „human brain reference RNA“(Applied Biosystems) was used to determine RNA expression of survivin in meningiomas compared to normal tissue more precisely. „Human brain reference RNA“ is pooled RNA from several donors and different brain regions, which is used as a high-quality standard for microarray analysis. Data displayed a significant increased survivin expression in meningiomas compared to arachnoidea and human brain reference, not dura mater, independent of likelihood of recurrence and malignity. These differences show that results strongly depend on both used methods and control tissues. The ideal control tissue for meningiomas is arachnoidea, from whose cells meningioma origin. Arachnoidea specimens are difficult to obtain as it envelopes the brain like a spider web. Surgical specimens are very small therefore merely a low amount of RNA can be isolated. This allowed only a comparison between arachnoidea and meningioma RNA. To obtain more reliable data dura mater was also used as control tissue, which forms the cerebral meninges together with arachnoidea and pia mater. Furthermore normal human brain was used as control tissue. Because it adjoins to meningiomas significant changes in expression profiles between brain and tumor could provide information for possible therapy starting points.

The basis for the third part of this thesis was a preliminary experiment, in which the effectiveness of various apoptosis-inducing substances (cycloheximide, Pac1, cyclophosphamide, dexamethasone, gambogic acid, epicatechin gallat, and farnesol) was

tested in meningioma primary cell cultures. Thereby farnesol and gambogic acid displayed the strongest apoptotic effect on meningioma cells. Both are herbal substances, which show significant stronger effect on tumor cells then normal cells *in vitro*. Gambogic acid is not only a potent anti-carcinogenic substance, but also exhibits an anti-angiogenetic effect. Quantitative Real-Time PCR and the new highly sensitive Taqman® Protein Assay were used to quantify vascular endothelial growth factor A (VEGFA), VEGF receptor 2 (KDR), platelet-derived growth factor (PDGFB), and PDGF receptor β (PDGFRβ) in meningiomas *in vitro* and *in vivo*. In addition, the effects of VEGFA, PDGFB and tyrosine kinase inhibitors such as gambogic acid on cell proliferation and tyrosine phosphorylation of PDGFRβ in primary meningioma cell cultures were examined[5]. The study shows that meningiomas express little to none KDR *in vitro* and *in vivo*. Instead they overexpress PDGFRβ. In addition, meningiomas showed a low PDGFB expression. Meningioma primary cell cultures, which were stimulated with exogenous VEGFA, displayed a significant higher proliferation rate. If PDGFRβ was inhibited before administration of exogenous VEGFA, this proliferative stimulus was abolished. Furthermore VEGFA induced concentration-dependent PDGFRβ tyrosine phosphorylation comparable to PDGFB-induced PDGFRβ tyrosine phosphorylation. Based on these results several tyrosine receptor inhibitors were tested. Sunitinib, an already approved chemotherapeutic agent, which inhibits preverentially KDR, but also inhibits PDGFRβ, equally impaired migration of meningioma cells as tandutinib, which displays a high activity towards PDGFRβ but a low activity towards KDR. Moreover gambogic acid equally impaired cell migration of meningiomas and also suppressed VEGFA-induced PDGFRβ tyrosine phosphorylation. In the first place the study established that VEGFA regulates cell migration mostly through PDGFRβ not KDR in meningiomas. Secondly it was able to show, that gambogic acid is a potent inhibitor of meningioma cell growth *in vitro*.

To examine the apoptotic effect of Farnesol in meningiomas *in vitro* more closely a new method of protein quantification to detect apoptosis had to be established. For reliable apoptosis detection two different methods should be used, which in addition measure at different points in the apoptotic process. Applied methods are highly dependent on the basis material. Using benign meningioma primary cell cultures presented several issues for detection of apoptosis: 1. Experiments are only reproducibly in a tight timeframe, because meningioma primary cell cultures change their phenotype with prolonged cultivation; 2. Just

a limited cell number is available, since meningioma primary cell cultures display a low proliferation rate and go into senescence after few passages. For the distinct detection of apoptosis the following methods were used: immunohistochemical staining of single-stranded DNA (ssDNA), determination of cleaved PARP1 using western blot, and the quantification of active caspase-3 using Taqman® Protein Assay. Whereas the appearance of active caspase-3 and cleaved PARP1 are a hallmark of early apoptosis, fragmentation of DNA characterizes late-stage apoptosis. The immunohistochemical detection of ssDNA was chosen over the commonly used TUNEL test, because TUNEL is not specific for apoptosis. Taqman® Protein Assay is a recently developed highly sensitive method for protein quantification, which utilizes antibodies and proximity ligation for quantitative Real-Time PCR. The study describes the development, verification, and usability of a Taqman® Protein Assay to detect active caspase-3 in primary meningioma cell culture[6]. The immunohistochemical staining of ssDNA showed the induction of apoptosis in meningiomas after treatment with 2 µM farnesol for 24 hours. Expression of active caspase-3, which was quantified using the Taqman® Protein Assay, correlated with cleaved PARP1 expression, which was determined using western blot. Thereby the Taqman® Protein Assay exhibited a significantly higher sensitivity than the western blot. In addition, low concentrations of farnesol affected meningiomas differently. While one group of meningiomas displayed a limited effect on cell viability after the treatment with 0.4 µM and 0.8 µM farnesol after 24 hours, the other group was more sensitive experiencing a 70% decrease in viability at 0.4 µM farnesol. After the treatment with 1.2 µM farnesol for 24 hours cell viability was reduced in both groups by over 90%. This effect also showed when detecting active caspase-3 using Taqman® Protein Assay. In group 1 the expression of active caspase-3 increased abruptly after treatment with 2 µM farnesol for 2 hours, whereas the level of active caspase-3 rose with increasing farnesol concentration. Summarizing the study showed, that Taqman® Protein Assay for active caspase-3 is able to reliably detect and quantify active caspase-3 in very low concentrations in meningiomas.

High sensitivity of meningiomas towards gambogic acid and farnesol indicates that both substances are predestined as potential chemotherapeutic therapies of meningiomas.

3 INTRODUCTION

3.1 MENINGIOMA

Meningiomas are the most common intracranial tumors. They originate from the arachnoidal cap cells of the meningeal cover of the spinal cord and brain, constituting approximately 13% to 26% of all intracranial neoplasms[7,8]. The conventional treatment for meningiomas is surgery[9]. However, meningiomas can recur due to incomplete resection, because they lie in delicate skull-based structures or because of their tissue character. Recurrence probability highly depends on radicality of tumor resection and histological classification. Average recurrence rates for radical removed meningiomas – the dural onset is also excised – are within 5 years between 4-7%, for subtotal removed tumors or anaplastic meningiomas average recurrence rates amount to 30-50% [10-13]. The definition of the potential for malignancy is unresolved, due to frequent discordance between histology and biology[14,15].

Meningiomas are categorized into 3 grades per the World Health Organization (WHO), for which there are several subtypes, based on histology. Clinical and biological behavior of benign and malign meningiomas differs with regard to their growth characteristics, recurrence rates, and adhesive and infiltrative character inter- and intraindividual. Therefore WHO classification describes predominantly morphological criteria for dignity assessment involving indices such as mitosis and necrosis, vascularity criteria, nuclear polymorphism or loss of architectal structures[16]. Accordingly immunohistological markers for determination of vascularity and proliferation are proven and accepted regulators by growth and invasion with predictive value[17-20]. However, the range of established proliferation indices is within the group of benign meningiomas too variable to make reliable predictions for the patient[21].

Radiotherapy is increasingly applied in case of recurrence and malign meningiomas. However, the resulting tumor growth control is on average lower than is the case for primary brain tumors or metastases[22-26]. So far an adequate chemotherapeutic treatment could not be provided for either recurrent or anaplastic meningiomas[27,28]. Therefore their treatment poses an unsolved interdisciplinary challenge.

3.2 Eicosanoid cascade

Arachidonic acid (AA) is a v-6 polyunsaturated fatty acid that is converted into biologically active lipid compounds called eicosanoids. Eicosanoids constitute a large family of biologically active lipid mediators that are produced by two enzyme classes, cyclooxygenases (COX-1 and COX-2) and lipoxygenases (5-LO, 12-LO, and 15-LO) (Figure 1). Both catalyze the same enzymatic reaction occurring in the synthesis of prostaglandine (PG) G_2 and PGH_2 which are successively metabolized to PGE_2, PGD_2, and PGF_2, to thromboxane (TX) A_2, and to prostacyclin PGI_2[29].

Various malign tumors overexpress eicosanoids. The resulting excess supply of eicosanoids can promote vascularity, tumorigenesis (COX-2) and cerebral edema (5-LO)[30-32]. Also COX-2 positive tumors demonstrate an association between invasiveness and metastasis. In the normal central nervous system (CNS) tissue neurons and glia cells express COX-2 constitutive in a two-to-one ration to COX-1. In addition physiological and pathological stimulation can induce further COX-2 expression[33].

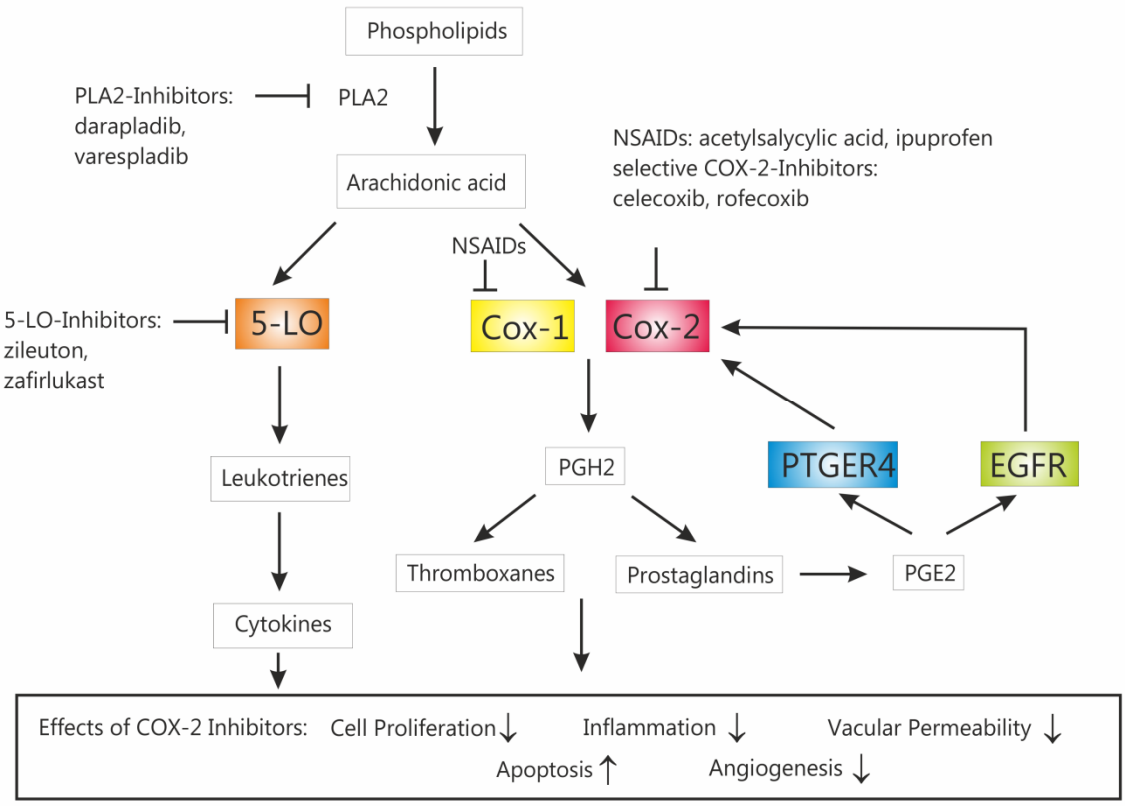

Figure 1: Simplified diagram demonstrating the arachidonic acid cascade and the effects of COX-2 inhibitors.

The crucial role of COX enzymes has been clearly demonstrated in human colon cancer, in which COX-2 is often overexpressed[34,35]. COX-inhibitors, such as non-steroidal anti-inflammatory drugs and COX-2 specific inhibitors, can reduce the incidence of colorectal cancer in humans and experimental animals, and can decrease the polyp number and size in patients with familial adenomatous polyposis[36]. Recently, gliomas and meningiomas were also found to overexpress both COX and LO enzymes compared with normal brain tissue[37-39]. Matsuo and co-workers reported that the staining intensity of glioblastomas was relatively weak, but that astrocytomas of WHO grades II/III and meningiomas were strongly positive for COX-2[40]. Although much attention has been focused on the role of COX derived metabolites in cancer development and progression, accumulating evidence suggests that 5-LO derived eicosanoids may play an equally important role. 5-LO RNA and/or protein was found to be over-expressed in human breast, pancreatic or colon cancers[41-43]. Boado et al reported increased expression of 5-LO RNA in three of three meningioma surgical specimens examined[44].

Most tumors that express cyclooxygenase have been found to contain high levels of PGE_2[45]. Presumably especially this bioactive lipid product of cyclooxygenase is responsible for some of the pro-neoplastic effects mediated by the enzymes, e.g. inducing growth, migration, and invasiveness of colorectal carcinoma cells[46]. Little is known of the production of prostaglandins in intracranial tumors, with some authors reporting increased PGE_2 levels in brain tumors and significant higher PGE_2 level in meningiomas[47,48]. By contrast, other authors reported no increase in production of PGE_2 in brain tumors[49]. A receptor of PGE_2 in the brain is prostaglandine E2 receptor EP4 subtype (PTGER4), which is coupled to protein kinase A/adenyl cyclase and mediates elevations in intracellular cyclic AMP. PTGER4 is implicated to promote early carcinogenesis. Its expression is associated with poor prognosis in upper urinary tract tumors[50]. Also PTGER4 expression is increased during colon cancer progression[51]. In malignant cells high COX-2 protein expression converts AA to PGE_2, which can act in an autocrine or paracrine manner to activate tumor cell PTGER4[52]. Inhibition of PGE_2 synthesis with nonsteroidal anti-inflammatory drugs or PGTER4 signaling with selective prostaglandine receptor antagonists could inhibit tumor metastasis.

There are therapeutical approaches with COX-inhibitors and LO-inhibitors carried out on several tumors types[53-56]. Therefore this approach should be also promising with

meningiomas. Clinical studies have shown, that commercial COX-inhibitors such as celecoxib are used in the anti-edemic treatment of primary brain tumors to supplement glucocorticoid treatment[57] or as „radiosensitizer“ to improve prognosis for radiotherapy[58-60]. *In vitro* and *in vivo* COX-2 inhibitors inhibit cell growth of meningiomas in a dose-dependent manner[1,61].

3.3 APOPTOSIS

Apoptosis, programmed cell death, is essential for normal development and maintenance of multicellular organisms[62]. Its dysregulation effects the ability to avert apoptosis, which has a significant function in cancer[63]. Abnormal apoptosis and deregulation of the genes controlling the apoptotic cascade is closely related to tumor development, progression and recurrence[64]. Apoptotic cell death can be triggered either via the intrinsic, mitochondrial pathway or the extrinsic, death receptor pathway[65]. Both pathways converge on the same terminal pathway, which is initiated by the cleavage of caspase-3, and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Figure 2).

Caspases, a class of cysteine-aspartyl proteases, are widely expressed in an inactive proenzyme form in most cells. Once activated caspases can activate other procaspases, allowing the initiation of a protease cascade[66-68]. This proteolytic cascade amplifies the apoptotic signaling pathway and thereby leads to rapid cell death. To date 14 distinct mammalian caspases have been identified, whereof 7 are central to the regulation of the apoptotic process. Those 7 caspases have been classified as either initiator caspases (caspase-2, -8, -9, and -10) or effector caspases (caspase-3, -6 and -7) based on their structure and function. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues. Caspases are the ultimate effectors of apoptotic cell death, which are tightly regulated. Over-activation of these enzymes has been implicated in certain neurodegenerative disorders. Inappropriate inactivation of caspases is implicated in the development of many cancers[69]. Caspases are regulated through their structure and activation being synthesized as inactive pro-enzymes and also through specific inhibition by naturally occurring inhibitors. Those inhibitors of apoptosis (IAP) function as intrinsic regulators of the caspase cascade.

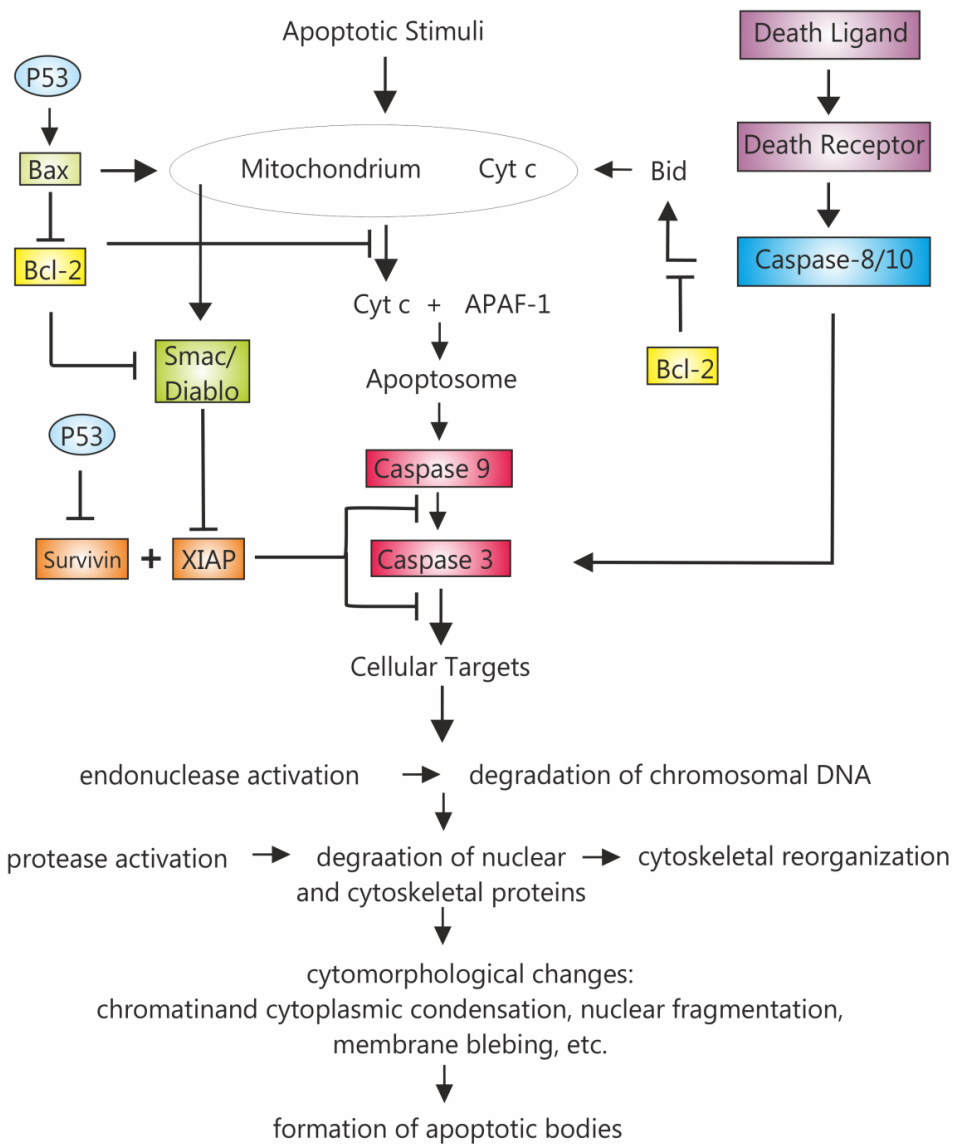

Figure 2: Schematic representation of apoptotic events with the two main pathways (extrinsic and intrinsic pathway) and morphological changes occurring during apoptosis.

3.3.1 Caspase-3

Caspase-3 is the most important effector caspase and is activated by any of the initiator caspases. Caspase-3 is synthesized as an inactive 32 kDa proenzyme and is processed during apoptosis into its active form, which is composed of two subunits, p17 – 20 and p10 – 12[66]. Its activation is a biochemical hallmark of early apoptosis, which renders the detection of active caspase-3 in cells a reliable method of measuring apoptosis, for which antibodies against cleaved caspase-3 are used in several methods such as immunostaining, enzyme-linked immunosorbent assay (ELISA), flow cytometry and western blot.

3.3.2 INHIBITORS OF APOPTOSIS (IAP)

The IAP family includes eight members: BIR containing gene 1 (BIRC1/NAIP); cellular IAP1/ human IAP2 (BIRC2/HIAP2); cellular IAP2/ human IAP1 (BIRC3/HIAP1); X-linked inhibitor of apoptosis (BIRC4/XIAP); Survivin (BIRC5/TIAP), BIR-containing ubiquitin conjugating enzyme (BIRC6/Apollon); Livin (BIRC7/KIAP); and testis-specific IAP (BIRC8/ILP-2). XIAP RNA is ubiquitously expressed in all adult and fetal tissue inhibiting apoptotic in response to a large variety of apoptotic stimuli[70,71]. In contrast, survivin is most highly expressed during fetal development. Also survivin is transcriptionally restricted to expression during the G2/M phase of the cell cycle[72,73]. There it functions as a mitotic checkpoint protein[74]. Survivin specifically binds caspase-3 and caspase-7 inhibiting their activities[75]. In addition, survivin also plays an important role in the angiogenesis process[76,77]

IAPs are elevated in several cancer cell lines and in the majority of human cancers[70,78-81]. Survivin has the most dramatic overexpression. Its absent in most healthy, adult tissue[78,79] and its expression is correlated with poor prognosis, increased rates of treatment failures, and relapse[82]. In addition, high levels of survivin prevent cells from responding to apoptotic stimuli, such as serum deprivation and administration of the anticancer drug etoposide[75,83]. However, the inhibitory action of survivin in caspase-3 activity has been debated[84]. Several groups have shown that survivin is overexpressed in meningiomas[85-89].

The general inhibitor of IAPs is Smac/DIABLO [second mitochondrial activator of caspases, or direct IAP binding protein with low PI) a nuclear encoded, mitochondrially localized protein which is released into the cytosol in response to apoptotic stimuli that disrupt the integrity of the mitochondria[90,91]. Smac/Diablo has been shown to bind to all IAPs except NAIP. Smac/ Diablo negatively regulates the caspase inhibitory properties of XIAP (X-linked IAP) by binding into the same pockets in XIAP which are used to bind caspases; when XIAP binds Smac/Diablo, the caspases are displaced and primed to effect the execution phase of apoptosis[92]. It has been demonstrated that tumor cells with reduced Smac/DIABLO expression resist apoptosis and retain clonogenicity[93]. In addition, it has shown that overexpression of Smac/DIABLO sensitizes tumor cells to apoptotic death[94,95]. Also several studies have established an inverse correlation between Smac/DIABLO expression and cancer progression[96-98]. Furthermore, Smac/DIABLO is able to stimulate induced apoptosis

by promoting caspase-3 activation and cytochrome-c release in colon cancer cells[99] or via an escalation of inhibition of the Survivin/Smac-DIABLO interaction[100]. These findings led to the development of peptides derived from the NH_2-terminal of Smac/DIABLO and small molecules that mimic Smac/DIABLO functions as therapeutic agents in order to induce death or to increase the apoptotic effect of chemotherapeutic agents. Synthetic Smac/DIABLO peptides showed an enhancing effect of etoposide-induced apoptosis in human glioblastoma cell lines[101]. This was also demonstrated with various chemotherapeutic drugs and with irradiation treated breast cancer cells, which either overexpressed the Smac/DIABLO gene or were treated with the Smac/DIABLO peptide[102].

3.3.3 FARNESOL

Many chemotherapeutic drugs induce apoptosis. Farnesol (FA) is a polyprenyl alcohol in plant essential oil and is used as anti-bacterial agent in cosmetics (Figure 3). FA has been shown to inhibit proliferation of a number of cell lines[103,104] and to induce apoptosis in several tumor-derived cell lines[105-108]. In addition, FA induces apoptosis in the lung adenocarcinoma cell line A-549 with an IC50 in the µM range[109,110]. FA preferentially caused apoptosis in malign cell lines rather than in lines derived from non-neoplastic cells[107]. FA has been reported to be effective in chemoprevention and chemotherapy in various *in vivo* cancer models[111,112]. FA also displays *in vivo* anti-tumor activity suppressing pancreatic tumor growth[103]. FA induces caspase-3, -6, -7 and -9 but not caspase-8. In addition FA inhibits survivin and Bcl2[113].

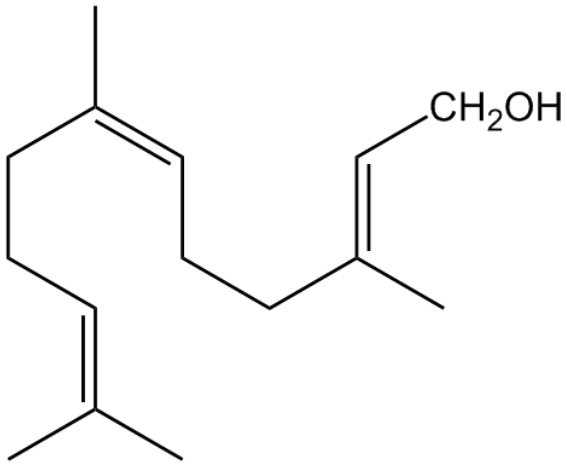

Figure 3: Chemical structures of farnesol.

3.4 Angiogenesis

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. At the beginning tumors are an avascular mass of host-derived cells, which proliferate atypically because they have lost the ability to control their growth[114]. Whereas tumors initially survive on vasculature that is available in the surrounding host environment, tumor growth beyond 2 – 3 mm² needs new blood vessels to prevent hypoxia[115]. Tumor angiogenesis forms abnormal blood vessels, in which the vessel walls are usually made of a combination of tumor cells and endothelial cells[116]. The frequent absence of functional pericytes from peripheral blood vessel resulting in an incomplete basement membrane[117], which causes those vessels to be leaky and dilated[118]. Vascular endothelial growth factor (VEGF) is the key regulator of pathological angiogenesis[119]. Increased levels of VEGF and VEGF receptor 2 have been observed in several cancers, including in various brain tumors[120,121].

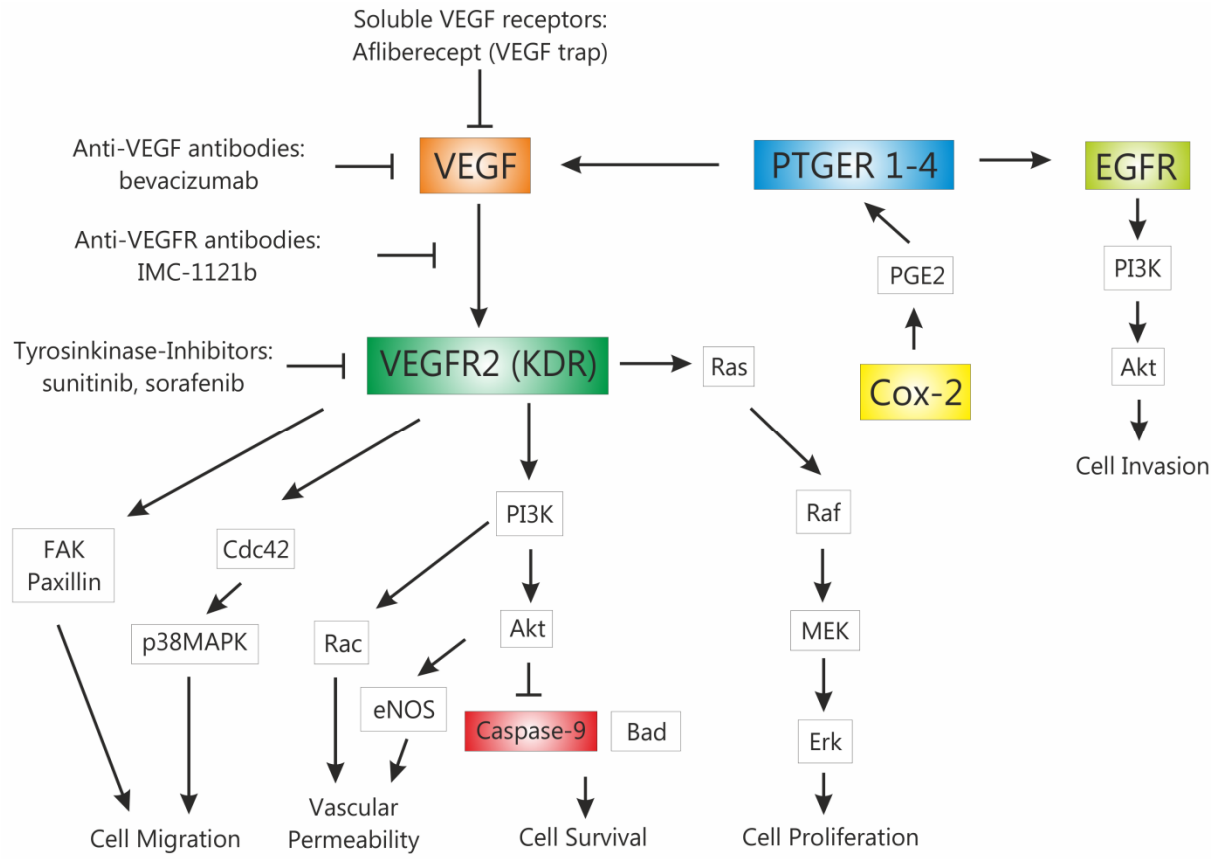

Figure 4: Simplified diagram demonstrating the VEGF pathway and the attacking points of various VEGF pathway inhibitors.

In meningiomas VEGF induces tumor angiogenesis independent from size and histological subtype and is regulated by hypoxia[122]. As hypoxia is only observed in atypical and anaplastic meningiomas[123], other pathogenic mechanisms may be involved in the up-regulation of VEGF. EGF and PDGF overexpression has been described in meningiomas and postulated to contribute to their growth[124-127]. Also arachidonic acid cascade may play a role in tumor angiogenesis in meningiomas. PGE_2 induces a trans-activation of epidermal growth factor receptor (EGFR). Thereby the activation of EGFR increases COX-2 expression through a positive feedback loop and also elevates VEGF expression via trans-activation of the EGFR-MAPK pathway, which in turn supports tumor angiogenesis via COX-2[128,129] (Figure 4).

3.4.1 VEGFA

The human VEGF family consists of five members: VEGF or VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PIGF)[130,131]. VEGFA is the most abundant member of the VEGF family[132,133], which comprises several isoforms through alternative splicing such as $VEGF_{165}$. VEGFA is a 45-kDa glycoprotein and is mainly responsible for most angiogenetic downstream effects. VEGFA is overexpressed in several tumors such as glioblastoma, hemangioblastoma, head and neck, breast, gastric, colorectal, cervical and ovarian carcinoma[130,134]. In certain tumors elevated levels of VEGFA correlates not only with increased microvascular density (MVD) but also with poorer prognosis[135-140]. The role of VEGFA has been extensively studied and reviewed[141].

Meningiomas are highly vascularized tumors. Several studies have demonstrated the significance of VEGF in the proliferation and migration in meningiomas in which VEGF has been upregulated[142-146]. Some groups have observed a correlation between the degree of VEGF expression and tumor vascularity or invasiveness[145,147]. Also some groups noted a strong correlation between meningioma histological grade and VEGF expression, wherein increased VEGF expression was linked to higher WHO grades[143,148]. In contrast, Pietsch *et al* and Nishikawa *et al* could not find a relationship between VEGF expression and tumor vasculature or histological grade[120,149]. Another study correlated VEGF expression and the recurrence of benign meningiomas[146].

3.4.2 VEGFR2/KDR

There are 3 vascular endothelial growth factor receptor tyrosine kinases (VEGFR1-3), but VEGFA only binds to VEGFR1 (fms-like tyrosine kinase; FLT1) and VEGFR2 (kinase insert domain receptor; KDR). FLT1 plays mostly an important role in development[150], whereas KDR is the predominant mediator of VEGF-stimulated endothelial cell migration, proliferation, survival and enhanced vascular permeability[151-153]. Though FLT1 has higher affinity for VEGF than KDR (≈10pM versus 75–750 pM)[150], KDR exhibits robust protein-tyrosine kinase activity in responds to its ligands. VEGF induces the dimerization of KDR, which leads to receptor autophosphorylation and activation. The two major VEGFA-dependent autophosphorylation sites in KDR are Y1775 and Y1214, but only autophosphorylation of Y1775 is essential for VEGF-dependent endothelial cell proliferation[152].

KDR was initially found on endothelial cells, but KDR is expressed at a low level in a fraction of hematopoietic cells[154]. Low expression levels of KDR are also observed in neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, and megakaryocytes[155], but the biological role of KDR in these- non-endothelial cells remains to be clarified. Plate *et al* showed that KDR expression is 3-5-fold higher in the tumor vasculature than in the normal vasculature[156]. Several studies have shown that tumor cells of different origins also express VEGFRs suggesting that VEGF may act as an autocrine signal[157-159]. In different cell lines (melanoma, prostate carcinoma, leukemia, and rhabdomyosarcoma) this presence of an autocrine loop has been demonstrated, which results in increased proliferation[160-164]. Although in other tumor models an anti-proliferative effect has also been observed[165].

Several studies have examined KDR and FLT1 in meningiomas[121,166-168]. Several studies determined KDR expression using either immunohistochemistry and/or in situ hybridization[166-168]. Hatva *et al* examined only 3 meningiomas, which cannot provide justifiable conclusions[166]. KDR protein expression was detected in 31.4% specimens[167] respectively KDR mRNA was demonstrated in 44.4% samples[168]. Those results show that KDR is not expressed in the majority of meningiomas. Also the data provides no information about the expression level of KDR.

3.4.3 PDGFRB

Platelet-derived growth factor (PDGF) is another critical factor that promotes the recruitment and proliferation of vascular cells[169]. PDGF signalling also indirectly regulates angiogenesis. Four different polypeptide chains (PDGF-A, -B, -C, and –D) have been described[170], which form either homo- or heterodimers: PDGF-AA, -AB, -BB, -CC, and –DD. PDGFs exert their cellular effects through PDGF-α and PDGF-β protein tyrosine kinase receptors, which are activated by forming receptor dimers[171]. The activated receptors phosphorylate several substrates, including themselves, initiating a network of signaling cascades (Figure 5). Platelet-derived growth factor receptor β (PDGFRβ) can induce the transcription and secretion of VEGF. PDGF and its receptors are expressed in several human cancers, such as gliomas, breast cancer, colorectal cancer, and myelomonocytic leukaemia[172]. Meningiomas preferentially express PDGF-BB (PDGFB) and PDGFRβ, and their overexpression correlates with WHO grade[127,173-175]. Several groups have proposed an autocrine mechanism in meningiomas, based on the coexpression of PDGFB and PDGFRβ, which regulate tumor growth[173,175-177].

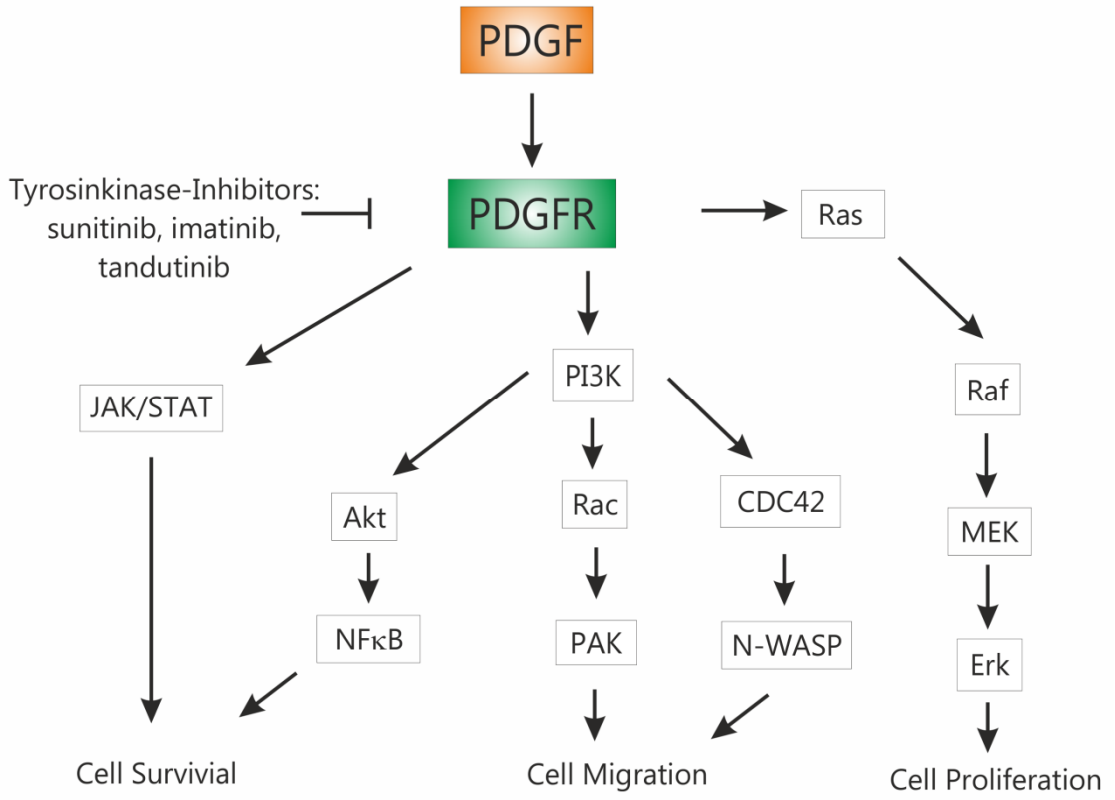

Figure 5: Simplified diagram demonstrating the PDGF pathway.

