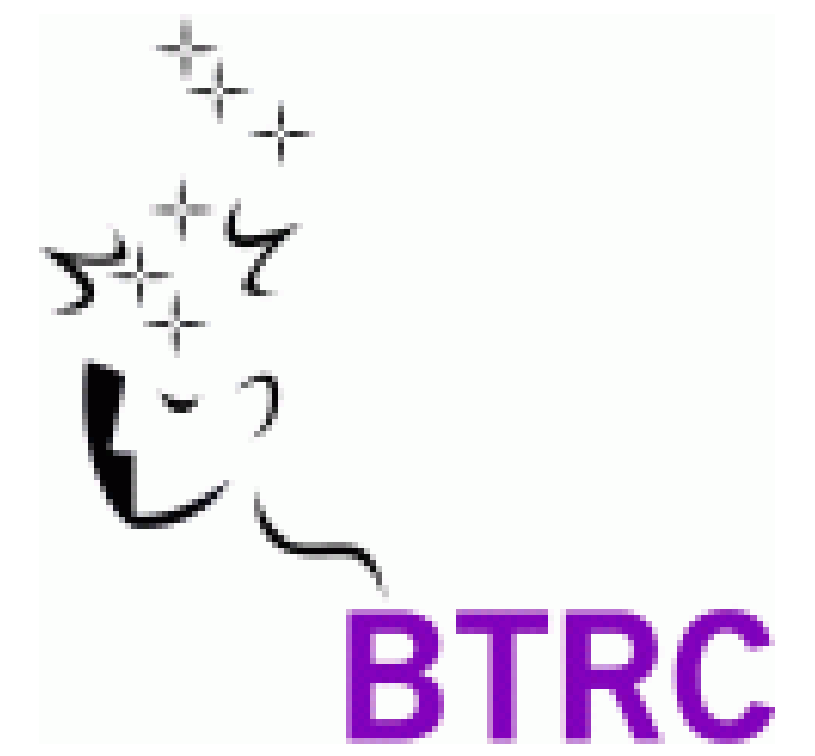


Transcriptional silencing disrupts two levels of arginine biosynthesis in glioblastoma multiforme: a novel targeted therapeutic strategy for high grade gliomas

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SUMMARY

Despite aggressive radio-chemotherapy, the prognosis for patients with glioblastoma multiforme (GBM) remains poor. New agents are therefore required. The molecular mechanisms, which are key in the pathogenesis and progression of GBM are still poorly understood. Documented mutations of isocitrate dehydrogenase 1 and 2 in more than 70% of astrocytomas (Yah H, 2009) brought the attention on metabolism as a primary target in the development of novel treatment modalities for these tumours.

Using de novo gene finding techniques and epigenetic profiling in human GBM cell lines and cultured explants of primary GBM, we observed frequent transcriptional silencing of the 2 most important enzymes of the arginine biosynthetic pathway: argininosuccinate synthetase 1 (ASS1) and argininosuccinate lyase (ASL). Down regulation in some cases correlated with aberrant, neoplasia specific methylation in the CpG islands located in the 5' regulatory regions of each gene and expression was restored by de-methylation. In a subset of cases we observed methylation specific down-regulation of both genes.

Loss of expression of ASS1, ASL or both, as determined by RT-PCR and immunohistochemistry in combination with methylation renders GBM cell lines and primary tumour explants auxotrophic for arginine. This confers sensitivity to highly selective, dose-dependent killing of the cells by arginine deprivation due to either nutritional starvation or pharmacological depletion with pegylated arginine deiminase (ADI-PEG20, Polaris Pharmaceuticals, Inc., San Diego, CA, USA). We have further demonstrated on tumour tissue sections that 20% of de novo GBMs have little or no expression of ASS1 and are methylated indicating that ASS1 expression tested by immunohistochemistry and methylation represents an excellent predictor of response to arginine deprivation with arginine deiminase treatment. These pharmacogenomic findings suggest that ASS1 negativity may serve as a biomarker to select a population sensitive to arginine depletion with ADI-PEG20. Further studies are warranted.

