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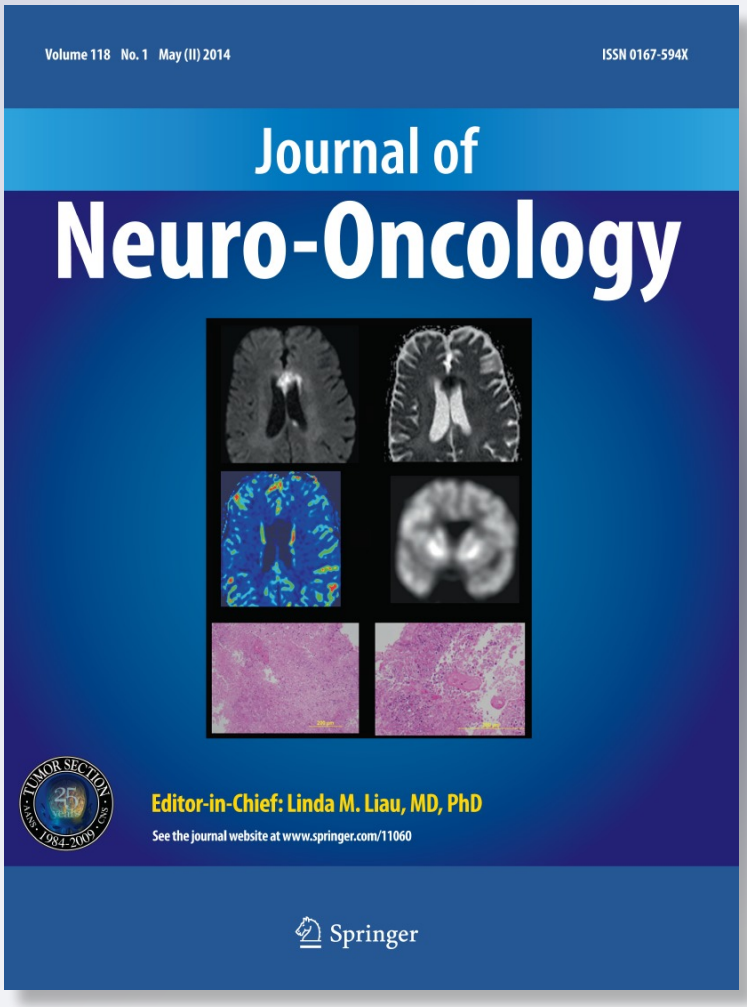

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LABORATORY INVESTIGATION

Vitamin D receptor expression is associated with improved overall survival in human glioblastoma multiforme

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Abstract Vitamin D and its analogs have been shown to display anti-proliferative effects in a wide variety of cancer types including glioblastoma multiforme (GBM). These anticancer effects are mediated by its active metabolite, 1α, 25-dihydroxyvitamin D_3 (calcitriol) acting mainly through vitamin D receptor (VDR) signaling. In addition to its involvement in calcitriol action, VDR has also been demonstrated to be useful as a prognostic factor for some types of cancer. However, to our knowledge, there are no studies evaluating the expression of VDR protein and its association with outcome in gliomas. Therefore, we investigated VDR expression by using immunohistochemical analysis in human glioma tissue microarrays, and analyzed the association between VDR expression and clinico-pathological parameters. We further investigated the effects of genetic and pharmacologic modulation of VDR on survival and migration of glioma cell lines. Our data demonstrate that VDR is increased in tumor tissues when compared with VDR in non-malignant brains, and that VDR expression is associated with an improved outcome in patients with GBM. We also show that both genetic and pharmacologic modulation of VDR modulates GBM cellular migration and survival and that VDR is necessary for calcitriol-mediated effects on migration. Altogether these results provide some limited evidence supporting a role for VDR in glioma progression.

Keywords Vitamin D receptor · Human GBM · Tissue microarray · Biomarker · Survival · Prognosis

Introduction

Tumors of the central nervous system (CNS) include a number of unique, often difficult to treat neoplasms [1], being the tumors of glial origin the most frequent intracranial neoplasia in adults [2]. Among these, GBM is the most prevalent and lethal type of primary tumors of the CNS, with a median survival of 10–12 months [3]. Current treatment for gliomas remains suboptimal, and the promise for improved therapies rests largely on a better understanding of the underlying biology and genetics of these tumors [4]. The molecular and genetic heterogeneity of gliomas undoubtedly contributes to this suboptimal response to treatments which are usually based on standard pathologic diagnoses [5–8]. Thus, glioma diagnosis has been historically based on examining the cellular morphology of the tumor to assess both its presumed cell of origin and surrogate markers of tumor aggressiveness in order to determine the tumor grade [9]. However, these tumor parameters are insufficient to predict the evolution of each individual patient since distinct biological subtypes

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may occur within identical histological grade tumors [10, 11]. Therefore, there is an urgent need to identify relevant prognostic and predictive biomarkers in primary brain tumors.

The active form of vitamin D, calcitriol, is well recognized as a modulator of cell proliferation, differentiation, apoptosis, angiogenesis and metastasis [12–14]. These activities are generally mediated by the vitamin D receptor (VDR) [15, 16], this being a transcription factor that belongs to the superfamily of nuclear receptors for steroid hormones [17, 18]. VDR expression seems to be important for the antineoplastic effects of calcitriol and analogues as well as a prognostic factor in some cancer types. Indeed, VDR expression has been demonstrated to be associated with patient prognosis in various tumor types [19–26]. With regard to gliomas, there is one study showing an increase in VDR mRNA in GBM compared with low-grade astrocytomas [27], and another demonstrating a decrease in VDR mRNA in GBM compared with normal brain [28]. However, to our knowledge, there are no studies of VDR protein expression in human gliomas and no information regarding the role of VDR in the prognosis of this tumor type. Although calcitriol and its synthetic analogues inhibit growth and induce apoptosis in the rat C6 glioma cell line [29], there are some other results demonstrating lack of effects in some GBM cell lines [28]. This resistance to treatment has been explained by the glioma cell line lacking the VDR receptor, resistance which was reverted when VDR was overexpressed [30]. Importantly, beneficial effects of the vitamin D_3 metabolite 1α-hydroxycholecalciferol have been reported for the treatment of GBM in a phase II clinical study [31]. The authors showed response in 20 % of the patients and suggested that VDR expression may be important for the observed outcome in the responders, although they did not analyze VDR expression in tumors. The presence of VDR in target tissues is considered a prerequisite for yielding a calcitriol-mediated effect, and that is why in order to assess the therapeutic potential of this agent, it is necessary to characterize VDR expression in tumors. Therefore we have investigated the expression of VDR in human glioma tissue microarrays, studied its association with clinico-pathological data and survival, and further analyzed the relevance of receptor expression for human glioma progression.

