Transcriptomic Profiling of Forkhead Box Transcription Factors in Adult Glioblastoma Multiforme

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Abstract. *Background: The Forkhead box transcription factor (FOX) family plays an essential role in embryogenesis, especially during brain development. Our hypothesis is that de-regulation of FOX genes may contribute to aggressive tumor biology and therapy resistance in patients with glioblastoma multiforme (GBM). Materials and Methods: Univariate and multivariate analyses were performed to evaluate prognostic significance of transcript levels of 31 FOX genes in a test set of GBM patients (n=191) and validated them in The Cancer Genome Atlas (TCGA) cohort comprising of 508 adult cases of GBM. The predictive significance of key FOX genes was investigated in patients who received chemotherapy or radiotherapy. Results: Low FOXA2 mRNA, low FOXN2 mRNA, low FOXN3 mRNA and high FOXG1 mRNA were associated with poor survival in the test and TCGA validation cohorts. In multivariate analysis, low FOXA2 mRNA, low FOXN2 mRNA, low FOXN3 mRNA and high FOXG1 mRNA remained independently associated with poor survival in the test and TCGA validation cohorts. In patients who received chemotherapy or radiotherapy, low FOXA2 mRNA, low FOXN2 mRNA and high FOXG1 mRNA correlated with adverse outcomes in the TCGA validation cohort. Conclusion: To our knowledge, our data provide the first comprehensive clinical evidence that FOXA2, FOXN2, FOXN3 and FOXG1 are promising biomarkers of GBM and warrant further investigation.*

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Despite advances in surgery, chemotherapy and concurrent chemoradiotherapy strategies, the overall prognosis of patients with glioblastoma multiforme (GBM) remains poor, with a 3-year survival of less than 10% (1). Forkhead box transcription factors (FOX) are responsible for regulating the transcription of several proteins involved in embryogenesis, cell proliferation, differentiation, DNA repair and cell survival (2-4). There exist at least 50 known FOX genes in the human genome, categorised into 19 sub-groups (from A to S) (2-4). FOX genes may have a role in cancer pathogenesis. *FOXOA1* has been linked to prostate cancer (5). Overexpression of *FOXM1* has been identified in cancer of the liver, brain, and pancreas (6). *FOXP1* may act as a tumor suppressor in breast cancer and paradoxically as an oncogene in certain types of lymphoma (7). In addition, *FOXA1* and *FOXG1* may also be involved in gliomagenesis (8, 9). We hypothesized that FOX genes may have a role in GBM pathogenesis.

Materials and Methods

We investigated FOX gene expression in GBMs in two datasets, a test set and a validation set.

Test set. The dataset E-GEOD-13041 (publically available from http://www.ebi.ac.uk/arrayexpress/) was used as a test set. This dataset contained microarray gene profiling data for 267 patients using three different Affymetrix platforms. A total of 191 patients with GBM were included in the subsequent data analysis for the test dataset, all of whom were profiled using the Affymetrix U133A array. The median age of the patients was 54 years, with a range of 18-86 years. One hundred and eighteen of the patients (61.8%) were male. The patients were followed-up for a median of 385 days (range=7-3353 days). At the end of the follow-up, 92.1% of patients had died (176/191). Limited treatment data was available in this dataset.

Validation set. The dataset obtained from The Cancer Genome Atlas (http://cancergenome.nih.gov/) was used as a validation set. The dataset consisted of 548 patients out of which 508 were selected for analysis after duplicates and patients with missing survival data were excluded. The median age in this set was 59 years, with a range of 10-89 years. 60.6% of the patients were male (308/508). Follow-up was undertaken

Table I. *FOX gene probes in test and validation sets.*

Probe set ID	Gene symbol	Gene name
204667_at	*FOXA1*	Forkhead box A1
210103_s_at	*FOXA2*	Forkhead box A2
214312_at		
40284_at		
208513_at	*FOXB1*	Forkhead box B1
213260_at	*FOXC1*	Forkhead box C1
214520_at	*FOXC2*	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
206307_s_at	*FOXD1*	Forkhead box D1
207653_at	*FOXD2*	Forkhead box D2
208500_x_at	*FOXD3*	Forkhead box D3
217032_at	*FOXD4 /// FOXD4L1*	Forkhead box D4 /// forkhead box D4-like 1
206912_at	*FOXE1*	Forkhead box E1 (thyroid transcription factor 2)
208239_at		
220621_at	*FOXE3*	Forkhead box E3
205935_at	*FOXF1*	Forkhead box F1
206377_at	*FOXF2*	Forkhead box F2
206018_at	*FOXG1*	Forkhead box G1
207658_s_at		
207644_at	*FOXH1*	Forkhead box H1
208006_at	*FOXI1*	Forkhead box I1
205906_at	*FOXJ1*	Forkhead box J1
203734_at	*FOXJ2*	Forkhead box J2
206015_s_at	*FOXJ3*	Forkhead box J3
217310_s_at		
203064_s_at	*FOXK2*	Forkhead box K2
216572_at	*FOXL1*	Forkhead box L1
220102_at	*FOXL2*	Forkhead box L2
202580_x_at	*FOXM1*	Forkhead box M1
207683_at	*FOXN1*	Forkhead box N1
206708_at	*FOXN2*	Forkhead box N2
205021_s_at	*FOXN3*	Forkhead box N3
205022_s_at		
218031_s_at		
202723_s_at	*FOXO1*	Forkhead box O1
202724_s_at		
204131_s_at	*FOXO3*	Forkhead box O3
204132_s_at	*FOXO3 ///*	Forkhead box O3 /// forkhead box O3B pseudogene
210655_s_at	*FOXO3B*	
217399_s_at		
205451_at	*FOXO4*	Forkhead box O4
221333_at	*FOXP3*	Forkhead box P3
221334_s_at		