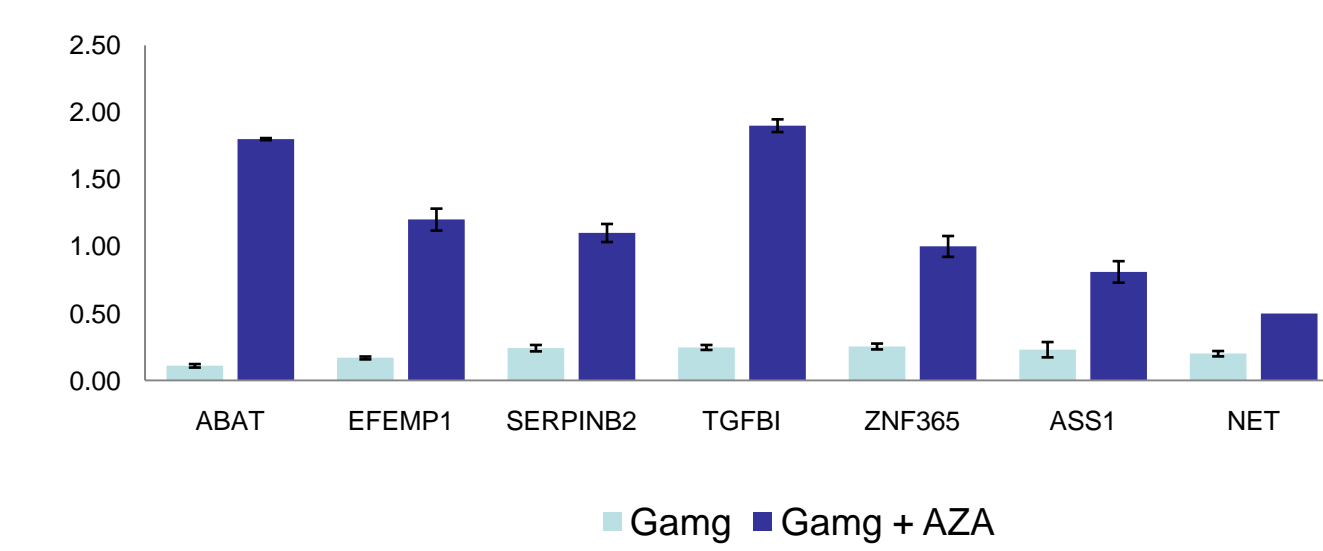
METHODS

Primary tumour explants and established GBM cell lines were treated with 5-Aza-2'-Deoxycytidine for one week. RNA was extracted from both treated and untreated samples and subjected to microarray analysis.