Materials and methods

Tumor tissues

Glioma tumor samples from 89 patients were obtained from the Glioma Biorepository at Henry Ford Hospital, Detroit, MI. The data of the samples is deposited in the repository of molecular brain neoplasia data (REMBRANDT) database (Madhavan et al. [32]; and National Cancer Institute. 2005. REMBRANDT home page. <http://rembrandt.nci.nih.gov>). This database is provided as a public service by the Neuro-Oncology Branch of the National Cancer Institute (NCI, NIH) and the National Institute of Neurological Disorders and Stroke (NINDS, NIH) and includes many samples of brain tumors including the ones that are part of the tissue microarray used in this work. All tumor samples were obtained with written consent in accordance with institutional guidelines. All tissue specimens were acquired between 1993 and 2003 at first resection from untreated patients and were classified morphologically and graded according to the current WHO system. Patients were only included if a gross total or subtotal tumor resection was possible. All patients received the same treatment after surgery and those who died had clear evidence of uncontrolled tumor growth at the time of death. Median follow-up for all patients was 560 days (9–5,910).

Additionally, a cohort of 38 individual whole sections that are not TMA was retrieved from the Pathology Service of Dr. José Penna Hospital, Bahía Blanca, ARG. with IRB approval, and was staged according to the American Joint Committee on Cancer Staging System [33]. They were acquired at first resection from untreated patients between 1997 and 2004.

Tissue microarray construction

Tissues microarrays (TMAs) were constructed at the Neuro-Oncology Branch of the NCI/NINDS (National Institutes of Health) with IRB approval. Two different array blocks were used that added to a total of 89 glioma cases, including 8 oligodendrogliomas WHO grades II and III (age median: 40 years; survival median: 626 days), 11 mixed tumors WHO grades II and III (age median: 37 years; survival median: 2,015 days), 9 astrocytomas WHO grades II and III (age median: 43 years; survival median: 1,923 days) and 61 astrocytomas WHO grade IV (GBM; age median: 58 years; survival median: 329 days). Additionally, 8 cores of non-malignant brain tissues obtained from epileptic brain resections and 16 other tissues were included as controls. Data on the initial diagnosis, staging, gender and survival were collected. The tissue used for arraying was 1 mm in diameter and was selected by a pathologist as representative of the whole tissue sample. The characteristics of the studied population are shown in Online Resource 1.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) staining was performed as previously described [34]. Tumor tissues were incubated

with anti-VDR antibody (1/100; C-20, Santa Cruz Biotechnology), followed by incubation with biotinylated anti-rabbit antibody (1/400; Vector Laboratories) and then incubation with VECTASTAIN ABC reagent (Vector Laboratories Inc). Sections were counterstained with Harri′s hematoxylin (Zymed Laboratories) for analysis by bright field microscopy (Olympus CX31 microscope).

Scoring and statistical analysis

All samples were evaluated and scored simultaneously by two investigators (DS, MF) and a pathologist (JA) as already described [35]. In brief, the specimens were assessed using the semi-quantitative immunoreactive score (IRS). In the TMAs, only representative tissue cores containing at least 200 tumor cells were scored. Sections with IRS > 0 were considered positive. The statistical significance of VDR expression levels with tumor grades or histologic subtypes was determined by Kruskal–Wallis test. The statistical significance of VDR expression levels and rate between groups was determined by the two-tailed Mann–Whitney U and the χ^2 tests, respectively. Survival intervals were measured from the time of surgery to death or until the last follow-up. Overall survival according to VDR expression was constructed using Kaplan–Meier survival curves, with data censored at 3 years, and the log-rank test was used for comparison of survival curves in univariate analyses.

Analyses were performed using Graph Pad 5.0 and MedCalc 12.0 softwares. *p* values of less than 0.05 indicated a significant result.

Cell culture

Human GBM cell lines U251, U87MG and T98G, and human breast adenocarcinoma cell line T47D were grown in DMEM (Sigma) supplemented with 10 % (v/v) FBS, L-glutamine (5 mM,), penicillin (100 U/ml), and streptomycin (100 µg/ml) Gibco.

Transfections

Transient and stable expressions of shRNA VDR were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. The pFIV-H1/U6 VDR shRNA plasmid was used and was kindly donated by Dr. Tokino T. from the Sapporo Medical University (Japan). A scrambled shRNA-expressing pFIV-H1/U6 vector (Scr sh RNA) was used as control. The stable cell lines were obtained by incubating them with puromycin 1 µg/ml for 20 days.

Western blotting (WB)

Immunoblotting was performed as previously described [36]. Briefly, blots were incubated with anti-VDR (1/1,000), $p21^{Cip/Waf}$ (1/100), $p27^{Kip1}$, (1/500), p53 (1/200) and p57 (1/250) (BD Pharmigen), Cyclin D1 (1/1,000; Thermo Scientific) and actin (1/1,000; Santa Cruz Biotechnology).

Cell survival

Cell survival assay was performed as previously described [37]. T98G, T98G VDR-shRNA and T98G scrambled-shRNA were seeded at a density of 2×10^3 cells/100 µl and treated with calcitriol (1 µM) or vehicle for 96 h. The cells were counted manually using a hemocytometer. Data were analyzed using the one-way analysis of variance (ANOVA) to determine the effects of calcitriol and vehicle or the effect of overall cellular survival. Bonferroni post-test was used to determine statistical significance between sample sets.

