

Inter-run alignment, not the normaliser, governs the glioblastoma microRNA landscape and reveals candidates validated in TCGA

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Abstract

Two decades of glioblastoma microRNA profiling have produced overlapping but inconsistent signatures, often for methodological reasons. We profiled 377 microRNAs in three recurrent glioblastomas and three normal brains, measuring each sample across four separate qPCR plates, and asked which analytical choice drives the result. It is not the choice between a reference pair and a global mean, but whether the four plates are aligned before normalisation. The plates behave as independent runs whose shared calibrator, miR-16-5p, drifts by up to seven cycles within a sample. Pooling raw cycle thresholds into a per-sample global mean without removing this drift makes the global mean and a reference pair disagree on direction for 40% of microRNAs and fabricates a recovery of canonical oncomiRs; once each plate is aligned, the two normalisers become identical up to a constant. Stability scoring shows that no microRNA is stable across these tissues and that a previously used reference is itself disease-regulated. The corrected pipeline returns 38 robust discriminants that recover known biology and nominates unstudied candidates, led by the clustered miR-501-5p and miR-502-3p, whose direction we confirm in TCGA-GBM (272 tumours, 5 normal; 74% concordance).

Introduction

Glioblastoma is the most common and most lethal primary brain tumour, and its diagnosis and surveillance still depend on imaging and surgery. MicroRNAs entered this field in the mid-2000s, when the first profiles established the broad outline of which microRNAs rise and fall in the tumour.¹ Because microRNAs are short, remarkably stable, and cross the blood–brain barrier into blood and cerebrospinal fluid, they promise something the clinic lacks: a non-invasive molecular readout of the disease.^{5,6} A handful of microRNAs now anchor the field—miR-21 blocks apoptosis and a network of tumour-suppressor pathways in glioblastoma cells,^{2,14} miR-10b is so consistently up-regulated that the tumour is effectively addicted to it,³ and the miR-221/222 cluster drives proliferation by silencing p27.⁴

Yet the measurements that underpin these signatures are more fragile than they appear. Most discovery work rests on a handful of formalin-fixed, paraffin-embedded (FFPE) specimens—often the only material a rare presentation or a tumour-versus-treatment comparison ever yields—and although microRNAs survive fixation well,⁹ the normalisation step in such small studies is not a technical afterthought. The wrong reference, or a global scaling computed on the wrong quantity, can flip a microRNA from up- to down-regulated.⁷ Reporting guidelines have asked for years that the assumptions behind quantitative PCR be stated and justified rather than left implicit,⁸ but a single small-RNA control is still chosen out of habit.

Panel assays add a layer that the literature rarely discusses. When a sample is profiled across several plates—four 96-assay plates of the TaqMan Advanced Panel A in our case—each plate is a separate amplification run with its own loading and efficiency. Unless the plates are first aligned to one another, run-to-run drift propagates silently into every fold-change. In this study we show that this inter-run step,

not the familiar choice of normaliser, decides the outcome of a small multiplate experiment, and that skipping it produces a biologically plausible but entirely spurious signature.

A second problem is sociological. A few microRNAs—miR-21, miR-10b and miR-221 among them^{2,3,4}—absorb most of the glioblastoma literature, while others that are just as strongly dysregulated have barely been examined. Early attention attracts further attention, and the field has no routine, objective way to surface the molecules this feedback loop has left behind.

We therefore pursued five linked aims: (i) to quantify how much the analytical pipeline changes the glioblastoma-versus-normal picture, isolating the contribution of inter-run alignment; (ii) to score reference-gene stability on the corrected data; (iii) to derive a corrected set of discriminants and test it against known biology; (iv) to rank the discriminants by effect size against how much each has been studied; and (v) to test the direction of the resulting candidates in an independent cohort, TCGA-GBM. We intend the study as a worked example rather than a definitive signature, and it leaves behind a pragmatic recipe and a short, externally supported shortlist of candidates.