3.4.4 GAMBOGIC ACID

Gambogic acid (GA) is the major active ingredient of gamboges, a resin from the Chinese plant, *Garcinia hanburyi* (Figure 6). Previous studies demonstrated that GA has potent anticancer activity both *in vivo* and *in vitro*[178-184]. Furthermore, GA exhibits low toxicity against normal tissue[185-187]. GA activates apoptosis by binding to the transferrin receptor[188] and suppressing nuclear factor-kappa B (NFκB) signaling pathway[189]. GA binds to the transferrin receptor independent of the transferrin binding site, which rapidly induces apoptosis in tumor cells[188]. In addition, GA inhibits the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family of proteins[190]. Its anti-proliferative effect GA exerts by inhibiting the catalytic activity of topoisomerase IIα[191]. GA also represses telomerase activity in lung carcinoma cells[179]. In addition, GA has an anti-angiogenesis effect by suppressing KDR signaling[192]. Recently, Liu et al. demonstrated that GA induces G0/G1 cell cycle arrest and inhibits cell migration by suppressing PDGFRβ tyrosine phosphorylation[193]. A phase I tolerability trial and an ongoing phase II trial recommend a daily treatment with 45 mg/m² gambogic acid for 5 consecutive days[194,195]. The dose-limiting toxicity showed an involvement with liver dysfunction and pain[194].

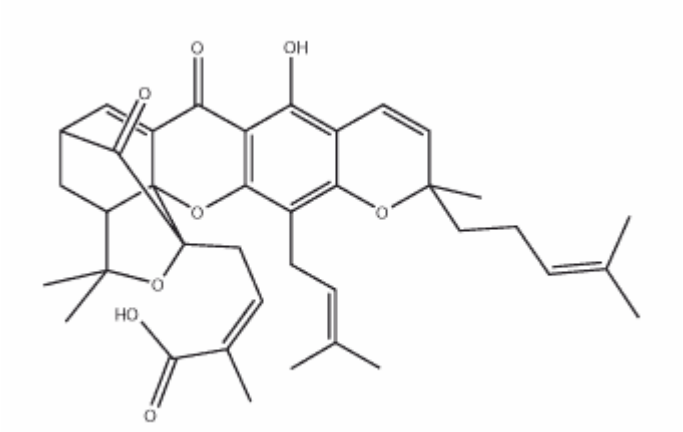

Figure 6: Chemical structures of gambogic acid.

4 CONCLUSION AND OUTLOOK

The topic of this doctoral thesis was deliberately defined broadly. There is little research about meningiomas especially basic research. Therefore existing data is often based on very few studies with limited methods, mostly immunohistochemical stainings, small patient pools, and insufficient controls. To begin with the existing data had to be verified. A promising approach such as selective COX-2 inhibitors had to be abandoned due to results contradicting present literature. It also became clear, that reproducible quantification methods for RNA and protein were essential.

Therefore the most stable reference gene for meningiomas and their control tissue was determined to quantify RNA using quantitative Real-Time PCR. Several Taqman® Protein Assays to quantify protein expression in meningiomas were established: firstly for active caspase-3 to determine farnesol-induced apoptosis, and secondly for several angiogenetic proteins including phosphorylated PDGFRβ. Thus it was possible to show that KDR protein expression was not detectable in most meningiomas even with a highly sensitive method as Taqman® Protein Assay. This method also enabled the quantification of phosphorylated PDGFRβ after stimulation with VEGFA and PDGFB. The inclusion of reliable controls and highly sensitive methods showed that VEGF primarily regulates VEGF-mediated migration and proliferation through PDGFRβ, not KDR, in meningiomas. This model is supported by findings, in which stimulation of meningiomas *in vitro* with VEGFA upregulated cell proliferation as well as induced PDGFRβ tyrosine phosphorylation. Selective PDGFRβ inhibitors such as gambogic acid and tandutinib were equally potent in inhibiting meningioma growth *in vitro* as unselective tyrosine kinase inhibitor sunitinib.

Based on these results gambogic acid is a promising candidate for a potential therapy of meningiomas as it combines anti-tumor activities with minimal toxic activities in pre-clinical experiments. Furthermore tyrosine kinase inhibitor (TKI) targeted therapies remain one of the most promising treatments against recurrent meningiomas with several clinical trials under way, which determine the efficacy of valatinib (VEGFR- TKI), sunitinib (VEGFR- and PDGFR - TKI), imatinib (PDGFR-TKI), and erlotinib (HER-1/EGFR-TKI) in meningioma patients. In the future it should be evaluated if KDR-negative and –positive meningiomas display different sensitivity towards gambogic acid. It should also be established at which

concentration gambogic acid induces apoptosis in meningiomas *in vitro* and *in vivo*. In addition, it should be analyzed if gambogic acid activates apoptosis by known targets such as transferrin receptor or NFκB. Therefore the expression of transferrin receptor in meningiomas should be determined. Also the efficacy of gambogic acid in meningiomas *in vivo* should be evaluated.

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6 ABBREVIATIONS

IC50	half maximal inhibitory concentration
pM	pico molar
µM	micro molar
A-549	adenocarcinomic human alveolar basal epithelial cells
AA	arachidonic acid
Akt	protein kinase B
AMP	adenosine monophosphate
APAF1	apoptotic protease activating factor 1
Bax	Bcl2 associated X protein
Bcl2	B-cell lymphoma 2
Bad	Bcl-2-associated death promoter
Bid	BH3 interacting-domain death agonist
BIR	baculovirus IAP repeat
Cdc42	cell division control protein 42 homolog
CNS	central nervous system
COX	cyclooxygenase
Cyt c	cytochrome c
DMEM	Dulbeccos modified Eagle Medium
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
Erk	extracellular-signal-regulated kinases
FA	Farnesol
FAK	focal adhesion kinase
FLT-1	vascular endothelial growth factor receptor 1
GA	Gambogic acid
IAP	inhibitors of apoptosis protein
IOMM-Lee	permanent malign meningioma cell line

JAK/STAT	janus kinase/signal transducer and activator of transcription
kDa	kilo Dalton
KDR	Kinase insert domain receptor; vascular endothelial growth factor receptor 2
LO	lipoxygenase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
NAIP	neuronal apoptosis inhibitory protein
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
N-WASP	neural Wiskott-Aldrich syndrome protein
PAK	p21 activated kinase
PARP1	poly [ADP-ribose] polymerase 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFB	PDGF-BB (for simplification; the gene encoding subunit B is called PDGFB)
PDGFRβ	platelet-derived growth factor receptor beta
PGE2	prostaglandine E2
PGH2	prostaglandine H2
PI3K	phosphatidylinositol 3-kinases
PIGF	placental growth factor
PLA2	phospholipase A2
qRT-PCR	quantitative real-time polymerase chain reaction
Rac	Ras-related C3 botulinum toxin substrate
Raf	RAF proto-oncogene serine/threonine-protein kinase
Ras	Rat sarcoma
RNA	ribonucleic acid
RPL37A	ribosomal protein L37a
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Smac/DIABLO	second mitochondrial activator of caspases; known as Diablo (direct IAP binding protein with low pI)
ssDNA	single-stranded DNA
TPA	Taqman® protein assay
TUNEL	terminal deoxynucleotidyl transferase-dUTP nick end labeling
TX	thromboxane
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis

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Are there attacking points in the eicosanoid cascade for chemotherapeutic options in benign meningiomas?

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Object. The current treatment for recurrent or malignant meningiomas with adjuvant therapies has not been satisfactory, and there is an intense interest in evaluating new molecular markers to act as therapeutic targets. Enzymes of the arachidonic acid (AA) cascade such as cyclooxygenase (COX)–2 or 5-lipoxygenase (5-LO) are upregulated in a number of epithelial tumors, but to date there are hardly any data about the expression of these markers in meningiomas. To find possible targets for chemotherapeutic intervention, the authors evaluated the expression of AA derivatives at different molecular levels in meningiomas.

Methods. One hundred and twenty-four meningioma surgical specimens and normal human cortical tissue samples were immunohistochemically and cytochemically stained for COX-2, COX-1, 5-LO, and prostaglandin E receptor 4 (PTGER4). In addition, Western blot and polymerase chain reaction (PCR) analyses were performed to detect the presence of eicosanoids in vivo and in vitro.

Results. Sixty (63%) of 95 benign meningiomas, 21 (88%) of 24 atypical meningiomas, all five malignant meningiomas, and all normal human cortex samples displayed high COX-2 immunoreactivity. All cultured specimens and IOMM-Lee cells stained positive for COX-2, COX-1, 5-LO, and PTGER4. The PCR analysis demonstrated no changes in eicosanoid expression among meningiomas of different World Health Organization grades and in normal human cortical and dura mater tissue.

Conclusions. Eicosanoid derivatives COX-1, COX-2, 5-LO, and PTGER4 enzymes show a high universal expression in meningiomas but are not upregulated in normal human cortex and dura tissue. This finding of the ubiquitous presence of these enzymes in meningiomas offers an excellent baseline for testing upcoming chemotherapeutic treatments. *(DOI: 10.3171/FOC-07/10/E8)*

Key Words • chemotherapy • eicosanoid • meningioma • polymerase chain reaction • Western blot

Meningiomas are usually benign tumors that arise from the leptomeningeal cells of the arachnoid membrane surrounding the brain and spinal cord. However, recurrence after seemingly complete surgical removal occurs in eight to 15% of cases, and tumor control rates achieved with radiotherapy are only 80%.[20,35,36,53] Moreover, a surgical cure is not always achievable in meningiomas involving the skull base or vital neurovascular structures. Results achieved with chemotherapeutic treatments in the past were not convincing, and even drugs such as temozolomide—which have shown high efficacy against malignant brain tumors—have failed to inhibit the growth of refractory meningiomas in Phase II studies.[7,26,39,45] The development of novel treatment strategies based on molecular information has not yet been successfully translated into common clinical practice.

Arachidonic acid is a v6 polyunsaturated fatty acid that is converted into biologically active lipid compounds called eicosanoids. Eicosanoids constitute a large family of biologically active lipid mediators produced by two enzyme classes: the cyclooxygenases (COX-1 and COX-2) and the lipoxygenases (5-LO, 12-LO, and 15-LO). Both classes catalyze the same enzymatic reaction that occurs in the synthesis of PG_2 and PGH_2, which are successively metabolized to PGE_2, PGD_2, PGF_2, thromboxane A_2, and prostacyclin PGI_2 (Fig. 1).[12]

The crucial role of COX enzymes has been clearly demonstrated in human colon cancer cells, in which COX-2 is often overexpressed.[30,40] Cyclooxygenase inhibitors,

Abbreviations used in this paper: AA = arachidonic acid; COX = cyclooxygenase; LO = lipoxygenase; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PG = prostaglandin; PTGER4 = prostaglandin E receptor 4; WHO = World Health Organization.

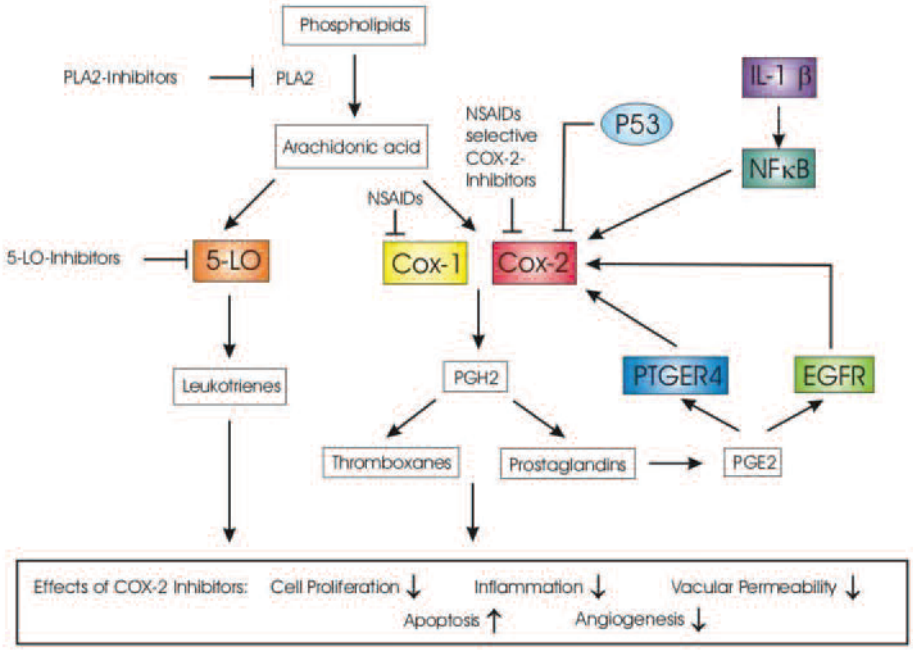

FIG. 1. Simplified diagram demonstrating the AA cascade and the enzymes produced. EGFR = epidermal growth factor receptor; IL = interleukin; NFκB = nuclear factor-κB; NSAIDs = nonsteroidal antiinflammatory drugs; PLA2 = phospholipase A2.

such as nonsteroidal antiinflammatory drugs and COX-2–specific inhibitors, can reduce the incidence of colorectal cancer in humans and experimental animal models, and can decrease the number and size of polyps in patients with familial adenomatous polyposis.[13] Recently, gliomas and meningiomas were also found to overexpress both COX and LO enzymes compared with normal brain tissue.[6,8,37] Matsuo and coworkers[32] reported that the staining intensity in glioblastomas was relatively weak, but that meningiomas and WHO Grade II/III astrocytomas were strongly positive for COX-2. Although much attention has been focused on the role of COX-derived metabolites in cancer development and progression, accumulating evidence suggests that 5-LO–derived eicosanoids may play an equally important role. 5-lipoxygenase mRNA and/or protein was found to be overexpressed in human breast, pancreatic, or colon cancer cells.[17,21,49] Boado et al.[3] reported increased expression of 5-LO mRNA in three of three meningioma surgical specimens examined.

Most tumors that express COX have been found to contain high levels of PGE_2.[10] Presumably, this especially bioactive lipid product of COX is responsible for some of the proneoplastic effects mediated by the enzymes such as inducing growth, migration, and invasiveness in colorectal carcinoma cells.[5] Little is known of the production of PGs in intracranial tumors; some authors have reported elevated PGE_2 levels in brain tumors in general and significantly higher PGE_2 level in meningiomas.[9,25] In contrast, other authors have reported no increase brain tumors' production of PGE_2.[14] In the present study, we demonstrate the ubiquitous expression of AA derivatives in human meningiomas with immunohistochemical staining, Western blot analysis, and PCR.

Materials and Methods

Tumor Specimens and Cell Culture

Meningioma surgical specimens were obtained from the Neurosurgical Department in accordance with regulations of the Ethics Committee of the University of Tübingen. Primary cultures were obtained from tumor tissue samples within 15 minutes of surgical removal. The samples were first washed in PBS, reduced and mashed through a filter and placed in Dulbecco modified Eagle's medium with fetal bovine serum, 2 mmol/L L-glutamine, and 0.1% 10 mg/ml gentamicin (Invitrogen). The cells were plated in 12.5-mm^2 tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 3 to 4 days, and cultures were split using 300-μl accutase (PAA). Viable cells were frozen in liquid nitrogen in 90% medium/10% dimethyl sulfoxide. As there is no PGE_2 antibody commercially available, the subtype 4 of the prostaglandin receptor E2 was selected for the studies because it is the only prostaglandin E2 receptor subtype that is expressed in brain. The IOMM-Lee cell line was a kind gift from Dr. Anita Lal (Department of Neurological Surgery, University of California, San Francisco).

Immunohistochemical Staining

Four-micron sections containing human meningiomas were cut from formalin-fixed tissue embedded in paraffin blocks. Normal cerebral cortex (Biochain Inc.), colon, and tonsil tissues were used as positive controls. Slides containing tissue were deparaffinized by bathing them in a series of histolene (Engelbrecht) and alcohol solutions. Vectastain Elite Universal Kits (Vector Laboratories) were used according to the manufacturer's protocol. Briefly, the

slides were treated with an antigen unmasking solution (citrate buffer solution at pH 6.0), rinsed with PBS, and bathed in a 3% H_2O_2 solution for 10 minutes, followed by blocking with avidinbiotin (Vector Laboratories). Slides were incubated overnight at 4˚C with primary COX-2 mouse monoclonal antibody (160112, Cayman Chemical) at a dilution of 1:50, COX-1 mouse monoclonal antibody (160110, Cayman Chemical) at a dilution of 1:100, 5-LO rabbit polyclonal antibody (101775, Cayman Chemical) at a dilution of 1:100, and PTGER4 rabbit polyclonal antibody (160402, Cayman Chemical) at a dilution of 1:50. After the samples were incubated with the primary antibody and washed twice with PBS, biotinylated secondary antibody was applied, and the samples were incubated with premixed avidin–biotin peroxide complex (Vector Laboratories). The final immunohistochemical staining was performed using Vector NovaRED (Vector Laboratories) to obtain a reaction to the avidin–biotin complex. Sections were counterstained with hematoxylin and examined under light microscopy.

The immunohistochemical samples were graded using a five-point scoring system to describe the percentage of cells that demonstrated positive staining, as previously published.[44] The scores were as follows: 0, no staining noted; 1, less than 1% of cells stained; 2, 1 to 10% of cells stained; 3, 11 to 50% of cells stained; and 4, more than 50% of cells stained.

Immunocytochemical Staining

Immunocytochemical analysis was performed on meningioma cells growing in a monolayer fashion in culture. The cells were subjected to treatment, plated in four-well glass slides, and allowed to remain in growth media for 2 to 3 days as described in the previous section. After removal of the growth media, the slides were rinsed with PBS for 5 minutes. Methanol maintained at –20˚C was added to each well for 10 minutes. After the methanol had been removed, the slides were rinsed twice with PBS and bathed in 3% H_2O_2 solution for 5 minutes to quench endogenous peroxidases. The incubation and staining protocols are identical to the immunohistochemical stainings described earlier.

Protein and RNA Isolation

Cytoplasmic and nuclear protein and RNA were isolated from surgical specimens and primary cell cultures using the protein and RNA isolation system (PARIS, Ambion, Inc.) according to the manufacturer's protocol. The nuclear and cytoplasmic protein and RNA was measured spectrophotometrically. The probes were stored at –80˚C until use.

Reverse Transcription and PCR

Ribonucleic acid samples were treated with DNase using the DNAfree kit (Ambion, Inc.) prior to reverse transcription–PCR analysis. A 0.4-μg sample of cytoplasmic and nuclear RNA was reverse-transcribed using random hexamer primers and the Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer's protocol. The PCR was performed with 50 ng of cDNA in a 50-μl reaction mixture containing FastStart Taq DNA Polymerase dNTPack (Roche) according to the manufacturer's protocol, with COX-2, COX-1, ACTB (loading control), PTGER4 primer (SuperArray), and 5-LO primer: forward, 5´-TCTCAAGCAACACCGACGTAAAG-3´; reverse, 5´-TGCATGAAGCGGTTGATGAA-3´ designed based on the sequence of human 5-LO cDNA.[40] The PCR conditions for COX-2, COX-1, ACTB, and PTGER4 were 95˚C for 15 minutes, 40 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 30 seconds, and one cycle of 72˚C for 10 minutes. For 5-LO the conditions were 40 cycles at 95˚C for 45 seconds, 60˚C for 1 minute, 72˚C for 1 minute, and one cycle at 72˚C for 10 minutes. Polymerase chain reaction products (16 μl) were subjected to electrophoresis on 10% TBE-Gel, and stained with the SilverQuest Silver Staining Kit (LC6070, Invitrogen, Inc.). The intensity of expression for each reaction was analyzed using a semiquantitative grading system as follows: Grade 0, no expression; Grade 1, minimal expression; Grade 2, moderate expression; Grade 3, marked expression; and Grade 4, strong expression.

Western Blot Analysis

Equal amounts of protein (~ 100 μg) were resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Nupage Bis-Tris-Gel 4–12%, Invitrogen, Inc.) and transferred to polyvinylidene difluoride Hybond-P membrane (Invitrogen, Inc.). The membrane was blocked and probed using a COX-2 mouse monoclonal antibody (160112, Cayman Chemical) at a dilution of 1:1000, COX-1 mouse monoclonal antibody (160110, Cayman Chemical) at a dilution of 1:100, 5-LO rabbit polyclonal antibody (101775, Cayman Chemical) at a dilution of 1:250, and PTGER4 rabbit polyclonal antibody (160402, Cayman Chemical) at a dilution of 1:200. An antibody to β-actin (1:1000; Ab8226, Abcam) was used to confirm equal loading among the samples. The Western blots were visualized using the Western Breeze Chromogenic Kit (Invitrogen, Inc.). The intensity of expression for each reaction was analyzed using the same semiquantitative grading system already described.

Statistical Analysis

Results are expressed as the means ± the standard error of the means. Statistical analysis was performed using the unpaired t-test. Probability values less than 0.05 were considered statistically significant.

Results

Patient Demographics and Tumor Characteristics

Tumor samples were obtained in 124 patients. The patients ranged from 22 to 88 years of age (mean 61 years), and 98 (79%) were women. Although 95 tumors (77%) were histologically benign, there were 24 atypical (19%) and five anaplastic meningiomas (4%). For the in vitro immunohistochemical staining studies, seven primary cell cultures were grown in monolayer cultures. Seventy-one percent of the cell lines had been obtained in female patients; the cell lines were derived from two benign, one atypical, one chordoid meningioma, and two malignant (IOMM-Lee) meningiomas. The primary cell cultures were immunohistochemically stained with epithelial membrane antigen and vimentin antibody to verify that they were

meningioma cells. A strong correlation between the Ki 67 proliferation index and the growth of tumor cells in culture has been seen ($p < 0.001$; Fig. 2).

Immunohistochemical Analysis of Eicosanoids in Meningiomas

Immunohistochemical staining for COX-2 was performed in 124 human meningioma surgical specimens, with tonsil tissue used as a positive control. Ninety-five benign (77%), 24 atypical (19%), and five (4%) anaplastic meningiomas stained positively for COX-2 (Table 1). The COX-2 immunoreactivity was found exclusively in the cytoplasm of all meningiomas studied. In fact 60 (63%) of the 95 benign meningiomas, and 21 (88%) of the 24 atypical meningiomas exhibited Grade 4 staining (Fig. 3D). Samples from 30 benign meningiomas (32%) and three atypical tumors (12%) displayed Grade 3 staining, and only five benign tumors (5%) displayed Grade 2 staining. No Grade 0 or 1 stainings were noted. Normal cerebral cortex tissue stained for COX-2 displayed strong staining of the cytoplasm (Grade 4; Fig. 3C). Meningioma tissue samples were also stained for COX-1, 5-LO (Fig. 3F), and PTGER4. All 5-LO- and PTGER4-stained samples exhibited Grade 4 staining, whereas all corresponding COX-1 samples displayed Grade 2 staining. As a control, the antibodies to COX-2, 5-LO, and PTGER4 were treated with matching blocking peptides. The meningioma tissues showed no staining for these antibodies (Fig. 3E).

Immunocytochemical Analysis of Eicosanoids in Meningiomas

The immunocytochemical analysis for COX-1, COX-2, 5-LO, and PTGER4 was performed in the IOMM-Lee malignant meningioma cell line and in six meningiomas grown in cell culture. All seven specimens exhibited extensive cytoplasmic staining of COX-2, 5-LO, and PTGER4 (Grade 4; Fig. 4D–F), whereas cells stained with COX-1 displayed slightly weaker coloring (Grade 3; Fig. 4C). The COX-2, 5-LO, and PTGER4 antibodies were treated with matching blocking peptides. The benign meningioma samples showed no staining (Fig. 3E).

Expression of Eicosanoid Protein in Meningiomas

Twenty meningioma specimens, 84 meningioma primary cell cultures, the IOMM-Lee cell line, normal cerbral cortex and one surgical specimen of human dura were processed for Western blot experiments. Cytoplasmic and nuclear protein extracts were isolated. Both the COX-1 and -2 proteins have a molecular weight of approximately 70 kD. The molecular weight of 5-LO is approximately 78 kD, and the loading control β-actin has a molecular weight of 42 kD. All meningiomas sampled exhibited staining of a large band of approximately 70 kD, and 78 kD in the cytoplasm extracts (Fig. 5). None of the nuclear protein extracts showed staining for COX-1, COX-2, and 5-LO as expected by cytoplasmic proteins.

Expression of Eicosanoid RNA in Meningiomas

There was expression of eicosanoid RNA in 20 meningioma surgical specimens, 84 meningioma primary cell cultures, the IOMM-Lee cell line, normal cerebral cortex, and two dura surgical specimens, as shown on reverse-transcriptase PCR analysis. The PCR products of COX-1, COX-2, 5-LO, and PTGER4 were 143, 168, 207, and 167 bp, respectively, and the product of the loading control ACTB was 183 bp. The semiquantitative derived mean value of COX-1 is significantly lower than the other investigated eicosanoids (compared with COX-2; $p < 0.01$, t-test). No change of expression in high-grade meningiomas was noted. The expression of COX-2 is comparable to normal cerebral parenchyma and dura in benign and atypical meningiomas. The higher expression of COX-2 in WHO

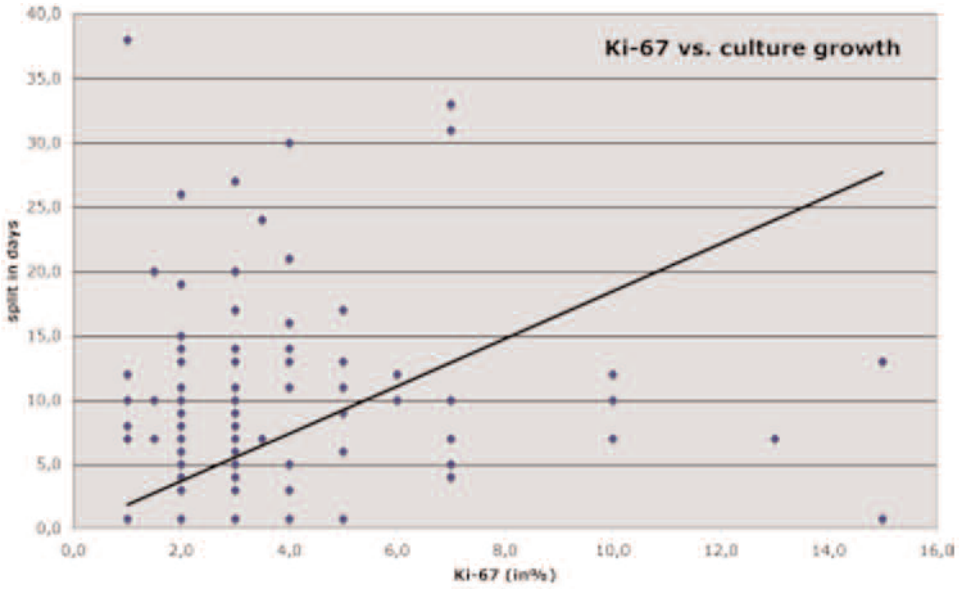

FIG. 2. Graph showing a linear regression of the Ki 67 proliferation index compared with culture growth.

TABLE 1

*Immunoreactivity to COX-2 in 124 meningiomas stratified by WHO grade**

	Level of Cox-2 Reactivity					
WHO Grade	0	1	2	3	4	Total
I (benign)	0	0	5 (4)	30 (24)	60 (48)	95 (77)
II (atypical)	0	0	0	3 (2)	21 (17)	24 (19)
III (anaplastic)	0	0	0	0	5 (4)	5 (4)

* No. of patients (%)

Grade III meningiomas might be due to a bias based on few tissue samples. There are no significant differences in the expression of 5-LO in meningiomas and in normal cerebral cortex and dura. The mean value of PTGER4 is the same for normal cerebral cortex and dura as for WHO Grades I and II meningiomas (Fig. 6).

Discussion

Meningiomas are the most frequent benign tumors of the central nervous system. Although generally slow growing, these lesions continue to be a major cause of complications and death due to recurrence and unresponsiveness to adjuvant therapies. The finding that AA derivatives are expressed in meningiomas is fundamental and might create potential therapeutic opportunities.[6,14,37,44] Few reports exist on the constitutive expression of these enzymes in healthy brain tissue, as this should be the baseline for tumor investigation.[18] In the central nervous system, COX-2 is expressed in components such as neurons, glia, and cerebrovascular elements,[2,19,28] or it can be induced by physiological or pathological stimuli.[52] In accordance with the findings of Castelli and coworkers,[6] who described similar expression of AA and metabolites in nine meningiomas and 10 healthy brain tissue specimens, we also found a high expression of AA derivatives in the healthy brain and in meningiomas of all grades. Ragel et al.[44] described an almost ubiquitous (95%) expression of COX-2 in 128 positively tested meningioma specimens. Because these researchers failed to compare their results with those from normal brain tissue, we validate their results adding data for healthy dura, normal brain tissue, and a substantial number of meningiomas of all different grades. Additionally, we verified the immunohistochemical findings through PCR, Western blot analysis, and immunocytochemistry, which should change the interpretation of previous reports proposing an upregulation of AA derivatives in meningiomas.[37] Although the investigated enzymes are not upregulated in meningiomas compared with normal brain and dura, future chemotherapeutic strategies could still have numerous starting points in attacking key molecules in tumor development.

Arachidonic acid metabolism via the COX and the LO pathways leads to the production of a number of metabolites that may modulate many of the mechanisms involved in tumor initiation, growth, and dissemination. Nathoo and associates[38] have demonstrated 5-LO expression solely in the cytoplasm of neurons in the healthy adult brain, whereas, in most astrocytoma surgical specimens, strong staining for 5-LO was seen in the nuclei of tumor cells. The authors argued that the activation of tumor cells may lead to the translocation of 5-LO to the nuclear membrane rather than the plasma membrane; furthermore, the nuclear import

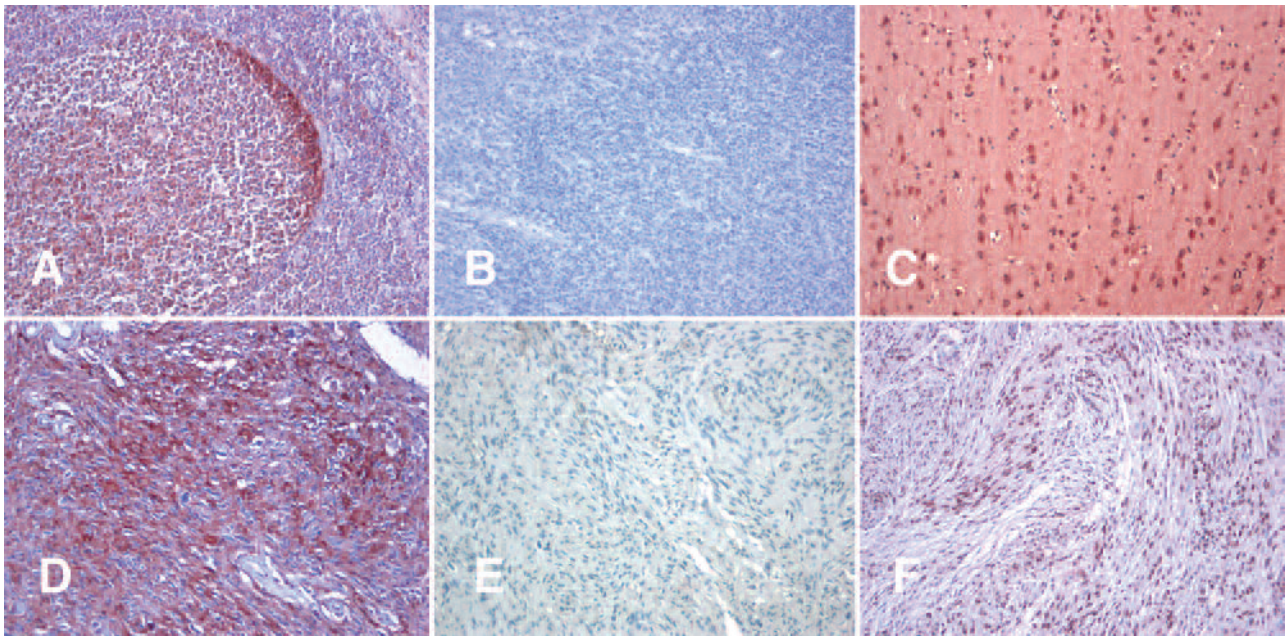

FIG. 3. Photomicrographs demonstrating eicosanoid expression. Positive immunoreactivity appears *red*. A: Positive control (human tonsil tissue) with strong COX-2 immunoreactivity in the cytoplasm. B: Negative control (tonsil tissue). C: Normal cerebral cortex showing strong COX-2 reactivity in neurons. D–F: Benign meningioma tissue. Strong COX-2 immunoreactivity is noted diffusely throughout the cytoplasm (D), no staining after incubation with COX-2 antibody and COX-2 blocking peptide (E), and strong immunoreactivity to 5-LO is noted diffusely throughout the cytoplasm (F). NovaRED, original magnification × 10.

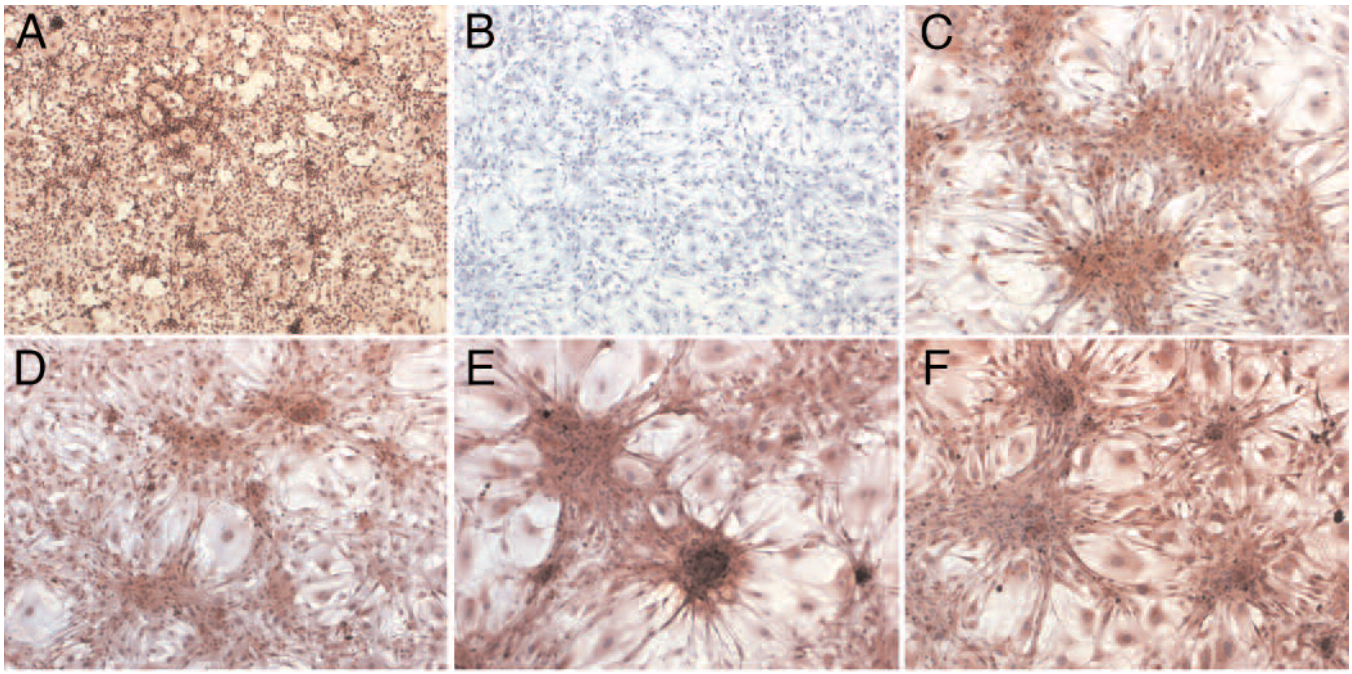

FIG. 4. Photomicrographs demonstrating expression of COX-2 in a culture of meningioma primary cells. Positive immunoreactivity appears *red*. A: Strong COX-2 immunoreactivity is seen in the cytoplasm of the IOMM-Lee cell line, used as the positive control. B: Negative control (meningioma tissue without primary antibody treatment). C: Grade 3 immunoreactivity to COX-1 is shown. D–F: Grade 4 COX-2 (D), 5-LO (E), and PTGER4 (F) immunoreactivity is noted diffusely throughout the cytoplasm in meningioma. NovaRED, original magnification × 4.

sequence of 5-LO protein has been identified in certain leukocytes.[22] We found high cytoplasmic expression of 5-LO in almost all meningioma samples, with no expression in the nuclei of the tumor cells—either on immunohistochemical slides or on protein analysis. It remains to be determined whether 5-LO protein in meningiomas is functionally active and whether tumor cells express leukotriene receptors that could mediate cellular responses to 5-LO eicosanoids. Because 5-LO inhibitors are cytotoxic to cancer cell lines derived from several types of malignant tumors, further research into the potential use of 5-LO inhibitors for cancer prevention and treatment is warranted.[1,15,16,38]

Staining for COX-1 had a significantly lower expression than for the other observed eicosanoids, in agreement with the results of the reverse-transcriptase PCR analysis. These observations correlate with the findings of Matsuo et al.,[32] who reported a weak expression of COX-1 compared to COX-2 in normal human brain tissues, meningiomas, and other brain tumors. This could be due to the constitutive expression of COX-2 in the normal brain and particularly in meningiomas, leading to a takeover of functions elsewhere assigned to COX-1. Pai and colleagues[41] reported that in addition to acting on its own receptor, PGE_2 could also activate the epidermal growth factor receptor, providing another potential mechanism for the tumor promotion effect of COX enzymes. Their results not only further support the role of COX-2 as a tumor promoter in the intestine, but also point to PGE_2 as the key mediator of the COX-2–related susceptibility to colon cancer. Wu and coworkers[51] published a study demonstrating inhibition of proliferation and induction of apoptosis by celecoxib in human cholangiocarcinoma cells. Their results suggest that COX-2 plays a central role in the production of PGE_2 and that therefore specific inhibition with celecoxib, as a chemopreventive and chemotherapeutic agent of COX-2, inhibits proliferation and induces apoptosis of cells via suppression of PGE_2 production. This study for the first time demonstrated that the brain intrinsic prostaglandin receptor PTGRE4 is strongly expressed in all meningioma specimens, pointing towards an important role of this enzyme in the development of meningiomas. Whether the compatible ligand of the receptor acts as a growth promotor or itself leads to molecular changes in the genetic program of the meningioma has to be elucidated.