Cell migration

Cell migration was measured by "wound healing" assay as previously described [38]. Briefly, T98G, T98G VDR-shRNA and T98G scrambled-shRNA were seeded in 35 mm Petri dishes and cultured until confluence and were treated with vehicle (isopropanol) or calcitriol 1 µM for 17 h. The cells were then scraped with a 200 µl micropipette tip and monitored at 0, 5 and 17 h. The uncovered wound area was measured and quantified at different intervals with ImageJ 1.37v (NIH). *T* test was used to determine statistical significance between sample sets.

RNA extraction and real-time qPCR

Total RNA was isolated using TRIzol reagent (Life Technologies) and purified using SV total RNA isolation system (Promega). cDNAs were synthesized with (MMLV RT, Promega) and used for real-time PCR amplification with Taq PCR Master Mix 2X (Qiagen). Primer VDR: FWD: 5-TCGGCATGATGAAGGAGTTC-3 REV: 5-AGTCGG AGTAGGTGGGGTCGTAGG-3 (Sigma Integrated DNA Technologies). Each PCR was done in duplicate and the experiment was repeated twice. Data were analyzed by Rotor Gene 6,000 and normalized to GAPDH.

Immunofluorescence (IF)

IF was performed as previously described [35]. T98G cells without treatment or treated with 1 µM of calcitriol or

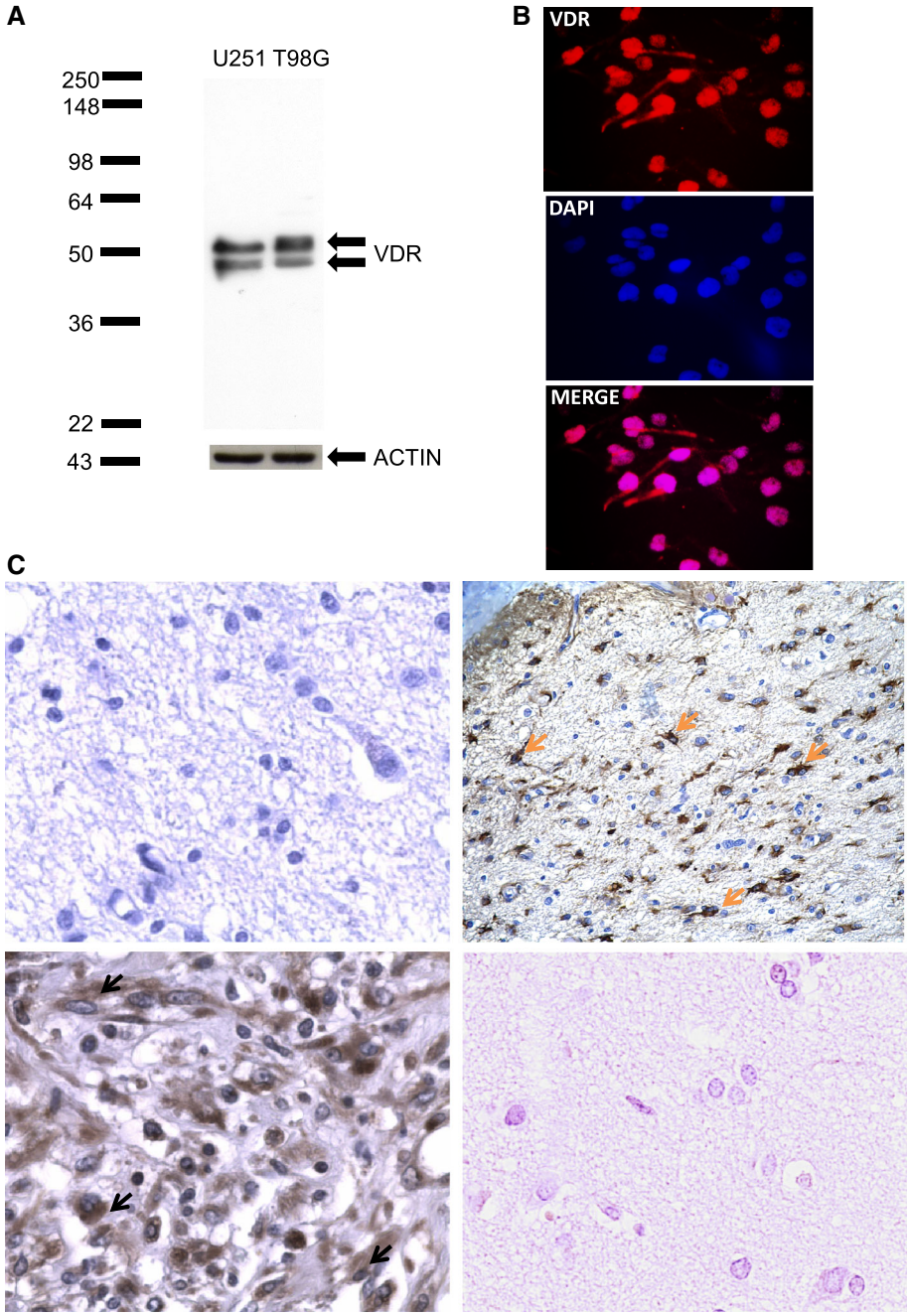

Fig. 1 VDR antibody specificity and protein expression in human gliomas. **a** U251 (lane 1) and T98G (lane 2) cells were lysed and immunoblotted against VDR as explained in the methods section. The electrophoretic mobilities of marker proteins are indicated to the left. Protein loading was normalized with actin. **b** Immunofluorescence showing nuclear and cytoplasmic localization of VDR in T98G cell line (×400). **c** Immunohistochemical analyses for VDR and GFAP in individual samples of human gliomas and non-malignant brain tissues. Grade IV astrocytoma (GBM) negative control performed omitting primary antibody (upper-left picture; ×400), grade II astrocytoma showing GFAP in astrocytes *(upper-right picture, orange arrows*; ×100), grade IV astrocytoma (GBM) showing cytoplasmic VDR (*lower-left picture*, *black arrows*; ×400), non-malignant brain tissue showing lack of VDR expression (lower-right picture; ×400)

vehicle (isopropanol) for 48 h were incubated with anti-VDR (1:100, Santa Cruz, C20) and with anti-$p27^{Kip1}$ (1:100, BD Pharmigen) following with incubation with the secondary anti-rabbit Alexa 568 fluoro-conjugated antibody (Molecular Probes, Invitrogen). Nuclei were stained with DAPI and then mounted on slides. Images were captured with a Nikon Eclipse TE 2000S fluorescence microscope equipped with a Digital Sony Coolpix 14 camera.