Results

Tumour and normal brain differ in total microRNA content, and the plates drift

We detected 377 microRNAs across the six samples—226 to 238 per glioblastoma and 208 to 216 per normal brain—so the tumour carried the richer repertoire, and the two tissues also differed in overall expression level. The four plates of a single sample, however, were far from interchangeable. miR-16-5p sits on every plate and is biologically constant within a sample, yet its cycle threshold varied by up to seven cycles between plates of the same sample (for example, 24.9 on one plate and 17.7 on another in a single normal brain). A seven-cycle swing corresponds to a roughly 128-fold technical difference for an RNA that has not changed, so any pipeline that pools cycle thresholds across plates without first removing this drift mixes run effects with disease effects. With only three samples per group, a single mis-scaled plate can push a microRNA across the boundary between up- and down-regulated.

Critically, this swing reflects the plate, not miR-16-5p itself. The four plates of a sample are loaded from the same pre-amplified cDNA pool, so the biological input is identical across them by construction and any between-plate difference in this calibrator must be technical. The numbers bear this out: replicate measurements of miR-16-5p within a plate agreed to about 0.4 cycle (mean within-plate standard deviation 0.38), whereas its per-plate means spanned up to seven cycles within a single sample (between-plate standard deviation 1.6), roughly an eighteen-fold excess of between-plate over within-plate variance (Fig. S1). The variation therefore lives at the level of the run, not of the measurement—the expected signature of plate-to-plate loading and amplification-efficiency differences—and this is what licenses a ubiquitous, biologically constant assay as the alignment anchor: what alignment removes is

the per-plate offset, not biological variation in the calibrator. We nonetheless treat the alignment as the principal assumption of the analysis and return to its limits in the Discussion.

Inter-run alignment, not the normaliser, rewrites the result

We first reproduced the analysis a researcher would naturally run. Among the 182 microRNAs detected in every sample, a per-sample global mean computed directly on the raw cycle thresholds—without inter-run alignment—agreed only loosely with a conventional reference pair (miR-191-5p/miR-484): Spearman $\rho = 0.44$, with opposite directions of change for about 40% of microRNAs (Fig. 1A). The disagreement landed squarely on the canonical oncomiRs, which appeared up-regulated only under the global mean. This is precisely the pattern one would report as the normaliser reshaping the microRNA landscape.

The effect is an artefact of the missing alignment step. When we aligned each plate on miR-16-5p and then took a single global mean per sample^{10,11}, the global mean and the reference pair became identical up to a constant offset ($\rho = 1.00$; the per-microRNA difference was a fixed 0.90 log2 with a standard deviation of 0.0000; Fig. 1B). This identity is not a coincidence but a necessity: with one normalisation scalar per sample, replacing the global mean with a reference pair shifts every microRNA by the same amount and therefore cannot reverse the tumour-versus-normal contrast. The genuine lever is the inter-run alignment, and the decades-old debate between a reference pair and a global mean is, in this single-scalar design, beside the point.

No microRNA is a stable reference here, but the best can still be chosen

We then scored reference-gene stability on the aligned data, across the 182 microRNAs present in every sample. Every candidate scored poorly on geNorm¹²: the most stable, miR-191-5p, still reached a stability value M of about 2.1, well above the usual 1.5 cut-off, so none qualifies as stable across tissues as different as tumour and normal brain. NormFinder¹³, which accounts for the group structure, ranked miR-16-5p as the single most stable candidate ($\rho = 0.19$), ahead of miR-214-3p, miR-106b-5p and miR-191-5p—an internal check that vindicates our use of miR-16-5p as the inter-run calibrator. Crucially, stability and suitability are not the same thing (Fig. 2). miR-191-5p combines a low instability score with a disease fold-change of essentially zero and is therefore a defensible reference, whereas miR-484—half of the reference pair used in the uncorrected analysis—is itself up-regulated in glioblastoma (log2 fold-change +1.8). Adopting a regulated microRNA as a reference would have biased every comparison built on it toward finding nothing. Being stable is necessary; being unregulated is what actually matters.

