Low Expression of FOXP2 is Associated with Better Prognosis in Glioblastoma.

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Abstract

FOXP2 expression has been associated with the prognosis of some tumors, but the role of FOXP2 in glioblastoma has not been studied in-depth until now. The aim of the present work is to study the role of FOXP2 as a prognostic biomarker in glioblastoma.

This is a retrospective observational case series study in which the expression of FOXP2 has been analyzed both at the protein level (immunohistochemistry) and at the mRNA level (RNAseq, in a cohort of glioblastoma patients from The Cancer Genome Atlas [TCGA] database). Other molecular and clinical data have also been included in the study, with special focus on miRNA expression data.

Survival analysis using Log-Rank test and COX-regression have been used. Non-parametric statistical tests were also used to study differences between low and high FOXP2 expression groups.

Patients with a high expression of FOXP2 protein showed a worse prognosis than those patients with low expression in both, progression free survival (PFS) (HR=1.711; p=0.034) and overall survival (OS) (HR=1.809; p=0.014). These associations were still statistically significant in multivariate analysis.

No prognostic association was found with FOXP2 RNA expression. Interestingly, two miRNAs that target FOXP2 (hsa-miR-181a-2-3p and hsa-miR-20a-3p) showed an interaction effect on OS with FOXP2 expression. A low level of these miRNAs expression was associated with a significantly worse prognosis in patients with high FOXP2 RNA expression.

Higher expression of FOXP2 at the protein level is associated with a worse prognosis. This protein expression may be regulated by the expression of specific miRNAs that target FOXP2 mRNA: hsa-miR-181a-2-3p and hsa-miR-20a-3p.

Introduction

Glioblastoma is the most common primary tumor in the adult brain. It is a tumor with an ominous prognosis, with a mean overall survival of approximately one year after diagnosis [1, 2]. Despite the progress in the treatment of most neoplasms, advances in the management of glioblastoma are still scarce. Because of this, new strategies and approaches should be performed to gain a better understanding of glioblastoma biology. Bearing this in mind, the study of the molecular means used by the normal brain to achieve plasticity (e.g. FOXP2) could help to identify new molecular pathways implicated in glioblastoma development and recurrence. This knowledge would help to improve the management of this tumor.

FOXP2 is a transcription factor that belongs to the FOX family (forkhead/winged-helix). It is endowed with activating and, most often, repressing transcriptional activities [3]. It is highly expressed in the adult brain and during fetal central nervous system development [4–6]. Furthermore, this gene seems to be
essential for the development of language [7, 8]. In fact, mutations in this gene have been associated with congenital aphasia syndromes [9].

The expression of FOXP2 in some tumors has previously been reported. On the one hand, high expression of FOXP2 has been associated with a bad prognosis in prostate cancer [10], neuroblastoma [11] and multiple myeloma [12]. On the other hand, other studies have reported an association between FOXP2 expression and a better prognosis in breast cancer [13], liver cell carcinoma [14], osteosarcoma [15] and gastric cancer [16]. Thus, whether FOXP2 is a tumor suppressor or tumor-promoting gene is matter of controversy. In any case, the role of FOXP2 in the development and progression of glioblastoma has not been completely studied to date. A recent work reports that low expression of FOXP2 was associated with better survival outcomes and down regulation of this transcription factor resulted in inhibition of glioma cell proliferation [17]. Other studies also report a tumor-promoting effect of this transcription factor in glioma cell cultures [18, 19]. However, more work is needed to establish the potential role of FOXP2 as a prognostic biomarker in glioblastoma.

The aim of the present study is to assess the role of FOXP2 expression as a prognostic biomarker in primary glioblastoma. Two cohorts of glioblastoma patients were analyzed. Firstly, the expression of FOXP2 was determined at the protein level using immunochemistry in a group of patients treated in our Neurosurgery Department. Secondly, the expression of FOXP2 at mRNA level was analyzed in a cohort of patients from the The Cancer Genome Atlas (TCGA) project.

**Methods**

**Patients**

Formalin-Fixed-Paraffin-Embedded (PFFE) tumor specimens were obtained from 62 patients (26 female; mean age 62.93 [SD = 9.9]) treated in our department from January 2015 to December 2016. All of them had a diagnosis of primary glioblastoma (Isocitrate Deshdrogenase [IDH] 1 and 2 wild-type). Sample features are summarized in supplementary table 1.

One hundred and forty-four patients (50 female; mean age 61.01 years [SD = 12.51]) were included from the TCGA database. All patients also presented a primary glioblastoma. Only patients whose tumor samples have RNAseq V2 data available were included. TCGA sample features are also shown in supplementary table 1.

The study was approved by the Hospital Universitario de Canarias Ethics Committee according to the Declaration of Helsinki.