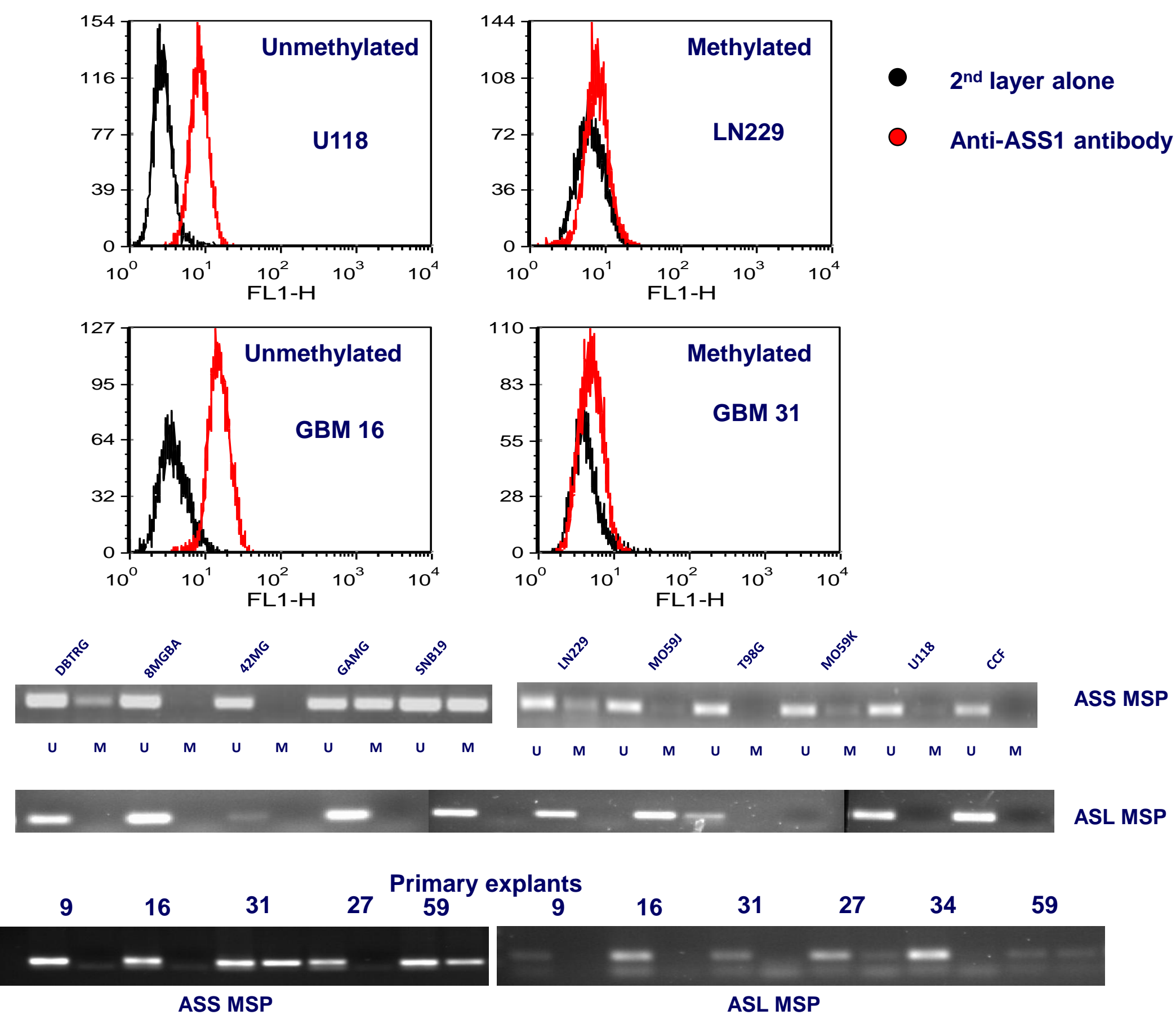
GBM cell lines, primary explants and 60 clinical GBM cases were analysed for methylation of ASS1 using methylation specific PCR (MSP) and pyrosequencing (data not shown). qPCR was used to detect ASS1/ASL RNA. Expression of ASS1 protein was assessed by immunohistochemistry and additionally by western blotting and flow cytometry in cell lines

Arginine depletion experiments were performed using the arginine depleting agent ADI-PEG20. For proliferation assays, cells were plated in triplicate in 96 well plates and analysed using sulphorhodamine B. For expression studies, cells were plated in 6 well plates and harvested for RNA or protein extraction