The corrected pipeline recovers the expected biology

With the aligned, per-sample global mean and a distribution-free rule suited to three-versus-three data—the three tumour values must not overlap the three normal values—39 microRNAs separated the groups, 38 of them with an absolute log2 fold-change above one (25 higher in glioblastoma, 13 higher in normal brain). The result reads like the textbook: the oncomiRs miR-21-5p^{2,14} and miR-221-3p⁴ were up, and tumour-suppressor microRNAs including miR-128-3p, miR-338-3p¹⁵, miR-101-3p¹⁶, miR-139-5p¹⁷ and miR-150-5p were down. We did not tune the pipeline toward this answer, so its agreement with prior work is a fair check that it behaves sensibly—and it contrasts with the uncorrected pipeline, which had to invoke a particular normaliser to recover the same oncomiRs.

Ranking effect against attention surfaces neglected candidates

We next plotted each discriminant’s corrected effect size against the number of indexed glioma or glioblastoma papers (PubMed, June 2026). The plot cleanly separated the well-worn markers—miR-221-3p with 94 papers, miR-338-3p with 33—from a cluster of strong but unexamined microRNAs (Fig. 3). Five stand out: miR-502-3p (+3.6; one indexed glioma paper), miR-501-5p (+4.9; three), miR-652-3p (+2.3; three), miR-193b-3p (+2.5; four) and miR-32-5p (−5.7; two). Outside glioma these microRNAs are not unknown—miR-501-5p promotes gastric cancer,¹⁸ miR-502-5p and miR-32-5p act as tumour suppressors elsewhere,^{19,21} and miR-193b is a dual-strand tumour suppressor in lung cancer²⁰—but their behaviour in glioblastoma is essentially unexplored. miR-501-5p and miR-502-3p belong to the same Xq26.3 cluster and rise together, a coherence that argues against either being noise. The full ranking accompanies the article as Supplementary Table S1.

An independent cohort confirms the direction of the candidates

To test these leads beyond our six samples, we retrieved TCGA-GBM microRNA-sequencing data from the NCI Genomic Data Commons (Data Release 45)^{22,23}—272 primary tumours and 5 solid-tissue normal samples—and compared the direction of change at the precursor level. The corrected directions agreed for 74% of discriminants overall (29 of 39) and for 77% of those with a strong effect (absolute log2 fold-change at least 2), despite an independent platform, a small normal group, and a primary-versus-recurrent difference in tumour type (Fig. 4, Table 1). All five neglected candidates were concordant, including miR-502-3p (TCGA +5.5) and both arms of the miR-501/502 cluster. The minority of discordant calls were dominated by precursor-level arm conflation and by microRNAs whose tissue level tracks immune infiltration, such as miR-150-5p and miR-342-3p, which differs between recurrent and primary disease.

Table 1. Neglected candidates (shaded) and canonical anchors among the corrected discriminants, with the independent TCGA-GBM direction. The full list of 39 discriminants is given in Supplementary Table

S1.

microRNA	Direction	log2FC (corrected)	log2FC (TCGA)	Concordant	Glioma papers
miR-502-3p	up in GBM	+3.60	+5.50	yes	1
miR-501-5p	up in GBM	+4.91	+3.71	yes	3
miR-193b-3p	up in GBM	+2.52	+2.26	yes	4
miR-652-3p	up in GBM	+2.34	+3.88	yes	3
miR-32-5p	up in normal	-5.67	-1.63	yes	2
miR-221-3p	up in GBM	+2.29	+2.67	yes	94
miR-423-5p	up in GBM	+5.84	+4.27	yes	13
miR-338-3p	up in normal	-6.06	-7.01	yes	33
miR-101-3p	up in normal	-4.18	-10.54	yes	39
miR-139-5p	up in normal	-6.18	-0.65	yes	36

Discussion

Our data make one methodological point and one constructive one. The methodological point upends a common intuition: in a small multiplate microRNA study, the normaliser is not the critical choice. With a single per-sample scalar, the global mean and a reference pair are interchangeable up to a constant, so they cannot, by construction, disagree about the direction of change. What decides the result is whether the separate runs are aligned beforehand.