**TCGA data extraction**

Data from TCGA was downloaded from Firebrowse (http://firebrowse.org/) (TCGA data version 2016_01–28). As mentioned above, only patients with RNAseq V2 data available were included in the study. Apart from RNA data, clinical, mutational, copy number variations, miRNA expression and
methylation data (for determination the O6-methylguanine-DNA-methyltransferase [MGMT]) from the selected patients were also downloaded and included in a new database. It should be noted that miRNA expression and methylation data were not available for all patients. Details of this data generation are described elsewhere [20, 21].

**IDH1 and IDH2 mutations identification**

In order to specifically select those patients with primary glioblastoma, the presence of isocitrate dehydrogenase 1 (IDH1) and/or isocitrate dehydrogenase 2 (IDH2) mutations were studied. In the case of the TCGA group, the mutational annotation for both genes were analyzed and those patients with any kind of mutation in these genes (7 patients with IDH1 mutation and 1 patient with IDH2 mutation) were excluded. In the case of the local cohort of patients, the most common mutation in IDH1 (R132H) and IDH2 (R172K) were tested by PCR amplification and pyrosequencing.

**MGMT methylation analysis in TCGA patients**

Methylation probes were available for 117 patients in the cohort of TCGA patients included in the present study. In 67 of them the methylation status was tested using the HumanMethylation27 (HM27) platform and the rest of the patients were tested with the HumanMethylation (M450) platform. Some patients presented data from both platforms. Thus, the absence of significant differences between those samples on the two Infinium platforms was confirmed by calculating the p-value using a student-t test [21]. Data was then merged by averaging the beta-values of the CpG probes of interest. The methylation status of MGMT was determined as explained in other reports [22]. In brief, the beta values were transformed in m-values using the following formula:

\[ M = \log_2(\text{Beta)/(1-Beta)}) \]

The logit(y) was then calculated using the model proposed by Bady et al (2012), where only the M-value of two MGMT CpG islands is considered (cg12434587 and cg12981137):

\[ \logit(y) = 4.3215 + 0.5271*cg12434587 + 0.9265*cg12981137 \]

As proposed by Bady et al. (2012) a probability cutoff of 0.358 was used which empirically maximized the sum of sensitivity and specificity [22].

**MGMT methylation analysis in local patients**

The methylation status of the four most informative CpG islands in the MGMT promoter was studied in DNA purified from glioblastoma areas dissected from PPFE tissue sections using a commercial kit for pyrosequencing (Qiagen).

**Immunohistochemistry (IHC) analysis**

The expression of FOXP2 was tested in the PPFE specimens (originating from surgical resections) of the local cohort of patients by using a specific antibody (Sigma-Aldrich, ref. HPA000382). Positivity was
evaluated by two independent observers using the ImageJ® software in hotspot areas. A semiautomatic protocol, validated for ki-67, was used. Spearman’s correlation coefficient was calculated between the positivity percentages of the two observers. FOXP2% positivity expression was included in regression analysis. Furthermore, this value was dichotomized (using different cutoffs: p25, p50 and p75) and this variable was used for the Kaplan-Meier curves and the Log-Rank test analysis.

**RNAseq data from TCGA patients**

FOXP2 RNA expression data were extracted as explained above and they did not have a normal distribution, and thus were transformed by using base 2 logarithm (Log2 (x + 1)), where x is the expression of FOXP2. This new variable (Log2FOXP2) was included in the univariate Cox regression analysis. Furthermore, FOXP2 RNA expression was also dichotomized (using different cutoffs: p25, p50 and p75) and this variable was used for the Kaplan-Meier curves and the Log-Rank test analysis.

Apart from FOXP2 RNA expression data, other gene expression profiles were also extracted to perform a molecular classification. As is widely described in previous reports, there are differences in gene expression in glioblastoma that allow their classification in proneural, neural, classical and mesenchymal transcriptomic subtypes [20, 23]. Using the list of input genes that are highly expressed in each subtype (http://tcga-data.nci.nih.gov/docs/publications/gbm_exp/), an unsupervised hierarchical cluster analysis was performed (MORPHEUS, Broad Institute, http://software.broadinstitute.org/morpheus/) and each patient was assigned to a molecular subgroup by cutting the resulted dendogram (supplementary Fig. 1).

**MicroRNA expression data from TCGA patients**

Bearing in mind that protein translation of FOXP2 mRNA is intensively regulated by miRNA [24], a number of these were selected to analyze their prognostic value. Firstly, miRNA public databases (mirDB [http://www.mirdb.org/] and mirANDA [http://www.microrna.org/microrna/home.do]) were used to identify the miRNA that target FOXP2. Secondly, from this list of miRNA (supplementary table 2), those with a proven role in glioblastoma pathogenesis acting as oncogenic or tumor suppressor miRNA were selected [25]. Finally, the expression of the miRNA from this list that was available in the TCGA data was analyzed (supplementary table 3).