for a median of 353 days (range=2-3880 days). At the end of follow-up, 81.9% of patients had died (416/508). Within this set, 69.1% received chemotherapy (351/508) and 73.4% received radiotherapy (373/508). Of these 65.7% received both chemotherapy and radiotherapy (334/508), 3.1% received just chemotherapy and no radiotherapy (16/508), 7.5% received only radiotherapy and no chemotherapy (38/508) and 23% (116/508) received neither treatment option.

Statistical analysis. Out of the 50 known human FOX genes, 31 genes, represented by 42 probes, were present in both datasets and included in subsequent analyses (Table I). Along with this expression data, demographic data including: age, gender and survival data were also included. X-Tile (version 3.6.1; Yale University, New Haven, Connecticut, USA) was used to classify gene expression data into high and low expression for the 42 probes. SPSS (IBM SPSS Statistics for Windows, Version 22.0; IBM Corp., Armonk, NY, USA) was used to generate Kaplan–Meier survival curves for each probe in both datasets. The Benjamini-Hochberg false-discovery rate (BH FDR) (10) was then applied to the values to allow for multiple comparisons. Cox multivariate regression models were constructed for each dataset including probes which were significant (with BH FDR correction) in both datasets. Four probes were found to be significant in both datasets. Subgroup analysis based on treatments received (chemotherapy, no chemotherapy, radiotherapy, and no radiotherapy) were also performed in the TCGA dataset.

Table II. *FOX genes and association with survival in test and validation datasets*.

Gene	Test set				Validation set		
	Probe	Expression associated with poor survival	*p*-Value	Adjusted *p*-value‡	Expression associated with poor survival	*p*-Value	Adjusted *p*-value‡
FOXA1	204667_at	Low	0.0560	0.1120	Low	0.0050	**0.0191**
FOXA2	210103_s_at	Low	0.0080	**0.0336**	Low	0.0230	**0.0460**
	214312_at	Low	0.3870	0.4064	Low	0.1810	0.1764
	40284_at	High	0.6320	0.6320	Low	0.1470	0.2001
FOXB1	208513_at	High	0.3680	0.3963	Low	0.0002	**0.0041**
FOXC1	213260_at	High	0.0020	**0.0280**	Low	0.0170	**0.0376**
FOXC2	214520_at	High	0.1180	0.1652	Low	0.0400	0.0730
FOXD1	206307_s_at	High	0.0940	0.1410	Low	0.0130	**0.0321**
FOXD2	207653_at	High	0.0340	0.0893	Low	0.1070	0.1400
FOXD3	208500_x_at	High	0.0030	**0.0280**	Low	0.0030	**0.0156**
FOXD4L	1217032_at	High	0.0390	0.0964	Low	0.0230	**0.0460**
FOXE1	206912_at	High	0.0900	0.1400	Low	0.0050	**0.0191**
	208239_at	High	0.2870	0.3256	Low	0.0130	**0.0321**
FOXE3	220621_at	High	0.0120	**0.0458**	Low	0.1550	0.1808
FOXF1	205935_at	Low	0.0060	**0.0280**	High	0.0080	**0.0258**
FOXF2	206377_at	Low	0.2230	0.2808	Low	0.0010	**0.0105**
FOXG1	206018_at	High	0.0050	**0.0280**	High	0.0030	**0.0156**
	207658_s_at	High	0.0190	0.0614	High	0.0020	**0.0156**
FOXH1	207644_at	High	0.1150	0.1652	Low	0.0050	**0.0191**
FOXI1	208006_at	Low	0.3530	0.3902	Low	0.0090	**0.0270**
FOXJ1	205906_at	High	0.0520	0.1120	High	0.0170	**0.0376**
FOXJ2	203734_at	Low	0.2170	0.2808	Low	0.1900	0.2046
FOXJ3	206015_s_at	Low	0.0320	0.0893	High	0.0030	**0.0156**
	217310_s_at	High	0.2340	0.2808	High	0.1100	0.1400
FOXK2	203064_s_at	High	0.0001	**0.0045**	High	0.1670	0.1896
FOXL1	216572_at	Low	0.2660	0.3103	Low	0.0010	**0.0105**
FOXL2	220102_at	High	0.0160	0.0560	High	0.0450	0.0756
FOXM1	202580_x_at	High	0.0060	**0.0280**	Low	0.1030	0.1400
FOXN1	207683_at	High	0.1650	0.2235	High	0.0430	0.0753
FOXN2	206708_at	Low	0.0020	**0.0280**	Low	>0.0001	**0.0018**
FOXN3	205021_s_at	Low	0.0040	**0.0280**	High	0.0070	**0.0245**
	205022_s_at	Low	0.0760	0.1277	Low	0.0370	0.0706
	218031_s_at	High	0.2330	0.2808	Low	0.0510	0.0824
FOXO1	202723_s_at	High	0.0060	**0.0280**	High	0.1030	0.1245
	202724_s_at	Low	0.0550	0.1120	High	0.0830	0.1400
FOXO3B	204132_s_at	Low	0.0820	0.1325	Low	0.2660	0.1400
	210655_s_at	Low	0.0280	0.0840	High	0.2310	0.1764
	217399_s_at	Low	0.0610	0.1150	Low	0.1470	0.2426
FOXO3	204131_s_at	Low	0.4220	0.4323	Low	0.1090	0.2725
FOXO4	205451_at	High	0.0560	0.1120	Low	0.0100	**0.0280**
FOXP3	221333_at	Low	0.0630	0.1150	Low	0.3850	0.0902
	221334_s_at	Low	0.0760	0.1277	Low	0.0580	16.1700