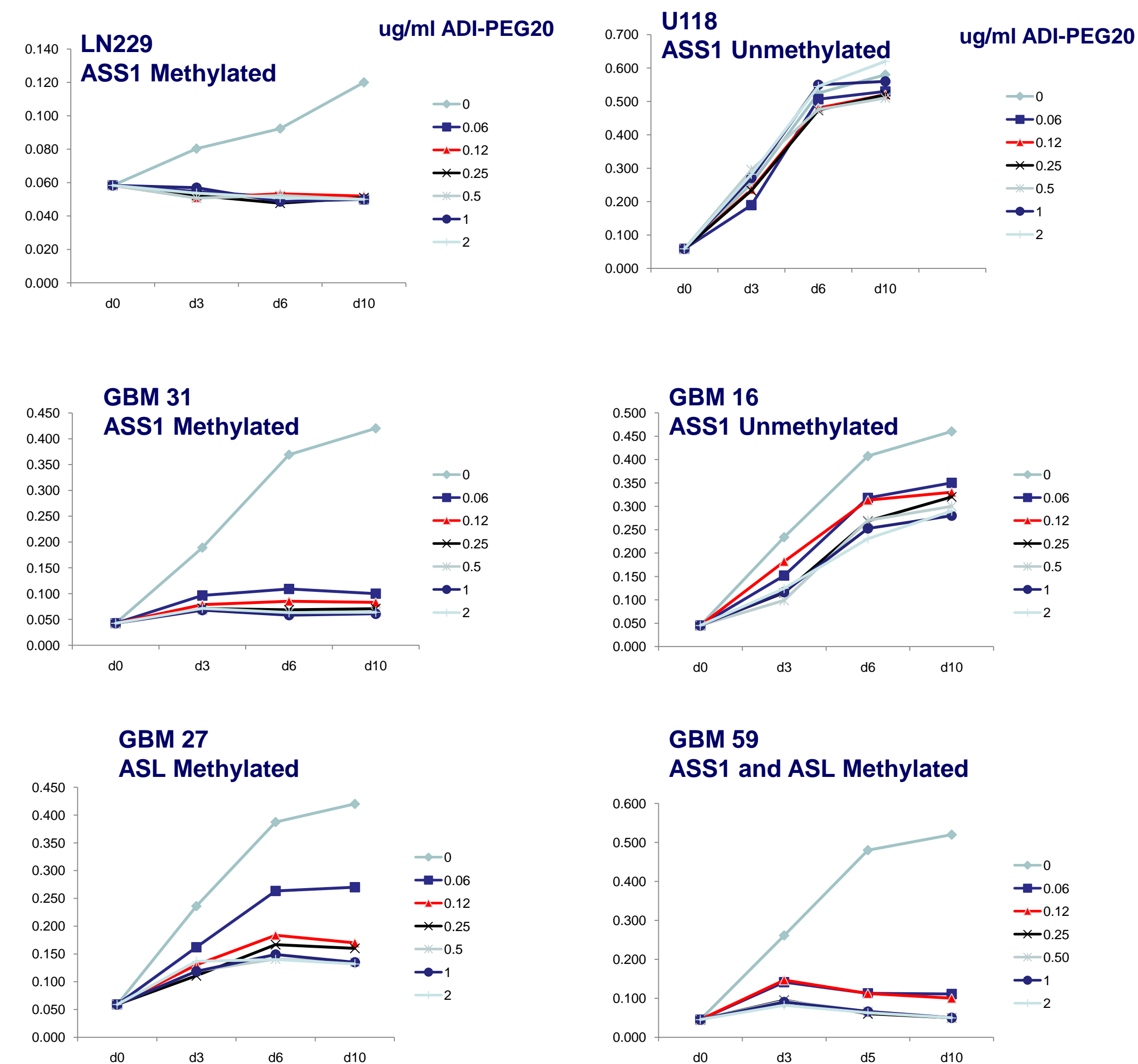
Validation of genes upregulated in GAMG



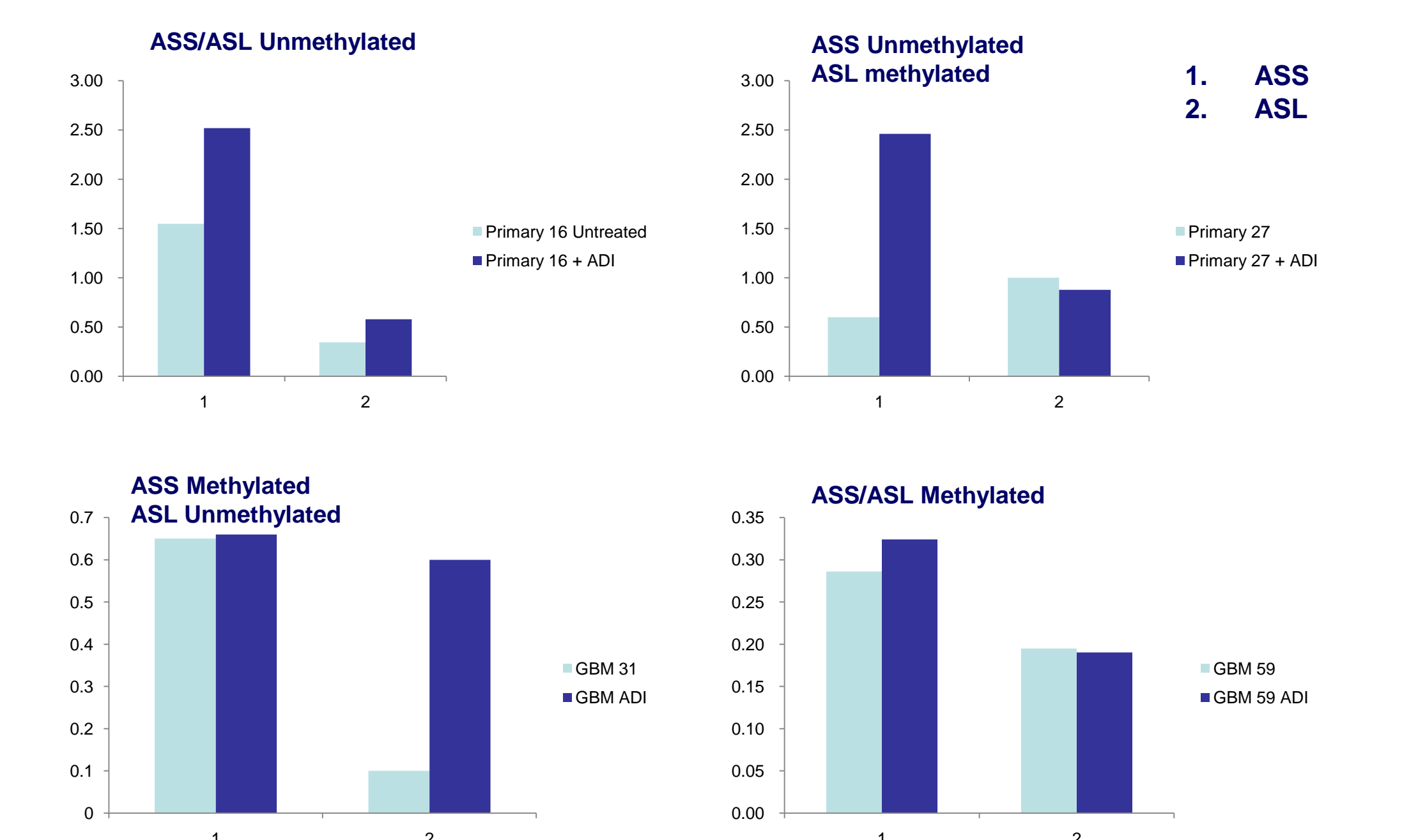
Flow cytometric (A) and MSP (B) analysis