Results

Validation of antibody and VDR expression in human gliomas

To validate the use of the antibody for detection of VDR in human GBM tissues we first analyzed protein expression in two GBM cell lines by WB. As shown in Fig. 1a, U251 and T98G cell lines displayed high VDR expression. The

antibody recognized only two bands, at 54 and 48 kDa, corresponding to the size variants reported for human VDR [39]. Online Resource 2A shows various controls for VDR expression including the silencing of the VDR receptor with a specific shRNA, breast adenocarcinoma T47D cell line [40, 41] and human white blood cells [41]. Additionally, immunofluorescence for VDR in the T98G cell line was performed, and both nuclear and cytoplasmic localization of the receptor was observed (Fig. 1b).

Subsequently, we analyzed tissue staining patterns by immunohistochemical evaluation of individual slides of human glioma tissues (representative pictures are shown in Fig. 1c). This analysis showed that the VDR antibody displayed strong staining in glial cells (Fig. 1c, lower left) and no staining was observed when the primary antibody was omitted (Fig. 1c, upper left picture). Non-malignant brain tissue adjacent to tumor tissue showed lack or very low levels of VDR expression (Fig. 1c, lower right). Online Resource 2B shows various controls for VDR immunostaining. The liver and lung alveolar cells are negative control tissues for VDR staining whereas spleen and colon are positive controls [41]. Serial sections were stained with an antibody to glial-fibrillary acidic protein (GFAP) in order to evaluate if glial cells (astrocytes) were the type of cell expressing VDR. Indeed, GFAP staining was observed in VDR-expressing cells, displaying the typical morphology of astrocytes (Fig. 1c upper right). Taken together, these analyses showed that VDR could be specifically detected by IHC in paraffin-embedded archived glioma tissues and by IF and WB in human glioma cell lines. Therefore this antibody was used for further studies of VDR expression.

We therefore assessed VDR expression in 38 independent tumor slides (whole sections that are not TMA) of gliomas. The description of the samples and prevalence of VDR expression is shown in Online Resource 3. Adjacent non-malignant brain tissues that were present in 4 cases showed lack or very low levels of VDR expression.

Screening of VDR expression in tissue microarrays

To screen widely for VDR expression in human gliomas, we subsequently performed IHC in tissue microarrays (TMAs). The VDR expression was variable in the tumors examined (representative selected array cores are shown in Fig. 2). The prevalence of positive expression of VDR between non-malignant brains and astrocytomas showed significant differences ($p = 0.04$). On the other hand, the prevalence of positive expression of VDR between non-malignant brains and WHO-II and WHO-III showed no significant differences (Online Resource 4). Interestingly, the prevalence of VDR positivity was lower in non-malignant brains (37.50 %, 3/8) than in WHO IV (GBMs) (75.41 %, 46/61) ($p = 0.026$). The VDR immunoreactivity scores (IRS) for non-malignant brains and for GBMs also showed significant differences, the tumor tissues displaying higher VDR levels (median IRS: 3) than non-malignant brains (median IRS: 0) (Fig. 2f; $p = 0.009$). Regarding the sub-cellular localization of VDR, significant differences were observed ($p = 0.003$) as all non-malignant brains (3/3 positive samples) showed nuclear localization whereas only 30.43 % (14/46 positive samples) of GBMs did.

The differences in VDR expression according to gender ($p = 0.529$) or age ($p = 0.662$) were not significant.

VDR positivity correlates with patient survival

It has been reported that VDR expression correlates with the prognosis of some types of cancer [19–26]. Therefore, it was important to investigate the prognostic significance of VDR in GBMs to assess if VDR expression was also playing a role in glioma progression. For this purpose, we used a cohort of 61 GBM tumors and performed a Kaplan–Meier survival analysis according to the expression of VDR. Interestingly, positive VDR expression was associated with longer overall survival time (log rank test; $p = 0.046$) as shown in Fig. 3a. The median overall survival time was 389 days for the VDR positive group and 230 days for the VDR negative group. Additionally, and since nuclear localization of VDR is necessary for its transcriptional effects [42], we analyzed if this sub-cellular localization was associated with a better long-term survival, and therefore we divided the patients into those presenting nuclear-, cytoplasmic- or both nuclear and cytoplasmic- VDR in their tumors. No statistically significant differences in survival times were observed when the sub-cellular localization of VDR was analyzed (Fig. 3b; $p = 0.079$).

VDR is involved in cellular survival and migration

Since we had observed an association of VDR expression with longer patient overall survival in patients with GBM, it seemed plausible that VDR were playing a role in cellular survival, migration and/or invasion, all processes important in glioma progression. We therefore investigated the effects of genetic and pharmacologic modulation of VDR on these processes. For this purpose, we performed transient and stable transfections of T98G cells with a specific shRNA for VDR and investigated the effect of silencing the receptor on cellular survival and migration. A 70 % reduction in the levels of VDR was achieved in the stable cell line (Fig. 4a). The decrease in VDR levels significantly increased the survival of T98G cells (Fig. 4b; $p < 0.001$). Additionally, we analyzed if calcitriol also had effects on cellular survival and VDR induction in this cell