The mechanism is concrete. The four plates of a sample are independent amplification runs whose shared calibrator drifts by up to seven cycles—technical variation that dwarfs most biological effects. Pooling raw thresholds across them lets that drift masquerade as biology, which is exactly what reversed roughly 40% of comparable microRNAs and manufactured the apparent recovery of canonical oncomiRs in the uncorrected analysis. Aligning each plate on a ubiquitous calibrator removes the drift; only then does the downstream normaliser become immaterial. The instruction that follows is simple: align the runs first, normalise second, and report both steps explicitly.⁸

The stability analysis carries its own lesson. A reference must be stable, but stability alone is not enough. miR-16-5p and miR-191-5p both qualify here—stable and, for miR-191-5p, biologically flat—whereas miR-484 scored acceptably yet is itself regulated, so using it would have flattened real

differences. Where a global mean cannot be computed, as in a targeted plasma assay, the safer route is a small panel of endogenous controls selected from the data with geNorm and NormFinder,^{12,13} supplemented where necessary by an exogenous spike-in, rather than a single off-the-shelf control; reference microRNAs validated this way have outperformed conventional small-nuclear-RNA controls in other solid tumours.²⁴ Because microRNA expression is strongly tissue-specific,²⁵ candidate references should be verified in a study's own samples rather than borrowed from another tissue.

Our second contribution—ranking effect size against attention—is deliberately simple and travels well, because it needs only a fold-change and a literature count and applies to any qPCR or sequencing dataset in any tumour. The candidates it returns are leads, not findings. A publication count reflects what has been indexed, not what matters biologically; microRNA names drift over time; and confirmation will demand independent samples and function. The clustered miR-501-5p and miR-502-3p are the most attractive: strongly up in our tumours, up again in TCGA, and almost absent from the glioma literature. That the same pipeline recovered the canonical markers is reassurance that the read-out is sound, not proof that any newcomer is important.

Several limitations bound these conclusions. Three samples per group and a single platform make the separation rule stringent on within-group consistency yet, at three-versus-three, still permissive in type-I terms (about a 10% per-test false-positive rate), which is why we treat the candidate list as a hypothesis rather than a signature and lean on external validation rather than the rule alone. Our external validation works at the precursor level, which conflates the two arms of a microRNA; it rests on only five TCGA normal samples; and it compares our recurrent tumours with a largely primary cohort. Direction is therefore more trustworthy than magnitude, and the recurrent-versus-primary contrast probably explains some of the discordant immune-related calls. None of this overturns the large sequencing-based datasets that defined glioblastoma microRNA biology, in which the normalisation problem largely dissolves; the difficulty we describe is specific to the small, single-platform FFPE series that neuro-oncology still depends on.

The value of the study lies in its explicit checks and two reusable outputs: an inter-run-alignment-first recipe for small multiplate microRNA work, and a short, externally supported list of glioblastoma microRNAs that merit a closer look. For laboratories working with archival blocks and panel assays, the message reduces to three steps—align the runs, then normalise, then screen new hits against what is already known.

Methods

Samples. We studied six archival FFPE specimens: three recurrent glioblastomas (grade 4, 2021 WHO classification²⁶) and three histologically normal brains. A neuropathologist reviewed every block before inclusion, retaining glioblastoma blocks only above 70% tumour content and normal blocks only when free of any abnormality. The study protocol was reviewed and approved by the Comité de Protection des Personnes (CPP) Est III, Nancy, France — an accredited French institutional ethics committee — which

issued a favourable opinion on 15 December 2023 (national registration number ID-RCB 2023-A01732-43; sponsor: CHU de Caen Normandie). The study was conducted under the French loi Jardé as category-3 research involving the human person. All patients gave written informed consent for the research use of their archived tissue, and all experiments were performed in accordance with the relevant guidelines and regulations, including the Declaration of Helsinki.