As previously stated, COX-2 was expressed in all meningioma tissue samples. Because COX-2–derived metabolites may increase resistance to apoptosis, promote angiogenesis, and impair immune system surveillance, early prophylactic intervention with a COX-2 inhibitor may decrease tumorigenic progression in patients with malignant or invasive neoplasms. The tendency toward overexpression of COX-2 in more aggressive phenotypes of meningiomas suggests that COX-2 may play an important functional role in the later stages of invasive disease. Another hypothesis about increased COX-2 expression in more malignant meningiomas is that COX-2 levels could be an indicator of ischemia,[3] supported by findings of necrotic areas within the tumor. Moreover, establishing a correlation between COX-2 expression and tumor recurrence or the Ki 67 proliferation index was not feasible, due to the overall high expression of COX-2 in meningiomas of

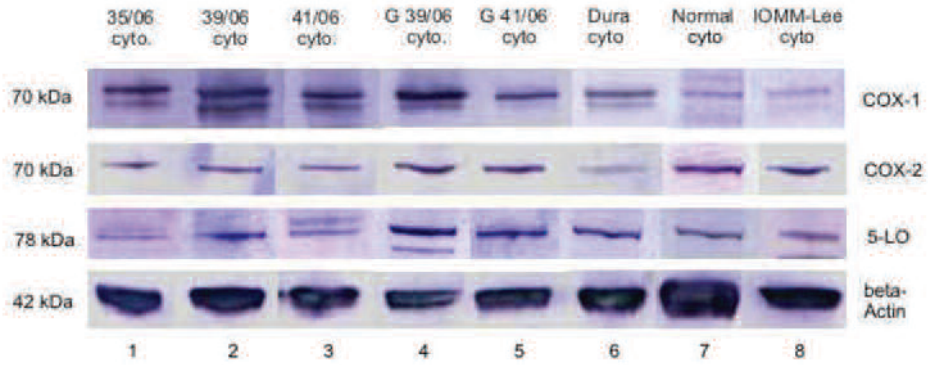

Fig. 5. Western blot analysis of three meningioma primary cells cultures, two meningioma surgical specimens, human dura, normal cerebral cortex, and IOMM-Lee cell line (numbered 1–8). Nuclear and cytoplasmic (cyto) protein extracts were isolated and probed with COX-1, COX-2, 5-LO, and beta-actin antibody (loading control). All nuclear protein extracts showed no staining, as expected by cytopasmic proteins. Lanes 1–5: All meningioma primary cell cultures and the fresh surgical specimens exhibited strong staining for COX-1, COX-2, and 5-LO. Lane 6: Human dura tissue stained positively for COX-1, COX-2 and 5-LO, although the band of COX-2 was weaker. Lane 7: Normal human cerebral cortex tissue showing corresponding staining for COX-2 and 5-LO, and weaker staining for COX-1. Lane 8: The positive control IOMM-Lee cell line exhibited strong staining for COX-2 and 5-LO, and weak staining for COX-1.

all grades. Based on the results of our study, it is not possible to declare COX-2 a prognostic indicator for tumor grading—as has been proposed by Lin et al.[28] in meningiomas and Deininger and coworkers[11] in oligodendrogliomas—because no significant differences have been detected in eicosanoid expression among meningiomas of different WHO grades.

Radiation therapy has an important role in the management of recurrent meningiomas. In preclinical models of different cancers and clinical studies of gliomas, the addition of COX-2 inhibitors to radiotherapy can delay tumor regrowth compared with the use of either drugs or radiation alone.[23,34,46,48] Pyo et al.[42] found that in colorectal tumors expressing COX-2 that had been treated with radiation therapy and COX-2 inhibitors, there was a greater therapeutic response than to radiation therapy alone. Inhibition of COX-2 is augmented by the antiangiogenic effect of ionizing radiation through inhibition of nuclear factor-κB.[4] The use of COX-2 inhibitors with ionizing radiation is a potentially attractive combination for recurrent meningioma.

In addition to the well-studied role of COX-2 in acute inflammatory processes, recent work clearly suggests that COX-2–derived metabolites contribute at multiple points in the early and late stages of carcinogenesis, including premalignant hyperproliferation, transformation, maintenance of tumor viability, growth, invasion, and metastatic spread.[24] We confirm that COX-2 might be a key player in a number of biological pathways leading not only to cancer in general, but also to meningiomas. Current evidence indicates that COX-2 promotes tumor-specific angiogenesis,[27,31] inhibits apoptosis,[50] and induces proangiogenic factors such as vascular endothelial growth factor,[29,47] inducible nitrogen oxide synthetase promoter,[33] and interleukin-6.[54] Taken together, the epidemiological data and preclinical studies in animal models have generated compelling interest in the potential use of COX-2 inhibitors in the prevention and chemotherapy of human tumors. Clinical trials are

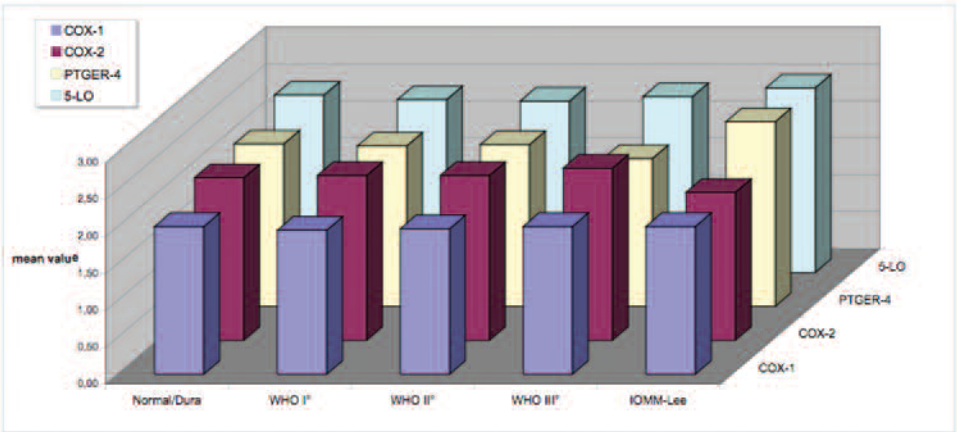

FIG. 6. Results of a PCR analysis of COX-1, COX-2, PTGER4, and 5-LO in meningiomas, normal cerebral cortex, human dura tissue, and the IOMM-Lee cell line. The mean values were determined by semiquantitative analysis. Beta-actin was used to confirm equal loading among samples.

necessary to determine whether COX-2 inhibitors will provide clinical benefit, as well as to define the intervention points during tumor progression that will allow for optimal efficacy.

Conclusions

The promising effect of COX-2 inhibitors in anaplastic meningiomas were recently reported by Ragel and colleagues,[43] although the effective dosage was far higher than the recommended plasma levels using standard dosages in humans. The association of COX-2 and meningioma is unique and represents a potential area for therapeutic intervention with selective COX-2 inhibitors, either as an adjunct to or in combination with radiation therapy. With the results of this study we confirm the existence of multiple attacking points in the eicosanoid cascade for a powerful chemotherapeutic treatment in recurrent meningiomas. Further studies are on the way to understand the influence and importance of single factors in this enzyme network so that treatment options can be explored.

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Evidence of ubiquitous *in vivo* and *in vitro* expression of pro-apoptotic Smac/DIABLO protein in meningioma cell lines

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Abstract. Although meningiomas are one of the most common tumors in the central nervous system, the adjuvant treatment for recurrent or malignant meningiomas is not satisfactory. An intense interest in evaluating new molecular markers that may serve as potential therapeutic targets exists. Changes in apoptosis mechanisms play important roles in tumor pathogenesis. One pro-apoptotic protein is Smac/DIABLO, which neutralizes the inhibitors of apoptosis (IAPs). As Smac/DIABLO has not been previously analyzed in meningiomas, We investigated the expression of Smac/DIABLO and survivin in primary meningioma cultures *in vivo* and *in vitro*. Expression of Smac/DIABLO, survivin and single-stranded (ss)DNA *in vivo* were determined immunohistochemically in 100 meningioma surgical specimens, dura and normal human cortex. The expression of the apoptotic enzymes *in vitro* was analyzed after RNA and protein isolation of all meningiomas via Western blotting and PCR. All examined meningiomas and normal cerebral cortex displayed intense positive cytoplasmic Smac/DIABLO immuno-reactivity. Survivin and ssDNA were expressed in all surgical specimens and showed weak staining overall. Examination of Smac/DIABLO protein via Western blotting showed distinct signals in the cytoplasmic extracts. PCR analysis displayed no changes of Smac/DIABLO and survivin expression in different meningioma grades, normal human cortical cortex or dura. Constant high-level Smac/DIABLO respectively low-level survivin expression in meningiomas and normal brain demonstrate similar apoptotic behavior of meningiomas compared to normal brain tissue. These findings indicate no pathological overexpression of survivin in meningiomas as is evident in several other cancer types impeding apoptosis.

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Key words: meningioma, apoptosis, Western blotting, PCR, survivin, Smac/DIABLO

Introduction

Meningiomas constitute one of the most intriguing and challenging tumors of the central nervous system, the definition of malignant potential is beset by the frequent discordance between histology and biologic behavior (1,2). Meningiomas originate from arachnoidal cap cells of the meningeal coverings of the brain, constituting approximatively 13-26% of all intracranial tumors (3,4). For most of these lesions, the conventional strategy of combining both surgery and radiation therapy for atypical or anaplastic meningiomas provides long-term effective and sufficient clinical results (5,6). However, some meningiomas recur or are resected subtotally due to their delicate location at skull-based structures. Additionally in a minority of patients, regrowth of tumor tissue after irradiation is a major clinical problem. Despite several chemotherapeutic trials, medical interventions in these tumors is unsatisfactory (7,8). The development of novel treatment strategies based on the molecular information has not yet been successfully translated to common clinical practice.

Abnormal apoptosis is a hallmark of human tumors and deregulation of the genes controlling the apoptotic cascade is closely related to tumor development, progression and recurrence (9). Among the many known regulators and effectors of apoptosis, the caspase family as well as survivin and opponent proteins play an important role in the execution phases of apoptosis (10,11). The function of survivin as a member of the family of inhibitors of apoptosis protein (IAP) has been explained by its ability to block the terminal effector cell-death proteases, caspase-3 and -7. In addition, high levels of survivin prevent cells from responding to apoptotic stimuli, such as serum deprivation and administration of the anticancer drug etoposide (12,13). However, the inhibitory action of survivin in caspase-3 activity has been debated (14).

Suppressing the activity of the IAPs themselves has been attributed to Smac [second mitochondrial activator of caspases; known as DIABLO (direct IAP binding protein with low pI) in mouse], a nuclear encoded, mitochondrially localized protein, which is released into the cytosol in response to apoptotic stimuli that disrupt the integrity of the mitochondria (15). Smac/DIABLO negatively regulates the caspase inhibitory properties of XIAP (X-linked IAP) by

Table I. Characteristics of 100 patients with meningioma.

Characteristics	Range
No. of patients	100
Sex, no. of patients (%)	
Male	26 (26)
Female	74 (74)
Age (years)	
Mean	62
Range	22-85
WHO grading, no. of patients (%)	
Benign	66 (66)
Atypical	30 (30)
Anaplastic	4 (4)

binding into the same pockets in XIAP which are used to bind caspases; when XIAP binds Smac/DIABLO, the caspases are displaced and primed to affect the execution phase of apoptosis (16).

It is of utmost importance to identify possible key players in this orchestra when aiming for clinical application of new chemotherapeutic agents (17). With this study we provide crucial information on several apoptotic proteins in meningiomas of different WHO grades, as these pro- and antiapoptotic enzymatic complexes in this tumor entity have not been previously studied in detail.

Materials and methods

Tumor specimens and cell culture. Meningioma surgical specimens were obtained from the Neurosurgical Department in accordance to regulations of the Ethics Committee of the University of Tübingen. Patient data associated with the *in vivo* and *in vitro* studies are characterized in Table I. Primary cultures were obtained from tumor tissue samples within 30 min of surgical removal. Samples were first washed in phosphate-buffered saline (PBS), reduced and mashed through a filter and placed in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS), 2 mmol/l L-glutamine and 0.1% 50 mg/ml Gentamicin (Invitrogen, Grand Island, NY). Cells were plated in 25-mm^2 tissue culture flasks and incubated at 37˚C in a humidified atmosphere of 5% CO_2. Medium was changed every 3-4 days and cultures were split using 600 μl Accutase (PAA, Pasching; Austria). Viable cells were stored in liquid nitrogen in 90% medium/10% dimethyl sulfoxide. The malignant meningioma IOMM-Lee cell line was a kind gift from A. Lal (Department of Neurological Surgery, University of California, San Francisco, CA, USA).

Immunohistochemical staining. Four-micron sections containing human meningiomas were cut from formalin-fixed tissue embedded in paraffin blocks. Normal cerebral cortex (Biochain, Inc., Hayward, CA), normal cerebral meninges and tonsil were used as positive controls. Slides containing tissue were deparaffinized by bathing them with a series of Histolene (Engelbrecht, Edermuende, Germany) and alcohol solutions. Vectastain Elite Universal Kits (Vector Laboratories, Burlingame, CA) were used according to the manufacturer's protocol. Briefly, the slides were treated with an antigen unmasking solution (citrate buffer solution pH 6.0), rinsed with PBS and bathed in a 3% H_2O_2 solution for 10 min, followed by blocking of avidin/biotin (Vector Laboratories). Slides were incubated overnight at 4˚C with primary Smac/DIABLO rabbit polyclonal antibody (Ab13817; Abcam, Cambridge, MA) at a dilution of 1:50, survivin rabbit polyclonal antibody (Ab469; Abcam) at a dilution of 1:100, EMA mouse monoclonal antibody (M0613; Dako, Hamburg) at a dilution of 1:100 and vimentin mouse monoclonal antibody (M7020; Dako) at a dilution of 1:200. Following the incubation with the primary antibody and a two-times PBS wash, biotinylated secondary antibody was applied, then incubated with premixed avidin-biotin-peroxide complex (ABC; Vector Laboratories). The final immunohistochemical staining was performed using Vector® Novared (Vector Laboratories) to obtain a reaction to the ABC. Sections were counterstained with hematoxylin and examined by light microscopic studies.

For immunohistochemical staining of ssDNA the deparaffinized slides were treated with 0.15 mg/ml saponin and 20 μg/ml proteinase K in PBS for 20 min. A three-time PBS wash was followed by an incubation with 50% formamide for 20 min at 60˚C. After a 5 min wash in ice-cold PBS the slides were blocked with 3% H_2O_2 solution for 5 and 15 min with 3% non-fat dry milk. Slides were incubated for 15 min with primary mouse monoclonal antibody ssDNA (ALX-804-192; Alexis, San Diego, CA) at a dilution of 1:100 in 1% non-fat dry milk. Following the incubation with the primary antibody and a two-time PBS wash, peroxidase-conjugated anti-mouse IgM secondary antibody (04-6820, Invitrogen, Inc., Carlsbad, CA) were applied for 15 min at a dilution of 1:100. The immunohistochemical staining was performed as described earlier.

Grading of immunohistochemical staining. The immunohistochemical samples were graded on the basis of a 5-point scoring system demonstrating the percentage of cells that showed positive staining, as previously published (18). The scores were as follows: 0, no staining noted; 1, <1% of cells stained; 2, 1-10% of cells stained; 3, 11-50% of cells stained; and 4, >50% of cells stained.

Immunocytochemical staining. An immunocytochemical analysis was performed on meningioma cells growing in a monolayer fashion in culture. The cells were subjected to treatment, plated in 4-well glass slides, and allowed to remain in growth media for 2-3 days as described earlier. After removal of the growth media, the slides were rinsed with PBS for 5 min followed by fixation for Smac/DIABLO and survivin with 4% PFA pH 7.0 in PBS for 15 min at room temperature. The slides were rinsed two-times with PBS and the PFA treated cells were incubated with 3% Triton-X 100 in PBS for 10 min. All cells were blocked with 10% goat serum in PBS for 30 min at room temperature. Slides were incubated 1 h at 37˚C with following primary antibodies:

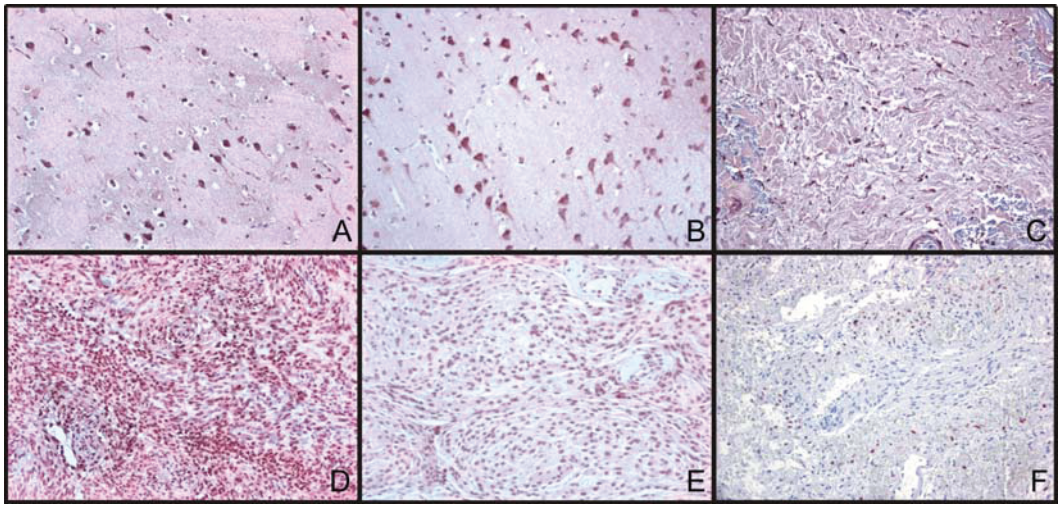

Figure 1. Photomicrographs demonstrating expression of apoptotic enzymes. Positive immunoreactivity appears as red staining (Vector® NovaRed™). Strong Smac/DIABLO immunoreactivity is seen in the cytoplasm of the neurons in normal brain (A) and in the cytoplasma of meningiomas (D). Survivin displayed a weak cytoplasmic and nuclear staining in normal brain (B) and meningiomas (E). Detection of ssDNA (F7-26) as positive apoptosis marker in cerebral meninges (C) and meningioma (F). Original magnification x10.

Smac/DIABLO rabbit polyclonal antibody (Ab13817; Abcam) at a dilution of 1:25, survivin rabbit polyclonal antibody (Ab469; Abcam) at a dilution of 1:50, EMA mouse monoclonal antibody (M0613; Dako) at a dilution of 1:100 and Vimentin mouse monoclonal antibody (M7020; Dako) at a dilution of 1:200. After a three-time wash with PBS, addition of the secondary fluorescence antibody Alexa Fluor 488 goat (1:200, Invitrogen, Inc.) for 1 h at room temperature in the dark. Finally after a two-time wash with PBS the slides were mounted with DAPI-containing fluorecence mounting medium.

Protein and ribonucleic acid isolation. Cytoplasmic and nuclear protein and ribonucleic acid (RNA) were isolated from the surgical specimens and primary cell cultures using Paris® kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol. The nuclear and cytoplasmic protein and RNA was measured spectrophotometrically. The probes were stored at -80˚C until use.

Reverse transcription and polymerase chain reaction. RNA samples were DNase treated using the DNA-free kit (Ambion, Inc.) prior to the RT-PCR analysis. Cytoplasmic and nuclear RNA (0.4 μg) were reverse-transcribed using random hexamer primers and Transcriptor First Strand cDNA-Synthesis kit (Roche, Mannheim, Germany), according to the manufacturer's protocol. PCR reaction was performed with 50 ng of cDNA in 50 μl reaction mixture containing FastStart Taq DNA Polymerase dNTPack (Roche), according to the manufacturer's protocol, with Smac/DIABLO, survivin and ACTB primer (loading control) (SuperArray, Frederick, MD). The PCR conditions were 95˚C for 15 min, 40 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, and 1 cycle of 72˚C for 10 min. PCR products (16 μl) were subjected to electrophoresis on 10% TBE-Gel and were stained with SilverQuest® Silver Staining Kit (Nr. LC6070, Invitrogen, Inc.). The intensity of expression for each reaction was analyzed by use of a semi-quantitative grading system: Grade 0, no expression; Grade 1, minimal expression; Grade 2, moderate expression; Grade 3, marked expression; and Grade 4, strong expression.

Western blot analysis. Equal amounts of protein (~100 μg) were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Nupage® Bis-Tris-Gel 4-12%; Invitrogen, Inc.) and transferred to polyvinylidene difluoride Hybond-P membrane (Invitrogen, Inc.). The membrane was blocked and probed using a Smac/DIABLO rabbit polyclonal antibody (AF789; R&D Systems, Minneapolis, MN) at a dilution of 1:2,000. An antibody against ß-actin (1:1,000; Ab8226, Abcam, Cambridge, UK) was used to confirm equal loading among the samples. The Western blots were visualized using Western Breeze® Chromogenic Kit (Invitrogen, Inc.). The intensity of expression for each reaction was analyzed by use of a semi-quantitative grading system: Grade 0, no expression; Grade 1, minimal expression; Grade 2, moderate expression; Grade 3, marked expression; and Grade 4, strong expression.

Statistical analysis. Results are expressed as mean ± SEM. Statistical analysis was performed using an unpaired t-test. P-values <0.05 were considered statistically significant.

Results

Patient demographics and tumor characteristics. Of the 100 patients studied, 74% were women and the patients ranged in age from 22 to 85 years with a median of 62 years (Table I). Most tumors were histologically benign; there were 30 atypical and 4 anaplastic meningiomas. For *in vitro* immunocytochemical staining studies, seven primary cell cultures were grown in monolayer cultures. The primary cell cultures were immunocytochemically stained with EMA and vimentin antibodies to verify presence of the meningioma tumor cells.

Immunohistochemical analysis of apoptosis enzymes in meningiomas. Immunohistochemical staining for Smac/DIABLO was performed on 100 human meningioma surgical specimens and tonsil tissue as positive control. All hundred meningiomas stained positively for Smac/DIABLO (Fig. 1D). The Smac/DIABLO immunoreactivity was found exclusively in the cytoplasm of all meningiomas studied. In fact, 61

Table II. Immunoreactivity grades of 100 human meningiomas immunohistochemically stained for Smac/DIABLO (%).

	Staining grade					
WHO	0	1	2	3	4	Total
Benign	0 (0)	1 (1)	4 (6)	30 (46)	31 (47)	66 (66)
Atypical	0 (0)	0 (0)	8 (27)	12 (40)	10 (33)	30 (30)
Malignant	0 (0)	0 (0)	0 (0)	2 (50)	2 (50)	4 (4)
Total	0 (0)	1 (1)	12 (12)	44 (44)	43 (43)	100 (100)

Table III. Immunoreactivity grades of 100 human meningiomas immunohistochemically stained for survivin (%).

	Staining grade					
WHO	0	1	2	3	4	Total
Benign	0 (0)	28 (43)	24 (36)	14 (21)	0 (0)	66 (66)
Atypical	0 (0)	18 (60)	6 (20)	6 (20)	0 (0)	30 (30)
Malignant	0 (0)	1 (25)	1 (25)	2 (50)	0 (0)	4 (4)
Total	0 (0)	47 (47)	31 (31)	22 (22)	0 (0)	100 (100)

Table IV. Immunoreactivity grades of 100 human meningiomas immunohistochemically stained for ssDNA (%).

	Staining grade					
WHO	0	1	2	3	4	Total
Benign	2 (3)	39 (59)	18 (27)	6 (9)	1 (2)	66 (66)
Atypical	3 (17)	20 (60)	4 (13)	0 (0)	1 (3)	30 (30)
Malignant	0 (0)	2 (50)	2 (50)	0 (0)	0 (0)	4 (4)
Total	7 (7)	61 (61)	24 (24)	6 (6)	2 (2)	100 (100)

(93%) of the 66 benign meningiomas and 22 (73%) of the 30 atypical meningiomas exhibited Grade 3 or 4 staining (Table II). Only seven benign meningiomas (7%) and eight atypical tumors (27%) displayed Grade 1 or 2 staining. Normal cerebral cortex stained for Smac/DIABLO displayed strong (Grade 4) staining of the cytoplasma (Fig. 1A).

The same tumors were examined for survivin and all displayed positive staining (Fig. 1E). Survivin immunoreactivity was found often in cytoplasm, sometimes in cytoplasm and nucleus and only rarely in the nucleus. In contrast to Smac/DIABLO no specimen displayed Grade 4 staining. Most of the benign meningiomas 52 (79%) and 24 atypical tumors (80%) exhibited Grade 1 or 2 staining (Table III). Fourteen (21%) of the 66 benign meningiomas and 6 (20%) of the 30 atypical meningiomas exhibited Grade 3 staining.

To verify the degree of apoptosis in meningiomas ssDNA was analyzed in the 100 specimens. Most meningiomas displayed a small amount of ssDNA. Forty-one benign (62%) and 22 atypical (77%) meningiomas showed no or almost no occurance of ssDNA (Table IV).

Immunocytochemical analysis of apotosis enzymes in meningiomas. The immunocytochemical analysis for Smac/DIABLO and survivin was performed on the IOMM-Lee malignant meningioma cell line and on six benign/atypical meningiomas grown in cell culture. All seven specimens exhibited extensive (Grade 4) cytoplasmatic staining of Smac/DIABLO (Fig. 2A, B, D and E), whereas cells stained with survivin displayed moderate colouring of the cytoplasm and sometimes of the nucleus (Grade 2) (Fig. 2C and F).

Expression of the Smac/DIABLO protein in meningiomas. One hundred meningioma primary cell cultures, the IOMM-Lee cell line, normal cerbral cortex and two surgical specimens of human dura were processed for Western blot experiments. Cytoplasmic and nuclear protein extracts were

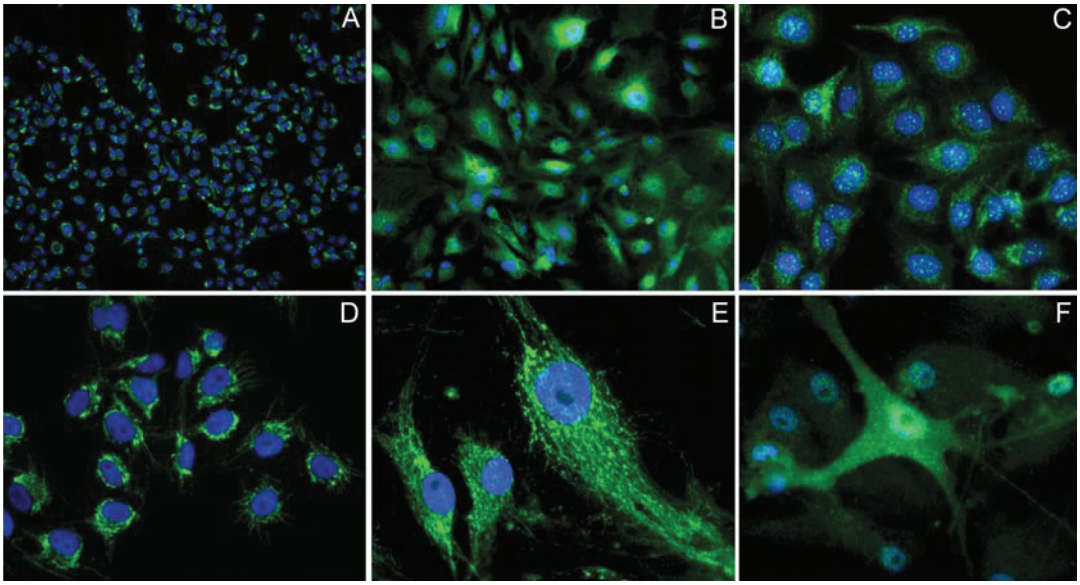

Figure 2. Expression of Smac/DIABLO in meningioma primary cell culture. Positive immunoreactivity appears as green staining (Alexa Fluor 488). Strong Smac/DIABLO immunoreactivity is seen in the cytoplasm of the IOMM-Lee cell line [(A), x10; (D), x40], and meningioma [(B), x10; (E), x40]. In contrast survivin displayed a weak cytoplasmic and sometimes nuclear staining in IOMM-Lee cell line (C) and meningioma (F). Original magnification x40 except for (A) and (B).

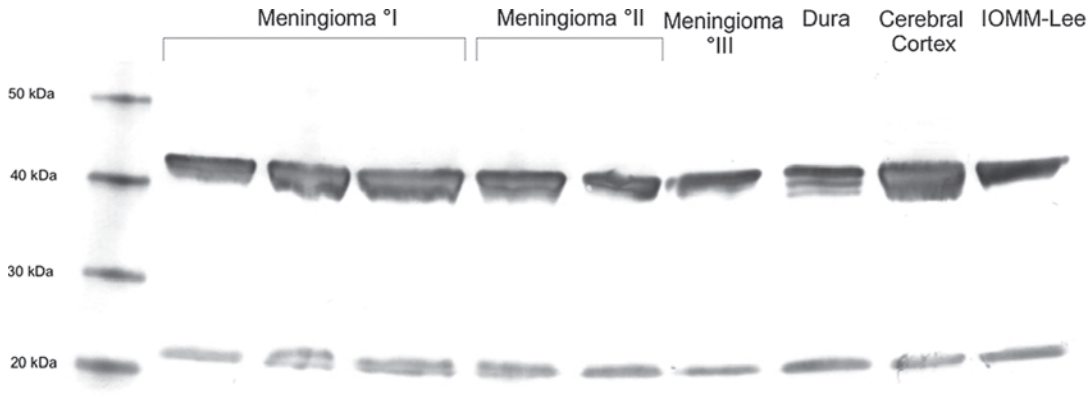

Figure 3. Western blot of Smac/DIABLO in meningiomas, IOMM-Lee cell line, normal cerebral cortex and human dura. Smac/DIABLO displayed a band at ~21 kDa in the cytoplasmic extracts and a large band at 42 kDa for ß-actin, which was used to confirm equal loading among samples. The mean values were determined by semi-quantitative analysis.

isolated. Smac/ DIABLO has a molecular weight of ~27 kDa and the loading control ß-actin 42 kDa. All meningiomas samples exhibited strong staining of a band at ~21 kDa in the cytoplasmic extracts for Smac/DIABLO and a large band at 42 kDa for the loading control ß-actin (Fig. 3). The nuclear protein extracts showed no staining for Smac/DIABLO as expected by cytoplasmic localization of the proteins.

Expression of apoptosis enzyme RNA in meningiomas. Expression of RNA was determined in 100 meningioma primary cell cultures, the IOMM-Lee cell line, normal cerebral cortex and three dura surgical specimens by reverse-transcriptase PCR analysis. PCR products of Smac/DIABLO and survivin were 118 and 73 bp, respectively. The product of the loading control ACTB was 183 bp. Smac/DIABLO showed a constant high-level expression in meningiomas, IOMM-Lee cell line, cerebral cortex and dura, in contrast to survivin, which was expressed throughout the specimens at a low-level. No changes in the expression of survivin with increasing WHO grade, in IOMM-Lee cell line, dura and cerebral cortex were seen (Fig. 4).

Discussion

Meningiomas are the most frequent tumors of the central nervous system. Although generally slow growing, they continue to be a major cause of morbidity and mortality due to recurrence and unresponsiveness to adjuvant therapies. The finding that apoptotic enzymes are expressed in meningiomas is fundamental and might create potential therapeutic opportunities. To identify the role of apoptosis in meningioma cell death regulation, we investigated macromolecular (RNA and protein) synthesis and activity in meningiomas of different malignancies.

A feature common to all successful multicellular organisms is the ability to control the cell number. This property is

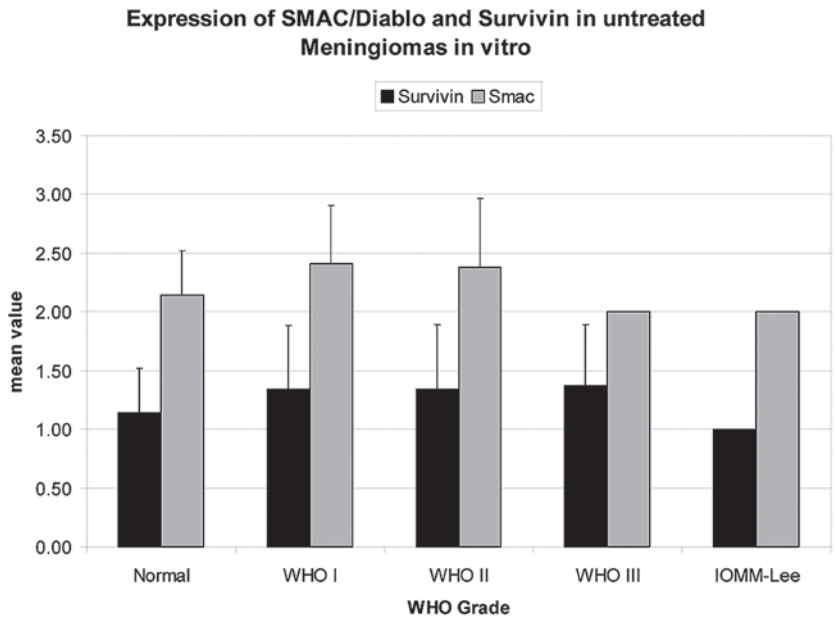

Figure 4. PCR analysis of Smac/DIABLO and survivin in meningiomas, IOMM-Lee cell line and normal cerebral cortex. Mean values where determined by semi-quantitative analysis. ß-actin was used to confirm equal loading among samples.

essential in developmental and adult phases, and is achieved by modulating both cellular birth and death (19). The latter event is largely affected through the activation of a strongly regulated series of events that lead to deconstruction, death and ultimately removal of the cell in a process known as apoptosis.

Survivin expression. Survivin is known to be expressed in fetal tissue and transformed cells in adults (20,21). It is expressed in most common human cancers of the lung, colon, pancreas, prostate, breast, esophagus, liver, stomach, uterus, bladder and skin, as well as in neuroblastomas and melanomas (21-27). The expression of survivin not only appears to correlate with aggressive tumor behavior and poor prognosis, but is also considered a risk factor for resistance to chemotherapy and radiation treatment (28). In the majority of primary nervous system tumors, particularly in glioblastomas and peripheral nerve sheath tumors survivin is also expressed (29). In glioblastomas, positive survivin expression was seen in immunohistochemistry and detected in high levels throughout the specimens, with a positive correlation to the Ki-67 proliferation index (30,31). However, no correlation between the intensity of survivin staining and the clinical course of the tumors has been seen (29). Moreover, simultaneous expression of survivin in both nucleus and cytoplasm has been interpreted as an important prognostic factor in astrocytoma (32).