‡Benjamini-Hochberg. Significant *p*-values are highlighted in bold.

Results

FOX gene expression and survival in adult patients with GBM (Table II). In the test set, 17 probes were found to be significantly associated with survival ($p<0.05$). In the TCGA validation cohort, 25 probes were significantly associated with survival ($p<0.05$). Following BH FDR assessments, the number of significant probes ($p<0.05$) was 11 in the test set and 21 in the TCGA validation cohort. As shown in Table II, *FOXA2* mRNA (probe 210103_s_at), *FOXC1* mRNA, *FOXD3* mRNA, *FOXF1* mRNA, *FOXG1* mRNA (probe 206018_at), *FOXN2* mRNA and *FOXN3* mRNA (probe 205022_s_at) were significantly expressed in both datasets. The probes for *FOXC1* mRNA, *FOXD3* mRNA and *FOXF1*

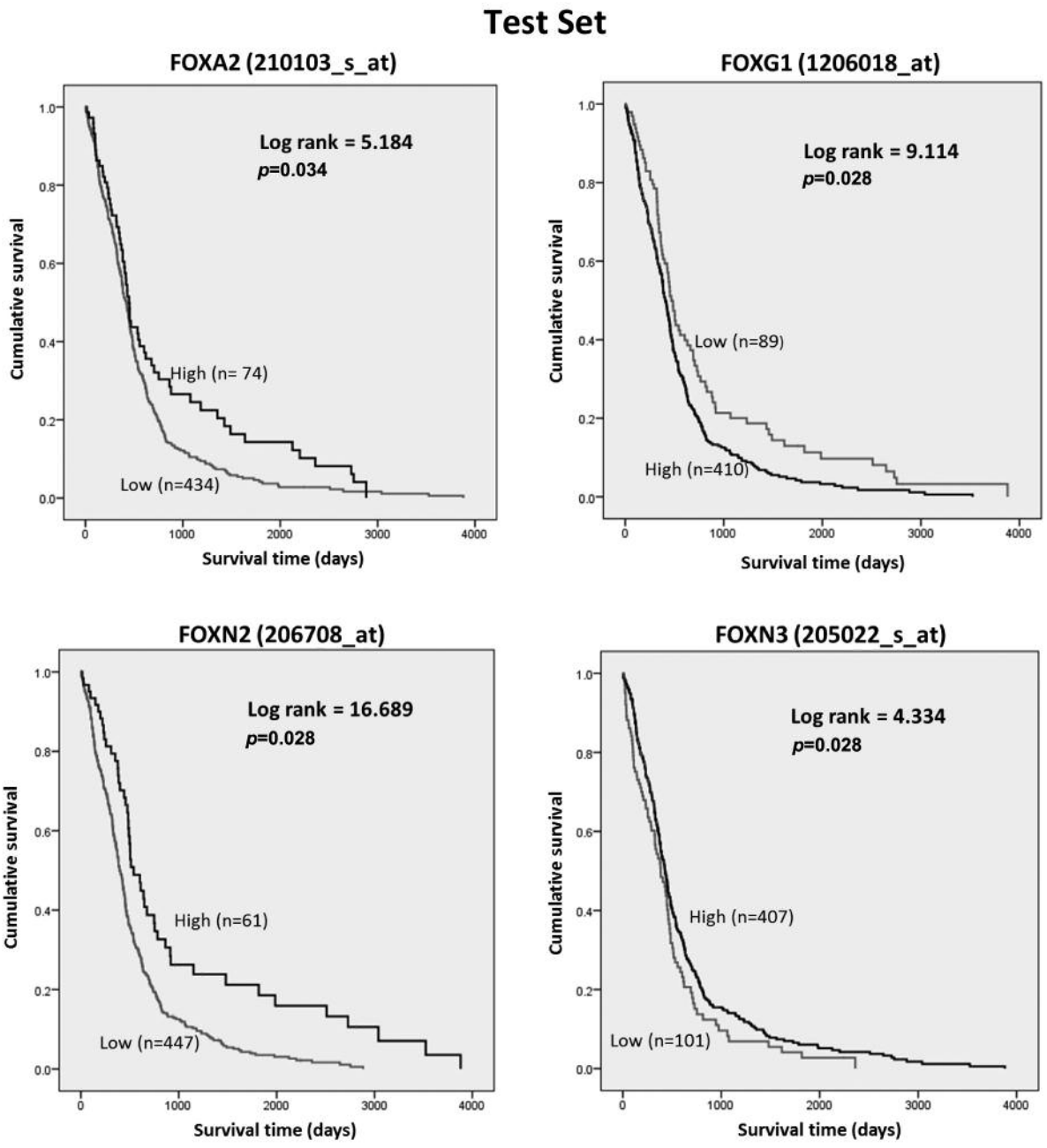

Figure 1. *Continued*