As most of the miRNA expression did not show a normal distribution, a dichotomization of each one was performed by using the median as cutoff. These variables were included in a stratified survival analysis with FOXP2 expression groups (supplementary table 4).

**DNA Copy-Number Variation and DNA mutations**

Copy number variation and mutation annotation files were downloaded as indicated above. After excluding those patients whose RNAseq data was not available, the ten-most-common cancer related genes showing copy number alteration (CNA) and/or mutation were studied. These genes (supplementary tables 5 and 6) were identified from the whole TCGA glioblastoma cohort whose analysis is available at cBioPortal (https://www.cbioportal.org/). A comparative analysis of the distribution of these genetic events between the two groups of FOXP2 expression was performed.
Statistical analysis

In order to study the differences between low and high expression of FOXP2, the median was used to generate two groups of expression. This approach was performed for both cohorts of patients. Nonparametric statistical tests were used (Mann-Whitney U for continuous variables and Chi-Square/Fisher exact test for discrete variables). Statistical significance was considered when the p-value < 0.05. However, bearing in mind the high number of comparisons during molecular analysis, a corrected p value was used for these variables using the False Discovery Rate (FDR) method. Differences were considered statistically significant when FDR < 0.1.

Univariate and multivariate Cox regression analysis were performed in both cohorts of patients. Clinical, radiological and molecular variables were included in the univariate analysis and those with a p-value < 0.1 were included in a multivariate model. Kaplan-Meier curves and the Log-Rank test were also used to study the differences in overall survival (TCGA and local group) and progression free survival (only the local group) between different FOXP2 expression groups. The statistical significance for survival analysis was considered when the p-value < 0.05.

Finally, miRNA expression data was also considered for post-hoc analysis. A stratified survival analysis bearing in mind the expression level of the selected FOXP2-targeting miRNAs and FOXP2 expression group was performed.

Results

Expression of FOXP2 in the local cohort of glioblastoma patients.

The IHC analysis, using a specific FOXP2 antibody, showed that the protein was mainly located in the nucleus and the cytoplasm. The mean percentage of positivity in hot-spot areas was 28.33% (SD=32.29), with an adequate correlation between the results of the two independent observers (Spearman’s rho=0.897; p<0.001).

Prognostic evaluation of FOXP2 expression in the local cohort of glioblastoma patients.

The univariate Cox regression analysis showed that higher FOXP2 expression was associated with a worse prognosis in both progression free survival (PFS) (HR=1.711, 95% C.I. [1.040 – 2.814]; p=0.034) and overall survival (OS) (HR=1.809, 95% C.I. [1.129 – 2.900]; p=.014). The univariate Cox regression analysis also included other variables that had previously been associated with the prognosis of glioblastoma (tables 1 and 2). The association of FOXP2 IHC positivity with worse PFS and OS was still significant when this variable was included in a multivariate Cox regression analysis with those variables that showed statistical significance in the univariate Cox regression analysis (p<0.1) (tables 1 and 2).

Comparison between high and low FOXP2 expression groups in the local cohort of glioblastoma patients.
Using the median (p50=14.0) of FOXP2 % positivity expression, a comparative analysis between patients with low FOXP2 expression (<=p50) and high FOXP2 expression (>p50) was performed. This cutoff was selected because it was considered the best way to show prognostic differences (figure 1). No clinical, radiological or molecular differences were identified between both groups (table 3). However, according to the Cox regression results, patients with high FOXP2 expression presented a worse PFS (176.1 vs. 339.9 days; p=0.024) and a worse OS (381.9 vs. 624.9 days; p=0.042) (table 3, figure 1).

Expression of FOXP2 in the TCGA cohort of glioblastoma patients.

The mean expression of FOXP2 RNA was 25.75 RPKM (SD=52.02) (from RNA seq V2 data). The mean expression of FOXP2 in normal brain had been established in previous assays at around 3.715 RPKM +/-0.605 (data extracted from https://www.ncbi.nlm.nih.gov/gene/). Bearing this figure in mind, one hundred and two glioblastoma patients (70.83%) presented an over-expression of FOXP2 RNA.

Prognostic evaluation of FOXP2 expression in the TCGA cohort of glioblastoma patients.

The univariate Cox regression analysis did not show any effect of FOXP2 mRNA expression on OS (HR=1.142, 95% C.I. [0.787 – 1.659], p=0.484) (table 4). Using the median of FOXP2 expression as cutoff, the Log-Rank test and the Kaplan-Meier curves were performed to test differences in OS. Similarly to the Cox regression results, no significant differences were identified in the median survival between low and high FOXP2 expression (419.0 vs. 343.0 days; Log-Rank; p=0.426) (figure 2). PFS was not evaluated here, because data from TCGA was not accurate enough. Other clinical and molecular data were evaluated, but only the age showed an association with OS (table 4).