of ASS1 and ASL in GBM lines and explants



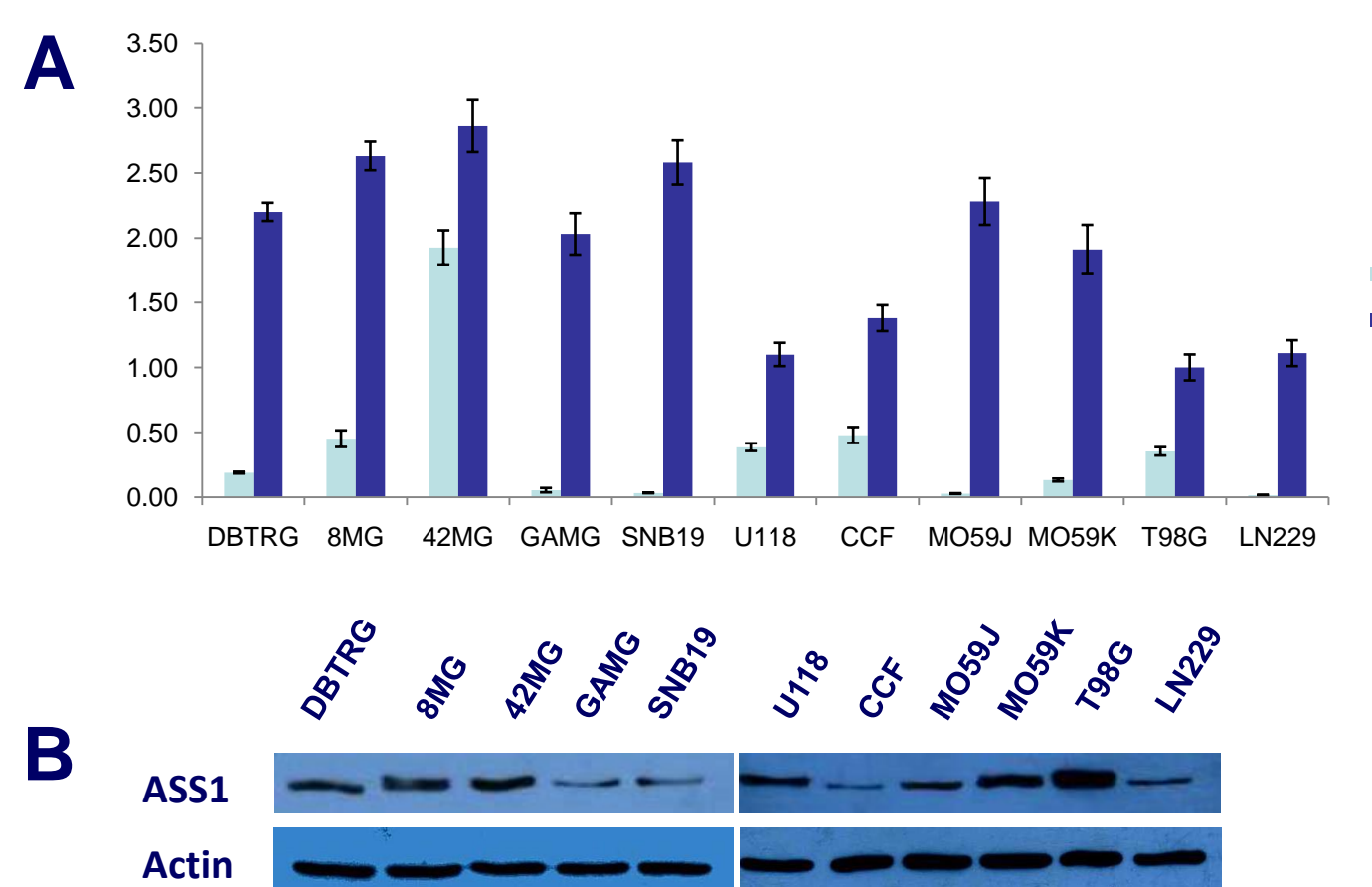
ADI-PEG20 sensitizes ASS1 and ASL methylated lines to cell death