mRNA were eliminated due to discordant significance, as they were represented by high expression in one dataset and low expression in the other. At the end of univariate analysis, *FOXA2* mRNA (probe 210103_s_at), *FOXG1* mRNA (probe 206018_at), *FOXN2* mRNA and *FOXN3* mRNA (probe 205022_s_at) were consistently significantly associated with poor survival in the test set as well as in the TCGA validation set. The Kaplan–Meier survival curves according to expression of these genes are shown in Figure 1. Low expression of *FOXA2* mRNA, *FOXN2* mRNA and *FOXN3* mRNA were associated with poor survival; conversely, high expression of *FOXG1* mRNA was associated with poor survival. We then proceeded to multivariate analysis.

FOXA2, FOXG1, FOXN2 and FOXN3 are independently associated with survival in adult patients with GBM (Table III).

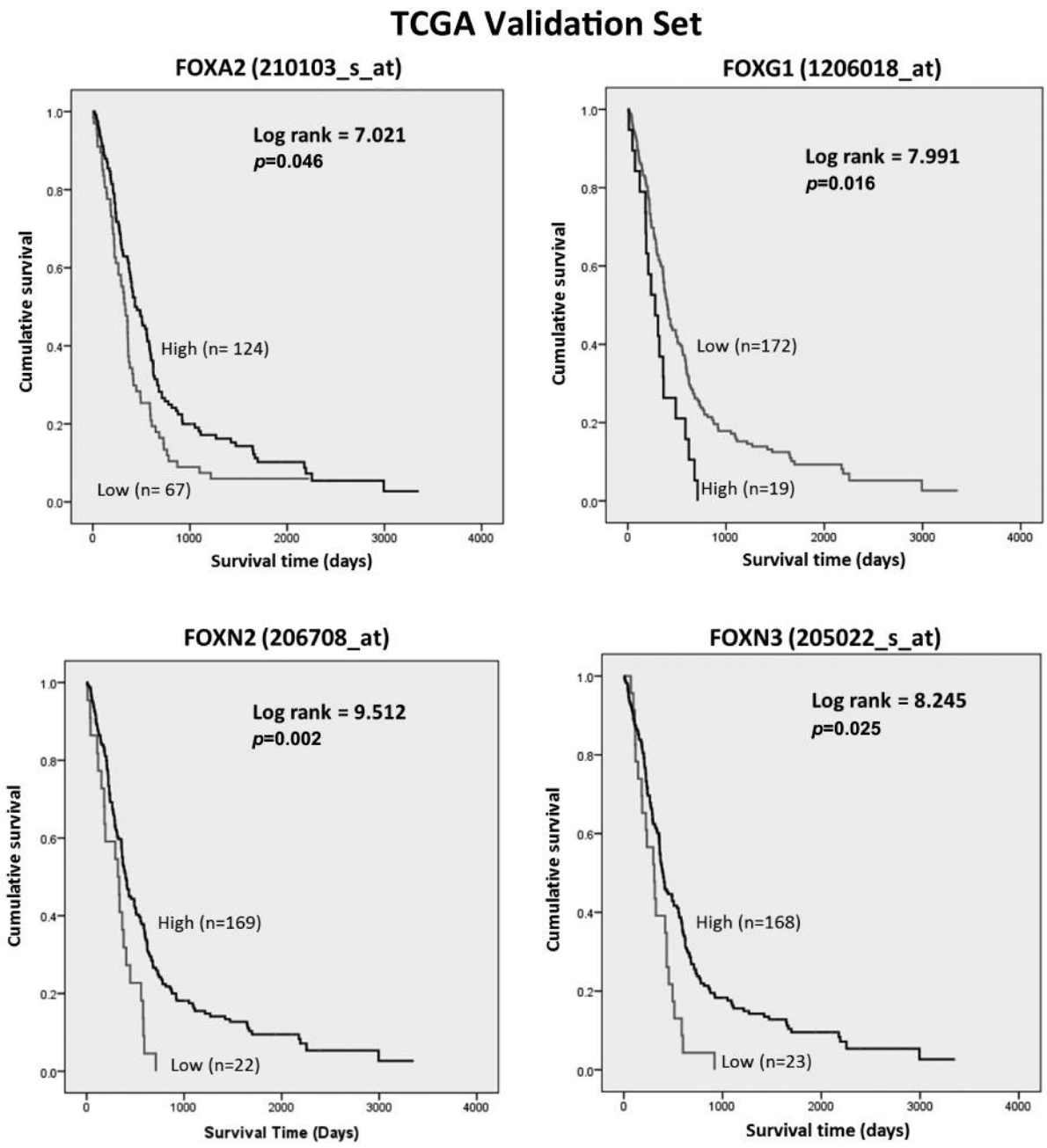

Figure 1. *Kaplan-Meier survival curves in Test and TCGA validation cohorts for genes shown to be significant after BH correction.*