Somewhat contrary results have been demonstrated regarding survivin expression in meningiomas: with immunoblotting techniques a high level of survivin protein has been shown (33), whereas flow cytometrical analysis showed that both spontaneous and radiation-induced apoptosis levels are very low in these neoplasms (34). Das *et al* found in benign meningiomas a survivin expression in 94% (85/90) of tissue samples and no correlation of expression with Bax/Bcl2 or clinicopathological factors (35). Kayaselcuk *et al* correlated survivin expression to recurrence by means of immunohistochemistry staining in 40 meningiomas. Although no or only mild expression in high grade meningiomas has been noted, there was a significant relation between grade 1 and expression of survivin and Ki-67 (36).

Combining *in vitro* and *in vivo* experiments we provide evidence of a low level expression throughout all grades of meningiomas, with no increase in meningiomas with a high cell turnover. The expression of survivin in benign slow growing tumors does not provide evidence that this anti-apoptotic protein might be related to tumor malignancy and poor prognosis in meningiomas. The suggested overexpression of survivin as an early event in the stepwise tumorigenesis and its responsibility for the growth advantage during tumorigenic progression can only be applied to malignant meningiomas (33). However, investigating the malignant IOMM-Lee cell line we suggest that these cells do not express typical features of a meningioma cell line.

Mediating survivin activity, either as a mitosis regulator and apoptosis inhibitor should be taken into account in therapeutic strategies using survivin (31). In an animal model, the topoisomerase-I-inhibitor irinotecan led to decrease in tumor growth in malignant menignioma that was accompanied by a decrease in Bcl-2 and survivin levels and an increase in apoptotic cell death. However, the treatment was much more effective against the IOMM-Lee cell line than against primary meningioma cultures. This focuses its treatment options to atypical or malignant meningiomas and should be evaluated further for this purpose (37).

ssDNA was stained *in vivo* to verify the amount of apoptosis in meningiomas, as the antibody only stains single-stranded apoptotic DNA but not necrotic cells (38-40), however no significant inverse correlation with Smac/DIABLO or surviving expression has been seen as previously shown in glioblastomas as well (31,41).

Smac/DIABLO expression. The functions of activated caspases are inhibited by the binding of inhibitors of apoptosis (IAPs). The function of IAPs is regulated by pro-apoptotic protein Second Mitochondria-Derived Activator of Caspases (Smac) or Direct IAP Binding Protein with low isoelectric point, pI (DIABLO). Induction of apoptosis leads to increased mitochondrial permeability to Smac/DIABLO, which adheres to IAPs inhibiting their caspase-binding activity. The role of Smac/DIABLO, therefore, may have significant diagnostic and therapeutic features in carcinogenesis (42). Tumor cells with reduced Smac resist apoptosis leading to the suggestion for an obligatory role for Smac/DIABLO in these tumor cells during several pathways of apoptosis induction (43). It has been shown to induce apoptosis in various types of cancer, including ovarian (44), renal cell (45), colon (42), bladder (46) and lung (47).

The presence of apoptosis initiating proteins in cells of the central nervous system have been verified in mammalian stroke studies (48-51). One report describes an inhibition of clonogenic tumor growth by Smac through blocking proliferation and enhancing apoptosis in a cell type-dependent manner in neuroblastoma cells (52). This is the first study detailing the findings of Smac/DIABLO in benign, atypical and anaplastic meningioma cells.

As demonstrated in renal cell carcinoma (53) mRNA expression levels of Smac/DIABLO in meningioma cells did not relate to tumor stage or grade, although Smac/DIABLO is expressed at a high level. The lower expression of Smac/DIABLO displayed in WHO grade III meningiomas did not appear in Western blotting or immunohistochemistry. This might be bias due to the small numbers of WHO III tissue samples in this series. The semi-quantitative derived mean value of survivin was significant lower than Smac/DIABLO (t-test, $P<0.01$).

Considering therapeutic options for treatment-resistant meningiomas synthetic Smac/DIABLO peptides have shown an enhancing effect of etoposide-induced apoptosis in human glioblastoma cell lines (54). This was also shown with various chemotherapeutic drugs and irradiation treated breast cancer cells, who either were overexpressed with the Smac/DIABLO gene or were treated with Smac/DIABLO peptide (55). Furthermore, Smac/DIABLO is able to stimulate induced apoptosis by promoting caspase-3 activation and cytochrome-c release in colon cancer cells (56) or via an escalation of inhibition of the Survivin/Smac-DIABLO interaction (57). Therefore it would be worthwhile to test this marker as a target for chemotherapeutic agents.

Verifying constant high-level Smac/DIABLO, respectively low-level survivin expression in meningiomas this study indicates that these apoptotic players might be beneficial as targets for adjuvant therapeutic strategies in recurrent atypical meningiomas, although no pathological overexpression of survivin was observed as in several other types of cancer impeding apoptosis.

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SHORT REPORT Open Access

Selection of suitable reference genes for quantitative real-time polymerase chain reaction in human meningiomas and arachnoidea

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Abstract

Findings: At first 32 housekeeping genes were analyzed in six randomly chosen meningiomas, brain and dura mater using geNorm, NormFinder, Bestkeeper-1 software and the comparative ΔCt method. Reference genes were ranked according to an integration tool for analyzing reference genes expression based on those four algorithms. Eight highest ranked reference genes (CASC3, EIF2B1, IPO8, MRPL19, PGK1, POP4, PPIA, and RPL37A) plus GAPDH and ACTB were then analyzed in 35 meningiomas, arachnoidea, dura mater and normal brain. NormFinder and Bestkeeper-1 identified RPL37A as the most stable expressed gene in meningiomas and their normal control tissue. NormFinder also determined the best combination of genes: RPL37A and EIF2B1. Commonly used reference genes GAPDH and ACTB were considered least stable genes. The critical influence of reference genes on qPCR data analysis is shown for VEGFA transcription patterns.

Background: In meningiomas quantitative real-time reverse transcription-polymerase chain reaction (qPCR) is most frequently used for accurate determination of gene expression using various reference genes. Although meningiomas are a heterogeneous group of tissue, no data have been reported to validate reference genes for meningiomas and their control tissues.

Conclusions: RPL37A is the optimal single reference gene for normalization of gene expression in meningiomas and their control tissues, although the use of the combination of RPL37A and EIF2B1 would provide more stable results.

Background

Meningiomas are the most frequent intracranial tumours. They originate from the arachnoidal cap cells of the meningeal coverings of the spinal cord and brain, constituting for approximatively 13 to 26% of all intracranial pathologies [1,2]. The conventional strategy for meningiomas is surgery [3,4]. However, some meningiomas recur as resection might be sub totally due to their delicate location at skull-based structures. The definition of malignant potential is beset by the frequent discordance between histology and biology [5,6]. Meningiomas are categorized in three WHO grades, in which there are several subtypes differentiated by their histological features.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a sensitive and reliable method for quantifying gene expression. Most frequently the relative quantification method is used, which requires the use of an internal control gene for normalization. Reference genes are mostly genes, which are involved in basic metabolism and maintenance of the cell. An ideal reference gene should be expressed at a constant level in all examined tissues and cells, and should not be influenced by experimental conditions. However several studies have shown, that genes used as reference gene display significantly different gene expression levels [7-9].

Established housekeeping genes in meningioma RT-qPCR experiments are genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-Actin (ACTB) [10-18] as well as ribosomal RNA (18S rRNA) and TATA binding box protein (TBP) [19-21]. As the application of these various housekeeping genes shows, there are no

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reports that candidate reference genes in meningiomas were validated. Due to the heterogeneity of meningioma tissue and the necessity to compare meningiomas and their control tissue reliably, the selection of an appropriate reference gene with stable gene expression throughout the various tissues is essential for further use of RT-qPCR in meningioma research.

In this study, we investigate the gene expression levels of 32 commonly used housekeeping genes in meningiomas and their control tissues arachnoidea, dura and normal brain. The RT-qPCR results were analyzed with four different algorithms, to select eight suitable reference genes. Those genes plus GAPDH and ACTB were compared in an increased number of meningiomas and control tissues. These RT-qPCR results were further analyzed with two different algorithms: NormFinder and Bestkeeper-1.

Methods

Tumour Specimens and Cell Culture

Meningioma surgical specimens as well as arachnoidea and dura mater were obtained from the Neurosurgical Department in accordance to regulations of the Ethic Committee of the University of Tuebingen. Primary cultures were obtained from tumour tissue samples within 30 minutes of surgical removal. Samples were first washed in phosphate-buffered saline (PBS), reduced and mashed through a filter and placed in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS), 2 mmol/L L-glutamine and 0,1% 10 mg/ml Gentamicin (Invitrogen, Grand Island, NY). Cells were plated in 25-mm^2 tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every 3 to 4 days and cultures were split using 600 μl Accutase (PAA, Pasching; Austria). Viable cells were stored in liquid nitrogen in 90% medium/10% dimethyl sulfoxide.

RNA isolation and reverse transcription

Meningioma total ribonucleic acid (RNA) was isolated directly from primary cell cultures before splitting and RNA of Arachnoidea and dura was isolated from fresh tissue using PARIS® kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol. RNA was treated with DNA-free™ (Ambion, Inc., Austin, TX) to remove residual genomic DNA. The concentration of the isolated RNA and the 260/280 absorbance ratio was measured in triplicates with Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). The integrity of RNA samples was confirmed by electrophoresis on a 2% Sybr Green agarose gel (Invitrogen Inc., Carlsbad, CA). The criterion to include RNA samples was 260/280 ~ 2 (1.9 to 2.2) and 28S/18S ratio ≥ 1.7. The probes were stored at - 80°C until use. For normal brain FirstChoice® Human Brain Reference RNA (Ambion, Inc., Austin, TX) was used, which pools RNA from different donors and several brain regions. RNA samples were DNase treated using DNAfree kit (Ambion Inc., Austin, TX). Total RNA (1 μg) was reverse-transcribed to cDNA using des High Capacity RNA-to-cDNA Kits (Applied Biosystems, Foster City, CA) in a total volume of 20 μl, according to the manufacturer's protocol.

Primer selection

For investigation to identify the most stable reference gene that could be used for normalization in RT-qPCR studies in meningiomas TaqMan® Express Plate Human Endogenous Control Plates (Applied Biosystems, Foster City, CA) were used, which contained 32 different genes plated in triplicates (Table 1). This collection of genes has been selected from literature searches and/or whole genome microarray tests carried out on numerous human tissues. They have been shown to be expressed constitutively and at moderate abundance across most test samples. All primers further evaluated spanned an exon junction to minimize inaccuracies due to genomic DNA contamination in RNA samples except the primer for S18. Additional information on assay optimisation and validation such as primer sequence for each TaqMan® Assay are available from Applied Biosystems. Priming conditions, primer concentration and annealing temperature was identical in all used TaqMan® Gene Expression Assays. TaqMan®-based detection was chosen, because this detection method detects only specific amplification products, whereas SYBR®-Green based detection detects all amplified double-stranded DNA, including nonspecific double-stranded DNA sequences, which may generate false positive signals. Melt curves were not assessed because they are only suitable for SYBR®-Green based detection.

For further evaluation single TaqMan® Gene Expression Assays for ACTB, CASC3, EIF2B1, GAPDH, IPO8, MRPL19, PGK1, POP4, PPIA, RPL37A (Applied Biosystems, Foster City, CA) were used, which were identical with the assays used in TaqMan® Express Plate Human Endogenous Control Plates.

Real-time PCR

TaqMan® real-time PCR was run in triplicates in 48-well reaction plates with a StepOne™ (Applied Biosystems, Foster City, CA). Real-time PCR reaction was performed with 1 μl cDNA (5 ng/μl) in 20 μl reaction mix containing 10 μl TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and 1 μl TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). The cycling conditions were as follows: initial holding period at 95°C for 10 min, followed by a two-step PCR program consisting of 95°C for 15 s and 60°C for 1 min for 40 cycles. Reverse transcriptase negative

Table 1 Candidate reference genes evaluated in this study

Gene Symbol	Gene Name	Genbank Acession No.	TaqMan®Assay ID	Amplicon length
18S	Eukaryotic 18S rRNA	X03205.1 (mRNA)	Hs99999901_s1	187
ABL1	v-abl Abelson murine leukemia oncogene homolog 1	NM_005157.3 + NM_007313.2	Hs00245445_m1	91
ACTB	**Actin, Beta, cytoplasmic**	NM_001101.3	**Hs99999903_m1**	**171**
B2M	Beta-2-microglobulin	NM_004048.2	Hs99999907_m1	75
CASC3	**cancer susceptibility candidate 3**	NM_007359.4	**Hs00201226_m1**	**67**
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_078467.1 + NM_000389.3	Hs00355782_m1	66
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	NM_004064.3	Hs00153277_m1	71
EIF2B1	**eukaryotic translation initiation factor 2B, subunit 1 alpha, 26 kDa**	NM_001414.3	**Hs00426752_m1**	**75**
ELF1	E74-like factor 1/ets domain transcription factor)	NM_172373.3 + NM_001145353.1	Hs00152844_m1	76
GADD45A	growth arrest and DNA-damage-inducible, alpha	NM_001924.2	Hs00169255_m1	123
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**	NM_002046.3	**Hs99999905_m1**	**122**
GUSB	Glucuronidase, Beta	NM_000181.3	Hs99999908_m1	81
HMBS	Hydromethylbilane synthase	NM_000190.3	Hs00609297_m1	64
HPRT1	Hypoxanthine guanine phospho- ribosyl transferase 1	NM_000194.2	Hs99999909_m1	100
IPO8	**Improtin 8**	NM_006390.2	**Hs00183553_m1**	**71**
MRPL19	**mitochondrial ribosomal protein L19**	NM_014763.3	**Hs00608519_m1**	**72**
MT-ATP6	mitochondrially encoded ATP synthase 6	NC_001807.ATP6.0	Hs02596862_g1	150
PES1	pescadillo homolog 1, containing BRCT domain (zebrafish)	NM_014303.2	Hs00362795_g1	56
PGK1	**Phosphoglycerate kinase 1**	NM_000291.3	**Hs99999906_m1**	**75**
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A, 220 kDa	NM_000937.3	Hs00172181_m1	61
POP4	**processing of precursor 4, ribonuclease P/MRP subunit**	NM_006627.2	**Hs00198357_m1**	**68**
PPIA	**Peptidylprolyl Isomerase A**	NM_021130.3	**Hs99999904_m1**	**98**
PSMC4	proteasome (prosome, macropain) 26S subunit, ATPase, 4	NM_153001.1 + NM_006503.2	Hs00197826_m1	83
PUM1	pumilio homolog 1 (Drosophila)	NM_001020658.1 + NM_014676.2	Hs00206469_m1	89
RPL30	ribosomal protein L30	NM_000989.2	Hs00265497_m1	149
RPL37A	**ribosomal protein L37A**	NM_000998.4	**Hs01102345_m1**	**125**
RPLP0	Ribosomal protein, large, P0	NM_053275.3 + NM_001002.3	Hs99999902_m1	105
RPS17	ribosomal protein S17	NM_001021.3	Hs00734303_g1	93
TBP	TATA binding box protein	NM_008907	Hs99999910_m1	127
TFRC	Transferrin receptor	NM_001128148.1 + NM_003234.2	Hs99999911_m1	105
UBC	Ubiquitin C	NM_021009.4	Hs00824723_m1	71
YWHAZ	Tyrosine 3-monooxygenase	NM_003406.3	Hs00237047_m1	70

Thickly printed reference genes were further evaluated. TaqMan® Assay ID ending with "_m" indicates an assay whose probe spans an exon junction and will not detect genomic DNA. "_g" indicates an assay that may detect genomic DNA. The assay primers and probe may also be within a single exon. "_s" indicates an assay whose primers and probes are designed within a single exon, such assays will, by definition detect genomic DNA. Additional information for each TaqMan® Assay is available from Applied Biosystems.

controls and "no template controls" (without cDNA in PCR) were included. Data were collected and quantitatively analyzed using StepOne™ Software v2.1. Relative quantitation analysis of gene expression data for VEGFA analysis was conducted according to the $2^{-\Delta\Delta Ct}$ method [22].

For PCR efficiency a 5-fold dilution series was created from a random pool of cDNA from our sample group ranging from 50 ng to 0.08 ng. PCR were performed as described above in triplicate. The PCR efficiency and correlation coefficients (R^2) of each TaqMan® Gene Expression Assay were generated using the slops of the standard curves. The efficiencies were calculated by the formula: efficiency (%) = $(10^{(-1/slope)} -1) * 100$. All assays displayed efficiencies between 93.2% and 100.2% (Table 2).

Statistical analysis

To compare the stability of candidate reference genes, four validation software programs were used according to their original publication: geNorm http://medgen.ugent.be/~jvdesomp/genorm[23], NormFinder http://www.mdl.dk/publicationsnormfinder.htm[24],

Table 2 Efficiency data for evaluated genes

Gene symbol	Slope	R^2	Efficiency (100%)
ACTB	- 3.420	1.000	96.1
CASC3	- 3.442	0.999	95.2
EIF2B1	- 3.434	0.997	95.5
GAPDH	- 3.430	1.000	95.7
IPO8	- 3.390	1.00	97.3
MRPL19	- 3.364	0.999	98.3
PGK1	- 3.266	0.994	100.2
POP4	- 3.410	0.999	96.4
PPIA	- 3.497	1.00	93.2
RPL37A	- 3.406	1.000	96.6

BestKeeper-1 http://www.gene-quantification.de/bestkeeper.html[25] and the comparative delta Ct method [26]. For geNorm and NormFinder the raw C_t values were transformed to quantities by using the delta C_t method [27]. The highest relative quantities for each gene were set to 1. Bestkeeper-1 and the comparative delta Ct method use raw C_t values. To evaluate the results from the four algorithms an integration tool for analyzing reference genes expression was used http://www.leonxie.com/referencegene.php. First according to the reference genes ranking by every algorithm from the most stable gene to the least stable gene, a series of continuous integers starting from 1 as weight to each reference gene is assigned. The geomean of each gene weights across the four algorithms is calculated and then these reference genes are re-ranked. The gene with the less geomean is viewed as more stable reference gene. Input data is value data from Real-Time qRT-PCR. Statistical analysis was performed with GraphPad Prism V5.03 (GraphPad Software, La Jolla, USA). Normality was assessed according to D'Agostino-Pearson tests with alpha = 0.05. For evaluation of statistical equivalence a confidence-interval version of the Two One-Sided Tests (TOST) procedure of Schuirmann was used [28]. The groups are considered equivalent at a 5% significance level if their difference has a 90% confidence interval that lies entirely inside the upper and lower equivalence limits. Therefore we considered $\pm \delta = \pm 1.5$ to be reasonable limits of equivalence.

Results

Expression levels of 32 reference genes in meningioma and normal tissue

To select suitable reference genes TaqMan® human endogenous control plates (Applied Biosystems, Foster City, CA, USA) were used containing 32 known housekeeping genes (Table 1). Four different meningiomas, the malignant meningioma cell line IOMM-Lee, normal brain, cerebral meninges and dura mater were analyzed regarding the gene expression levels of those housekeeping genes. The mean C_t values displayed a wide range of expression levels between 10.41 and 33.78 as shown in Figure 1. The most abundant transcript was S18 with median C_t value of 11.50 in meningiomas and a mean C_t value of 13.59 in normal tissue. In meningioma the lowest expressed genes were YHWAZ with median C_t value of 30.27 and TBP with 30.08. In normal tissue HMBS had the lowest expression with a median C_t value of 32.70. Tumour tissue and normal tissue group of five candidate reference genes (CASC3, CDKN1B, POLR2A, PUM1 and UBC) were statistically equivalent to within ± 1.5.

Expression stability of 32 candidate reference genes in meningioma and brain

All 32 housekeeping genes were analyzed using four different algorithms, geNorm NormFinder, Bestkeeper-2 and the comparative delta Ct method. An integration tool calculated the geomean of each gene across the four algorithms and ranked the reference genes according to their comprehensive gene stability (Figure 2). The three most stable reference genes were PGK1 > RPL37A > POP4. The least stable reference genes were CDKN1A > RPL0 > GADD45A. Three of four used algorithms ranked PGK1 highest, only Bestkeeper-1 ranked CASC3 highest and PGK1 only in fourteenth place. For further analysis eight of the highest ranked expression genes were chosen: PGK1, RPL37A, POP4, MRPL19, IPO8 and CASC3. Additionally the most used reference genes in meningioma qPCR experiments ACTB and GAPDH were also chosen although being only ranked in fifteenth respectively eighteens place and being considered inconsistent with a standard deviation (SD) higher than 1 by Bestkeeper-1. Three reference genes (CDKN1B, UBC and POLR2A) with equivalent tumour and normal tissue group were ranked low from position 19 to 22 respectively 13. CASC3 was the only one included for further investigation with statistically equal groups.

Expression stability of eight reference genes plus GAPDH and ACTB in meningiomas, arachnoidea, dura and normal brain

To validate the expression stability of CASC3, EIF2B1, IPO8, MRPL19, PGK1, POP4, PPIA, RPL37A plus GAPDH and ACTB, thirty-four randomly chosen primary cultured meningiomas, the meningioma cell line IOMM-Lee, two arachnoidea, six dura mater, one cerebral meninges and two pooled normal brain samples were screened for these reference genes. For analysis two different algorithms were chosen: NormFinder and Bestkeeper-1. NormFinder has a model-based approach whereas Bestkeeper-1 employs a pair-wise correlation analysis. NormFinder also estimates the variation between subgroups such as normal and cancer tissue.

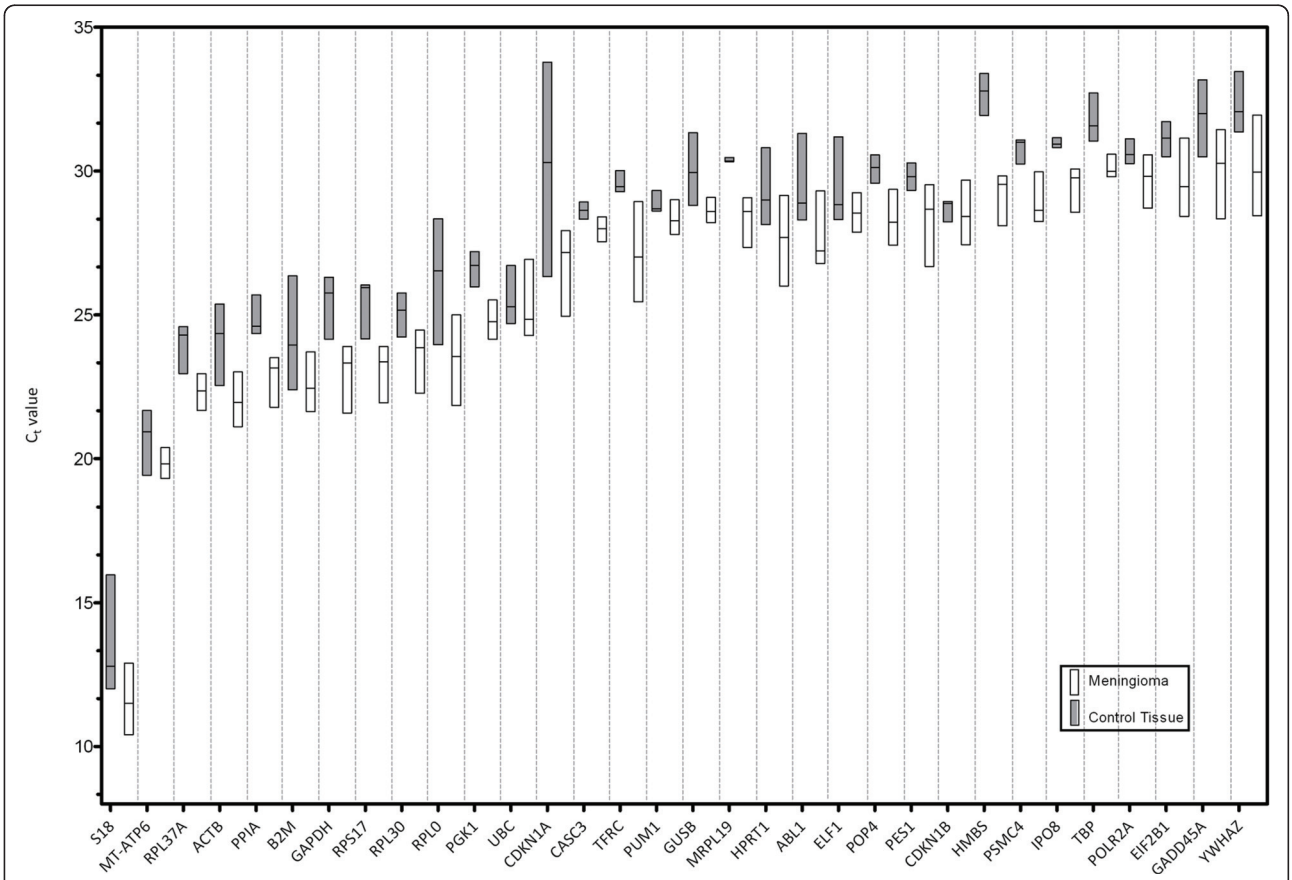

Figure 1 Expression levels of 32 candidate reference genes. Expression levels of 32 reference genes in four meningiomas, the malignant meningioma cell line IOMM-Lee, normal brain, cerebral meninges and dura mater. Real-Time PCR cycle threshold numbers are shown (C_t value). Box plots represent maximum and minimum values with median. Lower C_t values indicate higher gene expression.

Both algorithms identified RPL37A as the most stable gene in meningiomas and normal control tissue with an average expression stability value (M) value of 0.54 (Bestkeeper-1) respectively 0.12 (NormFinder). NormFinder not only determines the most stable gene, but also the best combination of two genes, which are RPL73A and EIF2B1 with a stability value of 0.088. The remaining ranking differed significantly for Bestkeeper-1 and NormFinder (Table 3). Bestkeeper-1 considered ACTB inconsistent with SD = 1.00 in meningiomas and their control tissue, whereas PGK1 was inconsistent in meningiomas (SD = 1.04). Both algorithms determined EIF2B1 and RPL37A as the two most stable genes in normal tissue (Table 5). In contrast there were significant differences between the ranking of Bestkeeper-1 and NormFinder in meningiomas (Table 4). Bestkeeper-1 ranked CASC3 and RPL37A highest. NormFinder identified MRPL19 and POP4 as the two most suitable genes, ranking CASC3 and RPL37A in eighth respectively ninth position.

TOST procedure showed statistical equivalence between normal tissue and meningiomas (± δ = ± 1.5) for three reference genes: CASC3 (+0.87), IPO8 (+0.57) and POP4 (+1.36). Those three genes were not normally distributed in meningiomas (CASC3 (P-value = 0.002), IPO8 (P-value < 0.0001) and POP4 (P-value = 0.0005). After inclusion of the normal tissue group IPO8 and POP4 remained not normally distributed.

Contribution of reference genes on expression levels of target genes

The selection of a reference gene for normalisation of qPCR can have a distinct influence on the expression profile of target genes [29]. To show the influence of different reference genes on the determination of gene expression levels, VEGFA expression levels in meningiomas and their control tissues were sequentially normalized with the analyzed ten reference genes. The C_t values for VEGFA were between 29 and 31. The expression level of VEGFA was normalized to each single reference gene as shown in Figure 3. The relative gene expression level (RQ) of VEGFA was calculated relative to the arachnoidea group ($RQ_{Arachnoidea}$ = 1). Subsequent bars represented the different expression levels of VEGFA in normal brain, dura and meningiomas normalized by

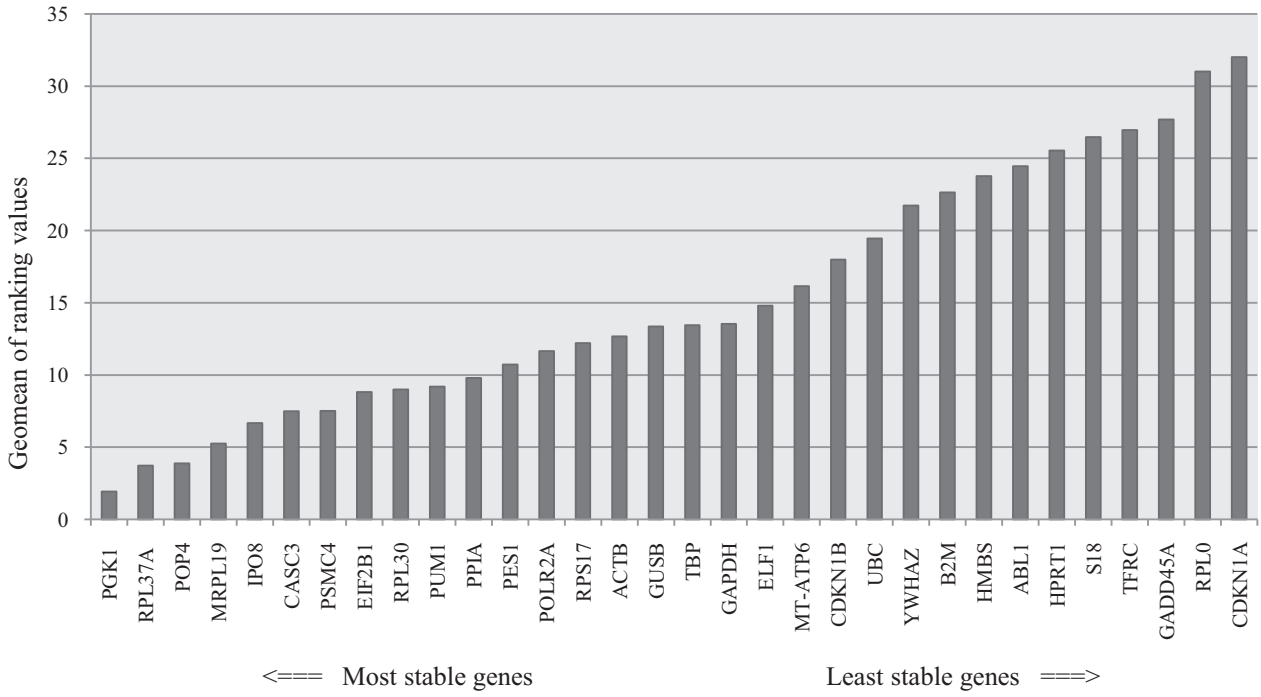

Figure 2 Comprehensive gene stability of 32 candidate reference genes in meningiomas and control tissue. Geomean of ranking values (y-axis) of 32 reference genes with their ranking from least to most stable gene expression (x-axis). Lower geomean values indicate more stable gene expression.

different reference genes. Most reference genes maintained the ratio between brain, dura and meningioma except IPO8 and CASC3. Normalization with IPO8 or CASC3 showed significantly increased ratio for brain to dura and brain to tumour.

Discussion

The requirement for distinct and reproducible results from quantitative gene expression analysis is accurate data normalization [23,24,29,30]. The application of an inappropriate reference gene can lead to false experimental conclusions [31-33]. Therefore one or more reference need to be chosen dependent on used tissue and experimental conditions.

To our knowledge, this is the first systematic analysis of average expression stability of reference genes in meningiomas for data normalisation in qPCR experiments. To evaluate the average expression stability four analysis software programs (geNorm, NormFinder,

Table 3 Ranking of ten candidate reference genes in meningiomas and their control tissue based on average expression stability value as calculated by Bestkeeper-1 and NormFinder

Rank	Bestkeeper-1		NormFinder	
	Gene name	Stability value	Gene name	Stability value
1	RPL37A	0.54	RPL37A	0.118
2	CASC3	0.54	EIF2B1	0.122
3	MRPL19	0.65	POP4	0.155
4	IPO8	0.66	MRPL19	0.163
5	POP4	0.70	PGK1	0.169
6	PPIA	0.73	PPIA	0.200
7	EIF2B1	0.74	GAPDH	0.286
8	PGK1	0.76	ACTB	0.287
9	GAPDH	0.83	CASC3	0.289
10	ACTB	1.00	IPO8	0.380

Table 4 Ranking of ten candidate reference genes in meningiomas based on average expression stability value as calculated by Bestkeeper-1 and NormFinder

Rank	Bestkeeper-1		NormFinder	
	Gene name	Stability value	Gene name	Stability value
1	CASC3	0.43	MRPL19	0.197
2	RPL37A	0.45	POP4	0.269
3	MRPL19	0.59	IPO8	0.277
4	IPO8	0.62	PPIA	0.312
5	PPIA	0.63	PGK1	0.332
6	POP4	0.63	EIF2B1	0.355
7	PGK1	0.67	GAPDH	0.386
8	EIF2B1	0.70	CASC3	0.388
9	GAPDH	0.75	RPL37A	0.447
10	ACTB	0.95	ACTB	0.521

Table 5 Ranking of ten candidate reference genes in normal control tissue based on average expression stability value as calculated by Bestkeeper-1 and NormFinder

Rank	Bestkeeper-1		NormFinder	
	Gene name	Stability value	Gene name	Stability value
1	EIF2B1	0.45	EIF2B1	0.306
2	RPL37A	0.56	RPL37A	0.318
3	PPIA	0.65	MRPL19	0.329
4	MRPL19	0.69	PPIA	0.404
5	ACTB	0.75	GAPDH	0.415
6	POP4	0.77	POP4	0.419
7	IPO8	0.82	CASC3	0.462
8	GAPDH	0.82	ACTB	0.542
9	CASC3	0.85	PGK1	0.590
10	PGK1	1.04	IPO8	0.621

Bestkeeper-1 and the comparative delta Ct method) based on different algorithms were used. So far various reference genes (GAPDH, ACTB, S18, TBP) were used in qPCR experiments in meningiomas [11-21,34], although GAPDH was mainly used for normalizations. This study demonstrates that none of these reference genes were ranked under the ten most stable genes of 32 analyzed reference genes. However GAPDH and ACTB as the most used reference genes in meningioma qPCR experiments were further analyzed. After reducing the number of reference genes and increasing the number of samples both reference genes were considered one of the least stable genes. Bestkeeper-1 considered ACTB unsuitable as reference gene in meningiomas and their control tissues.

Because there is so few data available for gene expression of reference genes in meningiomas a large number of reference genes were screened. Using four randomly chosen meningiomas, the malignant meningioma cell line IOMM-Lee, pooled normal brain, cerebral meninges and dura mater was sufficient to determine expression levels of all reference genes as shown in Table 1. Because the four algorithms use different approaches for their rankings of the 32 reference genes, the ranking differed significantly making a selection of genes for further investigation difficult. Using the integration tool which weighs the ranking of each algorithm made the selection easier and more comprehensible. The six most stable reference genes according to the integration tool (PGK1, RPL37A, POP4, MRPL19, IPO8 and CASC3) were chosen for further analysis. Additionally PPIA and EIF2B1 were selected. PPIA was the highest ranked gene, which displayed high expression levels. EIF2B1 was the most stable gene with low expression levels. Because RPL30 is potentially co-regulated with RPL37A, it was not chosen, so the outcome of the result would not be affected.

For a more detailed analysis the remaining ten reference genes were analyzed using an increased number of samples (n_{total} = 46 with n_{normal} = 11 and $n_{meningioma}$ = 35) but a decreased number of software (NormFinder and Bestkeeper-1). NormFinder was chosen because of

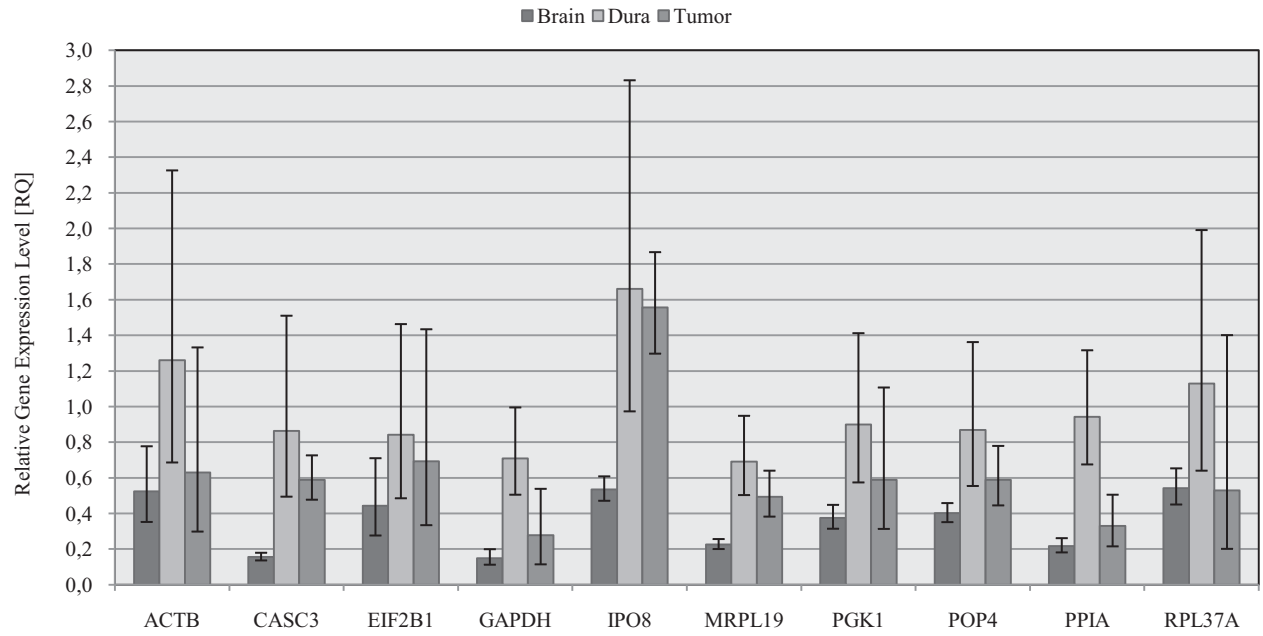

Figure 3 Contribution of reference gene selection to determination of target gene expression levels. Data is expressed as relative gene expression levels (RQ) to arachnoidea ($RQ_{arachnoidea}$ = 1). $RQ_{min.}$ and $RQ_{max.}$ are calculated with a confidence level of 95%.

the model-based approach and the additional estimation of variation between normal and cancer tissue. In contrast Bestkeeper-1 employs a pair-wise correlation analysis and uses raw C_t values whereas NormFinder uses transformed quantities. Also Bestkeeper-1 directly includes qPCR efficiency.