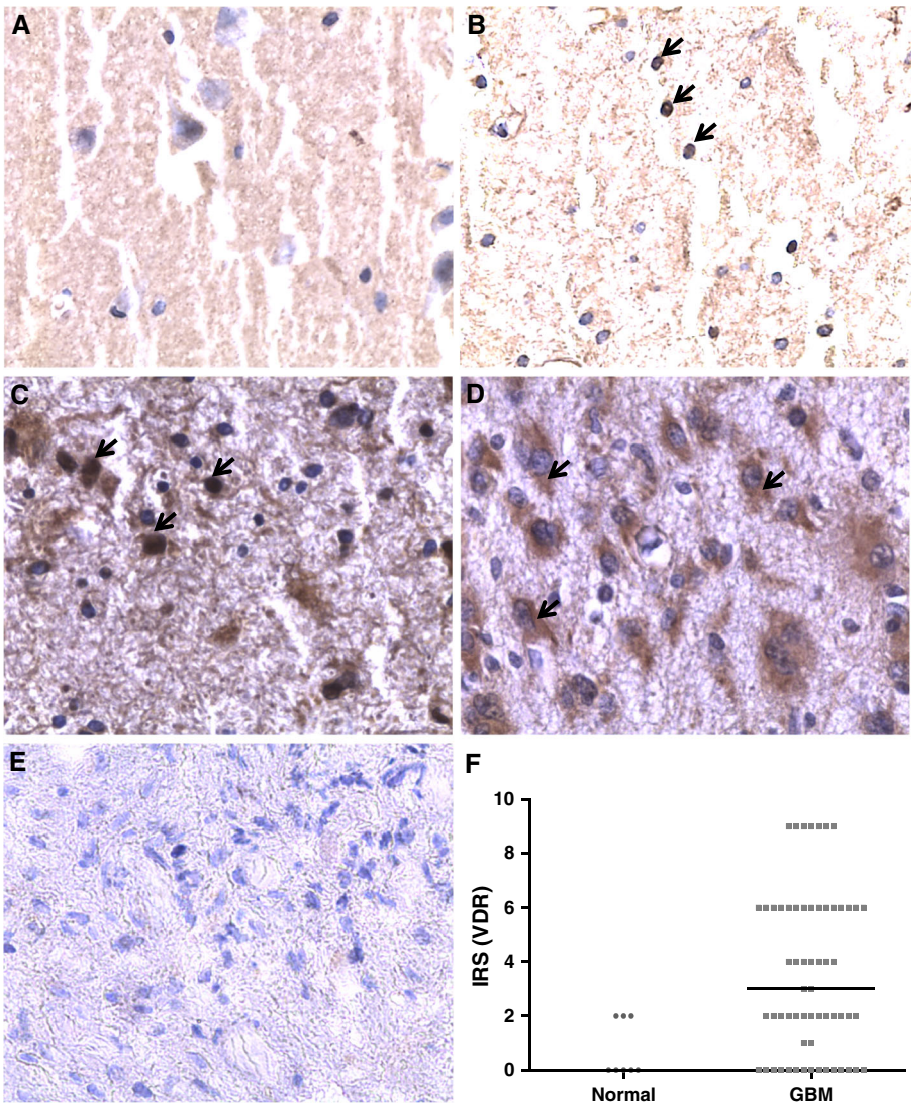

Fig. 2 VDR expression assayed in TMAs. **a** Section of a non-malignant brain showing lack of VDR expression. **b** Section of a non-malignant brain showing nuclear VDR (*arrows*). **c** Section of a GBM showing nuclear VDR (*arrows*). **d** Section of a GBM showing cytoplasmic VDR (*arrows*). **e** GBM, negative control performed omitting primary antibody. **f** *Dot-plot* depicting VDR expression according to the IRS. Significant differences between non-malignant brains ($n = 8$; median $= 0$) and GBMs ($n = 61$; median $= 3$) were observed ($p = 0.009$)

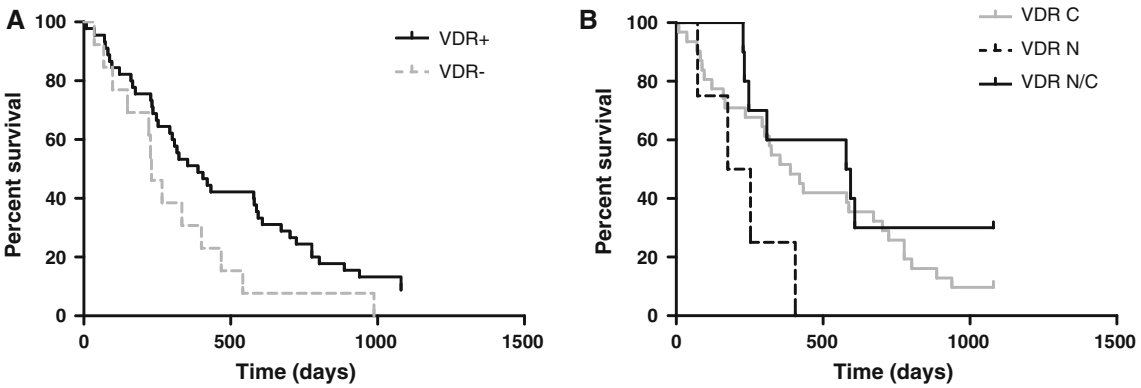

Fig. 3 Association of VDR expression with patient's overall survival time in GBMs **a** Association of VDR expression with patients' survival time in GBM. Tumors were divided into VDR-positive and VDR-negative as determined by immunohistochemical evaluation and Kaplan–Meier survival plots were performed (Log-rank test; $p = 0.046$). **b** Association of VDR sub-cellular localization with patients' survival time in GBM. Tumors that were positive for VDR were divided into those displaying cytoplasmic VDR **c**, nuclear VDR (N) and nuclear and cytoplasmic VDR (N/C) and Kaplan–Meier survival plots were performed (Log-rank test; $p = 0.079$)