Multivariate analysis in the test set demonstrated that *FOXA2* mRNA (p=0.006), *FOXG1* mRNA (p=0.044), *FOXN2* mRNA (p=0.004), *FOXN3* mRNA (p=0.001) were significant independent predictors of survival. Similarly, *FOXA2* mRNA (p=0.019), *FOXG1* mRNA (p=0.016), *FOXN2* mRNA (p=0.000101), *FOXN3* mRNA (p=0.013) were also significant independent predictors of survival in the TCGA cohort (Table III).

Predictive significance of FOXA2, FOXG1 and FOXN2 gene expression in adult patients with GBM. The data presented above provide evidence that *FOXA2* mRNA, *FOXG1* mRNA, *FOXN2* mRNA, and *FOXN3* mRNA have prognostic significance. To investigate if they also have predictive significance, we conducted analysis in various groups in the TCGA cohort that received chemotherapy

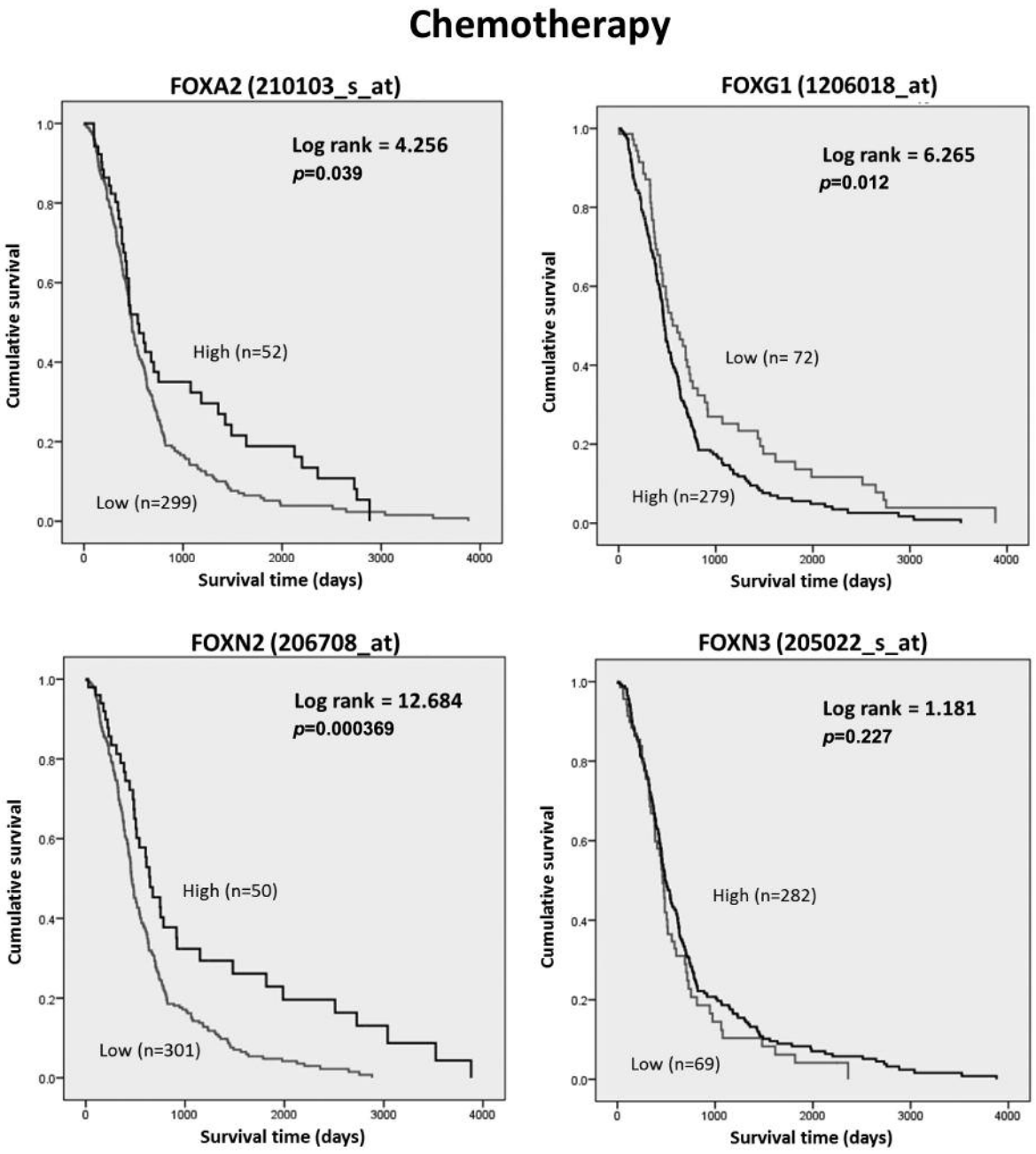

Figure 2. *Continued*

with/without radiotherapy. As shown in Figure 2, low *FOXA2* mRNA, low *FOXN2* mRNA and high *FOXG1* mRNA expression were significantly associated with poor survival in those patients who had received chemotherapy. There was no significance of the expression of these genes in patients who received no chemotherapy. Similarly, low *FOXA2* mRNA, low *FOXN2* mRNA and high *FOXG1* mRNA expression were significantly associated with poor survival in patients who had received radiotherapy (Figure 3). There was no significance in patients who received no radiotherapy. Interestingly, low *FOXN3* mRNA expression was significantly associated with poor survival only in the group not treated with chemotherapy or radiotherapy. Taken together, the data suggest that *FOXA2* mRNA, *FOXN2* mRNA and *FOXG1* mRNA have predictive significance in GBM.