Both algorithms considered RPL37A as the most suitable reference gene for normalization in qPCR in meningiomas and their control tissue. The following ranking differed significantly especially for CASC3, IPO8 and EIF2B1. Bestkeeper-1 considered CASC3 as the most stable genes in meningiomas, but ranked CASC3 only in ninth place for normal control tissue. This led to a second place in the combined ranking due to the higher number of tumour samples. In contrast NormFinder ranked EIF2B1 highest for normal control tissue and only in sixth place in meningiomas. Because NormFinder weighs the two subgroups, normal tissue versus meningiomas, the ranking of the control tissue has more influence on the combined ranking. This is also demonstrated with IPO8 and conversely with RPL37A. NormFinder ranks RPL37A in meningiomas only in ninth place and in normal control tissue in second place. But after including the variation between those subgroups NormFinder displays RPL37A as the most stable gene for both subgroups.

Considering the results of the normalization of VEGFA against every single reference genes with significantly altered results for CASC3 and IPO8, NormFinder displays a more accurate ranking for meningiomas and their control tissue.

Some researchers recommend the use of multiple reference genes for calculating a normalization factor [23]. NormFinder also determines the best combination of two genes, when subgroups are included. For meningiomas and their normal control tissue the combination is RPL37A and EIF2B1.

Conclusions

In conclusion, the results from the current study demonstrate that RPL37A is the most appropriate single reference gene for the normalization process of gene profiling studies in meningiomas and their normal control tissue arachnoidea, dura mater and normal brain. If a combination of reference genes is applicable RPL37A and EIF2B1 are most suitable. Additionally results from the current study indicate that widely used GAPDH and ACTB are both inappropriate reference genes for meningiomas.

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Authors' contributions

CP carried out cell cultivation and the Real-Time PCR studies, performed the statistical analysis and drafted the manuscript. MS supervised the study and reviewed the final version of the manuscript. FR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Vascular endothelial growth factor signals through platelet-derived growth factor receptor β in meningiomas *in vitro*

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BACKGROUND: Vascular endothelial growth factor (VEGF)-mediated angiogenesis mediates tumour growth and metastasis. Meningiomas are primarily benign, slow-growing, highly vascularised tumours. Aside from VEGF, there is little data on the function of major angiogenic proteins in meningiomas.

METHODS: The VEGFA, platelet-derived growth factor B (PDGFB), and their respective receptors – VEGF receptor 2 (KDR) and PDGF receptor β (PDGFRβ) – were quantified using real-time PCR and a TaqMan Protein Assay in meningiomas *in vivo* and *in vitro*. The effect of VEGFA and PDGFB on cell proliferation and the tyrosine phosphorylation of PDGFRβ were examined.

RESULTS: Most meningiomas displayed no KDR protein expression but elevated PDGFRβ levels. Exogenous VEGFA stimulation significantly increased cell proliferation. The PDGFRβ inhibition before stimulation with VEGFA abolished the proliferative stimuli. The VEGFA induced concentration-dependent PDGFRβ tyrosine phosphorylation comparable to PDGFB-induced PDGFRβ tyrosine phosphorylation. The PDGFRβ inhibitors gambogic acid, sunitinib, and tandutinib equally impaired the migration of meningioma cells. In addition, gambogic acid suppressed the VEGFA-induced PDGFRβ tyrosine phosphorylation.

CONCLUSION: Collectively, our data suggest that VEGFA primarily regulates VEGF-mediated migration through PDGFRβ in meningiomas. The inhibitory effect of gambogic acid and tandutinib against meningioma growth *in vitro* suggests that selective PDGFRβ inhibitors, in combination with VEGF inhibitors, should be evaluated further as potential therapies for recurrent and malignant meningiomas.

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Meningiomas are the most common type of intracranial tumours. They originate from the arachnoidal cap cells of the meningeal cover of the spinal cord and brain and account for ~13–26% of all intracranial neoplasms (Riemenschneider *et al*, 2006; Louis *et al*, 2007). The conventional treatment for meningiomas is surgery (McMullen and Stieber, 2004). The 5-year recurrence rates after complete resection in benign, atypical, and anaplastic meningiomas were 3%, 38%, and 78%, respectively, for World Health Organisation (WHO) grade I meningiomas, the recurrence rate increased after incomplete resection from 7.5% to 9.8% at 10 years and 9.3% to 25.6% at 20 years (Jääskeläinen, 1986; Nakasu *et al*, 2009). The potential for malignancy is unresolved, due to a frequent discordance between histology and biology (Mahmood *et al*, 1993; Schittenhelm *et al*, 2006). Meningiomas are categorised into three grades by the WHO, and there are several subtypes based on histology.

Angiogenesis is essential for the enlargement of any solid tumour. Vascular endothelial growth factor (VEGF) is the key regulator of pathological angiogenesis (Ferrara *et al*, 2003). The VEGFA is the most abundant member of the VEGF family (Holmes and Zachary, 2005; Yamazaki and Morita, 2006), which comprising several isoforms, such as $VEGF_{165}$, through alternative splicing. There are three VEGF receptor tyrosine kinases (VEGFR1–3), but VEGFA only binds to VEGFR1 (FLT1) and VEGFR2 (KDR), and most of the signal transduction is mediated by the latter (Cross *et al*, 2003).

The VEGF and VEGFRs mediate angiogenesis in various brain tumours (Nishikawa *et al*, 1998; Huang *et al*, 2005). Although VEGF is upregulated in meningiomas (Provias and Claffey, 1997; Christov *et al*, 1999; Lamszuz *et al*, 2000; Park *et al*, 2000; Yamasaki, 2000), it is unknown whether the degree of malignancy correlates with the expression of VEGF (Provias and Claffey, 1997; Lamszuz *et al*, 2000; Yamasaki, 2000).

Platelet-derived growth factor B (PDGFB) is another critical factor that promotes the recruitment and proliferation of vascular cells (Yancopoulos *et al*, 2000). The PDGF signalling indirectly regulates angiogenesis. Platelet-derived growth factor receptor β (PDGFRβ) can induce the transcription and secretion of VEGF. The PDGFB and its receptors (PDGFR) are expressed in several human cancers, such as gliomas, breast cancer, colorectal cancer, and myelomonocytic leukaemia (Yu *et al*, 2003). The PDGFRβ and PDGFB are expressed in meningiomas, and their overexpression correlates with the WHO grade (Wang *et al*, 1990; Yang, 2001).

Several therapeutic agents that specifically target the angiogenic pathway, such as sunitinib and tandutinib, have entered clinical development. Both compounds are tyrosine kinase inhibitors, but while sunitinib inhibits at least eight receptor protein tyrosine kinases, including KDR and PDGFRβ (Mendel *et al*, 2003), tandutinib only acts on PDGFRβ, Fms-like tyrosine kinase 3 (FLT3), and c-Kit (Kelly *et al*, 2002). Sunitinib is used to treat

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metastatic renal cell cancer and gastrointestinal stromal tumours (Motzer *et al*, 2006), and tandutinib is in early-phase clinical evaluation for acute myeloid leukaemia (Cheng and Paz, 2008).

Recently, Liu *et al* (2010) demonstrated that gambogic acid inhibits cell migration by suppressing PDGFRβ tyrosine phosphorylation. Gambogic acid is the major active compound in gamboge, a resin from *Garcinia hanburyi*. Gambogic acid has potent apoptotic activity against several cancer cell lines *in vitro* and *in vivo*, including glioblastoma cell lines (Gu *et al*, 2008; Qiang *et al*, 2008).

In this study, we analysed the expression of VEGFA, KDR, PDGFB, and PDGFRβ *in vitro* and *in vivo* using quantitative real-time PCR (qRT–PCR) and the TaqMan Protein Assay. We examined the biological significance of VEGFA signalling through PDGFRβ by comparing the effects of VEGFA and PDGFB on meningioma cell proliferation and PDGFRβ tyrosine phosphorylation. We also determined the effects of gambogic acid and two tyrosine kinase inhibitors of PDGFRβ (sunitinib and tandutinib) on meningiomas *in vitro*.

MATERIALS AND METHODS

Tumour specimens and cell culture

The meningioma surgical specimens, arachnoidea, and dura were obtained from the Neurosurgical Department of the University of Tuebingen according to the guidelines of the ethics committee. The primary cultures were established from tumour tissue samples within 30 min of surgical removal. Samples were washed in phosphate-buffered saline (PBS), reduced, passed through a filter, and placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1% 10 mg ml^{-1} gentamycin (Invitrogen, Grand Island, NY, USA). The cells were plated in 25-mm^2 tissue culture flasks and incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 3–4 days, and the cultures were split with 600 μl Accutase (PAA, Pasching, Austria). The viable cells were stored in liquid nitrogen in 90% medium/10% dimethyl sulphoxide (DMSO). To verify the presence of tumour cells, the primary meningioma cultures were tested immunocytochemically for the expression of epithelial membrane antigen and vimentin. Both proteins are expressed in most meningiomas. The cells were also tested for the expression of CD34, an endothelial cell marker. The malignant meningioma IOMM-Lee cell line was a kind gift from A Lal (Department of Neurological Surgery, University of California, San Francisco, CA, USA). The IOMM-Lee cell line was tested using cytogenic characterisation. The human umbilical vein endothelial cells (HUVECs) (Genlantis + Cell Applications, Inc., San Diego, CA, USA) were cultured in endothelial cell growth medium (Genlantis, San Diego, CA, USA). The human prenatal neural cells (HNCs; dv Biologics, Costa Mesa, CA, USA) were cultured in Neurobasal A medium supplemented with B27 and 10 ng ml^{-1} FGF basic (Invitrogen). The human adult mesothelial cells (HMCs; Zen-Bio, Research Triangle Park, NC, USA) were cultured in mesothelial cell growth medium (MSO-1; Zen-Bio).

TaqMan protein assays

The following biotinylated antibodies (R&D Systems, Minneapolis, MN, USA) were used: VEGFA (BAF293), KDR (BAF357), PDGFB (BAF220), and PDGFRβ (BAF385). Phospho-PDGFRβ (AF1767) and phospho-KSR (AF1766) were labelled with biotin using the Biotin-XX Microscale Protein Labelling Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The biotinylated antibodies were labelled with the TaqMan Protein Assay Open Kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions. The specificity of each assay was determined using the recombinant proteins VEGFA, PDGFB (Invitrogen Inc.), KDR, and PDGFRβ (R&D Systems). Applied Biosystems recommends ΔCt values for recombinant proteins greater than 5. All ΔCt values for the above-mentioned assays were greater than 5 (VEGFA ΔCt = 8.32; KDR ΔCt = 7.13; PDGFB ΔCt = 8.44; and PDGFRβ ΔCt = 5.17). The assays for phospho-PDGFRβ and phospho-KDR consisted of probe A PDGFRβ and probe B phospho-PDGFRβ and probe A KDR and probe B phospho-KDR, respectively. The specificity for phosphorylated PDGFRβ and KDR was determined using the recombinant proteins for PDGFRβ and KDR, which the respective assay could not detect.

The total meningioma protein *in vitro* was isolated directly from primary cell cultures before being split using the PARIS kit (Ambion, Inc., Austin, TX, USA). *In vivo* meningioma and dura proteins were isolated from fresh tissue using the Protein Quant Sample Lysis kit (Applied Biosystems) according to manufacturer's protocol. A two-fold serial dilution was prepared according to manufacturer's protocol with a cell lysate input per well of 5 μg for meningiomas and dura. Total human brain protein (Biochain, Hayward, CA, USA) was used as a reference with an input amount of 25 μg. A TaqMan Protein Assay was performed using the TaqMan Protein Assay Core Reagents Kit with Master Mix (Applied Biosystems) according to manufacturer's instructions. qRT–PCR was performed on the StepOne Real-Time PCR System (Applied Biosystems) using the standard instrument protocol for the TaqMan Protein Assay. The data were analysed using the ProteinAssist Software 1.0 (Applied Biosystems), which uses the ΔCt^2 method to calculate relative protein expression between untreated controls and treated samples. The sample dilutions were assayed, and the resulting Ct values were normalised to the sample input, which requires accurate protein quantitation. Every 48-well plate included a no-protein control (NPC) to calculate the ΔCt values (Ct value (sample) − Ct value (NPC)). Then, a linear range was generated for each sample, and a ΔCt threshold was designated. The fold change between samples was calculated between the crossover points of each linear trend line at the ΔCt threshold.

RNA isolation and reverse transcription

Total meningioma RNA was isolated *in vitro* directly from primary cell cultures before being split. Meningioma, arachnoidea, and dura RNA were isolated *in vivo* from fresh tissue using the PARIS kit (Ambion, Inc.) according to manufacturer's protocol. The RNA was treated with DNAfree (Ambion, Inc.) to remove residual genomic DNA. The RNA concentration and 260/280 absorbance ratios were measured in triplicate on an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). The integrity of the RNA samples was confirmed by electrophoresis on a 2% SYBR Green agarose gel (Invitrogen Inc.). The criteria for the inclusion of RNA samples were a 260/280 ratio of ~2 (1.9–2.2) and a 28S/18S ratio of ⩾1.7. The probes were stored at −80°C until use.

For normal brain samples, FirstChoice Human Brain Reference RNA (Ambion, Inc.), which pools RNA from various donors and several regions of the brain, was used. The RNA samples were treated with DNAfree (Ambion Inc.). The total RNA (1 μg) was reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) in 20 μl according to manufacturer's protocol.

Quantitative real-time PCR

TaqMan qRT–PCR was performed in triplicate in 48-well reaction plates with StepOne (Applied Biosystems). Each reaction contained 1 μl of cDNA (5 ng μl^{-1}) in 20 μl of reaction mix, containing 10 μl of TaqMan Gene Expression Master Mix (Applied Biosystems) and 1 μl of TaqMan Gene Expression Assay (Applied Biosystems).

Translational Therapeutics

The following TaqMan Gene Expression primers were used: VEGFA (Hs99999070_m1), KDR (Hs00911700_m1), PDGFB (Hs00234042_m1), and PDGFRB (Hs00387364_m1). All primers span an exon junction and will not detect genomic DNA. The cycling conditions were as follows: an initial hold at 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 40 cycles.

Reverse transcriptase-negative controls and no-template controls (without cDNA) were included. RPL37A (Hs01102345_m1), which is the optimal reference for meningiomas and control tissues (Pfister *et al*, 2011), was used as the reference gene for relative quantification. The data were collected and analysed quantitatively using StepOne, v2.2 (Applied Biosystems). The relative gene expression data were analysed using the $2^{-\Delta\Delta Ct}$ method (Erickson *et al*, 2007).

Immunohistochemistry

Four-micron sections that contained human meningiomas were cut from formalin-fixed paraffin-embedded tissue blocks. The slides were deparaffinised in a series of histolene (Engelbrecht, Edermuende, Germany) and alcohol solutions. The Vectastain Elite Universal Kit (Vector Laboratories, Burlingame, CA, USA) was used according to manufacturer's protocol. Briefly, the slides were treated with an antigen unmasking solution (citrate buffer, pH 6.0; 94°C; 30 min), rinsed with PBS, and bathed in 3% H_2O_2 for 10 min, followed by blocking with avidin/biotin (Vector Laboratories).

The slides were incubated overnight at 4°C with a primary VEGFA rabbit polyclonal antibody (sc-152; Santa Cruz, Santa Cruz, CA, USA) at 1 : 60, a KDR rabbit polyclonal antibody (Ab2349; Abcam, Cambridge, MA, USA) at 1 : 100, a PDGFB rabbit polyclonal antibody (sc-127; Santa Cruz) at 1 : 100, or a PDGFRβ rabbit polyclonal antibody (sc-339; Santa Cruz) at 1 : 200. Next, the slides were washed three times with PBS, a biotinylated secondary antibody was applied, and a premixed avidin-biotin-peroxide complex was added (ABC; Vector Laboratories). The stains were developed with Vector Novared (Vector Laboratories). The positive controls were selected for each specimen as follows: VEGFA + KDR (human kidney), PDGFRβ (human placenta), and PDGFB (human pancreas). The negative control slides were incubated either without primary antibody or with a blocking peptide. The sections were counterstained with haematoxylin and examined using light microscopy. The immunohistochemical samples were graded from 0% to 100% based on the percentage of cells that were positive relative to the total tissue.

Growth factors, inhibitors, and drugs

$VEGF_{165}$ (VEGFA, PHC9391; Invitrogen Inc.) was reconstituted in sterile distilled water, aliquoted, and stored at −20°C until use. PDGF-BB (PDGFB, PHG0041; Invitrogen Inc.) was reconstituted in 100 mM acetic acid and 0.1% BSA, aliquoted, and stored at −20°C until use. The VEGFR2 (KDR) kinase inhibitor VI (Ki8751) (676484; Merck, Darmstadt, Germany) inhibits KDR with an IC_{50} of 0.9 nM and has no effect on PDGFRβ at concentrations as high as 10 μM. PDGFR tyrosine kinase inhibitor III (521232; Merck) inhibits PDGFRβ with an IC_{50} of 80 nM with no inhibitory effect on KDR. Both inhibitors were dissolved in DMSO (Sigma-Aldrich, St Louis, MO, USA) and stored at −20°C. Sunitinib malate (SU-11248, Sutent) and gambogic acid were obtained from Santa Cruz, and tandutinib (MLN518) was purchased from Selleck Chemicals (Houston, TX, USA). The stock solutions of sunitinib, tandutinib, and gambogic acid were dissolved in DMSO, stored at −20°C, and diluted in fresh medium immediately before use.

Proliferation assay

The IOMM-Lee cells and primary meningioma cells in mid-log phase were seeded onto 24-well culture plates in DMEM containing 10% FBS for 24 h. Subsequently, the cells were treated with increasing concentrations of VEGFA (0, 5, 10, 20, 50, and 100 ng ml^{-1}) in serum-free medium for 4 h. After 3 h of treatment, 5-Bromo-2′-deoxy-uridine (BrdU) was added to each well (1 : 1000). After incubation, BrdU was detected using the BrdU Labelling and Detection Kit II (Roche, Mannheim, Germany) according to manufacturer's protocol. The cell number was evaluated under a light microscope.

GAP assay

Primary meningioma cells (0.3×10^6 cells per ml) were seeded onto 24-well tissue culture dishes using the Radius 24-Well Cell Migration Assay (Cell Biolabs, Inc., San Diego, CA, USA) according to manufacturer's instructions. After 24 h of incubation to allow spreading, the gel was removed. The compounds were added in a medium that contained 10% FBS, and the cells were incubated for 24 h with various concentrations of sunitinib, tandutinib, and gambogic acid (0.125–10 μg ml^{-1}) to determine their effect on cell migration. The meningioma cell migration was observed and photographed under an inverted microscope before and after 24 h of inhibition with sunitinib, tandutinib, or gambogic acid. The cells were analysed using CellProfiler (www.cellprofiler.org) (Carpenter *et al*, 2006).

Statistical analysis

Statistical analysis was performed with GraphPad Prism, V5.03 (GraphPad Software, La Jolla, CA, USA). All results are expressed as mean values ± standard deviation (s.d.). Statistical comparisons between two groups were made using the Mann–Whitney test. For statistical comparisons between a group and the human brain reference, the Wilcoxon signed-rank test was used. Significant differences between sets of data were determined using a paired *t*-test. The level of significance was set at $P<0.05$. All tests were two-sided.

RESULTS

Elevated PDGFRβ expression in meningiomas

The RNA and protein expression was measured in meningiomas *in vitro* and *in vivo* (Table 1). The RNA expression was quantified using qRT–PCR with RPL37A for normalisation. The protein levels were measured with a TaqMan Protein Assay. The human brain was used as a reference. Only three samples of arachnoidea, the cap cells from which meningiomas originate, were obtained. Due to the small sample size and the structure of the arachnoidea, only a small amount of RNA and protein was isolated. The RNA and protein levels of several angiogenesis genes in the arachnoidea and

Table 1 The number of meningiomas used for the *in vivo* and *in vitro* experiments

	RNA		Protein	
	In vivo	*In vitro*	*In vivo*	*In vitro*
Arachnoidea	3	–	3	–
Dura	10	–	6	–
Meningioma WHO °I	28	39	17	17
Meningioma WHO °II	21	34	17	17
Meningioma WHO °III	3	5	3	5

Abbreviation: WHO = World Health Organisation. *In vivo* RNA and protein were isolated directly from fresh surgical specimen. For the *in vitro* RNA and protein isolation, the surgical specimen were processed and cultured, and the RNA and protein were isolated before the cells were split. The RNA and protein were isolated from human dura directly due to the difficulties in handling.

dura were similar, with the exception of KDR, which was significantly higher in arachnoidea ($P=0.002$). Thus, we chose to measure the gene expression in the dura, from which ample RNA and protein could be isolated. Due to the small number of arachnoidea samples, the expression of angiogenesis genes in meningiomas was compared with the expression in dura to detect significant changes in expression levels, except for KDR. All results are expressed as the relative gene expression levels (RQ) to brain ($RQ_{Brain}=1$) for RNA or as the fold change with brain as reference (fold $change_{Brain}=1$) for protein.

The PDGFRβ RNA and protein expression was significantly higher in meningiomas compared with the brain ($P<0.0001$) (Figure 1A). The PDGFRβ RNA expression *in vitro* was significantly decreased in WHO °III ($P_{WHO\ °I\ vs\ WHO\ °III}=0.0198$ and $P_{WHO\ °II\ vs\ WHO\ °III}=0.0004$), but there was no difference between WHO °I and °II. The *in vivo* RNA expression of PDGFRβ was significantly decreased in meningiomas compared with dura ($P=0.0014$) but displayed no significant changes between WHO grades. In contrast to the *in vitro* RNA levels, the *in vitro* protein expression of PDGFRβ was significantly decreased in dura compared with meningioma ($P=0.042$). The PDGFRβ RNA and protein levels *in vitro* were significantly higher than those detected *in vivo* ($P_{RNA}=0.0002$; $P_{Protein}=0.0045$).

Most meningiomas displayed no KDR protein expression

The KDR RNA and protein expression was significantly elevated in arachnoidea compared with dura ($P_{RNA}=0.002$; $P_{Protein}=0.0167$) (Figure 1B). In addition, the KDR levels were higher in dura compared with the brain ($P_{RNA}=0.002$; $P_{Protein}=0.0156$). In contrast, the KDR RNA and protein expression in meningiomas was significantly decreased ($P_{RNA}=0.001$; $P_{Protein}=0.0014$). In fact, KDR could not be detected in most meningiomas. Only 4 of 38 (10.3%) primary meningioma cell cultures displayed positive KDR protein expression (Figure 1C). In all, 8 of the 37 (21.6%) meningiomas that were isolated directly from fresh surgical specimens were KDR positive. The KDR RNA expression was extremely low in most meningiomas ($median_{in\ vitro}=0.17$; $median_{in\ vivo}=0.28$). Only five *in vitro* (6.4%) and nine *in vivo* (17.3%) meningiomas displayed an RQ of >0.5. The KDR expression was two-fold higher in meningiomas *in vivo*, but a significant difference was only displayed between the *in vivo* and *in vitro* RNA levels ($P=0.0043$). There were no significant differences in the expression levels between WHO grades. For comparison, the RNA and protein expression levels of PDGFRβ and KDR in eight cancer cell lines were determined (data not shown). The PDGFRβ expression was on average 97% lower in cancer cell lines compared with brain expression, and KDR expression was on average 15% higher.

Broad distribution of VEGFA expression and low PDGFB expression in meningiomas

VEGFA RNA *in vivo* and *in vitro* was significantly downregulated in meningiomas compared with arachnoidea and dura (arachnoidea: $P_{in\ vitro}=0.0346$; $P_{in\ vivo}=0.0331$; dura: $P_{in\ vitro}=0.012$; $P_{in\ vivo}=0.0023$) (Figure 1D). In contrast, the *in vitro* VEGFA protein expression was significantly higher in meningiomas compared with dura ($P=0.034$). In addition, the VEGFA expression in meningiomas displayed a broad distribution, especially *in vivo*. For the *in vivo* RNA expression, the minimum was 0.02 and the maximum was 132.5 with a median of 0.725. *In vitro*, the lowest VEGFA expression was 0.22 and the highest expression was 38.34 with a median of 1.42. For the *in vivo* protein expression, the minimum was 0.001 and the maximum was 80 with a median of 2.6. The lowest VEGFA expression *in vitro* was 0.029 and the highest expression was 65 with a median of 0.92.

The PDGFB RNA and protein levels were significantly downregulated in meningiomas compared with dura (RNA: $P_{in\ vitro}=0.0002$; $P_{in\ vivo}=0.0076$; protein: $P_{in\ vitro}=0.003$; $P_{in\ vivo}=0.0082$), as shown in Figure 1E. There were no significant differences in expression levels between WHO grades.

Immunohistochemical staining of angiogenetic proteins

Immunohistochemistry was performed using 54 surgical human meningioma specimens that included – 28 WHO °I, 21 WHO °II, and 5 WHO °III meningiomas. Figure 2A shows a representation of the staining in a negative control (A), a positive control (kidney) for VEGFA (B), positive VEGFA expression in a meningioma (C), a positive control (kidney) for KDR (D), positive KDR expression in a meningioma (E), negative KDR expression in a meningioma (F), a positive control (placenta) for PDGFRβ (G), and positive PDGFRβ expression in two meningiomas (H + I). All angiogenetic proteins displayed cytoplasmic and membranous staining in neoplastic cells. Expression of KDR and PDGFRβ was also found in blood vessels. Figure 2B–E shows the expression of angiogenetic proteins based on the percentage of cells that were positive relative to the total tissue sample.

All meningiomas stained positive for VEGFA and PDGFRβ. The median percentage of PDGFRβ-positive cells in meningiomas was 20% (range 2–80%). As shown in Figure 2C, PDGFRβ expression differed between grades ($P_{WHO\ °I\ vs\ WHO\ °II}=0.0426$; $P_{WHO\ °II\ vs\ WHO\ °III}=0.0315$). The median percentage of VEGFA-positive cells was 30% (range 2–90%). The expression in meningiomas did not differ between WHO °I and WHO °II. In contrast, VEGFA expression was higher in grade WHO °III *vs* grade WHO °II meningiomas ($P=0.02$) (Figure 2D).

Forty-seven meningiomas (87%) were positive for KDR with a median percentage of 2.5%. Most meningiomas (67%) showed low KDR expression with up to 5% positive cells. Eleven meningiomas (20%) demonstrated 10–30% positive staining. Expression of KDR was not significantly different between the WHO grades (Figure 2E).

Fifty-three meningiomas (98%) were positive for PDGFB (median percentage 20% and maximum 90%). There were no differences in PDGFB expression between the WHO grades.

Proliferative response to exogenous VEGFA in meningiomas

To investigate the biological significance of the elevated PDGFRβ expression and the mostly non-expression of KDR in meningiomas IOMM-Lee cells, a malignant proliferating meningioma cell line, were cultured with increasing amounts (0, 5, 10, 20, 50, and 100 ng ml^{-1}) of VEGFA and PDGFB for 4 h in serum-free medium. As shown in Figure 3A, cell proliferation was significantly increased after treatment with 10 ng ml^{-1} VEGFA (+42% compared with control conditions, $P<0.05$), 20 ng ml^{-1} VEGFA (+87.4% compared with control conditions, $P<0.005$), 50 ng ml^{-1} VEGFA (+94.6% compared with control conditions, $P<0.005$), and 100 ng ml^{-1} VEGFA (+99% compared with control conditions, $P<0.001$). The control cells were also incubated with PDGFB. As shown in Figure 3B, cell proliferation was similarly increased after treatment with 10 ng ml^{-1} PDGFB (+46.2% compared with control conditions, $P<0.05$), 20 ng ml^{-1} PDGFB (+71.2% compared with control conditions, $P<0.005$), and 50 ng ml^{-1} PDGFB (+87.5% compared with control conditions, $P<0.005$). After treatment with 100 ng ml^{-1} PDGFB, proliferation decreased compared with treatment with 50 ng ml^{-1} PDGFB (+71.3% compared with control conditions, $P<0.005$).

To determine if these results also apply to benign meningiomas with low proliferation, two primary cell cultures of meningothelial meningiomas were treated as described. As shown in Figure 3C, the mean proliferation without growth factors was 5.4 ± 1.7%.

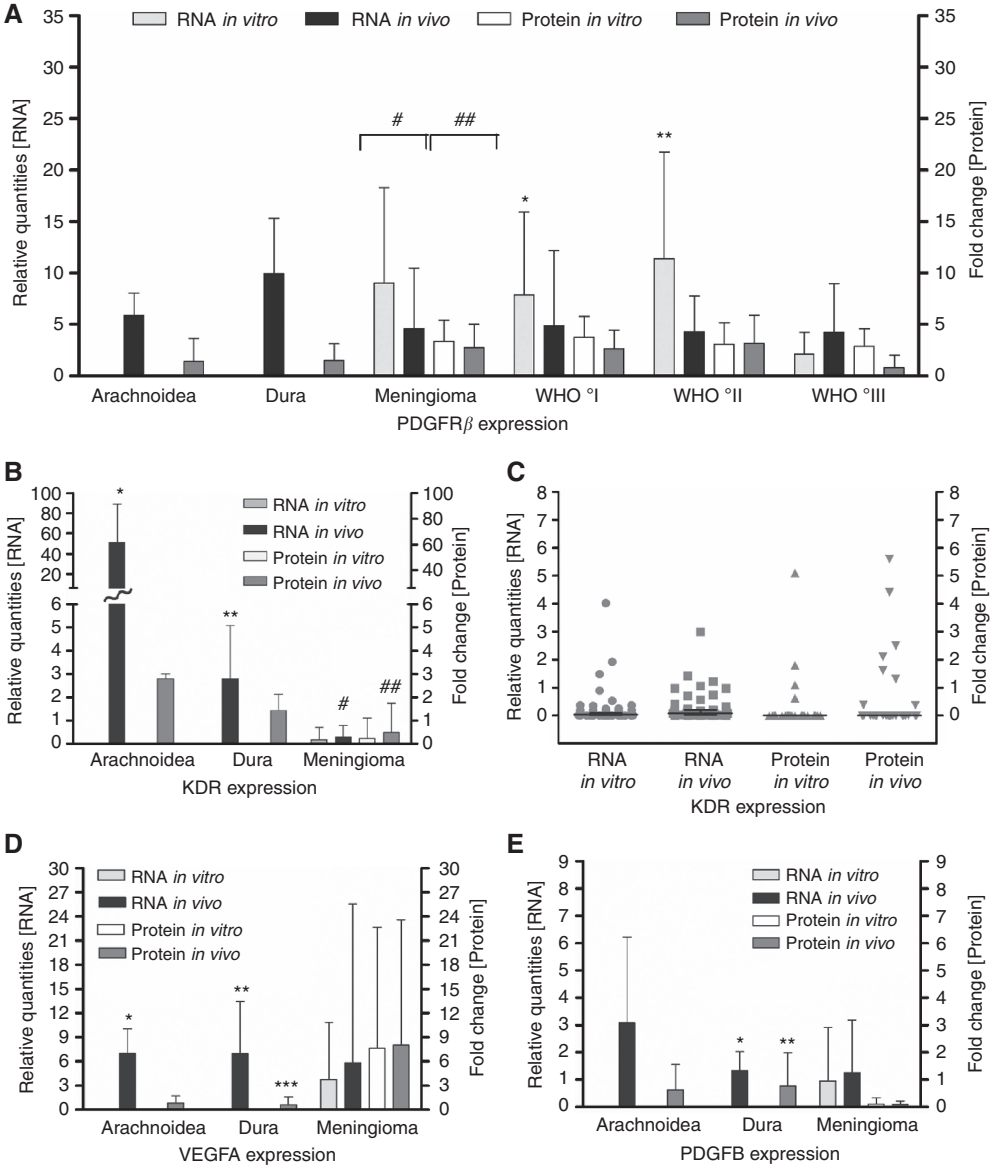

Figure 1 *The in vitro* and *in vivo* RNA and protein expression of angiogenetic proteins in meningiomas and dura. Expression of RNA was quantified using qRT–PCR with RPL37A for normalisation. The protein levels were measured using the TaqMan Protein Assay. The data for RNA are expressed as the relative gene expression level (RQ) compared with the brain ($RQ_{Brain} = 1$). The data for protein are expressed as fold change, with the brain as the reference. (**A**) The PDGFRβ RNA and protein expression was significantly higher in meningiomas compared with the brain. The *in vitro* PDGFRβ RNA expression was significantly decreased in WHO °III, $^{*}P_{RNA\ WHO\ °I\ vs\ WHO\ °III} = 0.0198$; $^{**}P_{RNA\ WHO\ °II\ vs\ WHO\ °III} = 0.004$; the PDGFR$\beta$ RNA and protein levels *in vitro* were significantly higher compared with those *in vivo*. $^{\#}P_{RNA\ in\ vitro\ vs\ in\ vivo} = 0.0002$; $^{\#\#}P_{Protein\ in\ vitro\ vs\ in\ vivo} = 0.045$. The bars represent the mean ± s.d. (**B**) The KDR RNA and protein expression levels were significantly elevated in arachnoidea compared with dura, $^{*}P_{RNA} = 0.002$; $^{**}P_{Protein} = 0.0167$. In meningiomas, the KDR RNA and protein expression levels were significantly decreased, $^{\#}P_{RNA} = 0.001$; $^{\#\#}P_{Protein} = 0.0014$. (**C**) Most meningiomas displayed no KDR protein expression. In addition, the KDR RNA expression was extremely low in most meningiomas ($median_{in\ vitro} = 0.17$; $median_{in\ vivo} = 0.28$). The symbols represent single data points. The horizontal bars represent the median. (**D**) The VEGFA RNA was significantly downregulated *in vivo* and *in vitro* in meningiomas compared with arachnoidea ($^{*}P = 0.0331$) and dura ($^{**}P = 0.0023$). In contrast, the VEGFA protein expression *in vitro* was significantly higher in meningiomas compared with dura ($^{***}P = 0.034$). (**E**) The PDGFB RNA and protein levels were significantly downregulated in meningiomas compared with dura, $^{*}P_{RNA} = 0.0076$; $^{**}P_{Protein} = 0.0082$.

Cell proliferation was significantly increased after treatment with 20 ng ml^{-1} VEGFA (+100% compared with control conditions, $P<0.05$), 50 ng ml^{-1} VEGFA (+126.2% compared with control conditions, $P<0.005$), and 100 ng ml^{-1} VEGFA (+114.7% compared with control conditions, $P<0.001$). Treatment with PDGFB significantly increased proliferation in benign meningiomas after treatment with 10 ng ml^{-1} PDGFB (+84.7% compared with control conditions, $P<0.05$), 20 ng ml^{-1} PDGFB (+99.1% compared with control conditions, $P<0.005$), and 50 ng ml^{-1} PDGFB (+134.4% compared with control conditions, $P<0.005$). After treatment with 100 ng ml^{-1} PDGFB proliferation slightly decreased compared with treatment with 50 ng ml^{-1} PDGFB (+130.8% compared with control conditions, $P<0.005$), as shown in Figure 3D.