RNA purification and RT-qPCR. We extracted total RNA, including the small-RNA fraction, after xylene deparaffinisation with the miRNeasy FFPE kit (Qiagen). Reverse transcription used the TaqMan Advanced miRNA cDNA Synthesis kit (Applied Biosystems), which adds a 3' poly-A tail and a 5' adaptor to mature microRNAs before universal reverse transcription and a miR-Amp pre-amplification step. Real-time PCR used TaqMan Fast Advanced Master Mix with the TaqMan Advanced miRNA Human Panel A, four 96-assay plates per sample. We counted a target as detected when an amplification call coincided with a numerical cycle threshold; No Amp, Undetermined and Inconclusive calls were treated as non-detected. The poly-A/ligation chemistry assays mature microRNAs only and returns none of the small nuclear or nucleolar RNAs (RNU48, RNU44, U6) used by older stem-loop assays, so normalisation rested on microRNAs or on the global mean.

Inter-run alignment and normalisation. Within each sample, we aligned every plate on miR-16-5p, present on all four plates ($\Delta Ct = Ct$ of the target minus the plate's miR-16-5p Ct), removing run-to-run drift. As the main normalisation, we then expressed each target relative to the mean ΔCt of all microRNAs detected in that sample across the four plates (per-sample global mean, or mean-centring).^{10,11} For comparison, we re-normalised the same aligned data to a reference pair, the geometric mean of miR-191-5p and miR-484.²⁴ To reproduce the uncorrected pipeline, we also computed the per-sample global mean on the raw cycle thresholds without inter-run alignment.

Reference-gene stability. We scored stability across the 182 microRNAs detected in every sample, on the aligned data. geNorm¹² stability M was the mean standard deviation of pairwise log2 expression ratios; NormFinder¹³ stability came from a two-way (gene-by-group) model combining the estimated intergroup bias with the pooled intragroup variance. We read each candidate's stability against its corrected disease fold-change to separate stable-and-neutral from stable-but-regulated controls.

Discriminant definition. With three samples per group we used a distribution-free rule rather than an under-powered test: a microRNA discriminated the groups when its three tumour values did not overlap its three normal values. Effect sizes are log2 fold-changes of the per-group means; we called a discriminant robust when it met the separation rule with an absolute log2 fold-change above one. Because the rule operates on three-versus-three data, perfect group separation arises in 1 of every 10 random orderings (72 of the 720 permutations of six values, counting both directions), giving a per-microRNA false-positive probability of about 0.10 under the null and predicting on the order of 18 chance separations across the 182 microRNAs tested, before the fold-change filter. The discriminant list is therefore a screen rather than a hypothesis test, and its credibility rests not on the rule alone but on the recovery of canonical glioblastoma biology and on the TCGA-GBM direction concordance, which at 29 of

39 discriminants (74%) exceeds the 50% expected by chance well beyond sampling error (binomial $P \approx 0.002$).

Literature-aware prioritisation. For every discriminant we queried PubMed/MEDLINE (June 2026) as <microRNA> AND (glioma OR glioblastoma) at the precursor/family level and recorded the number of indexed records, plotted on a log scale against the corrected log2 fold-change.

External validation. We retrieved TCGA-GBM miRNA-Seq expression-quantification files from the NCI GDC API (Data Release 45)^{22,23}: 272 primary tumours and 5 solid-tissue normal samples. Precursor-level reads-per-million were log2-transformed (pseudocount 1), and we computed the tumour-minus-normal difference of means per microRNA, summing multi-locus precursors. We assessed direction concordance with the corrected discriminants by sign agreement. Analyses and figures were produced in Python (NumPy, SciPy, pandas, Matplotlib).

Large language model use. During preparation of this manuscript the authors used a large-language-model assistant to support language editing. The authors reviewed and verified all content, data and code, and take full responsibility for the work.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

A.L. N.L. and A.Lc. performed the experiments; A.L. analysed the data and wrote the original draft; M.F. carried out the pathological review; E.E. and G.L. reviewed and edited the manuscript; G.L. supervised the study. All authors approved the final manuscript.