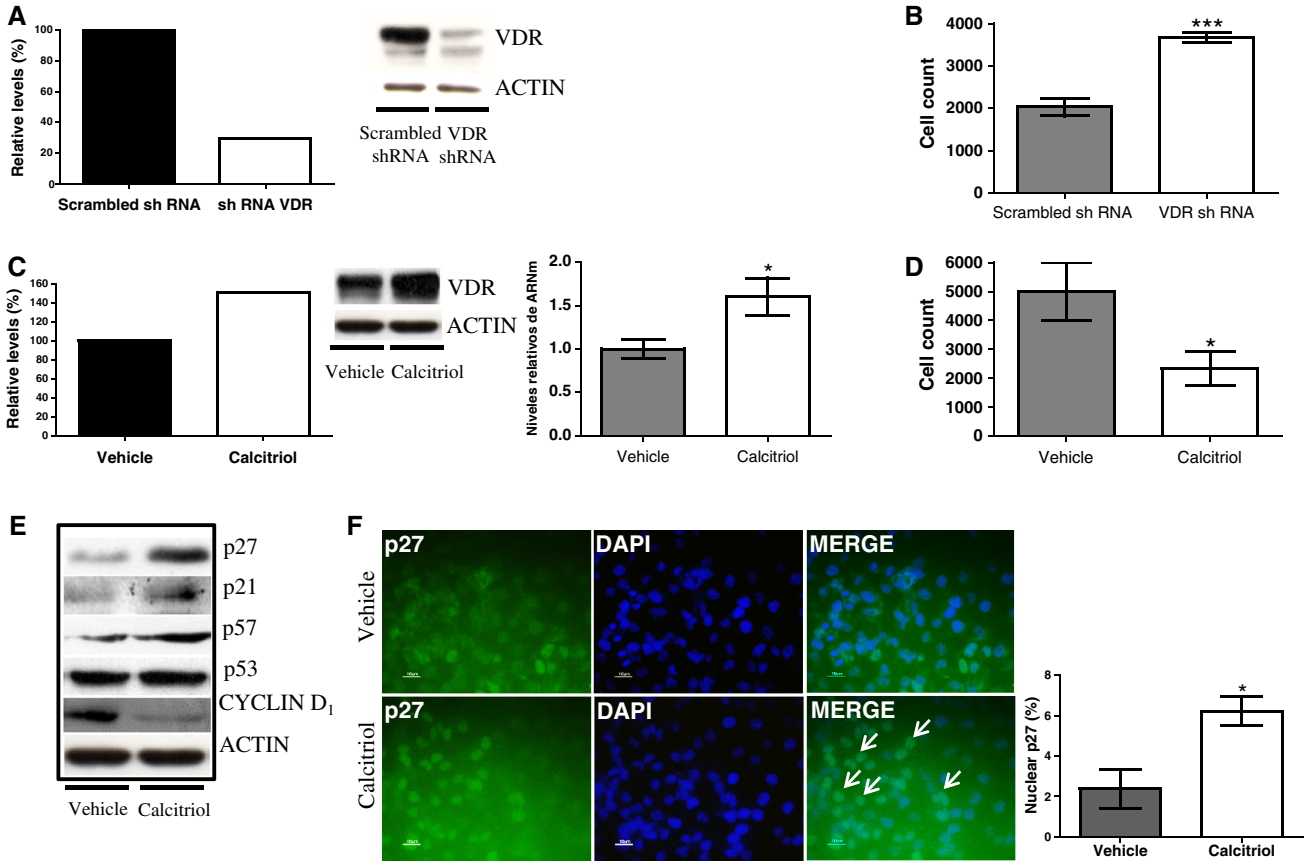

Fig. 4 Effect of genetic and pharmacologic modulation of VDR on T98G cellular survival. **a** Expression of VDR in T98G cells stably transfected with VDR shRNA. **b** Cell count in T98G cells stably transfected with VDR shRNA or scrambled shRNA ($p \leq 0.001$). **c** Expression of VDR protein and relative VDR mRNA levels in T98G cells after treatment with calcitriol (1 μM) for 48 h ($p \leq 0.05$). **d** Cell count in T98G cells after treatment with calcitriol 1 μM for 96 h ($p \leq 0.05$). **e** WB for p27, p21, p57, p53 and cyclin D_1 after treatment with calcitriol 1 μM for 48 h. Protein loading was normalized with actin. **f** Immunofluorescence showing nuclear p27 (*arrows*) in T98G cells after treatment with calcitriol 1 μM for 48 h ($p \leq 0.05$) (400X). Each experiment was repeated three times

line. Indeed, calcitriol decreased cellular survival (Fig. 4d; $p < 0.001$) and this effect was accompanied by augmented VDR mRNA (Fig. 4c; $p = 0.035$) and protein levels (Fig. 4c) in T98G cells. In order to evaluate if this reduction in cellular survival could be due to modulation of the cell cycle, we studied the expression of proteins known to regulate cell cycle progression and which had been previously reported to be modulated by calcitriol treatment in other types of cancer [43]. As shown in Fig. 4e, an increase in p57, p27 and p21 and a decrease in cyclin D_1 levels were observed, thus suggesting an effect of calcitriol on T98G cell growth arrest. Moreover, nuclear p27 was augmented following calcitriol treatment, as studied by IF (Fig. 4f; $p = 0.012$), thereby demonstrating that the induction of p27 was actively involved in decreasing cellular survival.

Infiltration throughout the brain is a prominent feature of low and high grade malignant gliomas [44] usually involving the activation of several processes, including the migratory properties of the cells. Therefore we analyzed the involvement of VDR on human glioma cellular migration by stably silencing this gene in T98G cells and analyzing its effect on cellular migration by using the wound closure assay. We observed that the cells that expressed lower levels of VDR owing to the silencing with the shRNA displayed a higher migratory rate than the control cells (Fig. 5a; $p = 0.017$). In contrast, calcitriol treatment (accompanied by augmented expression of VDR) reduced the migration rate of T98G cells (Fig. 5b; $p = 0.029$). Importantly, when VDR was silenced in T98G cells, calcitriol was not able to reduce the migration rate (Fig. 5c), thus suggesting that VDR is necessary for the modulation of cellular migration by calcitriol.