To evaluate the functional effect of these findings, PDGFRβ and KDR were inhibited with an anti-PDGFRβ neutralisation antibody

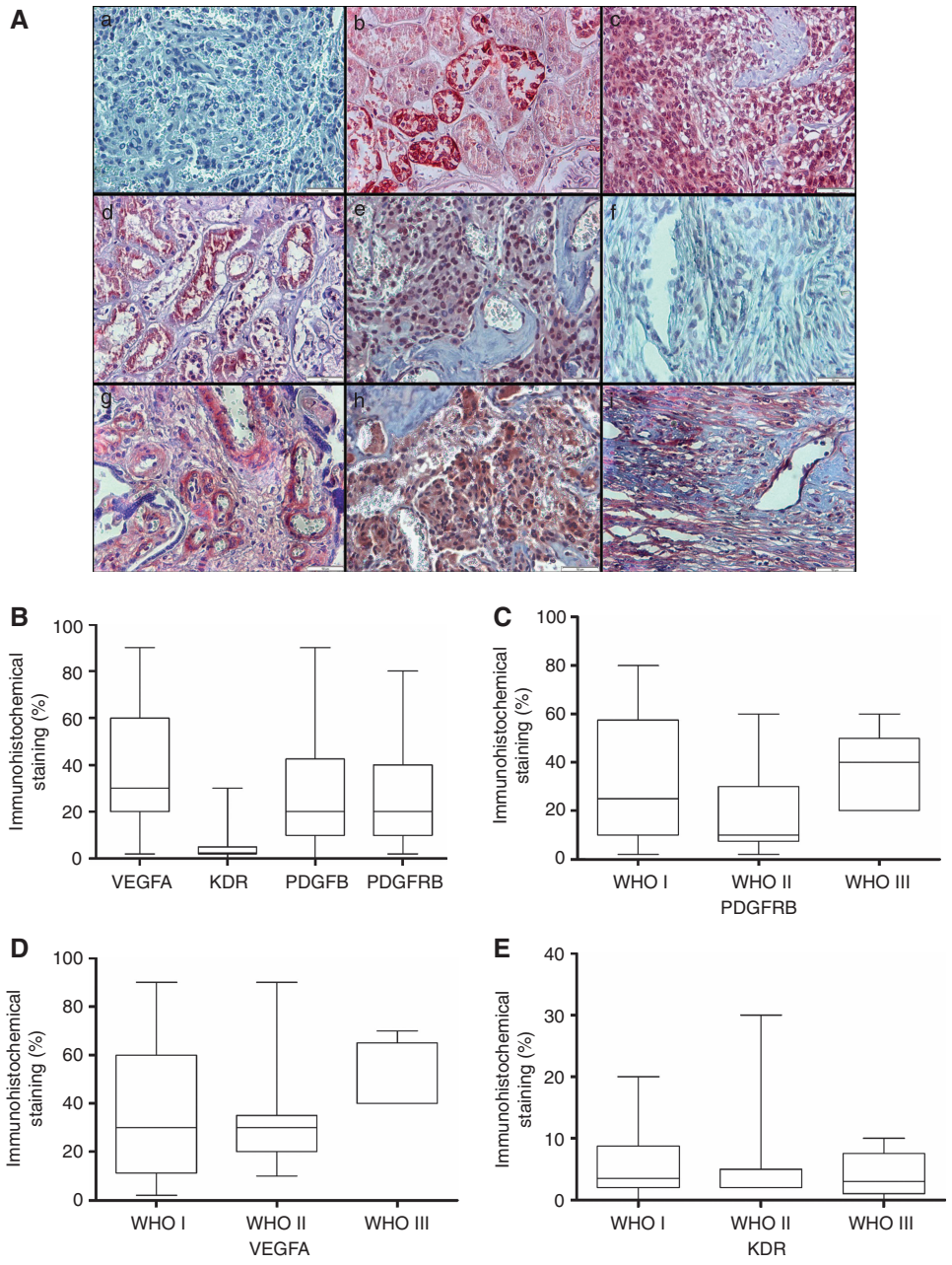

Figure 2 Immunohistochemical staining of angiogenic proteins in meningiomas. The paraffin-embedded, formalin-fixed sections of meningiomas were stained with antibodies against VEGFA, KDR, PDGFB, and PDGFRβ. Positive immunoreactivity appears as red staining (Vector NovaRed). The stains were graded from 0% to 100% based on the percentage of cells that were positive relative to the total tissue. (**A**) The panels are a representation of staining in a negative control (a), a positive VEGFA control (kidney) (b), positive VEGFA expression in a meningioma (c), a positive KDR control (kidney) (d), positive KDR expression in a meningioma (e), negative KDR expression in a meningioma (f), a positive PDGFRβ control (placenta) (g), and positive PDGFRβ expression in two meningiomas (h + i). (**B**) All 54 meningiomas (28 WHO °I, 21 WHO °II, and 5 WHO °III) stained positive for VEGFA and PDGFRβ. Forty-seven meningiomas (87%) were positive for KDR with a median percentage of 2.5%. (**C**) The PDGFRβ expression differed between grades ($P_{\text{WHO °I vs WHO °II}} = 0.0426$; $P_{\text{WHO °II vs WHO °III}} = 0.0315$). (**D**) The VEGFA expression did not differ between WHO °I and WHO °II. In contrast, the VEGFA expression was higher in grade WHO °III *vs* grade WHO °II meningiomas ($P = 0.02$). (**E**) The KDR expression displayed no significant differences between WHO grades. The box plots represent maximum and minimum values with the median.

or a PDGFRβ tyrosine kinase inhibitor or an anti-KDR neutralisation antibody or a KDR tyrosine kinase inhibitor, respectively, before stimulation with VEGFA.

Pre-treatment with either 10 μg ml^{-1} anti-PDGFRβ neutralisation antibody or 2 μM PDGFRβ tyrosine kinase inhibitor (PDGFRβ-TK) for 1 h followed by incubation with 10 ng ml^{-1} VEGFA for 4 h resulted in a significant decrease in IOMM-Lee cell growth compared with VEGFA grown without pre-treatment (−38.7% after anti-PDGFRβ pre-treatment, $P < 0.005$ and −32.6% after PDGFRβ-TK pre-treatment, $P < 0.05$, Figure 3E). In contrast, pre-treatment with either 10 μg ml^{-1} anti-KDR neutralisation antibody or 100 nM KDR tyrosine kinase inhibitor (KDR-TK) before incubation with VEGFA displayed no anti-proliferating effect.

In addition, two benign meningiomas were pre-treated as described before and then incubated with 50 ng ml^{-1} VEGFA

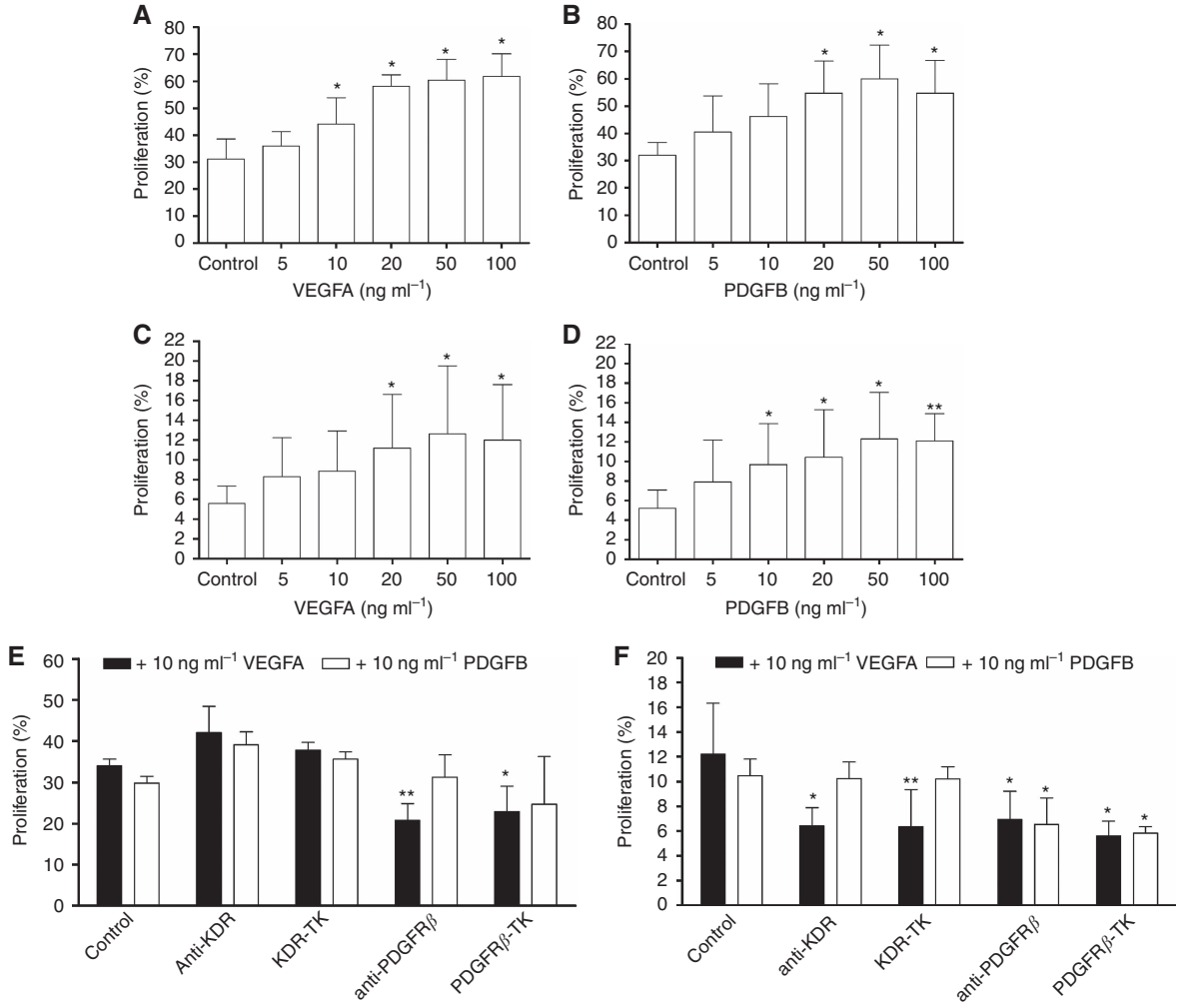

Figure 3 The exposure to VEGFA increased proliferation in meningiomas *in vitro*: Proliferation was determined on the basis of the malignant meningioma cell line IOMM-Lee (**A** and **B**) and two benign meningiomas (**C** and **D**). The cells were incubated for 4 h with serum-free medium and fresh growth factor. The control represents proliferation independent of supplemented growth factor. Proliferation was measured using a BrdU assay. (**A** and **C**) VEGFA (**B** and **D**) PDGFB. (**E**) The inhibition of PDGFRβ decreased the VEGFA-induced proliferation. The IOMM-Lee cells were pre-treated for 1 h with 10 μg ml^{-1} of either anti-KDR or -PDGFRβ neutralisation antibodies, 100 nM KDR tyrosine kinase inhibitor (KDR-TK) and 2 μM PDGFRβ tyrosine kinase inhibitor (PDGFRβ-TK), followed by either 10 ng ml^{-1} VEGFA or 10 ng ml^{-1} PDGFB for 4 h. The control represents VEGFA- or PDGFB-induced proliferation. (**F**) The inhibition of PDGFRβ or KDR decreased VEGFA-induced proliferation in benign meningiomas. After pre-treatment, the cells were incubated with either 50 ng ml^{-1} VEGFA or 50 ng ml^{-1} PDGFB. The control represents VEGFA- or PDGFB-induced proliferation. The data shown are the mean number of proliferating cells ± s.d. determined from five random fields from each of three independent experiments. *$P<0.05$; **$P<0.005$.

for 4 h, because the primary meningioma cell cultures displayed the highest proliferation effect after treatment with this growth factor concentration. Cell growth significantly decreased after the inhibition of PDGFRβ (-43.1% after anti-PDGFRβ pre-treatment, $P<0.05$ and -54.2% after PDGFRβ-TK pre-treatment, $P<0.05$; Figure 3F). The control cells were incubated with 50 ng ml^{-1} PDGFB. Cell proliferation significantly decreased after anti-PDGFRβ pre-treatment 37.7% and decreased 44.4% after PDGFRβ-TK pre-treatment ($P<0.05$). In addition, meningioma cell growth significantly decreased after the inhibition of KDR and incubation with VEGFA (-47.5% after anti-KDR pre-treatment, $P<0.05$ and -48.1% after KDR-TK pre-treatment, $P<0.005$).

VEGFA induced PDGFRβ tyrosine phosphorylation

To provide further evidence that PDGFRβ is stimulated by VEGFA, six randomly chosen primary meningioma cells (four WHO °I + two WHO °II) were starved for 24 h in serum-free medium. The cells were stimulated with increasing amounts of either VEGFA or PDGFB for 10 min at 37°C. PDGFRβ expression and PDGFRβ phosphorylation were measured with the TaqMan Protein Assay. The HUVEC was used as the control. PDGFRβ and KDR expression was determined in different cell types (Figure 4A). As expected, the HUVECs displayed high KDR and very low PDGFRβ expression. Because meningiomas are of mesenchymal origin cultured normal mesothelial cells (HMCs) were also examined. These cells showed both PDGFRβ and KDR expression. In contrast, HNCs displayed very low KDR expression and decreased PDGFRβ levels compared with the brain.

After the HUVECs were stimulated with VEGFA, the tyrosine phosphorylation of KDR was evaluated. The tyrosine phosphorylation of KDR reached a maximum with 10 ng ml^{-1} VEGFA and decreased with higher VEGFA concentrations (Figure 4B). The tyrosine phosphorylation level of PDGFRβ was determined after stimulation with PDGFB, which was three-fold higher after stimulation with 10 ng ml^{-1} PDGFB.

All meningiomas tested displayed high PDGFRβ expression, and KDR was not detectable. The PDGFRβ expression and PDGFRβ

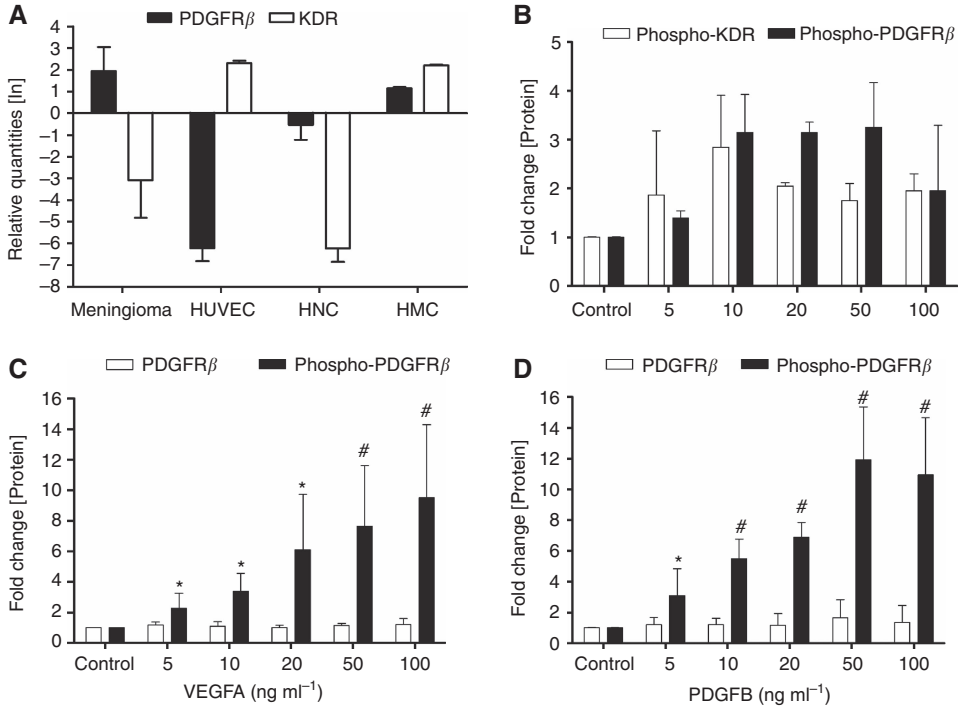

Figure 4 VEGFA stimulated PDGFRβ tyrosine phosphorylation is comparable to PDGFB in meningiomas *in vitro*. (**A**) PDGFRβ and KDR expression was compared between meningiomas, HUVECs, HNCs, and HMCs. The data are expressed as relative quantities (RQs) compared with the brain ($RQ_{Brain} = 0$). The HUVECs were used as a PDGFRβ-negative and KDR-positive control. (**B**) The tyrosine phosphorylation was determined in HUVECs as a control. The HUVECs were starved for 24 h with serum-free medium. The cells were stimulated with increasing concentrations of either VEGFA or PDGFB for 10 min at 37°C. The tyrosine phosphorylation was measured using a TaqMan Protein Assay. The data are shown as the mean number of two independent experiments using different HUVEC cultures. (**C** and **D**) Six primary meningioma cell cultures were given the same treatment as the HUVECs. The PDGFRβ expression and PDGFRβ phosphorylation were determined. $^{*}P < 0.05$; $^{\#}P < 0.005$, compared with the respective unstimulated cells. The bars represent the mean ± s.d.

phosphorylation were determined from VEGFA-treated primary meningioma cells. As shown in Figure 4C, the phosphorylation of PDGFRβ by VEGFA increased with increasing concentrations. The tyrosine phosphorylation level was three-fold higher after stimulation with 10 ng ml^{-1} VEGFA (fold change = 3.4 ± 1.18, $P < 0.05$) and doubled after stimulation with 20 ng ml^{-1} VEGFA (fold change = 6.1 ± 3.62, $P < 0.05$). The tyrosine phosphorylation of PDGFRβ reached its maximum with 100 ng ml^{-1} VEGFA (fold change = 9.5 ± 4.79, $P < 0.005$). The control cells were also stimulated with PDGFB. As shown in Figure 4D, the tyrosine phosphorylation of PDGFRβ increased higher after stimulation with 5 ng ml^{-1} PDGFB (fold change = 3.1 ± 1.75, $P < 0.05$) and 10 ng ml^{-1} PDGFB (fold change = 5.5 ± 1.28, $P < 0.005$). After stimulation with 20 ng ml^{-1} PDGFB, the tyrosine phosphorylation of PDGFRβ was similar to VEGFA (fold change = 6.9 ± 0.98, $P < 0.005$). With 50 ng ml^{-1} PDGFB, the tyrosine phosphorylation of PDGFRβ reached its maximum (fold change = 11.9 ± 3.43, $P < 0.005$).

Gambogic acid, sunitinib, and tandutinib inhibit migration of primary meningioma cells

The Radius cell migration assay was used to examine the migration of six randomly chosen primary meningioma cells (four WHO °I + two WHO °II) after treatment with the angiogenesis inhibitors sunitinib and tandutinib. Sunitinib is a tyrosine kinase inhibitor that inhibits at least eight receptor protein tyrosine kinases, including KDR and PDGFRβ, at the same IC_{50} (10 nM) (Mendel *et al*, 2003). In contrast, tandutinib is a more selective tyrosine kinase inhibitor that does not inhibit KDR up to $IC_{50} = 30\ \mu$M but affects PDGFRβ *in vitro* at $IC_{50} = 30$ nM and in cellular assays at $IC_{50} = 200$ nM (Kelly *et al*, 2002). The effect of gambogic acid on meningioma cell migration was also examined. Gambogic acid inhibits the transferrin receptor with an IC_{50} of 1 μM, induces apoptosis and inhibits cell growth with an EC_{50} of 0.5–1.5 μM (Kasibhatla *et al*, 2005).

Sunitinib, tandutinib, and gambogic acid suppressed cell migration (Figure 5A). With increasing doses of gambogic acid (b–i), meningioma cells became unable to close the uncoated gap in 24 h. Figure 5B shows the dose-dependent inhibition of cell migration by meningiomas following incubation with 31.25–2000 ng ml^{-1} gambogic acid. Cell migration was inhibited in a concentration-dependent manner with a mean of 9.3% for 31.25 ng ml^{-1} gambogic acid to 72.1% for 2000 ng ml^{-1} gambogic acid. Migration was suppressed by 50% on average with 1 μg ml^{-1} gambogic acid (1.6 nM). Sunitinib and tandutinib inhibited cell migration similar to gambogic acid. Migration was suppressed by 50% on average with 3.8 nM sunitinib and with 3.6 nM tandutinib (Supplementary material online).

Gambogic acid inhibits tyrosine phosphorylation of PDGFRβ

Gambogic acid inhibits cell migration by suppressing PDGFRβ tyrosine phosphorylation, as demonstrated by Liu *et al* (2010). Thus, three randomly chosen primary meningothelial meningioma cells were pre-treated with increasing concentrations (0.125–2 ng ml^{-1}) of gambogic acid for 1 h. Then, cells were stimulated with either 100 ng ml^{-1} VEGFA or PDGFB for 10 min at 37°C. PDGFRβ expression and PDGFRβ phosphorylation were measured using a TaqMan Protein Assay. As shown in Figure 5C, gambogic acid inhibited the tyrosine phosphorylation of PDGFRβ after

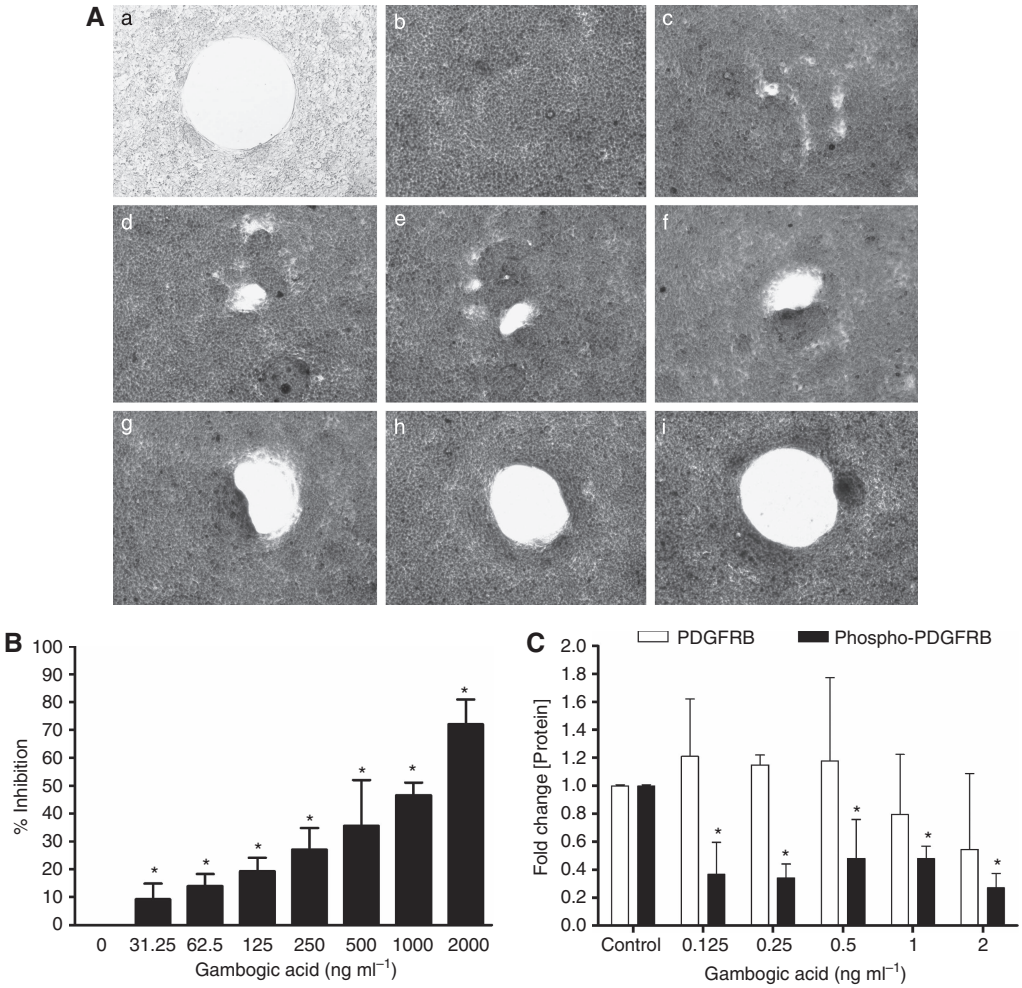

Figure 5 Gambogic acid inhibits the migration of meningioma cells *in vitro*. Eight different primary meningioma cells were plated in 24-well plates. The next day, the coating of the gap was removed followed by treatment for 24 h with either gambogic acid or vehicle (DMSO). Each gap was photographed after 24 h, except for the first, which was photographed after 0 h. (**A**) A representative experiment is shown. (a) The control group after 0 h. (b) The control group after 24 h. (c) Treatment with 31.25 ng ml^{-1} gambogic acid. (d) Treatment with 62.5 ng ml^{-1} gambogic acid. (e) Treatment with 125 ng ml^{-1} gambogic acid. (f) Treatment with 250 ng ml^{-1} gambogic acid. (g) Treatment with 500 ng ml^{-1} gambogic acid. (h) Treatment with 1000 ng ml^{-1} gambogic acid. (i) Treatment with 2000 ng ml^{-1} gambogic acid. (**B**) The concentration-dependent effect of gambogic acid on primary meningioma cells in a gap assay. The pictures were analysed using CellProfiler. The data are shown as the mean number of the inhibitory effect of gambogic acid compared with the uninhibited meningioma cells. (**C**) The primary meningioma cells were pre-treated with increasing concentrations of gambogic acid for 1 h. Then, the cells were stimulated with 100 ng ml^{-1} VEGFA for 10 min at 37°C. The PDGFRβ expression and PDGFRβ phosphorylation were measured using a TaqMan Protein Assay. (**B** and **C**) *$P<0.05$, compared with the respective uninhibited cells. The bars represent the mean ± s.d.

stimulation with VEGFA and PDGFB (data not shown) in a concentration-independent manner (− 67% on average compared with uninhibited cells, $P<0.05$).

DISCUSSION

In this study, we demonstrated that VEGFA signals through PDGFRβ in meningioma cells *in vitro*. We measured the RNA and protein levels of angiogenesis-related genes with sensitive and novel techniques. The KDR was barely detectable in meningiomas with either qRT–PCR or the TaqMan Protein Assay. In contrast, PDGFRβ was significantly upregulated in meningiomas compared with normal human brain samples. To provide evidence that VEGFA signalling through PDGFRβ has biological significance, we compared the effects of VEGFA and PDGFB on cell proliferation. Additionally, we demonstrated that stimulation with VEGFA induced PDGFRβ tyrosine phosphorylation in meningiomas similar to PDGFB-stimulated PDGFRβ. We also noted that the PDGFRβ inhibitors sunitinib, tandutinib, and gambogic acid inhibit the migration of meningioma cells *in vitro*.

Meningiomas are highly vascularised tumours. Several studies have demonstrated the significance of VEGF in the proliferation and migration of meningiomas in which VEGF has been upregulated (Provias and Claffey, 1997; Christov *et al*, 1999; Lamszuz *et al*, 2000; Park *et al*, 2000; Yamasaki, 2000). Some groups have observed a correlation between the degree of VEGF expression and tumour vascularity or invasiveness (Samoto *et al*, 1995; Provias and Claffey, 1997). In contrast, Pietsch *et al* (1997) and Nishikawa *et al* (1998) could not find a relationship between VEGF expression and tumour vasculature (Pietsch *et al*, 1997; Nishikawa *et al*, 1998). Lamszuz *et al* (2000) noted a strong correlation between meningioma histological grade and VEGF expression, wherein increased VEGF expression was linked to higher WHO grades. However, Barresi and Tuccari (2010) showed the absence of correlation between VEGF expression and WHO

grade (Barresi and Tuccari, 2010). Another study correlated VEGF expression and the recurrence of benign meningiomas (Yamasaki, 2000).

Uesaka *et al* (2007) quantified VEGFA expression in meningiomas compared with normal human brain samples using qRT–PCR and did not observe any changes in expression (Uesaka *et al*, 2007). In contrast, we observed that VEGFA RNA levels *in vitro* and *in vivo* were significantly increased in meningiomas compared with the brain but were downregulated compared with the arachnoidea and dura. The VEGFA expression *in vitro* and *in vivo* did not differ between WHO grades. Only the immunohistochemical staining of VEGFA showed increased levels in WHO °III meningiomas. This result was not confirmed by qRT–PCR or the Taqman protein assay results. Due to the low sample numbers of anaplastic meningiomas results can be biased especially when using only a single method. While VEGFA was upregulated on average, VEGFA expression in meningiomas displayed a broad distribution especially *in vivo*, which may explain opposing observations.

Several studies have examined KDR and FLT1 in meningiomas (Hatva *et al*, 1995; Otsuka *et al*, 2004; Huang *et al*, 2005; Preusser *et al*, 2012). Hatva *et al* (1995), Otsuka *et al* (2004), and Preusser *et al* (2012) determined KDR expression using either immunohistochemistry or *in situ* hybridisation. Hatva *et al* (1995) examined only three meningiomas, which cannot provide justifiable conclusions. The KDR protein expression was detected in 31.4% of specimens (Otsuka *et al*, 2004), and KDR mRNA was demonstrated in 44.4% of samples (Preusser *et al*, 2012). Those results show that KDR is not expressed in the majority of meningiomas. The data also provide no information about the expression level of KDR. Our finding that most examined meningiomas displayed no KDR protein expression does not contradict previous studies. In addition, the methods used may influence the results. Our immunohistochemical data for KDR expression show 87% positive specimens, but most meningiomas displayed low KDR levels.

Contradicting results were reported by Huang *et al* (2005), who measured KDR and FLT-1 expression in various brain tumours with qRT–PCR, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalise and compare the results with those in HUVECs. Of the brain tumours that were examined, meningiomas had the highest KDR and FLT-1 levels. In contrast, we determined very low KDR RNA expression in most examined meningiomas.

Huang *et al* (2005) also observed higher VEGF receptor levels in meningiomas compared with the brain. Our data showed that KDR expression was significantly decreased in meningiomas compared with the brain. These RNA findings were verified by measuring KDR with a TaqMan Protein Assay. The reason for these contradictory results cannot be satisfactorily explained because the study does not state the number of examined samples. A few meningiomas show higher KDR expression compared with the brain. If a small specimen number was analysed, then that could account for the high KDR RNA levels.

Low or absent KDR expression suggests that mechanisms other than VEGFA signalling through KDR influence angiogenesis in meningiomas.

In addition to VEGF, PDGF signalling indirectly regulates angiogenesis. Yang (2001) showed that nearly all meningiomas express PDGFB and PDGFRβ *in vitro*, and both were increased in WHO °II meningiomas. Several groups have proposed an autocrine mechanism in meningiomas, based on the co-expression of PDGFB and PDGFRβ, which regulate growth (Wang *et al*, 1990; Figarella-Branger *et al*, 1994; Kuratsu *et al*, 1994; Mauro *et al*, 1995).

In contrast, we showed that PDGFB expression *in vitro* and *in vivo* was significantly decreased compared with the brain and dura. Further, PDGFRβ expression *in vivo* and *in vitro* rose in meningiomas compared with the brain. The significantly decreased *in vitro* PDGFRβ RNA levels in WHO °III meningiomas were probably influenced by the small sample numbers ($n = 5$). The low number is due to their rarity among meningiomas. Moreover, surgically resected anaplastic meningiomas are almost always previously irradiated, which influences the cell quality.

High PDGFRβ expression combined with low KDR and PDGFB levels in meningiomas suggests that PDGFRβ has a significant function in tumorigenesis in meningiomas.

We determined that the addition of exogenous VEGFA doubled cell proliferation in malignant IOMM-Lee and benign primary meningioma cells, comparable to exogenous PDGFB, suggesting that VEGFA signalling through PDGFRβ is biologically significant in meningiomas. To evaluate the functional effect of these findings, PDGFRβ was inhibited with an anti-PDGFRβ neutralisation antibody or a PDGFRβ tyrosine kinase inhibitor before stimulation with VEGFA. The proliferative stimuli were abolished under these conditions. The control experiments with PDGFB stimulation showed that PDGFRβ inhibitors also neutralised PDGFB-induced proliferation. Why the presence of the PDGFRβ inhibitor did not significantly influence the PDGFB-induced proliferation rate in IOMM-Lee cells remains to be determined.

The cells were also inhibited with an anti-KDR neutralisation antibody or a KDR tyrosine kinase inhibitor before stimulation with VEGFA or PDGFB, respectively. As expected, the PDGFB-induced proliferation was not influenced by KDR inhibition. The VEGFA-induced proliferative stimuli were abolished after KDR inhibition in benign meningiomas but not in IOMM-Lee cells. There are two possible ways to interpret these results. First, the VEGFA-induced proliferative stimuli may be dependent on the binding of VEGFA to PDGFRβ as implied by the IOMM-Lee data. Second, the VEGFA-induced cell proliferation may rely on both KDR and PDGFRβ binding to VEGFA. Because some meningiomas express detectable KDR the second possibility is preferable to the first.

To further investigate the effect of VEGFA on PDGFRβ in meningiomas, we determined whether VEGFA caused the tyrosine phosphorylation of PDGFRβ. The protein lysates from VEGFA-treated primary meningioma cells were analysed with a TaqMan Protein Assay, which allowed for the quantification of tyrosine phosphorylation in meningiomas. We demonstrated that VEGFA induces concentration-dependent PDGFRβ tyrosine phosphorylation in meningiomas after 10 min. A comparison with PDGFB-induced meningioma cells showed a similar increase in the phosphorylation of PDGFRβ. The effect of VEGFA on KDR was not investigated because KDR was not detectable in the meningiomas used. Thus, the data suggest that VEGFA mediates proliferation and migration through PDGFRβ, but not KDR, in meningiomas.

To determine if selective PDGFRβ inhibitors, such as tandutinib, have a similar effect as sunitinib, which also inhibits KDR and several other receptor protein tyrosine kinases, we treated primary meningioma cells with these two tyrosine kinase inhibitors. Tandutinib suppressed cell migration in primary meningiomas as effectively as sunitinib, in spite of the fact that tandutinib only inhibits PDGFRβ.

We also investigated the effect of gambogic acid on primary meningiomas. Recently Liu *et al* (2010) reported that gambogic acid inhibits cell migration by suppressing PDGFRβ tyrosine phosphorylation. The treatment of meningiomas *in vitro* with gambogic acid significantly inhibited cell migration. Furthermore, gambogic acid inhibited the tyrosine phosphorylation of PDGFRβ after stimulation with VEGFA, independent of the gambogic acid concentration. These results support those of Liu *et al* (2010), showing that gambogic acid inhibits cell migration by suppressing PDGFRβ tyrosine phosphorylation.

The low expression or absence of KDR in meningiomas both *in vitro* and *in vivo*, combined with the elevated level of PDGFRβ, suggests that VEGFA primarily regulates VEGF-mediated migration and proliferation through PDGFRβ, not KDR, in

meningiomas. This model is supported by our findings in which the stimulation of meningiomas *in vitro* with VEGFA upregulates cell proliferation and induces PDGFRβ tyrosine. Gambogic acid, tandutinib, and sunitinib were equally potent at inhibiting meningioma growth *in vitro*, suggesting that selective PDGFRβ inhibitors, such as gambogic acid and tandutinib, should be evaluated as potential therapies for recurrent and malignant meningiomas, possibly in combination with VEGF inhibitors.