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Data availability

Supplementary Table S1 (the full effect-versus-literature ranking with TCGA directions), Supplementary Figure S1 (per-plate miR-16-5p variance) and the reference-stability scores accompany this article; the raw RT-qPCR data are available from the corresponding author on reasonable request. TCGA-GBM data are openly available from the NCI Genomic Data Commons.

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Figures

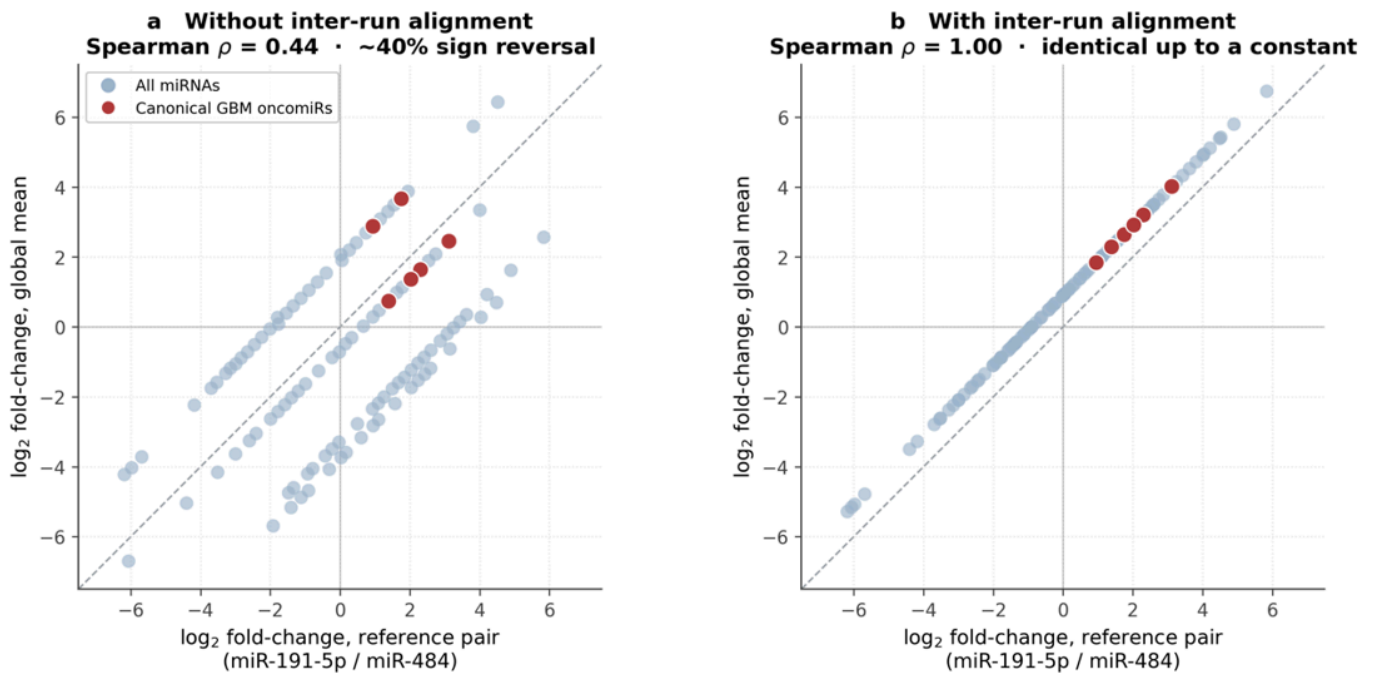


Figure 1

Inter-run alignment is the lever. Log₂ fold-changes (glioblastoma vs normal brain) under the reference pair (x-axis) versus the global mean (y-axis), across microRNAs detected in all six samples; red points are canonical oncomiRs. (a) Without inter-run alignment the two schemes diverge (Spearman rho = 0.44; ~40% reverse direction) and the oncomiRs are displaced. (b) After aligning each plate on miR-16-5p, the two schemes are identical up to a constant (rho = 1.00).