Comparison between high and low FOXP2 expression groups in the TCGA cohort of glioblastoma patients.

A comparison between patients with high and low FOXP2 RNA expression was performed using the median expression of FOXP2 as cutoff (p50=7.1668). Differences were identified in the molecular classification distribution, where the “high RNA FOXP2” expression group presented a higher percentage of patients classified in the neural and proneural groups (table 5). These groups presented higher FOXP2 RNA levels than the classical and mesenchymal subtypes, but this difference was only statistically significant for the comparisons neural vs. mesenchymal and proneural vs. mesenchymal (ANOVA; p=0.024 and p=0.030, respectively) (supplementary figure 2).

Regarding the broad molecular information available in the TCGA patients, additional comparisons between low and high FOXP2 expression groups were performed. These comparisons were focused on DNA copy-number variation (CNV) and DNA mutations. Firstly, the frequency of chromosomal gains or losses was analyzed in both FOXP2 RNA expression groups. It should be noted that twelve patients (19.7%) from the high FOXP2 RNA expression group showed losses in 15q. This genomic loss was significantly different to those in the low FOXP2 RNA expression group (uncorrected p value=0.038), but
did not reach significance with corrected FDR values (FDR>0.1) (supplementary table 7). No other statistical differences in the frequency of chromosomal alterations between both groups were identified.

Secondly, DNA CNV analysis was performed. Analysis of focal amplifications and deletions in the top-10 glioblastoma cancer-related genes with focal CNVs showed a larger number of amplifications of CDK4 (24.7% vs. 9.9%; p=0.027; FDR>0.1) in the high FOXP2 RNA expression group (supplementary table 8). CDK4 is located in chromosome 12 (cytoband: 12q14.1) and it encodes a protein member of the Ser/Thr protein kinase family. This kinase is responsible for the phosphorylation of retinoblastoma gene product (Rb). On the other hand, more patients in the low FOXP2 RNA expression group showed a notable deletion of CDKN2B (59.2% vs. 42.5%; p=0.048; FDR>0.1) (supplementary table 8). The protein encoded by this gene is also involved in cell growth and in the control of the cell cycle.

Finally, the mutational burden of the selected TCGA patients was analyzed. The most common driver mutations among the selected genes in the TCGA cohort of patients affected PTEN, TP53 and EGFR (supplementary table 5). Comparisons between the incidence of mutations in the two FOXP2 RNA groups are reported in supplementary table 9. No differences in the incidence of PTEN and TP53 mutations in low and high FOXP2 RNA expression groups were identified, but more patients with a missense mutation in EGFR gene were identified in the low FOXP2 RNA expression group (38.0% vs. 16.4%; p=0.042). Only one patient presented a driver mutation in ATRX. PCLO gene showed a higher incidence of passenger mutations in the low FOXP2 RNA expression group (15.5% vs. 2.8%; p=0.016; FDR=0.16). This gene encodes a protein of the presynaptic cytoskeletal matrix and is involved in establishing active synaptic zones and in synaptic vesicle trafficking. Its role in glioma pathogenesis has not been fully studied.

Expression and prognostic evaluation of hsa-miR-181a-2-3p and hsa-miR-20a-3p: two FOXP2-targeted miRNAs.

Regarding the results described above and bearing in mind the intense regulation of FOXP2 expression by different miRNAs, post-hoc analysis using the expression of some miRNAs were performed. Two miRNAs that targeted FOXP2 were selected (see Methods and supplementary table 4): hsa-miR-181a-2-3p and hsa-miR-20a-3p.

The OS of patients with a low expression of hsa-miR-181a-2-3p was influenced by FOXP2 expression. Those with a high FOXP2 expression have a median OS of 239.0 days (95% C.I. [49.5 – 428.5]) and patients with low FOXP2 expression have a median OS of 419.0 days (95% C.I. [308.7–529.2]). This difference was statistically significant (p=0.028) (figure 3a). On the other hand, patients with a high expression level of has-miR-181a-2-3p did not show differences in OS between low and high expression groups of FOXP2 (333.0 vs. 395.0 days; Log-Rank test; p=0.604) (figure 3a).

The OS of patients with a low expression of hsa-miR-20a-3p was also influenced by FOXP2 expression. Those with a high FOXP2 expression have a median OS of 239.0 days (95% C.I. [62.2 – 415.8]) and patients with low FOXP2 expression have a median OS of 448.0 days (95% C.I. [368.7–527.3]). This difference was statistically significant (p=0.020) (figure 3b). On the other hand, patients with a high
expression level of has-miR-20a-3p did not show differences in OS between low and high expression groups of FOXP2 (333.0 vs. 454.0 days; Log-Rank test; p=0.511) (figure 3b).