Discussion

It is well-known that vitamin D mediates anti-proliferative and pro-differentiation effects in various tissues, including gliomas [29, 45–47]. Importantly, vitamin D3 naturally circulates throughout the body and crosses the blood–brain-

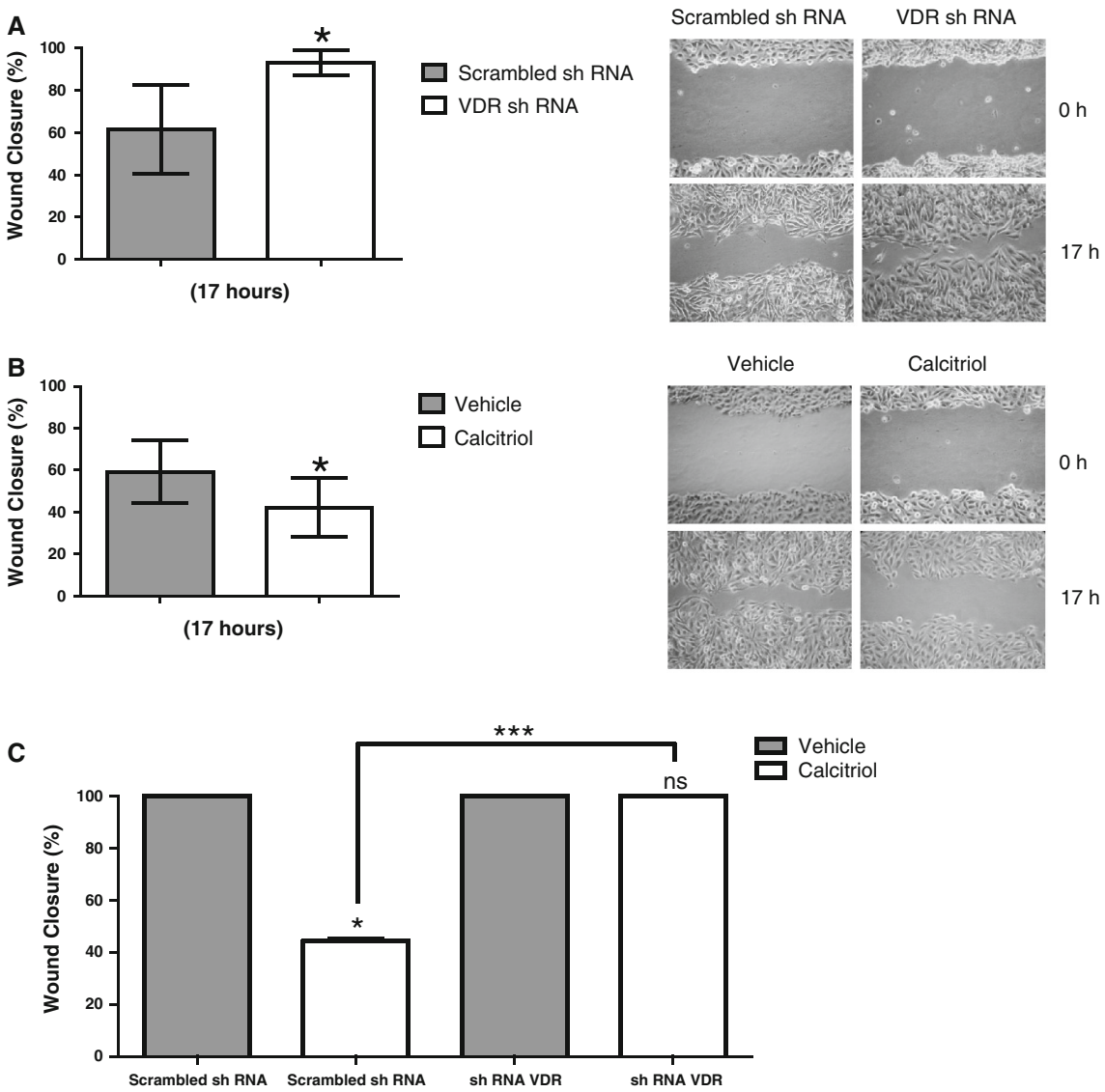

Fig. 5 Effect of genetic and pharmacologic modulation of VDR on T98G cellular migration. Confluent monolayers of cells were wounded and wound closure was monitored every hour for 17 h. The uncovered wound area was measured and quantified at different intervals with ImageJ 1.37v. **a** T98G cells stably transfected with VDR shRNA or scrambled shRNA ($p = 0.017$). **b** Calcitriol- and vehicle- treated T98G cells ($p = 0.029$). **c** T98G cells stably transfected with VDR shRNA or scrambled shRNA, and treated with calcitriol or vehicle. Shown is a representative experiment of three independent assays

barrier, making it an attractive therapeutic agent for GBM. In this regard, a clinical study that evaluated vitamin D analog calcidiol as a therapeutic approach in glioma treatment has yielded interesting results as the analog has been shown to induce long-lasting complete regression in 20 % of patients [31]. The authors suggest that the observed percentage of responders might be related to genetic specificities including VDR expression. However they did not analyze the expression of the receptor in tumor tissues. In fact, to our knowledge this work is the first to analyze VDR protein expression in glioma tissues. Furthermore, there is no information regarding the role of VDR in the prognosis of gliomas. Instead there are two previous reports analyzing VDR mRNA levels. Magrassi and col. [27] showed an increase in VDR mRNA in GBM when compared with lower grade astrocytomas, and Diesel and col. demonstrated a decrease in VDR mRNA in GBM when compared with normal brain [28]. Our data are in line with the former report as it shows there is a higher VDR protein expression in GBM tumors than in non-malignant brain tissues, as observed when VDR staining in both TMA and individual whole sections is performed. Tissue microarrays allow the screening of molecules in a large collection of normal and tumor tissues simultaneously, which allows homogenized staining conditions. However, the qualitative information concerning patterns of

expression that can be obtained with TMAs is limited and therefore complementing TMA with whole sections of tumor samples is necessary. Our results of VDR expression patterns in 38 whole sections that are not TMA confirmed those obtained with TMA and showed that VDR is homogeneously distributed across the whole section when present, except for areas of necrosis. This suggests that VDR staining in the TMA core reflects what occurs in the whole section.