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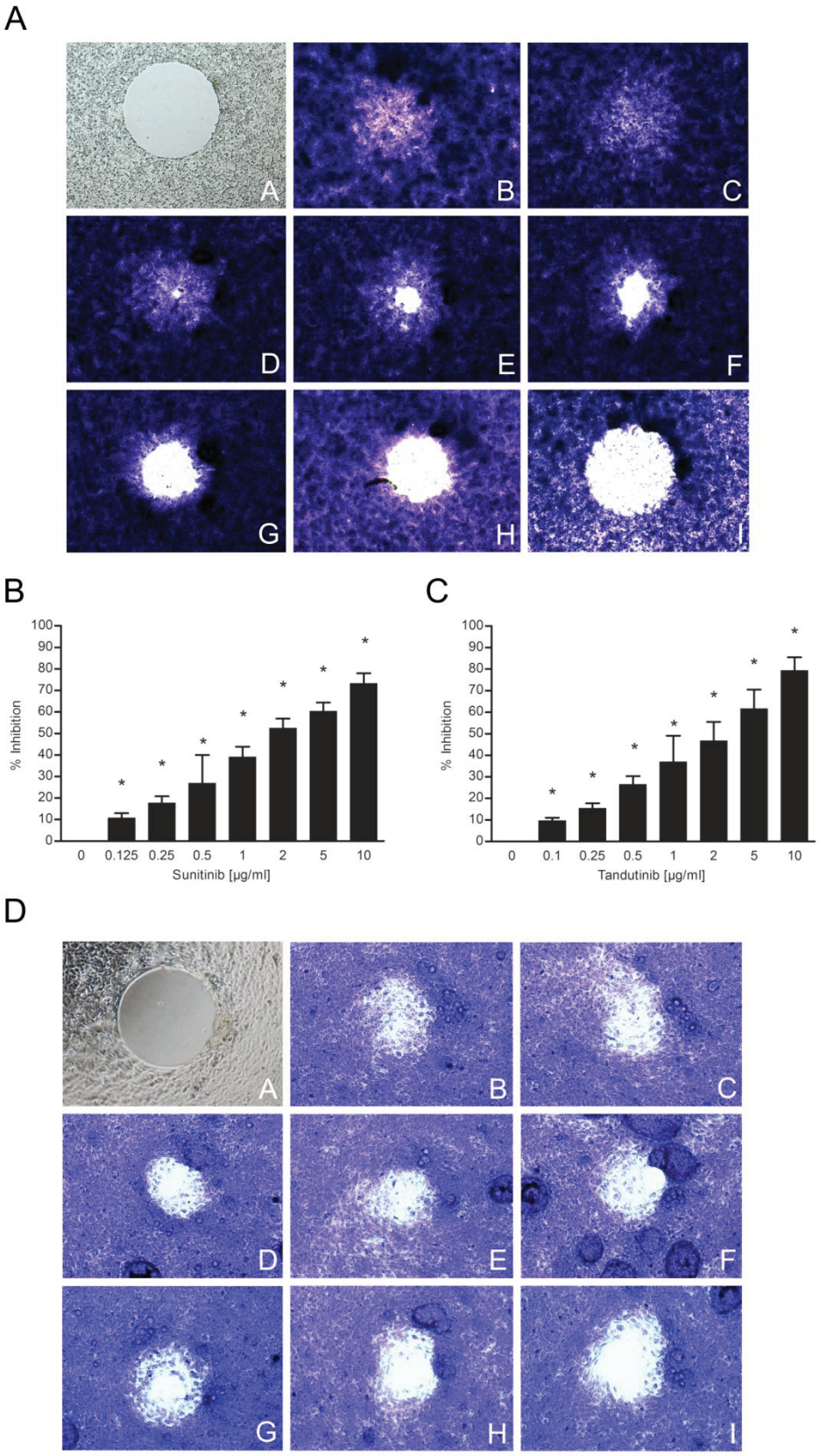
Supplement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Sunitinib and tandutinib inhibits the migration of meningioma cells *in vitro*. Eight different primary meningioma cells were plated in 24-well plates. The next day, the coating of the gap was removed followed by sunitinib, tandutinib or vehicle (DMSO) treatment for 24 h. Each gap was photographed after 24 h except for the first, which was photographed after 0 h. (**A**) A representative experiment for sunitinib is shown. A: The control group after 0 h. B: The control group after 24 h. C: Treatment with 0.125 µg/ml sunitinib. D: Treatment with 0.25 µg/ml sunitinib. E: Treatment with 0.5 µg/ml sunitinib. F: Treatment with 1 µg/ml sunitinib. G: Treatment with 2 µg/ml sunitinib. H: Treatment with 5 µg/ml sunitinib. I: Treatment with 10 µg/ml sunitinib. The concentration-dependent effect of sunitinib **(B)** and tandutinib **(C)** on primary meningioma cells in a gap assay. The pictures were analysed using CellProfiler™. The data shown are the mean number of the inhibitory effect of sunitinib or tandutinib compared with the uninhibited meningioma cells. (**D**) A representative experiment for tandutinib is shown. A: The control group after 0 h. B: The control group after 24 h. C: Treatment with 0.125 µg/ml tandutinib. D: Treatment with 0.25 µg/ml tandutinib. E: Treatment with 0.5 µg/ml tandutinib. F: + Treatment with 1 µg/ml tandutinib. G: Treatment with 2 µg/ml tandutinib. H: Treatment with 5 µg/ml tandutinib. I: Treatment with 10 µg/ml tandutinib. (**B**) + (**C**) *, $P < 0.05$, compared with the respective uninhibited cells. The bars represent the mean ± SD.

Apoptosis
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ORIGINAL PAPER

Detection and quantification of farnesol-induced apoptosis in difficult primary cell cultures by TaqMan protein assay

Christina Pfister · Heike Pfrommer · Marcos S. Tatagiba · Florian Roser

Abstract Apoptosis can be detected reliably by assaying for cleaved caspase-3, for which active caspase-3 antibodies are used in several methods, such as immunocytochemistry, enzyme-linked immunosorbent assay, and western blot. In this study, we used TaqMan protein assay (TPA), a novel method for protein detection and quantification that detects proteins by amplification of substitute DNA templates. TPA uses antibodies and proximity ligation for quantitative real-time PCR. Meningiomas are primarily benign intracranial tumors. Primary cell cultures of meningiomas are often unsuitable for sensitive protein detection methods. We optimized a TPA to detect active caspase-3 and evaluated its ability to detect farnesol-induced apoptosis in primary meningioma cells. The specificity and sensitivity of the inactive and active caspase-3 assay were determined using recombinant caspase-3. Apoptosis was induced in meningiomas in the presence of 0.2 μM farnesol as shown by immunocytochemistry of single-stranded DNA. Also, viability decreased by over 90 % after treatment with 1.2 μM farnesol for 24 h. The TPA detected a significant increase in active caspase-3 after treatment with 2 and 4 μM farnesol for 2 h, which could not be detected using standard methods such as western blot and immunofluorescence. In addition, TPA determined that meningiomas show disparate sensitivities to low concentrations of farnesol. Caspase-3 expression fell significantly in cells that were treated with 0.25 μM farnesol for 2 h. Further, by TPA, active caspase-3 peaked after 2 h and declined with longer incubation times. This study demonstrates that cleaved caspase-3 is detected and quantified reliably in meningiomas by TPA.

Keywords Meningioma · Apoptosis · Caspase 3 · Farnesol · Taqman protein assays

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Introduction

Apoptosis, programmed cell death, is essential for normal development and maintenance of multicellular organisms [1]. Its dysregulation effects the ability to avert apoptosis, which has a significant function in cancer [2]. Caspase-3 is the most important executioner caspase. Its activation is a biochemical hallmark of early apoptosis, which renders the detection of active caspase-3 in cells a reliable method of measuring apoptosis, for which antibodies against cleaved caspase-3 are used in several methods such as immunostaining, enzyme-linked immunosorbent assay (ELISA), flow cytometry, and western blot (WB).

Recently, a new method for protein detection and quantitation was developed. TaqMan protein assay (TPA) is a quantitative PCR-based method for detecting proteins, based on proximity ligation assay [3]. Each TPA comprises a pair of protein-specific antibodies, polyclonal or a matched monoclonal antibody pair that are linked to reporter oligonucleotides (Fig. 1). On binding to target TPA probes in proximity, they are joined by DNA ligase, forming DNA templates, which are amplified and analyzed by real-time PCR.

Meningiomas are the most common intracranial tumor. They originate from the arachnoidal cap cells of the meningeal cover of the spinal cord and brain, constituting approximately 13 to 26 % of all intracranial pathologies [4, 5]. Meningiomas are categorized into three grades per the world health organization (WHO), for which there are

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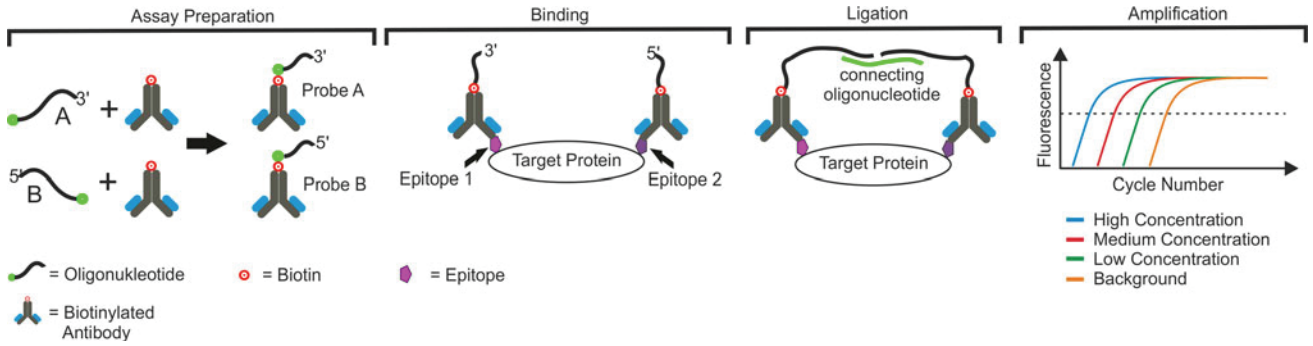


Fig. 1 Schematic overview of Taqman protein assay method. The workflow consists of four steps. 1. Assay preparation: 3′- and 5′-oligonucleotides are bound to either a polyclonal antibody or a monoclonal antibody pair using a biotin-streptavidin interaction. 3′-oligonucleotide + antibody → probe A + 5′-oligonucleotide + antibody → probe B. 2. Binding step: *probe A* and *probe B* bind to their specific epitope in a cell lysate. 3. Ligation step: the assay probe oligonucleotides in close proximity hybridize to a short connecting oligonucleotide using DNA ligase. 4. Amplification step: the ligation product is amplified and detected using Taqman-based real-time PCR

several subtypes, based on histology. WHO I tumors are benign meningiomas, atypical meningiomas are classified WHO II and malignant meningiomas are WHO III.

There are few stable meningioma cell lines, which are primarily malignant [6]. Thus, most meningioma cell culture research is conducted with primary meningioma cells. Comparable test conditions are difficult to achieve, because primary meningioma cells undergo senescence in early passages and alter their phenotype with ongoing culture [7]. Also due to their low proliferation rates cell numbers are limited. Thus, the use of commonly used methods in such cells to detect cleaved caspase-3 is restricted. TPA combines small cell numbers with increased sensitivity, which could provide a reliable tool to detect and quantify apoptosis in meningiomas.

Many chemotherapeutic drugs induce apoptosis. Farnesol is a polyprenyl alcohol in plant essential oil. Farnesol induces caspase-3, -6, -7 and -9 but not caspase-8. In addition farnesol inhibits survivin and Bcl2 [8]. Farnesol induces apoptosis in several tumor-derived cell lines [9–11] and has anti-tumor effects in vivo, suppressing pancreatic tumor growth [12].

In this study we optimized a TPA to detect and quantify inactive and active caspase-3. To evaluate this assay we induced apoptosis in primary meningioma cells using farnesol. On demonstrating that farnesol induces apoptosis in meningiomas by immunocytochemical staining of single stranded DNA, we observed that TPA detects farnesol-induced apoptosis in meningiomas at low concentrations.

Materials and methods

Tumor specimens and cell culture

Meningioma surgical specimens, arachnoidea, and dura were obtained from the Neurosurgical Department per the ethical committee of the University of Tuebingen. Primary cultures were established from tumor tissue samples within 30 min of surgical removal. Samples were washed in phosphate-buffered saline (PBS), reduced, passed through a filter, and placed in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 0.1 % 10 mg/ml gentamycin (PAA, Pasching; Austria).

Cells were plated in 25-mm^2 tissue culture flasks and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. Medium was changed every 3–4 days, and cultures were split with 600 µl Accutase (PAA, Pasching; Austria). Viable cells were stored in liquid nitrogen in 90 % medium/10 % dimethyl sulfoxide (DMSO).

To verify the identity of the tumor cells, primary meningioma cultures were examined immunocytochemically for vimentin, CD44, and epithelial membrane antigen (EMA), which are expressed in most meningiomas (Fig. 2a–c). Also, the cells were tested for negative expression of CD34, an endothelial cell marker (not shown). The malignant meningioma cell line IOMM-Lee was a kind gift of A. Lal (Department of Neurological Surgery, University of California, San Francisco, CA, USA). A karyogram of IOMM-Lee cells was established using GIEMSA, which coincides with literature [13]. IOMM-Lee expressed vimentin and CD44 (Fig. 2d + e). As control cell system a primary glioblastoma culture was used, which was a kind gift of S. Noell (Department of Neurosurgery, University of Tuebingen, Germany). This cell culture was tested for positive expression of neurofilament (not shown) and glial fibrillary acidic protein (GFAP) (Fig. 2f).

Reagents and antibodies

The following antibodies were used to design TaqMan® Protein Assays: caspase-3 (Cat. No. AF-605-NA, R&D Systems, Minneapolis, MN) and active caspase-3 (Cat. No.

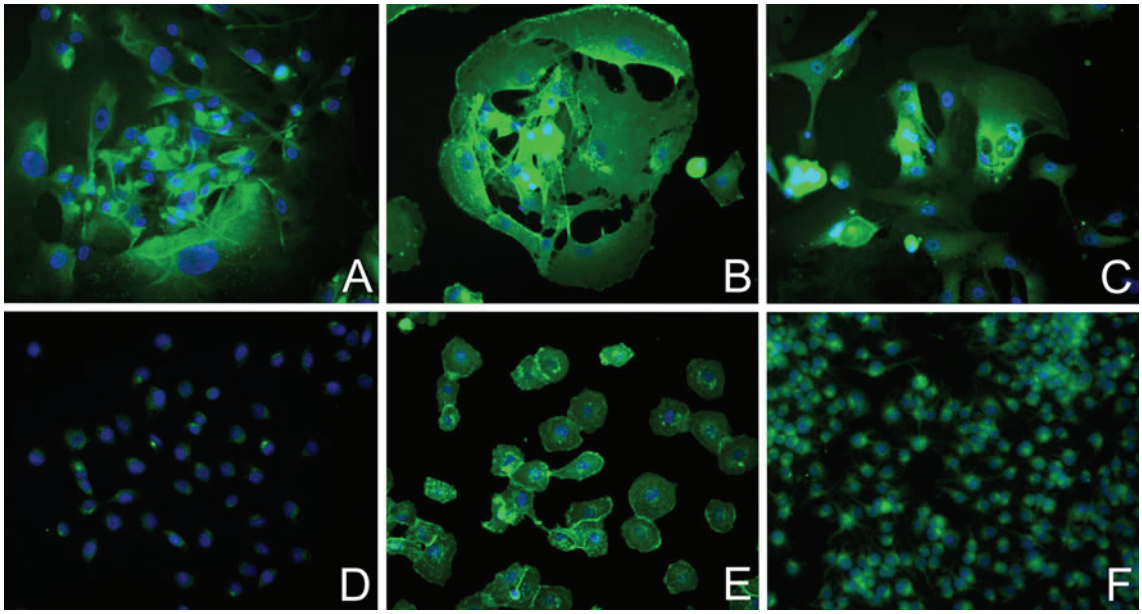


Fig. 2 Marker expression of primary cell cultures for **a–c** representative meningioma, **d** + **e** IOMM-Lee, and **f** glioblastoma. Positive immunoreactivity appears as green staining (Alexa Fluor 488). **a** + **d** vimentin, **b** + **e** CD44, **c** EMA, **f** neurofilament. Original magnification ×20

700182, Invitrogen, Carlsbad, CA). The secondary antibody was streptavidin–alkaline phosphatase (R&D Systems, Minneapolis, MN). Anti-ssDNA was obtained from Enzo Life Sciences Inc. (Farmingdale, NY), the secondary antibody for which was horseradish peroxidase-rat anti-mouse IgM (Invitrogen, Carlsbad, CA).

Anti-Vimentin, -GFAP, -neurofilament, -CD44, and -CD34 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary fluorescence antibodies were Alexa Fluor 488 goat anti-mouse, goat anti-rabbit, and donkey anti-goat (Invitrogen, Carlsbad, CA). Anti-EMA was purchased from DAKO (Glostrup, Denmark). Secondary antibody was Universal Biotinylated Anti-Mouse/Rabbit IgG secondary antibody from Vector Laboratories (Burlingame, CA).

Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and -cleaved PARP1 (poly [ADP-ribose] polymerase 1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), for which the secondary antibody was goat anti-mouse/rabbit IgG- alkaline phosphatase (Santa Cruz, Santa Cruz, CA). Jurkat whole cell lysate and Jurkat whole cell lysate treated with staurosporine were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

WB reagents (Vectastain® ABC-AmP) were purchased from Vector Laboratories (Burlingame, CA). Farnesol was obtained from Sigma–Aldrich Corporation (St. Louis, MO).

Immunofluorescence

An immunocytochemical analysis was performed on primary cell culture growing in a monolayer fashion in culture. The cells were plated in 4-well glass slides, and allowed to remain in growth media for 24 h. After removal of the growth media, the slides were rinsed with PBS for 5 min followed by fixation in methanol for 15 min at −20 °C. The slides were rinsed two-times with PBS and all cells were blocked with 10 % horse serum in PBS for 30 min at room temperature (RT). Slides were incubated with primary antibodies over night at 4 °C. After a three-time wash with PBS, addition of the secondary fluorescence antibody Alexa Fluor 488 (1:200) for 1 h at RT in the dark. After a two-time wash with PBS the slides were mounted with DAPI-containing fluorescence mounting medium (Vector Laboratories, Burlingame, CA).

Cell treatment

Primary meningioma cells ($\sim 10^6$ cells/ml) were used to seed 6-well culture plates in DMEM medium containing 10 % FBS, for 48 h. Next, the cells were treated with increasing concentrations of farnesol (0, 0.25, 0.5, 1, 2, 4 μM) in medium for 2, 4, and 6 h. The cells were washed twice with PBS and lysed in cold lysis buffer [protein quant sample lysis kit (Applied Biosystems, Foster City, CA)] per the manufacturer's protocol. Lysates were clarified by centrifugation at 1,200×*g* for 10 min at 4 °C and quantified on an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany).

TaqMan® protein assays

Antibodies were labeled with biotin using the Biotin-XX microscale protein labeling kit (Invitrogen, Carlsbad, CA)

per the manufacturer. Biotinylated antibodies were labeled using the TaqMan® Protein Assay Open Kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. The specificity of the caspase-3 assay was determined using recombinant caspase-3 (Cat.No. H00000836-P02, Above, Taipei City, Taiwan).

TaqMan protein assay was performed using protein lysates in triplicates. The initial concentration of meningioma protein lysate was 12.5 μg/μl and the initial input amount was 5 μg. Samples were diluted to 5000, 2500, 1250, 625, 312.5, and 0 ng protein per reaction. TPA was performed using the TaqMan® protein assay core reagents kit with master mix (Applied Biosystems) per the manufacturer's instructions [14]. Real-time-PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) using the standard instrument protocol for the TPA.

Data were analyzed using ProteinAssist™ 1.0 (Applied Biosystems, Foster City, CA), which uses the ΔCt^2-method to calculate relative protein expression between untreated controls and treated sample. Sample dilutions were assayed, and the resulting Ct values were normalized to the sample input, which requires accurate protein quantitation. Every 48-well plate included a no-protein control (NPC) to calculate ΔCt values (Ct-value [sample]−Ct-value [NPC]). Then, a linear range was generated for each sample, and a ΔCt-threshold was designated. The fold-change between samples was calculated between the crossover points of each linear trend line at the ΔCt threshold.

Western blot

Equal amounts of protein (20 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Nupage® Bis–Tris-Gel 4–12 %; Invitrogen, Inc., Carlsbad, CA) and transferred to polyvinylidene difluoride Hybond-P membrane (Invitrogen, Inc., Carlsbad, CA). Western blots were enhanced using the Vectastain® ABC-AmP per the manufacturer's instructions. Membranes were blocked for 5 min at RT and immunoblotted with primary antibodies for 2 h at RT. Anti-GAPDH was used to confirm equal loading between samples. The blots were visualized with 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitroblue tetrazolium (NBT) per the manufacturer's instructions and the bands were analyzed using ImageJ 1.45 s (http://rsbweb.nih.gov/ij/index.html).

Immunocytochemistry of single-stranded DNA (ssDNA)

Monolayer cultures of meningioma cells were analyzed by immunocytochemistry. The cells were treated, plated on 4-well glass slides, and allowed to grow in DMEM, containing 10 % FBS, for 48 h. Next, the cells were treated with increasing concentrations of farnesol (0, 1, 2, 4, and 8 μM) in medium for 24, 48, and 72 h. After the growth media was removed, the slides were rinsed with cold PBS, and the cells were fixed with ice-cold methanol for 20 min at −20 °C. The slides were rinsed twice with PBS and incubated with 100 % formamide (Roth, Karlsruhe, Germany) for 20 min at 75 °C.

After cooling, the slides were incubated with 3 % H_2O_2 for 5 min at RT and blocked with 3 % goat nonfat dry milk in distilled water for 15 min at RT. The slides were incubated for 15 min at RT with primary monoclonal anti-ssDNA per the manufacturer's protocol and with the secondary antibody (1:100 in PBS) for 15 min at RT. Stains were visualized using Vector® Novared (Vector Laboratories, Burlingame, CA), and the slides were counterstained with hematoxylin (Merck, Darmstadt, Germany) and examined by light microscopy.

Viability assay

Primary meningioma cells in mid-log phase were used to seed 4-well culture slides in DMEM medium, containing 10 % FBS, for 24 h. Then, the cells were treated with increasing concentrations of farnesol (0, 0.4, 0.8, 1.2, 1.6 and 2 μM) in medium for 24 h. Cells were stained using trypan blue solution 0.4 % (Invitrogen Inc., Carlsbad, CA) per the manufacturer's protocol and were analyzed under a light microscope.

Statistical analysis

Statistical analysis was performed with GraphPad Prism, V5.03 (GraphPad Software, La Jolla, USA). All results were expressed as mean ± standard deviation (SD). Statistical comparisons between two groups were made by Mann–Whitney test. The level of significance was set to $P < 0.05$. All tests were 2-sided. All data are expressed as mean ± SD.

Results

Development of TaqMan® protein assays for caspase-3 and active caspase-3

To optimize a TaqMan® Protein Assay, the selected antibody has to be polyclonal or a qualified ELISA antibody pair. Polyclonal antibodies must be antigen-purified and raised against full-length antigen.

The used caspase-3 antibody was antigen affinity-purified polyclonal goat IgG against *E. coli* derived recombinant human caspase-3. After labeling biotinylated

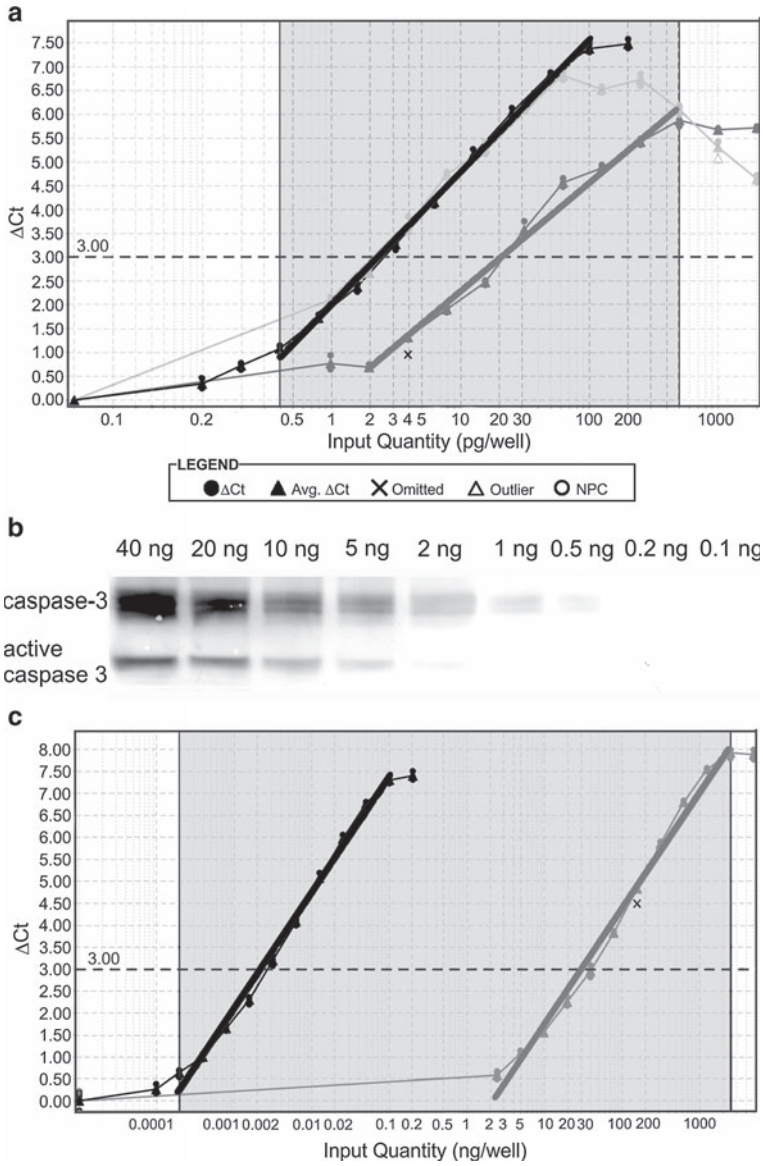


Fig. 3 Development of TPAs for active and inactive caspase-3 using recombinant caspase-3. **a** *Linear regression lines* of caspase-3 assay with initial input amounts of 2,000 pg (*grey*) and 200 pg (*black*). Quantitation threshold set to 3.0. Linear range for caspase-3 assay was 0.394–100 and 1.95–500 pg for active caspase-3 assay. Lower ΔCt values for caspase-3 quantities over 100 pg are due to an excess of target. By specific TPA, active caspase-3 was significantly lower than caspase-3. **b** This result correlates with the WB analysis of recombinant caspase-3. The same biotinylated antibodies were used for western blot analysis as in the TPA. Inactive and active caspase-3 was detectable up to 1 and 5 ng, respectively. **c** Caspase-3 quantity in random meningioma cell lysate (*grey*) compared with recombinant protein (*black*)

caspase-3 with oligonucleotides, we determined its specificity using full-length recombinant caspase-3. The input amount of caspase-3 was 200 and 2,000 pg. As shown in Fig. 3a, the linear range for the caspase-3 assay was 0.394–100 pg, resulting in a ΔCt value of 6.4. The used active caspase-3 antibody was a purified recombinant rabbit monoclonal antibody. The immunogen was a peptide corresponding to the amino acids 171–175 of caspase-3. The TPA for active caspase-3 consisted of probe A caspase-3 and probe B active caspase-3.

The specificity for active caspase-3 was also determined using recombinant caspase-3. Active caspase-3 was detected in a linear range of 1.95–500 pg. The ΔCt value for active caspase-3 was 5.19.

The passing criteria for TPA per Applied Biosystems are $\Delta Ct > 5$ for recombinant proteins and $\Delta Ct > 3$ for cell lysates. Thus, the assays detected active and inactive caspase-3, but the ability to detect active caspase-3 by its specific TPA was significantly lower versus caspase-3, correlating with our WB analysis of recombinant caspase-3 (Fig. 3b).

For WB analysis, the same biotinylated antibodies were used in the TPA. Inactive caspase-3 was detectable to 1 ng in untreated meningioma cell lysates. Active caspase-3 was detected up to 5 ng in apoptosis-induced treated meningiomas.

Using a random untreated meningioma sample, the ΔCt for caspase-3 was determined. As shown in Fig. 3c, the

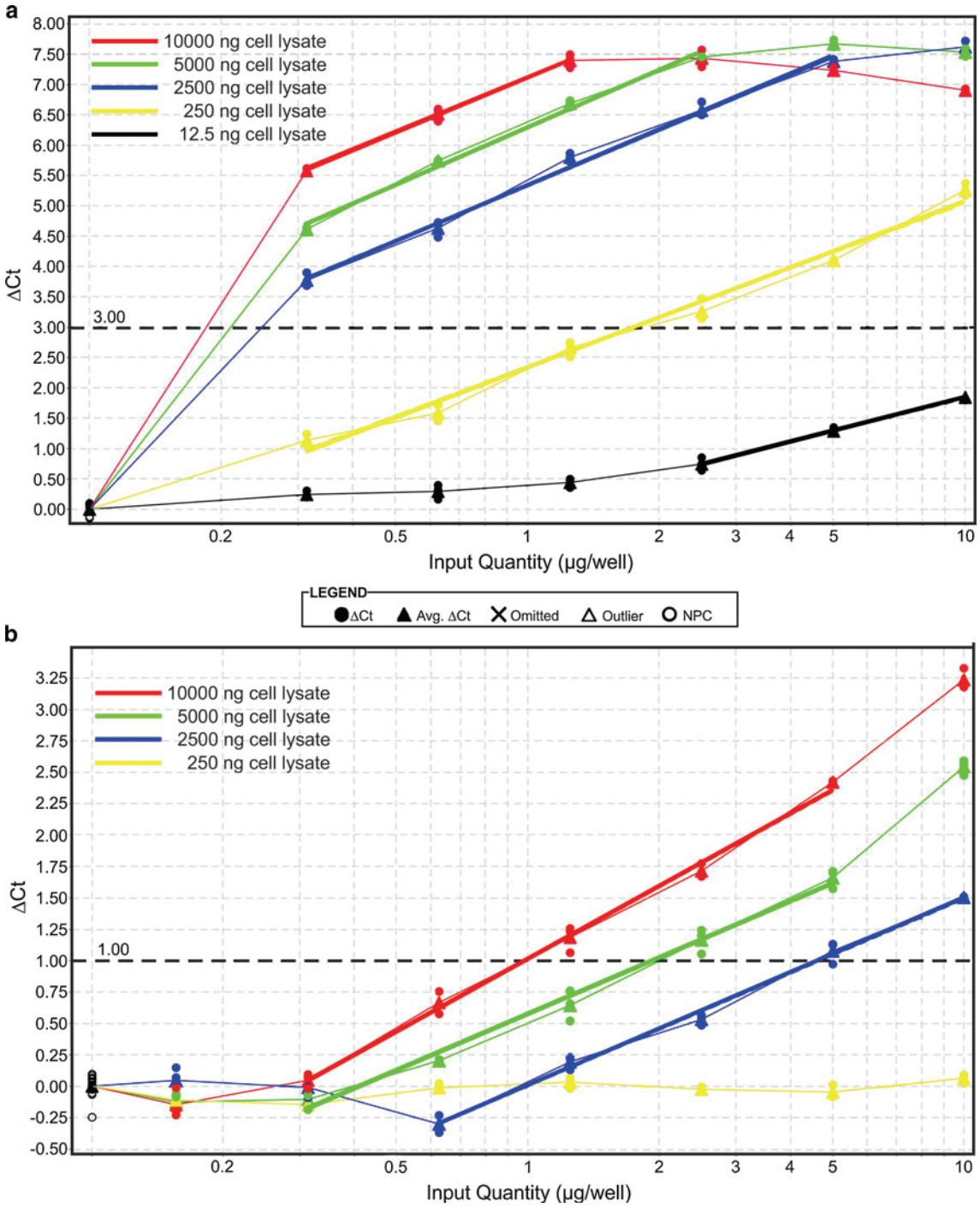


Fig. 4 Calibration of input quantity and quantitation threshold for inactive and active caspase-3 in meningiomas. **a** To calibrate the input quantity for the caspase-3 assay four untreated random meningiomas were examined using five input amounts (10000 (*red*), 5000 (*green*), 2500 (*blue*), 250 (*yellow*), and 12.5 (*black*) ng cell lysate). Results of a representative meningioma are shown at a quantitation threshold of 3.0. Slope of *linear regression lines* were comparable for 2.5–10 µg input (3.01, 3.04, and 3.14) but flattened with lower input amounts. **b** For input determination of active caspase-3 250 to 10,000 ng cell lysate was used. Active caspase-3 was detectable using 2.5–10 µg input. An initial input quantity of 5 µg cell lysate was selected for both assays

linear range was 2.44–2,500 ng cell lysate resulting in a ΔCt of 7.3. In eight meningiomas, the concentration of caspase-3 was 21.5 ± 10.5 pg/µg protein lysate. The concentration of active caspase-3 was 4.4 ± 2.1 pg/µg protein lysate in apoptosis-induced treated meningiomas.

Table 1 Influence of quantitation threshold settings on fold-change for **a** caspase-3 TPA and **b** active caspase-3 TPA in a representative meningioma

a

Meningioma cell lysate (%)	Theoretical fold-change	Observed fold-change at threshold setting					
		0.5	1	2	3	4	5
100	1	1	1	1	**1**	1	1
50	0.5	0.44	0.45	0.46	**0.48**	0.5	0.51
25	0.25	0.25	0.25	0.25	**0.25**	0.25	0.26
2.5	0.025	0.03	0.029	0.027	**0.025**	0.023	0.021
0.125	0.00125	0.0035	0.0027	0.0016	**0.001**	0.0006	0.0004

b

Meningioma cell lysate (%)	Theoretical fold-change	Observed fold-change at threshold setting					
		0.5	0.8	0.9	1	1.5	2
100	1	1	1	1	**1**	1	1
50	0.5	0.61	0.55	0.53	**0.51**	0.43	0.36
25	0.25	0.25	0.23	0.22	**0.21**	0.18	0.15
2.5	0.025	–	–	–	–	–	–

Fold-changes at used quantitation threshold appear in bold

Calibration of input quantity and quantification threshold

To calibrate the ideal input quantity for the caspase-3 assay, four untreated random meningiomas were examined with five input amounts (10000, 5000, 2500, 250 and 12.5 ng cell lysate). Figure 4a shows a representative meningioma. The slope of the linear regression lines were comparable for 2.5–10 µg of input (3.01, 3.04, 3.14) but flattened at lower amounts. Caspase-3 was detected in one meningioma at 12.5 ng cell lysate. In three of four meningiomas, caspase-3 was detected at input amounts of 250 ng but the results differed significantly compared to with higher input amounts.

To calibrate the ideal input quantity for the active caspase-3 assay, four farnesol-treated random meningiomas were examined with five input amounts (10000, 5000, 2500, 250 and 12.5 ng cell lysate). Active caspase-3 was detectable at 2.5–10 µg of input amounts, as shown in Fig. 4b. Thus, an input quantity of 2.5–10 µg cell lysate was considered. Because 10 µg cell lysate led to lower ΔCt values for higher amounts of cell lysates due to the availability of excess target, we selected an initial input quantity of 5 µg cell lysate for both assays.

The quantification threshold is used to calculate fold-changes between unknown and control samples. To calibrate the quantification thresholds for both assays, cell lysates were diluted to 100, 50, 25, 2.5, and 0.125 % meningiomas. Table 1a shows the fold-change results at various quantification thresholds (0.5, 1, 2, 3, 4, and 5) for caspase-3.

The fold-changes at a threshold setting of 3.0 (1.0, 0.5, 0.25, 0.025, 0.00125) most closely approximated the theoretical values (1.0, 0.48, 0.25, 0.025, 0.001). The active caspase-3 assay detected its target at 100, 50, and 25 % meningioma. As shown in Table 1b, a quantification threshold setting of 0.9 or 1.0 resulted in fold-change values that were closest to the theoretical values. To determine the fold-change in subsequent experiments, we used a threshold of 3.0 for caspase-3 and 1.0 for active caspase-3.

Determination of staurosporine-induced apoptosis using active caspase-3 TPA

To verify active caspase-3 as apoptotic marker the IOMM-Lee cell line and primary glioblastoma cells were cultured in the presence of 5 µM staurosporine (STR) for 3 h. Staurosporine is a widely used apoptosis inducer. As shown in Fig. 5a, STR induced apoptosis in both cell types. IOMM-Lee displayed positive immunofluorescence staining for active caspase 3 (Fig. 5B) and cleaved PARP1 (Fig. 5C). Primary glioblastoma cells only stained positive for active caspase-3 (Fig. 5E + F).