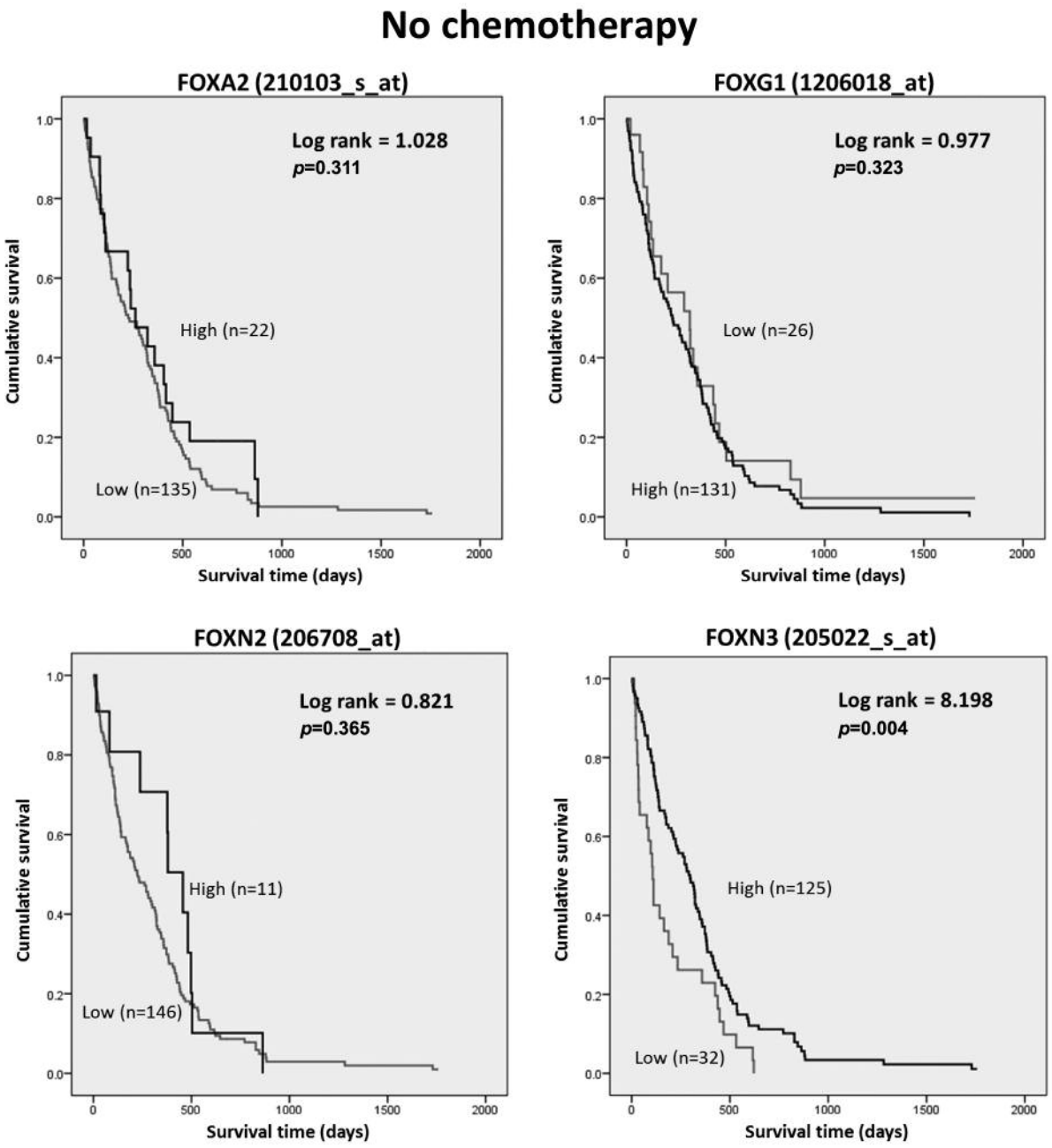

Figure 2. *Kaplan-Meier survival curves in TCGA validation cohort patients that either received Chemotherapy or no chemotherapy for genes shown to be significant after BH correction.*

Discussion

As far as we are aware, this is the first study to comprehensively evaluate the FOX gene family in GBM. FOXG1 is involved in the early development of the brain and has been linked to CNS tumors, including GBM (9). During normal development in mice, FOXG1 has been shown to be crucial in many aspects of the development of the forebrain, more specifically the telecephalon. It acts as a transcriptional repressor, not only during early development, but also into adulthood. In adulthood, it is thought to influence neuronal survival (11). A variant of Rett syndrome, known as FOXG1 syndrome, is characterised by mutation in the *FOXG1* gene and manifests as severe mental retardation, severe post-natal microcephaly, lack of language development, epilepsy and autism-like features (12). FOXG1 has also been implicated in