Discussion

The aim of the present work was to study the role of FOXP2 as a prognostic biomarker in glioblastoma. Higher expression of FOXP2 at the protein level was significantly associated with worse outcomes (PFS and OS). However, this result was not replicated when considering the level of FOXP2 mRNA expression. No significant association between the expression of FOXP2 at mRNA level and prognosis was identified for glioblastoma. The explanation for these contradictory results may lie in the influence of certain miRNAs that target FOXP2. All of these findings are discussed below.

FOXP2 expression in glioblastoma

The expression of FOXP2 in glioma have been previously reported [17–19]. This expression is higher than the FOXP2 expression in normal brain and the level of expression seems to increase with the tumor grade [19]. In the present study, the majority of the patients showed some expression of FOXP2, either at the RNA or protein level. The expression of FOXP2 in glioma cells may promote the development and recurrence of the tumor. For example, He Q et al (2017) found an upregulation of FOXP2 in U87 glioma – exposed endothelial cells [18]. This upregulation was associated with an enhanced viability, migration and tube formation of these cells. Thus, FOXP2 seems to promote the angiogenesis in glioma [18]. Another in vitro study showed that FOXP2 overexpression in U87 and U251 glioma cells increases their migration, proliferation and invasion, with reduced apoptosis rates [19]. Finally, in a recent study, Zhang H et al (2019) reported that low expression of FOXP2 slowed down the cell cycle and inhibited the proliferation of U251 glioma cells [17]. Interestingly, these authors also confirmed these results in vivo in a nude mouse model [17]. Furthermore, it should be noted that FOXP2 has also been associated with the promotion of migration and invasion of tumor cells in breast cancer [26, 27], hepatocellular carcinoma [14], prostate cancer [10] and colorectal carcinoma [28], among others. Therefore, results from in vitro studies suggest that FOXP2 may promote the malignant biological behavior of glioma cells.

In the present study, higher expression of FOXP2 was associated with two specific transcriptomic glioblastoma subtypes: proneural and neural. Although some molecular differences were identified (e.g. a different incidence of mutations in specific genes or CNVs), they did not reach statistical significance when a corrected p-value (FDR) was considered. Furthermore, no other clinical or radiological data were distributed differently between the expression groups of FOXP2 of the two cohorts. Therefore, FOXP2 is expressed in glioblastoma and its expression is not clearly associated with specific molecular, clinical and radiological features (apart from the mRNA expression profile).

FOXP2 as a prognostic factor

Bearing in mind the results of in vitro studies, one could expect that those glioblastoma patients with a higher expression of FOXP2 would have a worse prognosis. Indeed, the present work showed an
association between a worse OS and PFS in glioblastoma patients with higher expression at protein level (Table 1 and 2, Fig. 1). Zhang et al (2019), using a different method (western-blot) and a different antibody (Abcam, ab16046), also reported a worse prognosis for patients with higher FOXP2 expression in terms of mortality and tumor recurrences [17].

However, no significant association between FOXP2 expression at mRNA level and prognosis was identified (Table 4, Fig. 2). It should be noted that in many biological systems, gene expression at mRNA and protein levels are not perfectly correlated. In fact, significant differences have been detected by others [29–31]. In many cases, this discrepancy is mediated by the effect of microRNAs (miRNAs). miRNAs are small, highly conserved, non-coding RNA molecules found in most eukaryotes, including in humans [32]. miRNAs have a central role in the regulation of gene expression and, in the context of neoplastic cells, they are involved in malignant transformation [33, 34]. A single miRNA may target several dozen or even hundreds of messenger RNAs (mRNA) and can cause an altered phenotypic state at the cellular level [35]. They normally affect gene expression (gene silencing) via translational inhibition and/or mRNA degradation [36]. Furthermore, data suggest that miRNAs play an essential role in hallmark biological features of glioblastoma [25], and they can act as potential oncogenes or tumor suppressors in gliomas [25, 37].