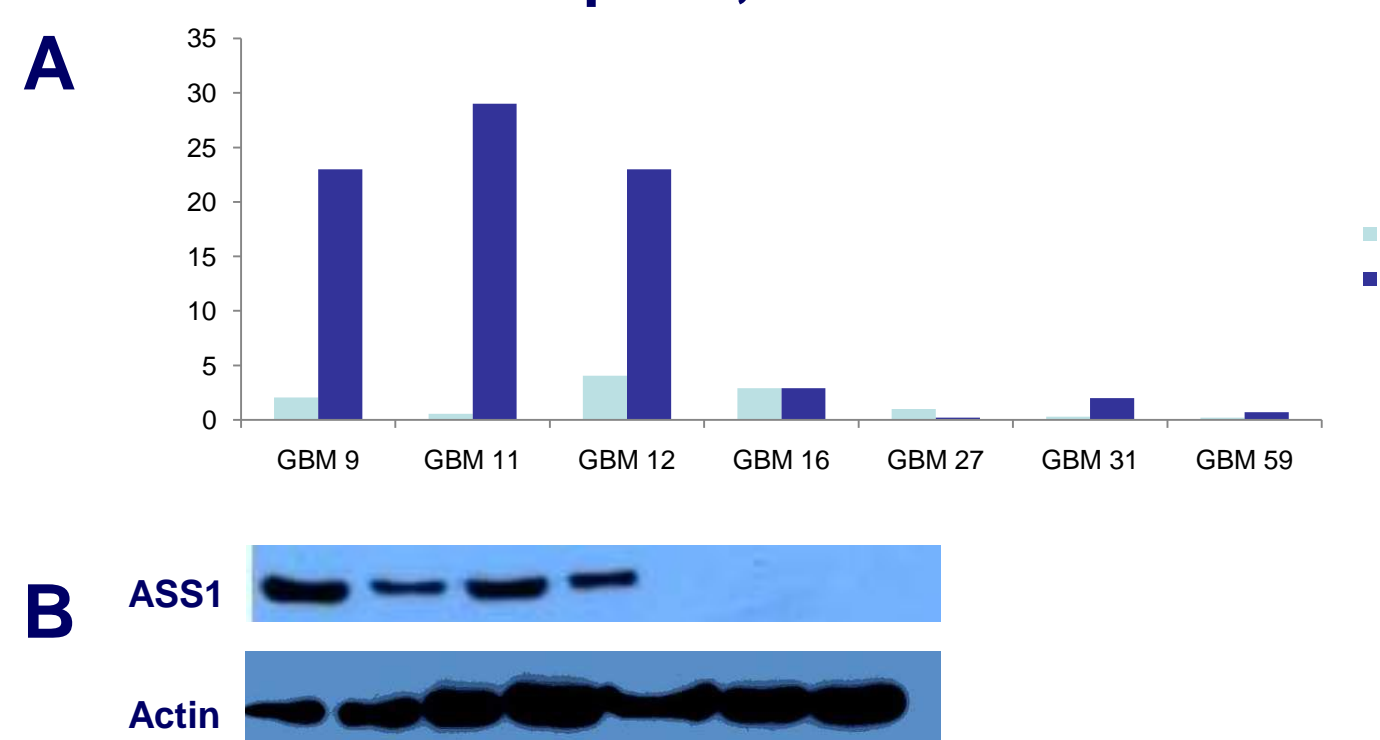
Arginine deprivation leads to upregulation of ASS/ASL in unmethylated lines