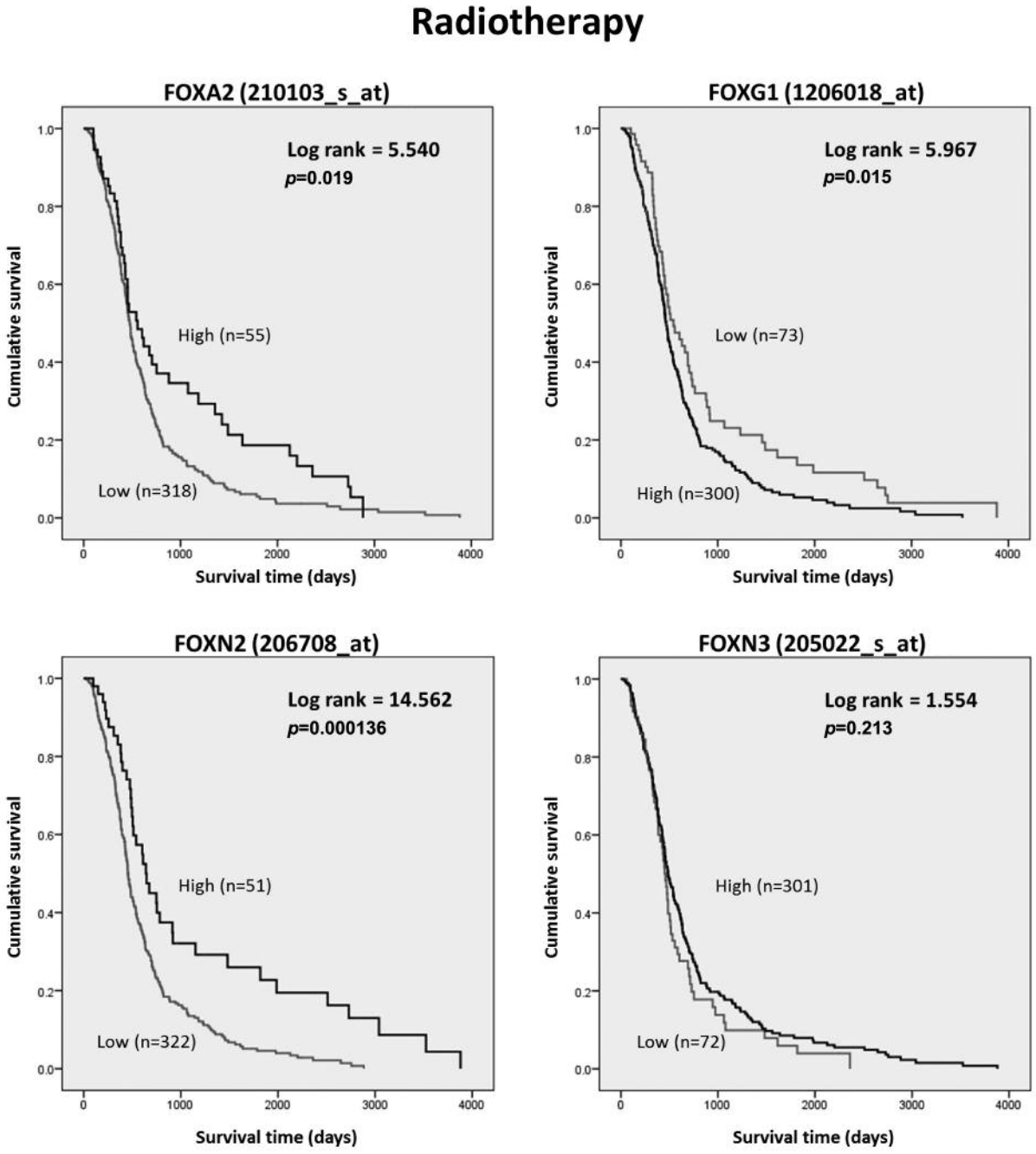

Figure 3. *Continued*

medulloblastoma, hepatoblastomas (13, 14) and ovarian cancer (15). A direct role for FOXG1 in GBM was recently described (9). A link between the FOXG1 protein and the member of the Groucho family human transducin-like enhancer of split (TLE) proteins has been shown. Verginelli *et al*. observed that FOXG1 and TLE form a complex in brain tumor-initiating cells that have stem cell-like properties (9). Interestingly, inhibiting the function of this complex reduced tumor growth. Our data would concur with pre-clinical observations.