Post-transcriptional regulation of FOXP2 mRNA is mainly mediated by miRNAs [24]. More than 280 miRNA are predicted to target human FOXP2 [24]. In the present study, miRNA public databases (mirDB and mirANDA) were used to identify miRNA that target FOXP2 and, from this list, a selection of those miRNAs that have a proven role in the pathogenesis of glioblastoma was compiled (supplementary table 4). An interaction effect of hsa-miR-181a-2-3p and hsa-miR-20a-3p expression with FOXP2 RNA expression was identified. Higher FOXP2 RNA expression was associated with worse OS in each of the groups with low hsa-miR-181a-2-3p and low hsa-miR-20a-3p expression. On the contrary, no difference in OS between FOXP2 RNA expression groups was identified in the high hsa-miR-181a-2-3p and hsa-miR-20a-3p expression groups. In other words, these miRNAs may negatively regulate FOXP2 expression and only when they are in a low level of expression FOXP2 mRNA could they be translated to protein and FOXP2 expression would have an oncogenic effect. In this regard, one can consider that the lack of significant prognostic value of FOXP2 mRNA expression levels (as opposed to FOXP2 protein expression level) is associated with the effect of these miRNAs. Both, hsa-miR-181a-2-3p and hsa-miR-20a-3p would act as tumor suppressors. hsa-miR-181a has already been considered as tumor suppressor in the context of glioblastoma [25]. Low expression of hsa-miR-181a has been associated with chemotherapy resistance in glioblastoma [38] and is considered a down-regulated miRNA in glioblastoma [39]. On the other hand, hsa-miR-20a-3p has not been specifically studied in gliomas (unlike the 5p strand, which has been reported to show an oncogenic role [40, 41] but does not target FOXP2). hsa-miR-20a-3p has been reported as a downregulated miRNA in gastric cancer [42] it seems to negatively regulate the TGF-b1 pathway or vascular endothelial growth factor (VEGF) expression in different diseases [43, 44]. These pathways are important in glioblastoma development [45]. Therefore, the previously reported activity of these miRNAs would be compatible with the findings of the present work.
Limitations

Some limitations should be mentioned concerning the present study. Firstly, the lack of FOXP2 protein expression information in the TCGA database hindered the direct validation of the results of the local cohort of glioblastoma patients. Although, the combination of information from RNAseq and miRNA expression were used to solve this lack of protein data, confirmation of the prognostic role of FOXP2 (at the protein level) in larger cohorts of patients is recommended.

Secondly, no information about the functional role of FOXP2 in glioblastoma has been provided here. Previous studies have reported on this issue, but only with commercial glioma cell cultures [17–19]. Bearing in mind that FOXP2 is a transcription factor, ChiP-Seq approaches (among others) in primary cultures should be considered in future studies to analyze the specific target genes of FOXP2 in the context of glioblastoma.

Conclusion

Higher expression of FOXP2 at protein level is associated with a worse prognosis in glioblastoma patients. In the present study, results from protein expression were reproduced when considering the expression of FOXP2 at RNA level together with the expression of hsa-miR-181a and hsa-miR-20a-3p, two of the multiple miRNAs targeting FOXP2. Slight differences between patients with high or low FOXP2 expression in molecular features were identified. The expression of FOXP2 and its functional role in the pathogenesis of glioblastoma should be evaluated in future studies.

Declarations

Ethics approval and consent to participate

The study was approved by the Hospital Universitario de Canarias Ethics Committee according to the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Funding

Not applicable.

Authors’ contributions

JPB, SAL and ES had conceived the project.

JPB, HFJ and IB had collected all the data.

JPB, SAL and ES had performed the statistics.

All authors had drafted the manuscript.

All authors had approved the final version of the manuscript.

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Tables
Table 1. Cox regression analysis evaluating the effect of clinical, radiological and molecular variables in progression free survival in the local cohort of patients.

**UNVIARIATE COX REGRESSION**

| Variable                             | Hazard Ratio | 95% C. I.  | p-value |
|--------------------------------------|--------------|------------|---------|
| **Age**                              | 0.994        | 0.953 – 1.037 | .784   |
| **Gender**                           |              |            |         |
| Female                               | 1.055        | 0.548 – 2.030 | .874   |
| Male                                 | 0.948        | 0.493 – 1.825 | .874   |
| **Neurological deficit at diagnosis**| 1.821        | 0.902 – 3.677 | .095*  |
| **Epilepsy**                         | 0.532        | 0.239 – 1.184 | .478   |
| **Resection**                        |              |            |         |
| Partial/Biopsy                       | 2.032        | 0.532 – 7.768 | .300   |
| Subtotal                             | 0.447        | 0.207 – 0.965 | .040*  |
| Total                                | 0.218        | 0.074 – 0.641 | .006*  |
| **Subventricular zone involvement**  | 1.107        | 0.544 – 2.256 | .779   |
| **MRI gadolinium enhancement**       |              |            |         |
| Heterogeneous                        | 4.213        | 2.075 – 8.554 | .0000* |
| Ring                                 | 0.237        | 0.117 – 0.482 | .0000* |
| **Ki-67 expression (ICH)**           | 2.722        | 0.605 – 12.249 | .192   |
| **MGMT promoter methylation**        | 0.454        | 0.234 – 0.879 | .019*  |
| **FOXP2 expression (ICH)**           | 1.711        | 1.040 – 2.814 | .034*  |