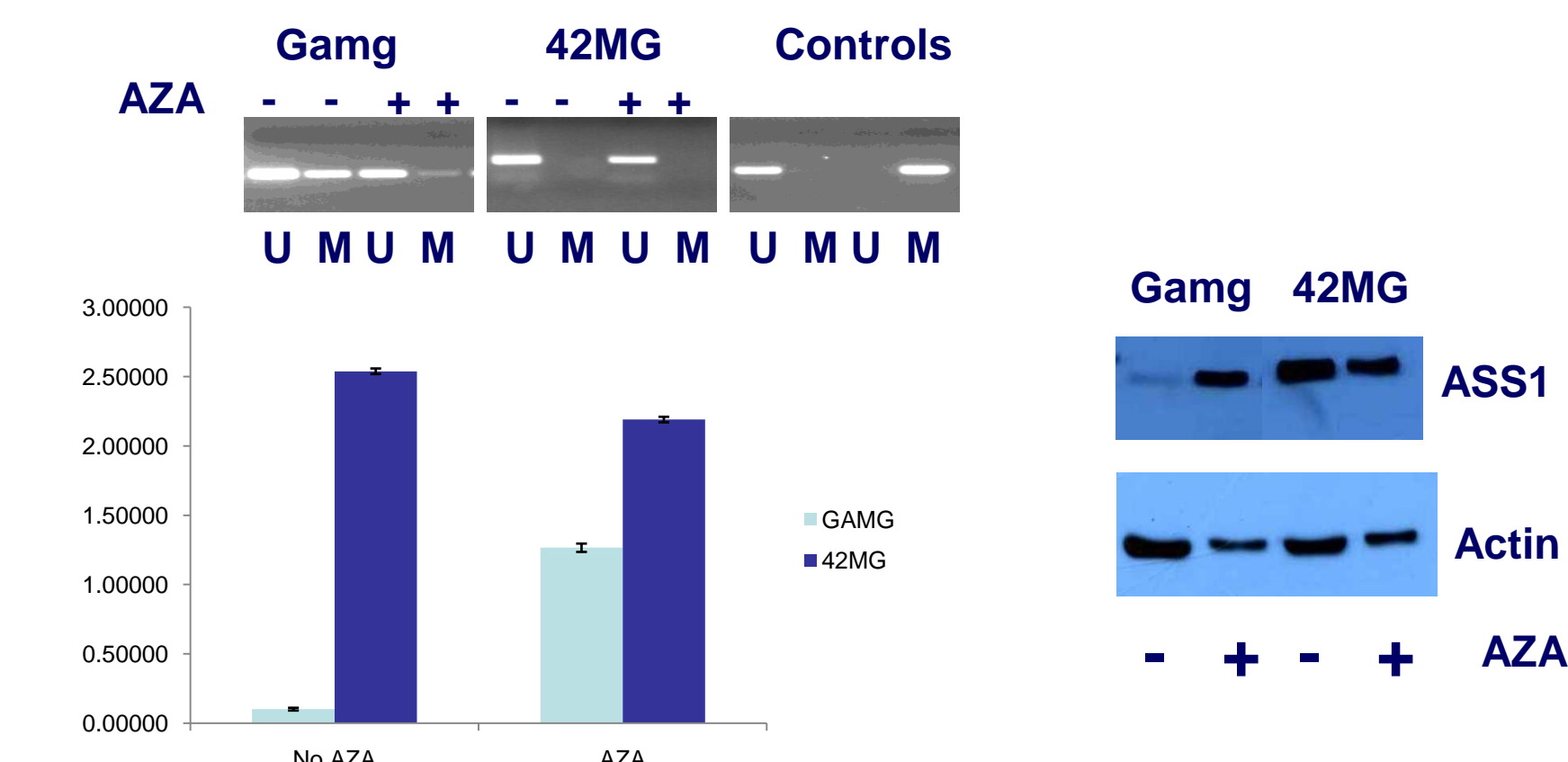
Expression of ASS1 and ASL in GBM cell lines A: qPCR, B: western blot