**Reference stability is not biological neutrality
a good reference is stable AND unregulated (lower-left)**

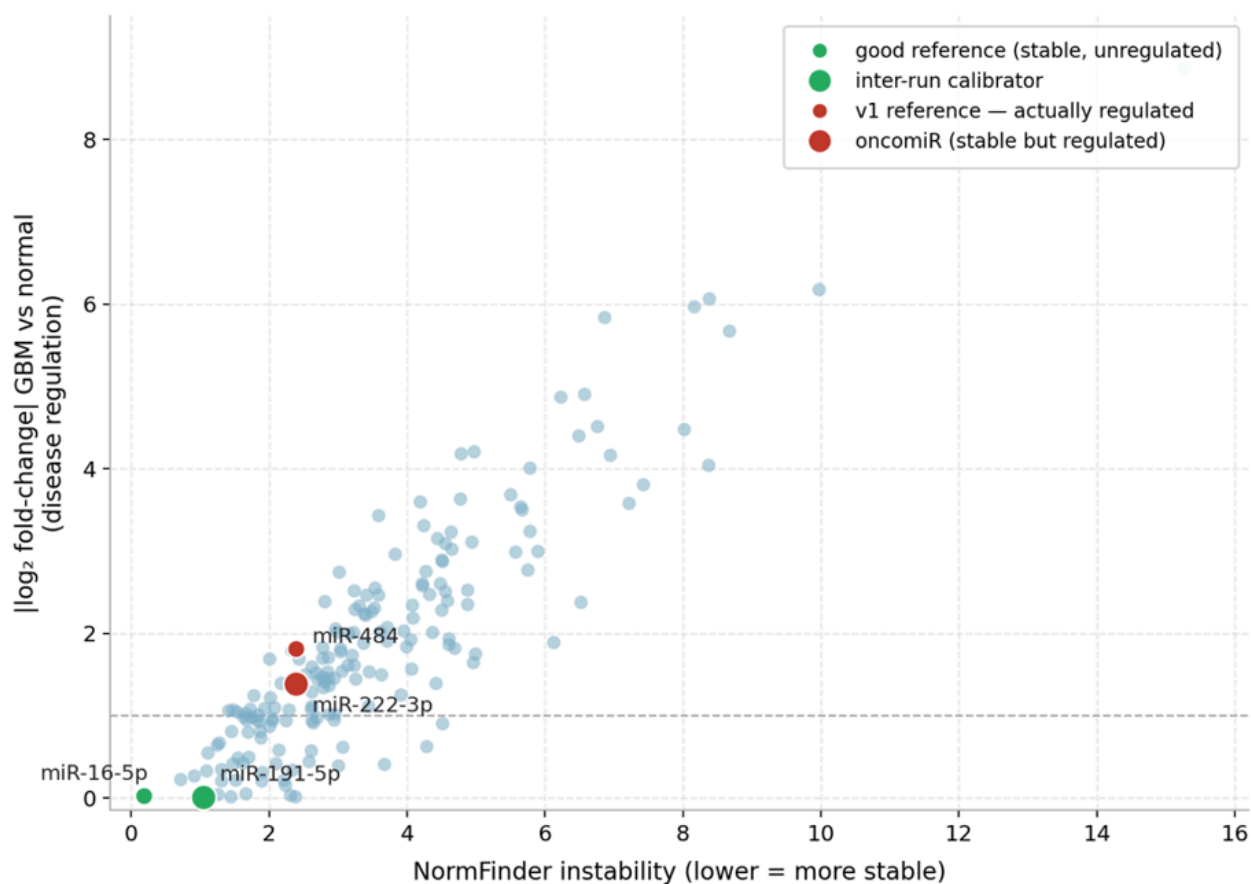


Figure 2

Reference stability is not biological neutrality. NormFinder instability (x-axis; lower = more stable) versus disease regulation (y-axis; absolute log₂ fold-change). A good reference lies at the lower left (stable and unregulated): miR-191-5p and the calibrator miR-16-5p qualify, whereas miR-484 (a reference in the uncorrected analysis) and the oncomiR miR-222-3p are regulated and disqualified.