**MULTIVARIATE COX REGRESSION**

| Variable                             | Hazard Ratio | 95% C. I.  | p-value |
|--------------------------------------|--------------|------------|---------|
| **Neurological deficit at diagnosis**| 0.887        | 0.410 – 1.919 | .887   |
| **Resection**                        |              |            |         |
| Partial/Biopsy                       | 1.541        | 0.323 – 7.359 | .588   |
| Subtotal                             | 0.609        | 0.244 – 1.521 | .288   |
| Total                                | 0.335        | 0.102 – 1.104 | .072   |
| **MRI gadolinium enhancement**       |              |            |         |
|                      |     |                  |      |
|----------------------|-----|------------------|------|
| **Heterogeneous**    | 4.213 | 2.075 – 8.554   | .0001* |
| **Ring**             | 0.226 | 0.104 – 0.489   | .0001* |
| **MGMT promoter methylation** | 0.586 | 0.277 – 1.237 | .161   |
| **FOXP2 expression (ICH)** | 1.964 | 1.061 – 3.634 | .032*   |
Table 2. Cox regression analysis evaluating the effect of clinical, radiological and molecular variables in overall survival in the local cohort of patients.

| Variable                        | Hazard Ratio | C. I. for 95%    | p-value  |
|---------------------------------|--------------|------------------|----------|
| Age                             | 1.011        | 0.979 – 1.045    | .499     |
| **Gender**                      |              |                  |          |
| Female                          | 1.357        | 0.727 – 2.534    | .338     |
| Male                            | 0.737        | 0.395 – 1.376    | .338     |
| Neurological deficit at diagnosis | 1.434        | 0.729 – 2.820    | .296     |
| Epilepsy                        | 1.036        | 0.436 – 2.465    | .936     |
| **Resection**                   |              |                  |          |
| Partial/Biopsy                  | 0.827        | 0.325 – 2.108    | .921     |
| Subtotal                        | 0.302        | 0.151 – 0.606    | .001*    |
| Total                           | 0.107        | 0.031 – 0.364    | .0003*   |
| Subventricular zone involvement | 2.473        | 1.096 – 5.580    | .029*    |
| **MRI gadolinium enhancement**  |              |                  |          |
| Heterogeneous                   | 2.016        | 1.092 – 3.723    | .025*    |
| Ring                            | 0.496        | 0.269 – 0.916    | .025*    |
| **Ki-67 expression (ICH)**      |              |                  |          |
| 1.017                           |              | 0.994 – 1.041    | .155     |
| **MGMT promoter methylation**   |              |                  |          |
| 0.515                           |              | 0.280 – 0.948    | .033*    |
| **FOXP2 expression (ICH)**      |              |                  |          |
| 1.809                           |              | 1.129 – 2.900    | .014*    |

**MULTIVARIATE COX REGRESSION**

| Resection                        |              |                  |          |
| Partial/Biopsy                  | 0.503        | 0.189 – 1.341    | .170     |
| Subtotal                        | 0.257        | 0.116 – 0.571    | .001*    |
| Total                           | 0.146        | 0.039 – 0.544    | .004*    |
| Subventricular zone involvement | 2.738        | 1.143 – 6.560    | .024*    |
| **MRI gadolinium enhancement**  |              |                  |          |
| Heterogeneous                   | 2.016        | 1.092 – 3.723    | .025*    |
| Ring                            | 0.559        | 0.283 – 1.104    | .094     |
|                              |       |              |      |
|------------------------------|-------|--------------|------|
| **MGMT promoter methylation**| 0.481 | 0.239 – 0.970 | .041 |
| **FOXP2 expression (ICH)**   | 2.358 | 1.380 – 4.028 | .002*|
Table 3. Comparative analysis of clinical, radiological and molecular features between low FOXP2 expression (<=p50) and high FOXP2 expression patients.

|                      | Low FOXP2 (% <= 14.00) (n=31) | High FOXP2 (% > 14.00) (n=31) | p-value |
|----------------------|---------------------------------|---------------------------------|---------|
| Age (years)          | 63.8 (SD=9.21)                 | 62.0 (SD=10.58)                 | .4991   |
| Gender (female:male) | 15:16                           | 11:20                           | .0732   |
| Neurological deficit | 26 (83.9%)                     | 20 (64.5%)                      | .5682   |
| Epilepsy             | 2 (6.5%)                       | 7 (22.6%)                       | .0732   |
| Resection            |                                 |                                 |         |
| Partial/Biopsy       | 13 (41.9%)                     | 11 (35.5%)                      | .1853   |
| Subtotal             | 12 (38.7%)                     | 14 (45.2%)                      |         |
| Total                | 6 (19.4%)                      | 6 (19.4%)                       |         |
| MRI gadolinium enhancemnt |                          |                                 |         |
| Ring                 | 18 (58.1%)                     | 16 (51.6%)                      | .3992   |
| Heterogeneous        | 13 (41.9%)                     | 15 (48.4%)                      |         |
| Subventricular zone involvement | 23 (74.2%)                 | 23 (74.2%)                      | 1.002   |
| MGMT promoter methylation | 15 (48.4%)                 | 19 (61.3%)                      | .2222   |
| Ki-67 (%) expression | 22.2 (SD=10.00)                | 27.5 (SD=18.9)                  | .9401   |
| Progression free survival (days) | 339.9 [126.3 – 553.5]     | 176.1 [9.9 – 343.2]             | .024*4  |
| Overall survival (days) | 624.9 [274.6 – 975.2]     | 381.9 [217.1 – 546.6]           | .042*4  |