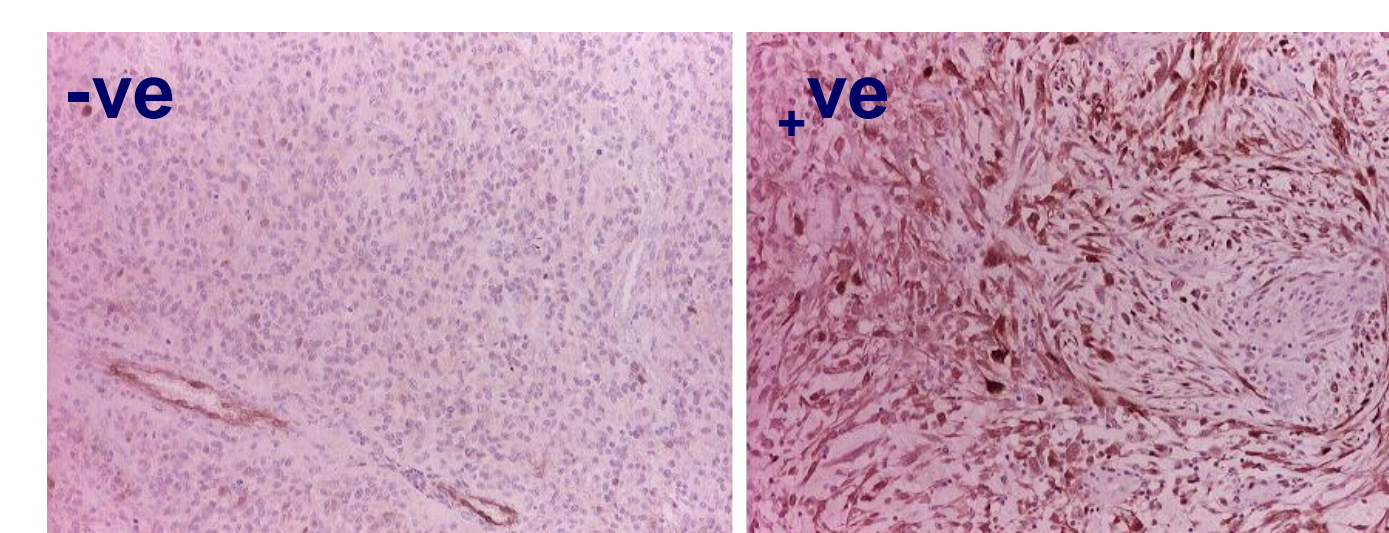
Expression of ASS1 and ASL in primary explants A: qPCR, B: western blot



De-methylation restores ASS1 mRNA and protein expression



Immunohistochemical analysis of ASS1 in primary tumour



RESULTS AND CONCLUDING REMARKS

Depletion of peripheral blood arginine levels by ADI-PEG20 represents a novel and viable therapeutic strategy for subset of patients with GBM. This strategy has several advantages over conventional approaches:

1. Passage of the drug across the blood: brain barrier is not required.
2. Systemic toxicity is limited because normal tissue expresses ASS1 and ASL. Furthermore the CpG islands of both genes are unmethylated in normal cells and are able to transcriptionally up-regulate in response to arginine starvation.
3. Methylation in ASS1 and/or ASL has predictive utility as a biomarker to identify responding patients and this can be confirmed by *in vitro* primary culture.