Next IOMM-Lee cell line and primary glioblastoma cell culture were seeded in 12-well culture plates in DMEM for 24 h for WB and TPA analysis. Cells were treated with increasing concentrations of STR (0.5–8 µM) for 4 h. Also cells were treated with 5 µM STR for 1, 2, 3, 4, 5, and 6 h. All experiments were conducted in replicates. WB analysis of STR-treated IOMM-Lee cells displayed only barely detectable bands for cleaved PARP1, as shown in Fig. 5b. As negative control a

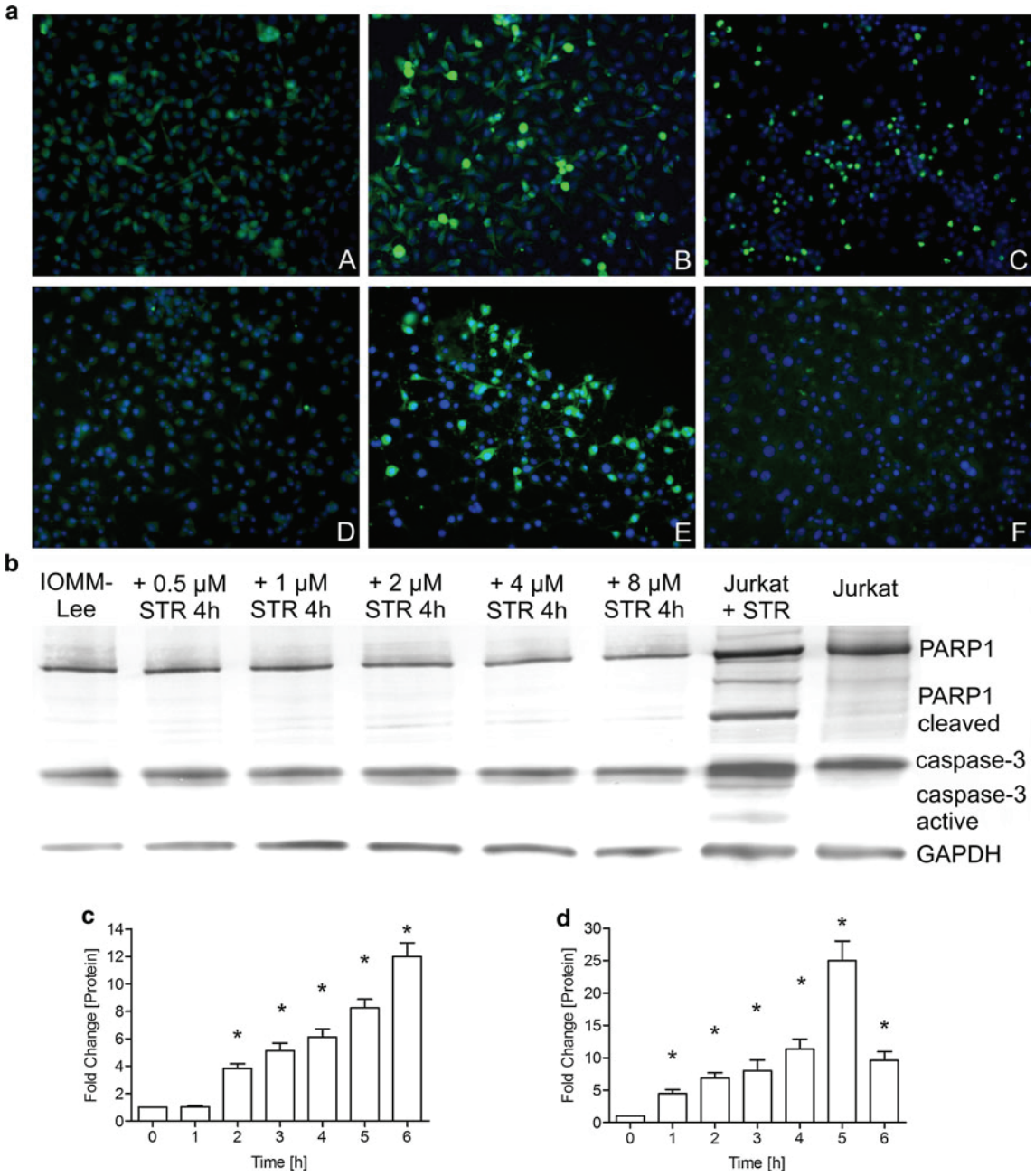


Fig. 5 TPA detected staurosporine (STR) induced apoptosis in malignant meningioma cell line IOMM-Lee and primary glioblastoma cell culture. **a** Immunofluorescence staining of active caspase-3 and cleaved PARP1. Positive immunoreactivity appears as green staining (Alexa Fluor 488). Original magnification ×20. **A–C** IOMM-Lee, **D–F** glioblastoma, **A** + **D** active caspase-3 in untreated cells, **B** + **E** positive active caspase-3 cells after treatment with 5 µM STR for 3 h, **C** IOMM-Lee cells stained positive for cleaved PARP1 after treatment with 5 µM STR for 3 h, **F** negative staining of glioblastoma cells after treatment with 5 µM STR for 3 h. **b** WB analysis of PARP1, cleaved PARP1, inactive, and active caspase-3 in STR-treated IOMM-Lee cells. As negative control a purchased Jurkat whole cell lysate was used. As positive control a purchased Jurkat whole cell lysate treated with STR was used. GAPDH was used to confirm equal loading between samples. STR-treated IOMM-Lee cells displayed only barely detectable bands for cleaved PARP1 and no bands for active caspase-3. **c** Active caspase-3 TPA showed, that 5 µM STR induced apoptosis in IOMM-Lee cells in a time-delayed manner increasing with ongoing time. **d** Primary glioblastoma cells displayed a 4-fold increased active caspase-3 expression after 1 h, which peaked after 5 h. *Bars* represent mean ± SD determined from triplicate assays. $*P < 0.05$ versus control

purchased Jurkat whole cell lysate was used. As positive control a purchased Jurkat whole cell lysate treated with STR was used. Confirming the immunofluorescence stainings primary glioblastoma displayed no bands for cleaved PARP1 and weak bands for PARP1 (not shown). Active caspase-3 was not detectable in both cell types using WB (Fig. 5b).

Analysis of apoptosis by active caspase-3 TPA showed, that 5 μM STR induced apoptosis in IOMM-Lee cells in a time-delayed manner (Fig. 5c). Active caspase-3 increased 4-fold after 2 h. After 5 h active caspase-3 expression doubled and increased further to a fold change of 12 after 6 h. In contrast, primary glioblastoma cells displayed a 4-fold increased caspase-3 expression after 1 h, which peaked after 5 h (fold change = 25.0 ± 3.0), as shown in Fig. 5d.

Farnesol induces apoptosis in meningiomas

To determine the concentration of farnesol that induces apoptosis, three primary meningioma cell cultures (WHO grade I–III, Table 2: 1–3) and primary glioblastoma cell culture were cultured in the presence of 0.2–8 μM farnesol. After 24, 48, and 72 h, apoptotic cells were measured by antibody-based assay to detect DNA damage (ssDNA); control cells were incubated with equal volumes of DMSO. Farnesol is a water-insoluble oil; thus, it was added directly to growth medium.

None of the meningiomas or glioblastoma underwent apoptosis after incubation with DMSO for 24 h, as shown in Fig. 6a, (A–C). Farnesol induced apoptosis in each meningioma sample and glioblastoma after 24 h at a concentration of 0.2 μM. Figure 6a, (D–F) shows that the number of apoptotic cells rose with increasing farnesol concentrations in malignant meningioma. Further, apoptosis increased after 48 h, as shown in Fig. 6b. After 72 h, more than 50 % cells were dead in meningioma and glioblastoma cell cultures that were given 4–8 μM farnesol.

Low concentrations of farnesol impair viability of meningiomas

To determine the effects of farnesol on meningioma, eight primary meningiomas (seven WHO grade I and one WHO grade II meningioma; Table 2: 4–11), IOMM-Lee cell line, and primary glioblastoma were treated with increasing concentrations of farnesol for 24 h and stained with trypan blue; the control was incubated with 2 μl DMSO. All experiments were conducted in replicates.

The control group displayed a viability rate of 92.11 ± 4.1 %. After treatment with 0.4 and 0.8 μM farnesol meningiomas were divided into two groups. In group 1, comprising five meningiomas, farnesol had minor effects on viability. Viable cell number decreased from 92.6 ± 2.9 % to 87.0 ± 4.1 % and 74.0 ± 21.6 %, respectively (Fig. 7a). Group 2 reacted more severely with 0.4 and 0.8 μM farnesol (30.25 ± 8.6 %; 14.34 ± 9.3 %), as shown in Fig. 7b. Both groups showed highly impaired viability on treatment with 1.2 μM farnesol (3.1 ± 2.4 %; 9.2 ± 5.2 %). After treatment with higher farnesol concentrations, nearly all meningioma cells were dead, as shown in Fig. 7a + b.

In addition, the effect of farnesol on control cells malignant meningioma cell line IOMM-Lee and primary glioblastoma were evaluated. IOMM-Lee displayed a viability rate of 87.4 ± 6.1 % (Fig. 7c). After treatment with 0.4 μM farnesol viability decreased to 28.1 ± 16.0 %, showing a similar reaction as meningioma group 2. IOMM-Lee cells treated with 1.2 μM farnesol showed no viability. Primary glioblastoma displayed a different behavior. Viability decreased from 90.8 ± 4.6 % to 10.7 ± 10.7 % after treatment with 0.4 μM farnesol (Fig. 7d). But after treatment with higher farnesol concentrations primary glioblastoma still showed low viability.

Determination of farnesol-induced apoptosis by TPA

To determine if active caspase-3 TPA detects farnesol-induced apoptosis in meningiomas, malignant meningioma cell line IOMM-Lee and as control a primary glioblastoma cell culture were treated with either 60 μM farnesol for 1, 2, 3, and 4 h or with 8–200 μM farnesol for 3 h. As control two other methods (immunofluorescence and WB) were used to detect apoptosis markers active caspase-3 and cleaved PARP1. IOMM-Lee cells displayed positive immunofluorescence staining for active caspase-3 (Fig. 8a, D) and cleaved PARP1 (Fig. 8a, E) after treatment with 60 μM farnesol for 3 h. In addition, farnesol-treated glioblastoma cells stained positive for active caspase-3 (Fig. 8a, F). WB analysis detected bands of cleaved PARP1 in farnesol-treated IOMM-Lee cells after treatment with 60, 80, 100, and 200 μM farnesol for 3 h, as shown in Fig. 8b. Also cleaved PARP1 bands were detected after treatment with 60 μM farnesol for 3 and 4 h and after treatment with 100 μM farnesol for 3 h (Fig. 8c). After treatment with 100 μM farnesol for 4 h most IOMM-Lee cells were dead. WB analysis of inactive and active caspase-3 showed similar results. Inactive caspase-3 bands weakened with increasing farnesol concentrations, but showed no bands for active caspase-3 (Fig. 8b). In addition, active caspase-3 bands were detected after treatment with 60 μM farnesol for 3 and 4 h and after treatment with 100 μM farnesol for 2 and 3 h (Fig. 8c). Inactive caspase-3 bands weakened with increasing incubation time corresponding with the detection of active caspase-3 bands.

Figure 8d shows the active caspase-3 TPA diagram. Active caspase-3 TPA confirmed the WB analysis displaying highest active caspase-3 expression after 3 h. Also TPA showed higher sensitivity displaying an increased fold change after 1 h (fold change = 2.9 ± 0.4) and 2 h (fold change = 9.2 ± 0.8) (Fig. 8e). Active caspase-3 peaked after 3 h (fold change = 27.0 ± 2.0). In addition, TPA detected increased active caspase-3 level after treatment

Table 2 Histopathological data of used primary meningioma cell cultures, meningioma cell line IOMM-Lee, and control primary glioblastoma cell culture

Case no.	Gender	Age	WHO grade	Histological subtype	Recurrence	Brain invasion	Ki-67 [%]
Glioblastoma	F	60	4	–	–	+	20
IOMM-Lee [13]	M	61	3	–	+	+	36
1	M	75	3	Anaplastic	+	+	20
2	F	53	2	Atypical	+	+	10
3	F	62	1	Meningothelial	–	–	3
4	F	76	2	Atypical	+	+	20
5	F	86	1	Meningothelial	–	–	4
6	F	52	1	Meningothelial	–	–	3
7	F	61	1	Meningothelial	–	–	2
8	M	55	1	Meningothelial	–	–	4
9	F	58	1	Transitional	–	–	3
10	F	47	1	Transitional	–	–	3
11	M	48	1	Transitional	–	–	2

M male, *F* female

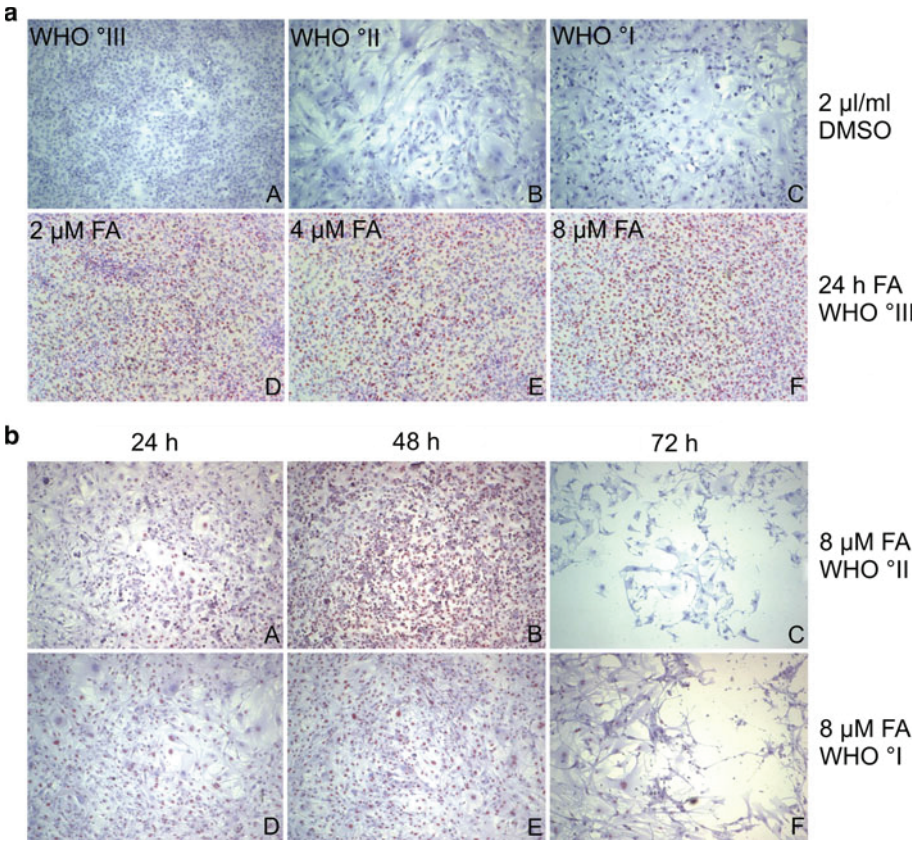


Fig. 6 Farnesol induced apoptosis in primary meningioma cell cultures. **a** + **b** Immunocytochemical staining of ssDNA of three primary meningioma (WHO grade I–III) cells treated with 1–8 µM farnesol. Cells were analyzed for ssDNA after 24, 48, and 72 h of incubation. Positive immunoreactivity appears as red staining (Vector® NovaRed™). **a** (**A**–**C**) Control cells were incubated with equal volumes of DMSO. After 24 h, none of the DMSO-treated meningiomas showed apoptosis. (**D**–**F**) Apoptotic cell number rose with increasing farnesol concentrations in meningiomas, as shown in a representative malignant meningioma. **b** Apoptosis increased after 48 h in both (**A**–**C**) WHO II and (**D**–**F**) WHO I meningioma. After 72 h, up to 50 % of all meningioma cells were dead. Original magnification ×10

with 8 µM farnesol after 3 h. Active caspase-3 rose with increasing farnesol concentrations and peaked at 80 µM farnesol (not shown).

As shown in Fig. 8f, primary glioblastoma cells displayed a 4.5-fold increased active caspase-3 expression after 1 h, which peaked after 3 h (fold change = 7.4 ± 1.7).

Active caspase-3 TPA detects low concentrations of active caspase-3 in meningiomas

Six primary meningiomas (five WHO grade I and one WHO grade II meningioma; Table 2: 4–9) were used to seed 6-well culture plates in DMEM, containing 10 % FBS, for 48 h. Next, the cells were treated with increasing

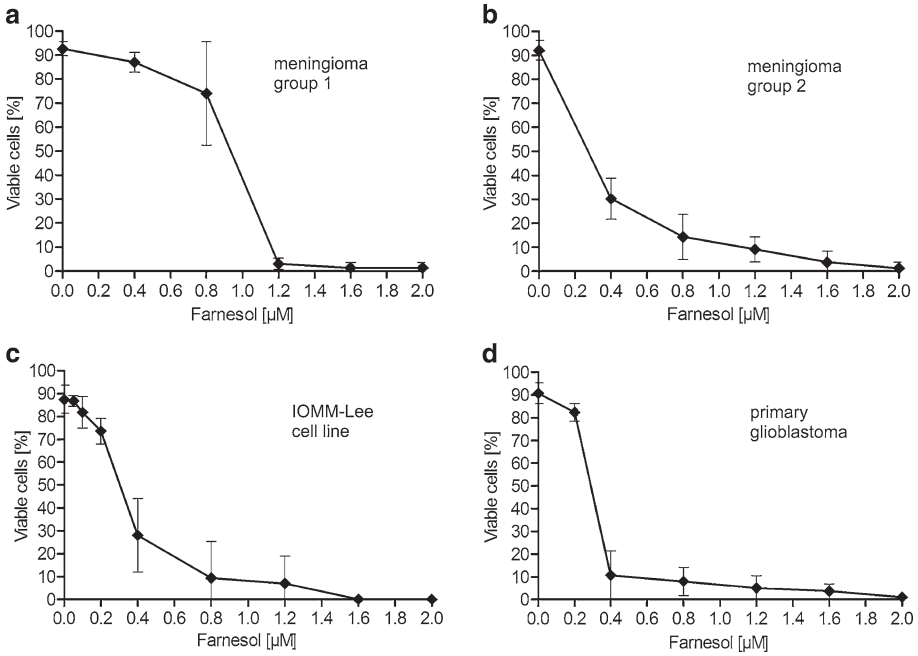


Fig. 7 Farnesol impaired viability in eight primary meningioma and glioblastoma cell culture, and IOMM-Lee cell line. The effect of farnesol on viability was determined using trypan blue. The control was incubated with 2 µl DMSO. **a** Group 1 ($n = 5$) showed minor effects on viability after treatment with 0.4 and 0.8 µM farnesol (87.0 ± 4.1 %; 74.0 ± 21.6 %). Less than 5 % of cells were viable after incubation with 1.2 µM farnesol. **b** Farnesol significantly impaired viability in group 2 ($n = 3$) already at a concentration of 0.4 and 0.8 µM (30.25 ± 8.6 %; 14.34 ± 9.3 %). Viability was reduced more than 90 % at 1.2 µM farnesol. After treatment with higher farnesol concentrations, nearly all meningioma cells in both groups were dead. **c** After treatment with 0.4 µM farnesol viability of IOMM-Lee was decreased to 28.1 ± 16.0 %, showing a similar reaction as meningioma group 2. IOMM-Lee cells treated with 1.2 µM farnesol showed no viability. **d** Primary glioblastoma displayed a different behavior. Viability decreased from 90.8 ± 4.6 to 10.7 ± 10.7 % after treatment with 0.4 µM farnesol. But after treatment with higher farnesol concentrations primary glioblastoma still showed low viability. Data shown are the mean cell number ± SD determined from triplicate assays ($n_{\text{meningioma}} = 8$)

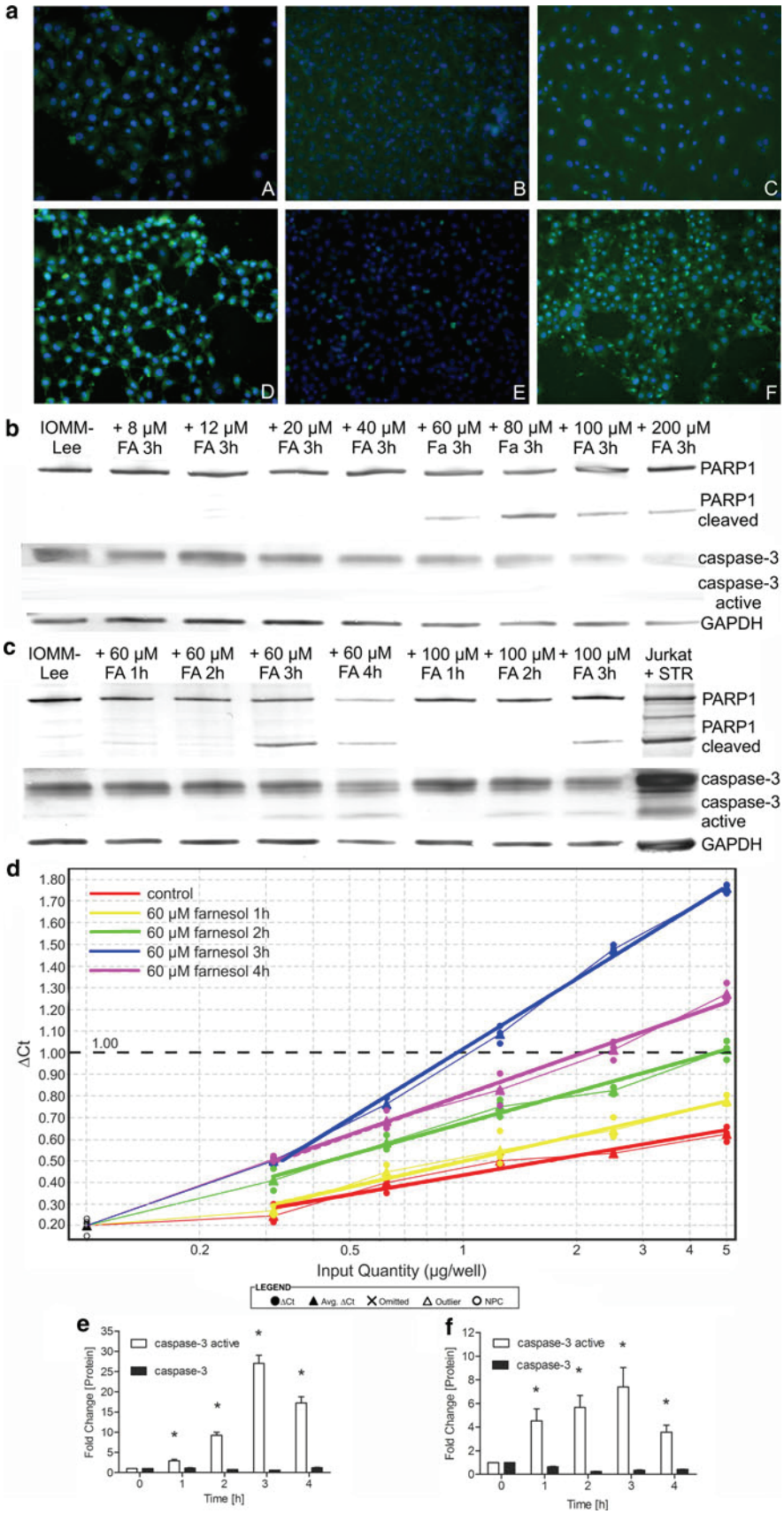
concentrations of farnesol (0, 0.25, 0.5, 1, 2, 4 µM) for 2, 4, and 6 h. Control cells were incubated with equal volumes of DMSO. Cells were lysed and quantified, and protein lysates were diluted to a 12.5-µg/ml stock solution. TPA was performed using an initial amount of 5 µg protein lysate.

As shown in Fig. 9a, after 2 h meningiomas that were treated with 0.25–1 µM farnesol showed no significant increase in active caspase-3. Treatment with 2 and 4 µM farnesol for 2 h up-regulated active caspase-3, indicating early apoptosis. Three of six meningiomas displayed similar behavior. Active caspase-3 expression rose abruptly after treatment with 2 or 4 µM farnesol. In contrast, the remaining three meningiomas upregulated active caspase-3 with increasing farnesol concentrations (Fig. 9b). To compare, inactive caspase-3 was also measured in these samples. Caspase-3 expression declined after treatment with 0.25–2 µM farnesol (fold-change 0.41–0.71), but the 4 µM farnesol downregulated caspase-3 with a fold change of 0.064 (−93.6 %), as shown in Fig. 9c.

Active caspase-3 increased in all six meningiomas after treatment with 2 and 4 µM farnesol for 2 h ($P_{2\ \mu M} = 0.014$; $P_{4\ \mu M} = 0.012$; Fig. 9e). Inactive caspase-3 declined in meningiomas that were treated with 0.25 µM farnesol ($P = 0.0001$; Fig. 9f). As shown in Fig. 9d + g, active caspase-3 peaked at 2 h with 4 µM farnesol and fell with longer incubations to baseline; the same pattern was observed on treatment with 1 and 2 µM farnesol (data not shown).

Discussion

In this study, we determined whether early apoptosis can be detected and quantified reliably using TPA by optimizing an assay for caspase-3 and active caspase-3. Traditional protein detection methods such as WB or ELISA displayed several limitations when used with primary meningioma cell cultures. WB requires a high input quantity and has a low sensitivity. Primary meningioma cell cultures have a slow growth rate and show replicative senescence, due in part to low or absent telomerase activity. The few stable meningioma cell lines that exist are highly malignant, whereas meningiomas are primarily benign or genetically altered. These cell lines were established by telomerase-mediated immortalization [15, 16]. ELISAs require less



◀ **Fig. 8** TPA detected farnesol-induced apoptosis in malignant meningioma cell line IOMM-Lee and primary glioblastoma cell culture. **a** Immunofluorescence staining of active caspase-3 and cleaved PARP1. Positive immunoreactivity appears as green staining (Alexa Fluor 488). Original magnification ×20. **A** Active caspase-3 in untreated IOMM-Lee cells, **B** cleaved PARP1 in untreated IOMM-Lee cells. **C** Active caspase-3 in untreated glioblastoma cells. **D** Positive active caspase-3 IOMM-Lee cells after treatment with 60 μM farnesol for 3 h. **E** Positive nuclear staining of cleaved PARP1 in 60 μM farnesol-treated IOMM-Lee cells. **F** Positive active caspase-3 glioblastoma cells after treatment with 60 μM farnesol for 3 h. **b** + **c** WB analysis of PARP1, cleaved PARP1, inactive, and active caspase-3 in farnesol-treated IOMM-Lee cells time- and concentration dependent. As positive control a purchased Jurkat whole cell lysate treated with STR was used. Bands for cleaved PARP1 were detected after treatment with 60 μM farnesol for 3 and 4 h, also after treatment with 80, 100, and 200 μM farnesol for 3 h. Caspase-3 bands weakened with increasing farnesol concentrations and incubation time. The WB for concentration-dependent farnesol treatment showed no positive active caspase-3 bands (**b**). Active caspase-3 bands were barely detectable showing positive bands after treatment with 60 μM farnesol for 3 and 4 h and with 100 μM for 2 and 3 h (**c**). **d** Active caspase-3 TPA diagram. **e** Active caspase-3 TPA confirmed WB analysis (**c**). TPA showed higher sensitivity and allowed accurate quantification. IOMM-Lee displayed a 3-fold increase of active caspase-3 expression after 1 h. Active caspase-3 peaked after 3 h (fold change = 27.0 ± 2.0). **f** Primary glioblastoma cells displayed a 4.5-fold increased active caspase-3 expression after 1 h, which peaked after 3 h (fold change = 7.4 ± 1.7). *Bars* represent mean ± SD determined from triplicate assays. $^{*}P < 0.05$ versus control

sample input and show a higher sensitivity than WB. ELISAs are usually conducted using 96-well plates. In each well an exact cell number is pipetted. Primary meningioma cells display tight cell–cell junctions. When these cells are completely separated meningioma cells show severely impaired growth behavior, go into senescence, or die. Especially proliferating primary meningioma cells are affected. This behavior influences further experiments negatively leading to false conclusions.

The benefits of using primary meningioma cell cultures are their morphological and genetical similarity to meningioma cells in vivo and the variety in cell cultures subtypes. Thus, primary meningioma cell cultures remain the closest samples to in vivo conditions.

Despite the limitations of primary meningioma cell cultures, recently developed TPAs have provided the possibility to quantify protein. TPA detects protein by quantitative PCR, based on proximity ligation assay. TPA requires considerably less sample input, which is an advantage when slow growing primary cell cultures are used. In this study TPA input quantity was 5 μg, whereas Western blot input quantity was 4-fold higher. In addition, TPA displays a high sensitivity of TPA detecting 0.4 pg caspase-3 and 2 pg active caspase-3, respectively. In comparison Western blot detection was limited to 0.5 ng caspase-3 and 5 ng active caspase-3. Thus TPA showed a 1,000-fold higher sensitivity than Western Blot. Compared with other quantitative apoptosis detection methods, such as ELISA and flow cytometry, protein lysates from an experiment can be used later to determine several protein expressions with regard to apoptosis. Also, TPA compares protein quantification and quantitative mRNA analysis directly.

Although most meningiomas are benign, some recur or are resected subtotally due to their delicate location in skullbased structures. Additionally, in a minority of patients, regrowth of tumor tissue after irradiation is a major clinical problem. Despite several chemotherapeutic trials, medical interventions for these tumors are unsatisfactory [17]. Thus, chemotherapeutic drugs without severe side effects are favorable.

Farnesol induced apoptosis in several tumor-derived cell lines [9–11, 18] and has anti-tumor effects in vivo [12]. Further, tumor cells are considerably more sensitive to farnesol-induced inhibition of growth and apoptosis than normal cells [19–21].

After evaluation of TPA specificity the widely applied apoptosis inducer staurosporine was used to determine active caspase-3 as apoptotic marker in two different cell systems (IOMM-Lee and primary glioblastoma cells). Apoptosis was verified using immunocytochemical staining of ssDNA and determination of cleaved PARP1. Active caspase-3 and cleaved PARP1 were analyzed by several methods (TPA, immunofluorescence, and WB). Immunofluorescence staining of active caspase-3 was positive for both staurosporine-treated cell systems, whereas only IOMM-Lee showed positive immunofluorescence for cleaved PARP1. WB analysis confirmed the cleaved PARP1 results, but active caspase-3 was not detectable in both cell systems. TPA was able to detect and quantify active caspase-3 in staurosporine-treated IOMM-Lee and glioblastoma cells. TPA analysis showed that staurosporine induced apoptosis in IOMM-Lee cells in a time-delayed manner in contrast to primary glioblastoma cells.

The effects of farnesol on primary meningioma cells were determined using immunocytochemical staining of ssDNA and a viability test (trypan blue). As control IOMM-Lee and primary glioblastoma cells were also treated with farnesol. Analysis of ssDNA showed that farnesol induced apoptosis in all three cell systems at a concentration of 0.2 μM after 24 h. Furthermore apoptosis increased with farnesol concentration and incubation time. Viability test of primary meningioma cells, IOMM-Lee, and glioblastoma cells displayed a high sensitivity towards farnesol. Meningiomas reacted differently after treatment with low farnesol concentrations. In Group 1 proliferation decreased slowly with increasing farnesol concentrations (0.4–0.8 μM) and showed a highly impaired viability with 1.2 μM farnesol. Group 2 was more sensitive experiencing a 70 and 85 % decrease in proliferation at 0.4 μM and

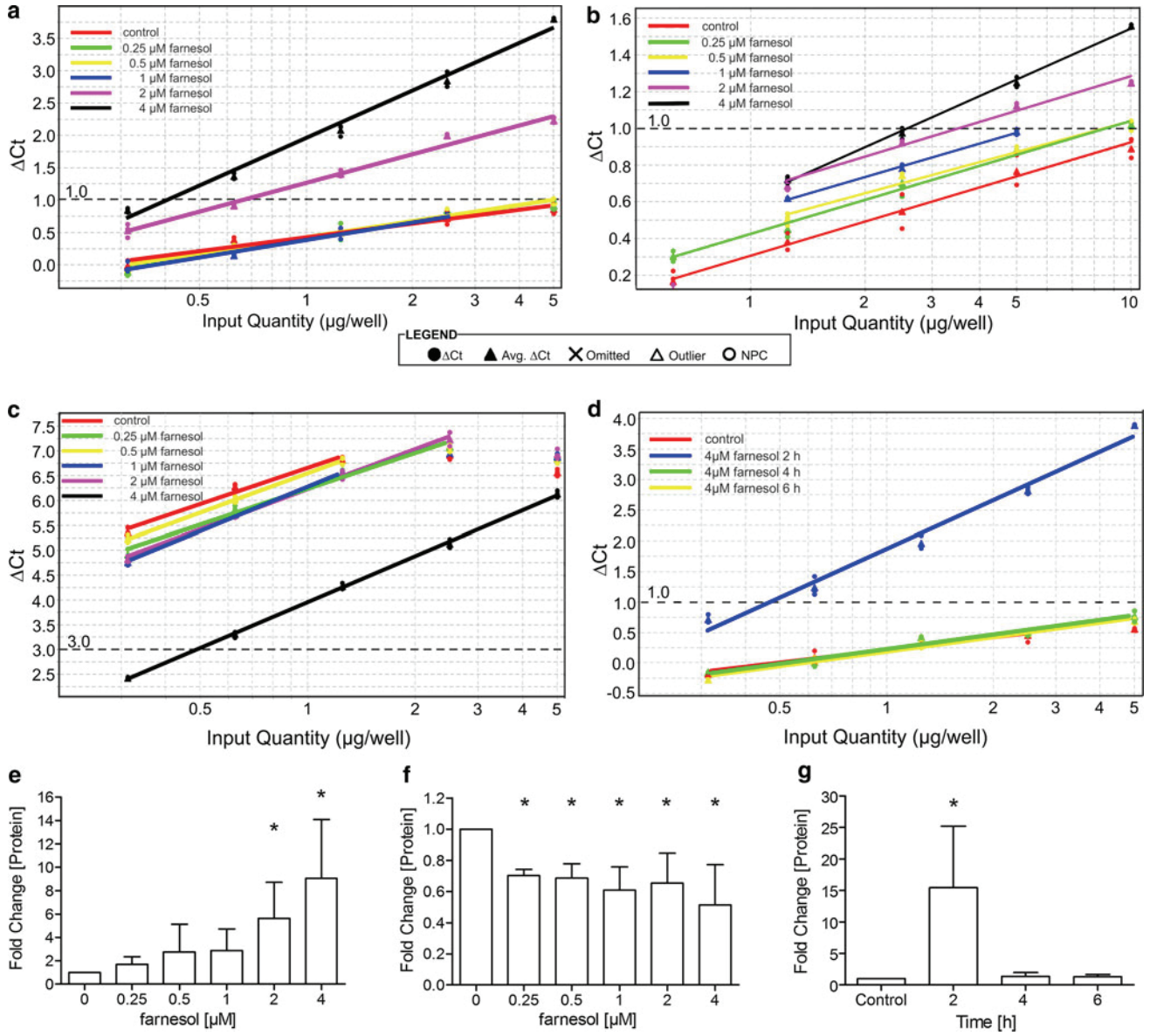


Fig. 9 TPA results on farnesol-induced apoptosis. Six primary meningioma cell cultures were treated with increasing concentrations of farnesol (0, 0.25, 0.5, 1, 2, and 4 µM) for 2, 4, and 6 h. **a** + **b** Active caspase-3 expression in farnesol-treated meningiomas after 2 h representing the different behavior of meningioma cells. **a** Group 1 ($n = 3$) showed a significant increased active caspase-3 expression after treatment with 2 or 4 µM farnesol. **b** In group 2 ($n = 3$) active caspase-3 was upregulated with increasing farnesol concentrations. ΔCt values and the resulting linear regression lines are shown. Quantitation threshold was set to 1.0 **c** Caspase-3 expression in a representative farnesol-treated meningioma after 2 h. Quantitation threshold was set to 3.0. **d** Active caspase-3 expression in a representative 4 µM farnesol-treated meningioma after 2, 4, and 6 h. Summary of TPA results of **e** active caspase-3 and **f** inactive caspase-3. Treatment with 2 and 4 µM farnesol for 2 h significantly increased active caspase-3. Inactive caspase-3 expression decreased at 0.25 µM, with similar levels at higher concentrations. **g** Active caspase-3 increased significantly after 2 h with 4 µM farnesol and declined with longer incubations to baseline. *Bars* represent mean ± SD ($n = 6$). *$P < 0.05$ versus control

0.8 µM farnesol, respectively. After treatment with 1.2 µM farnesol viability in group 2 was similar to group 1. IOMM-Lee cells displayed a similar behavior towards farnesol as group 2 meningiomas.

To evaluate active caspase-3 TPA for determination of farnesol-induced apoptosis IOMM-Lee and glioblastoma cells were treated with high concentrations of farnesol. Active caspase-3 and cleaved PARP1 were detected using immunofluorescence, WB, and TPA. Immunofluorescence stainings of active caspase-3 and cleaved PARP1 corresponded to those of staurosporine-induced apoptosis. In addition, cleaved PARP1 was detected by WB in IOMM-Lee after treatment with 60 µM farnesol showing the highest active caspase-3 level at 80 µM farnesol and after

treatment for 3 h. WB detection of inactive and active caspase-3 showed partly similar results as cleaved PARP1, but active caspase-3 was barely detectable using WB. In addition, several bands of active caspase-3 were not detectable and the band intensity did not correspond with the cleaved PARP1 bands. Active caspase-3 TPA confirmed the WB analysis of cleaved PARP1 also displaying the highest active caspase-3 level at 80 μM farnesol and after treatment for 3 h. In addition, TPA displayed significant higher sensitivity. Active caspase-3 was detectable after treatment with 8 μM farnesol for 3 h.

The effect of low farnesol concentrations were evaluated using TPA. By TPA, elevated active caspase-3 levels were determined in all treated meningiomas after a short incubation time at 2 μM farnesol. Similar to its effects on viability, the meningiomas showed disparate sensitivities to low concentrations of farnesol. In one group, active caspase-3 did not up-regulate until 2 μM, increasing abruptly. In the other group, active caspase-3 expression rose with increasing farnesol concentrations.

Active caspase-3 was also measured at various incubation times. Significantly elevated active caspase-3 levels were noted in all meningiomas after 2 h, independent of farnesol concentration. With longer incubations, active caspase-3 levels declined to baseline level.

Our results suggest that meningiomas are sensitive to farnesol-induced apoptosis and inhibition of growth. Further, meningiomas behave differently after treatment with low concentrations of farnesol, which should be examined further. TPA for active caspase-3 showed a significant higher sensitivity compared to standard protein detection methods such as WB and immunofluorescence. The combination of TPA to detect active caspase-3 and immunocytochemistry of ssDNA is a valuable tool that can be used to analyze and quantify apoptosis in primary cell cultures.

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Conflict of interest No potential conflicts of interest disclosed.

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