We also found that low mRNA expression of *FOXA2*, *FOXN2*, and *FOXN3* was associated with worse clinical outcomes. FOXA2 is involved in the embryonic development of the liver (2) and pancreas (16). In lung cancer models, FOXA2 acts to suppress metastasis by preventing epithelial-

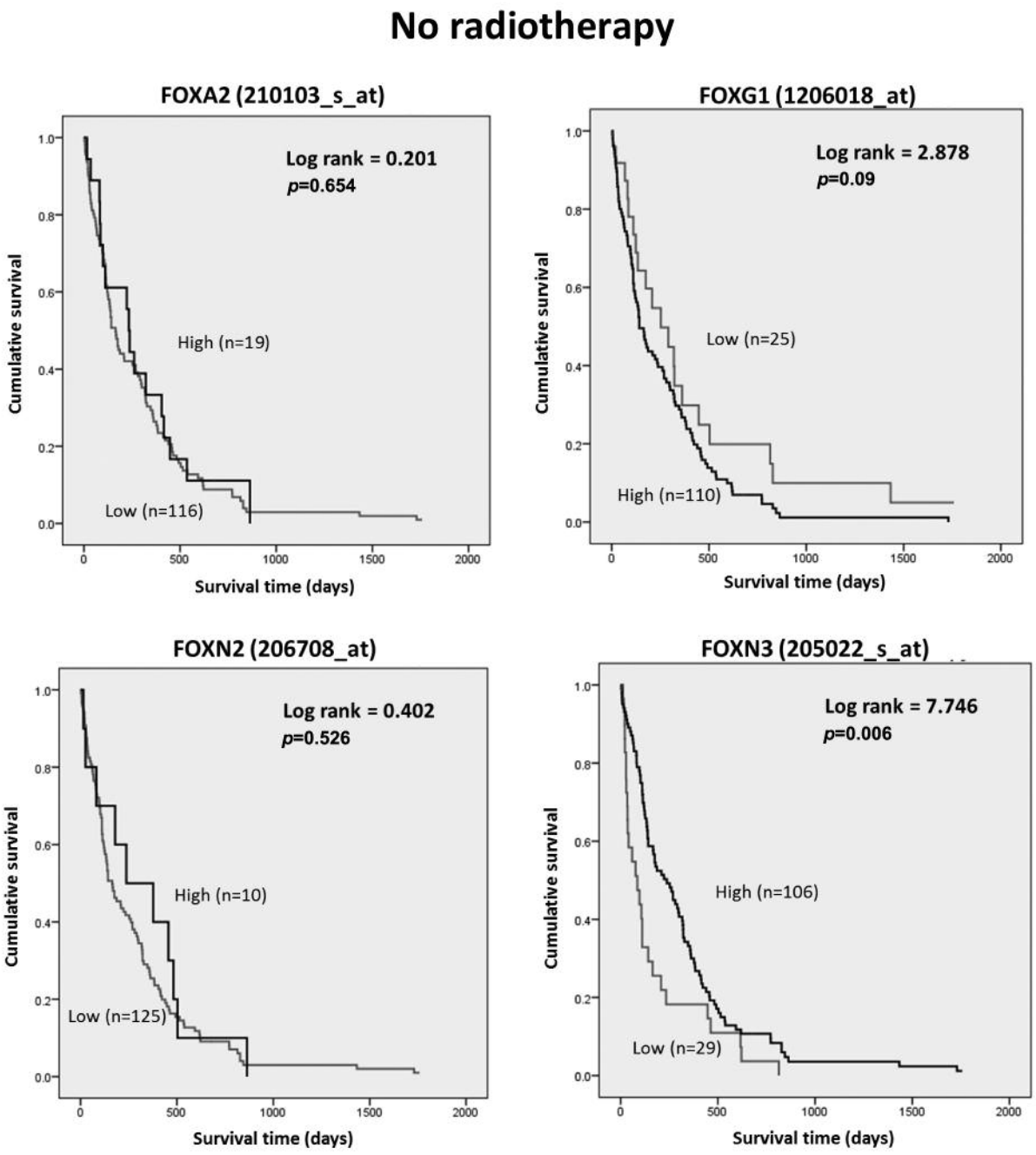

Figure 3. *Kaplan-Meier survival curves in TCGA validation cohort patients that either received radiotherapy or no radiotherapy for genes shown to be significant after BH correction.*

to mesenchymal transition (17). FOXN2 (murine) is known to be involved in the embryogenesis of the central nervous system (18). In humans, FOXN2 is also known as human T-cell leukaemia virus enhancing factor (19) and may be involved in the pathogenesis of adult T-cell leukaemia (19). FOXN3, if mutated in mice, causes craniofacial defects which are very similar to those seen in humans that have FOXN3 mutations (20). In humans, reduced FOXN3 expression has been described in carcinomas of the mouth, larynx, liver (16) and Hodgkins lymphoma (21).

Taken together, the above data provide clinical evidence for a potential role for FOX genes in gliomagenesis.

Table III. *Multivariate analysis in the test and the validation sets.*

Gene (probe set)	Test set		Validation set	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
FOXA2 (210103_s_at)	1.557 (1.136-2.134)	**0.0060**	1.395 (1.055-1.844)	**0.0190**
FOXG1 (206018_at)	0.601 (0.366-0.985)	**0.0440**	0.734 (0.572-0.943)	**0.0160**
FOXN2 (206708_at)	2.101 (1.253-3.226)	**0.0040**	1.883 (1.369-2.591)	**0.0001**
FOXN3 (205022_at)	2.120 (1.339-3.357)	**0.0010**	1.352 (1.064-1.717)	**0.0130**

CI: Confidence interval; HR: Hazard Ratio; Significant *p*-values are shown in bold.

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