Regarding VDR expression in non-malignant brain tissues, our results are in agreement with previously observed nuclear VDR staining in both neurons and glial cells [48]. Our data showing low VDR expression in non-malignant brain and up-regulation in tumors is also in agreement with previous reports of VDR expression in cervical [49], mammary [50] and ovarian [51] carcinomas and Hodgkin's lymphoma [52]. However, our results differ from those showing lower levels in tumors [18].

More corroborative are the studies evaluating the prognostic significance of VDR expression in tumors, as almost all of them report an association of VDR expression with better outcome. In agreement with this, we demonstrated an association of VDR expression with longer overall survival times of patients with GBM. This has also been demonstrated for cholangiocarcinomas [19], colorectal cancer [20], ovarian cancer [23], and lung adenocarcinoma [22], in which associations of VDR positive expression with longer survival times were observed. In line with this association of VDR with good prognosis, low VDR expression was found to be associated with recurrence of the tumor in oral squamous cell carcinomas [53] and negative VDR was demonstrated to be correlated with shorter disease-free interval in breast cancer [21]. On the contrary, no significant correlations between VDR status and histopathological data were observed in breast cancer [50] and cervical cancer [49]. Altogether these reports show that VDR expression is associated with improved outcome of patients in most tumor types and highlight the importance of evaluating VDR status in tumors. Of note, the glioma samples were obtained between 1993 and 2003 and patients were not treated with temozolomide. All cases were obtained before the patients had received any therapy, thus the change in standard of treatment occurred after the introduction of Temozolomide would not have affected the values here reported. Although unlikely, it is possible that the correlation between VDR expression and survival observed in our study might not apply to patients currently being treated with this drug.

Previous studies showed that nuclear localization of VDR was associated with improved survival in non-small cell lung cancer [24]. Contrary to this report, we observed no statistically significant differences in survival times when the sub-cellular localization of VDR was analyzed, although a trend to early death was observed in the group of patients that presented VDR nuclear localization that could have gained significance if a higher number of samples were used. It will indeed be interesting to study, in a higher number of samples, whether this association is significant, which may indicate that VDR is exerting its antitumoral effects in glioma through non-genomic pathways. These non-genomic effects, although less defined than the genomic ones, have been demonstrated in several cancer cells [43, 54]. We also demonstrated that there is no significant correlation of VDR expression with gender or age, as previously reported for other tumors [19, 25] although a difference in VDR expression was observed among the various histological subtypes and among the various tumor grades.

Since we observed an association of VDR expression with longer overall survival time in patients with GBM, it seemed plausible that VDR were playing a role in cellular survival, migration and/or invasion, all important processes in glioma progression. Therefore we analyzed if VDR could affect any of these processes by pharmacological and genetic modulation of the receptor. Our data show that silencing VDR in T98G cells significantly increased cell survival. In contrast, the treatment of T98G cells with calcitriol which increased VDR mRNA and protein levels reduced cellular survival. In concordance with a role in cell cycle arrest, we found that calcitriol up-regulates p21, p27, p57 and down-regulates cyclin D1. These results are in accordance with previous reports showing the modulation by calcitriol of the expression of proteins known to regulate cell cycle progression [43]. Moreover, we observed that the cyclin-dependent kinase (CDK) inhibitor p27 was localized to the nucleus after calcitriol treatment, which suggests that hormone treatment promotes activation of p27, since cell cycle inhibition is a nuclear p27 function. In many cancers, not only is p27 reduced in the nucleus, but tumors also exhibit different degrees of cytoplasmic p27 mislocalization [55]. The calcitriol-induced nuclear localization of p27 has already been demonstrated in a mouse model of metastatic follicular thyroid cancer [56]. The antiproliferative effects of calcitriol had been previously demonstrated for some rat [29, 45] and human glioma cell lines [46, 47], though no effect of calcitriol on the survival of some other glioma cell lines has been shown [47]. This disparity in responses to the hormone could be due to the differential expression of the VDR among the cell lines. Indeed, restoration of the sensitivity to calcitriol occurs in C6 glioma cell line when the VDR is overexpressed [30]. However, the involvement of other factors (e.g. co-factors, calcitriol metabolizing enzymes) which participate in vitamin D signaling and which may be differentially expressed in the cell lines cannot be excluded. Similarly, potential single nucleotide polymorphisms (SNPs) which may limit the

function of the VDR or of other gene of the vitamin D pathway and which may be associated with a specific type of glioma must be taken into account, since it has been demonstrated that there is an association between various SNPs in VDR and other genes of the vitamin D pathway and risk of various cancer types [43, 57].

Infiltration throughout the brain is a prominent feature of low and high grade malignant gliomas [44] and is the principal basis for the lack of complete tumor resection. This infiltration involves activation of several processes, including migration. In this study we demonstrate that T98G cells that express lower level of VDR due to the silencing with a specific shRNA, displayed higher migratory rates than the control cells. Additionally, calcitriol treatment reduced the migration of T98G cells. Importantly, VDR was actively involved in calcitriol reduction of cellular migration as silencing the receptor prevented this effect. This inhibition of migration by calcitriol and VDR could be attributed to p27 localization to the nucleus, since it has been demonstrated that cytoplasmic localization of p27 promotes glioma cell invasion [58]. However, further experiments are needed to assess if calcitriol and/or VDR effects on migration are due to re-localization of p27.

In conclusion, we demonstrate that VDR expression is up-regulated in glioma tissues and that it is associated with a better long-term survival of patients. We also show that VDR is important in cellular survival as well as in cellular migration, and that VDR expression is necessary for calcitriol-mediated inhibition of the migratory rate of T98G cells. Altogether these results provide some limited evidence supporting a role for VDR in glioma progression.

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Conflict of interest The authors declare that there are no conflicts of interest.

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