1 Mann – Whitney U.
2 Fisher exact test.
3 Chi Square.
4 Log Rank test.
Table 4. Cox regression analysis evaluating the effect of clinical and molecular in overall survival in the TCGA cohort of patients.

| Variable                        | Hazard Ratio | 95% C. I.   | p-value |
|---------------------------------|--------------|-------------|---------|
| Age                             | 1.038        | 1.017 – 1.058 | .0000* |
| **Gender**                      |              |             |         |
| Female                          | 1.304        | 0.868 – 1.958 | .201   |
| Male                            | 0.767        | 0.511 – 1.152 | .201   |
| **Kamofsky performance status > 70** | 0.746  | 0.347 – 1.601 | .452   |
| **Molecular classification**    |              |             |         |
| Classic                         | 0.713        | 0.456 – 1.113 | .137   |
| Mesenchymal                     | 1.132        | 0.727 – 1.764 | .584   |
| Proneural                       | 1.183        | 0.728 – 1.923 | .497   |
| Neural                          | 1.137        | 0.700 – 1.847 | .604   |
| **MGMT promoter methylation**   | 0.714        | 0.443 – 1.152 | .167   |
| FOXP2 expression                | 1.142        | 0.787 – 1.659 | .484   |
| **Fraction of genome altered**  | 0.594        | 0.123 – 2.873 | .517   |
| **Aneuploidy Score**            | 0.994        | 0.980 – 1.009 | .426   |
| **Mutation Count**              | 1.001        | 0.999 – 1.003 | .254   |
Table 5. Comparative analysis of clinical and molecular features between low FOXP2 RNA expression (<=p50) and high FOXP2 RNA expression patients.

| Feature                                | Low FOXP2 (n=74) | High FOXP2 (n=74) | p-value |
|----------------------------------------|-------------------|-------------------|---------|
| **Age (years)**                        | 61.2 (SD=11.98)   | 61.0 (SD=12.81)   | .880\(^1\) |
| **Gender (female:male)**               | 24:50             | 28:46             | .606\(^2\) |
| **Karnofsky performance score**        | 76.1 (SD=15.57)   | 74.4 (SD=13.83)   | .489\(^1\) |
| **Molecular subtype**                  |                   |                   |         |
| Classic                                | 25 (35.7%)        | 16 (22.2%)        | .032\(^3\) |
| Mesenchymal                            | 25 (35.7%)        | 18 (25.0%)        |         |
| Proneural                              | 11 (15.7%)        | 19 (26.4%)        |         |
| Neural                                 | 9 (12.9%)         | 19 (26.4%)        |         |
| **MGMT promoter methylation**          | 26 (44.1%)        | 21 (34.4%)        | .350\(^2\) |
| **FOX1P expression**                   | 293.5 (SD=165.04) | 343.8 (SD=181.13) | .028\(^1\) |
| **FOX1P4 expression**                  | 395.8 (SD=177.07) | 483.1 (SD=462.25) | .215\(^1\) |
| **Fraction of genome altered**         | 0.21 (SD=0.12)    | 0.22 (SD=0.12)    | .414\(^1\) |
| **Aneuploidy Score**                   | 10.2 (SD=14.15)   | 9.9 (SD=10.82)    | .960\(^1\) |
| **Mutation Count**                     | 70.8 (SD=130.21)  | 48.3 (SD=24.13)   | .124\(^1\) |
| **Overall survival (days)**            | 419.0 [363.5 – 474.5] | 343.0 [283.1 – 402.9] | .426\(^4\) |

1 Mann – Whitney U.  
2 Fisher exact test.  
3 Chi Square.  
4 Log Rank test.

Figures
Figure 1

Survival analysis using different FOXP2 expression cutoffs. The upper row shows images with different FOXP2 positivity level expressions (25, 50 and 75 percentiles). The corresponding Kaplan-Meier curves for progression free survival (intermediate row) and overall survival (last row) using the cutoffs are shown below each image.
Figure 2

Kaplan-Meier curves of low and high FOXP2 RNA expression for overall survival.
Figure 3

Kaplan-Meier curves representing overall survival for low and high FOXP2 RNA expression groups in different expression groups of has-miR-181a-2-3p (a) and has-miR-20a-3p (b).

Supplementary Files

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