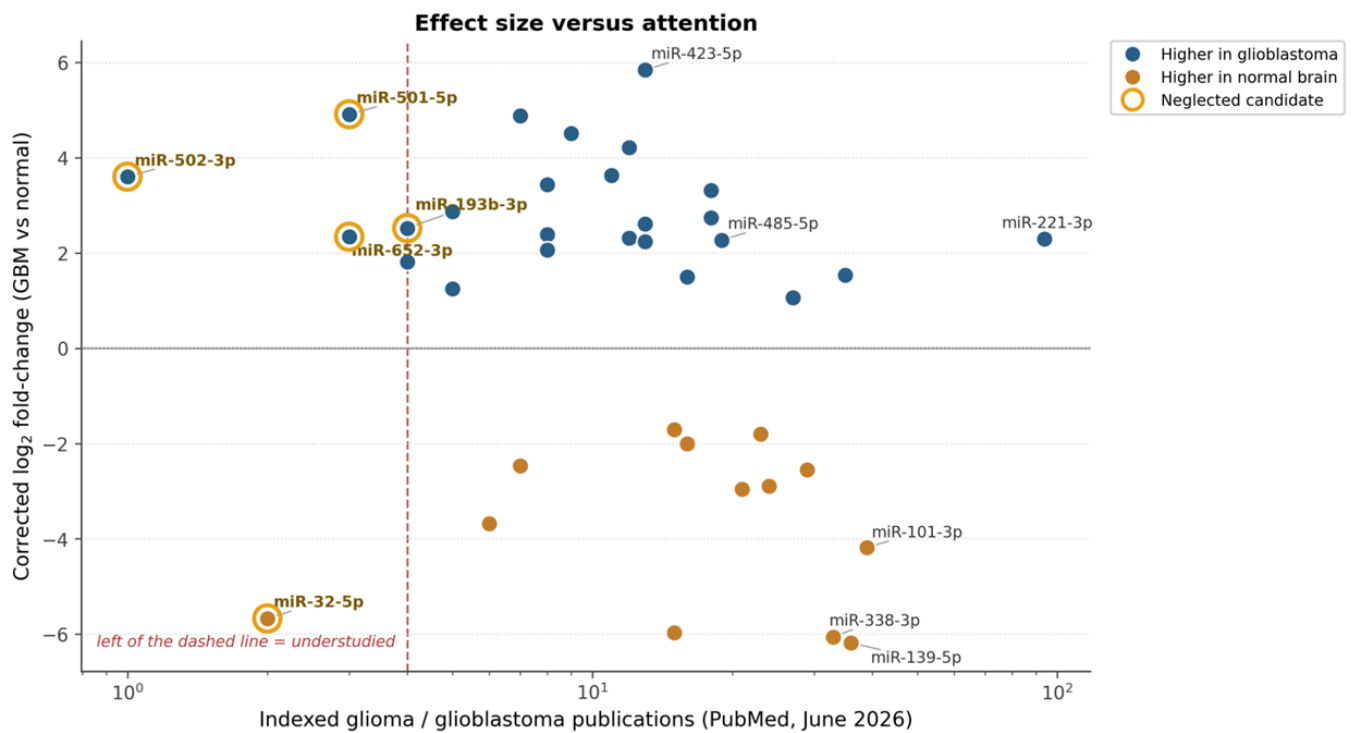


Figure 3

Effect size versus attention. Corrected log₂ fold-change for the 38 robust discriminants against indexed glioma/glioblastoma publications (PubMed, log scale; blue = higher in glioblastoma, orange = higher in normal brain). Points left of the red line are the neglected candidates, including miR-502-3p and miR-501-5p.

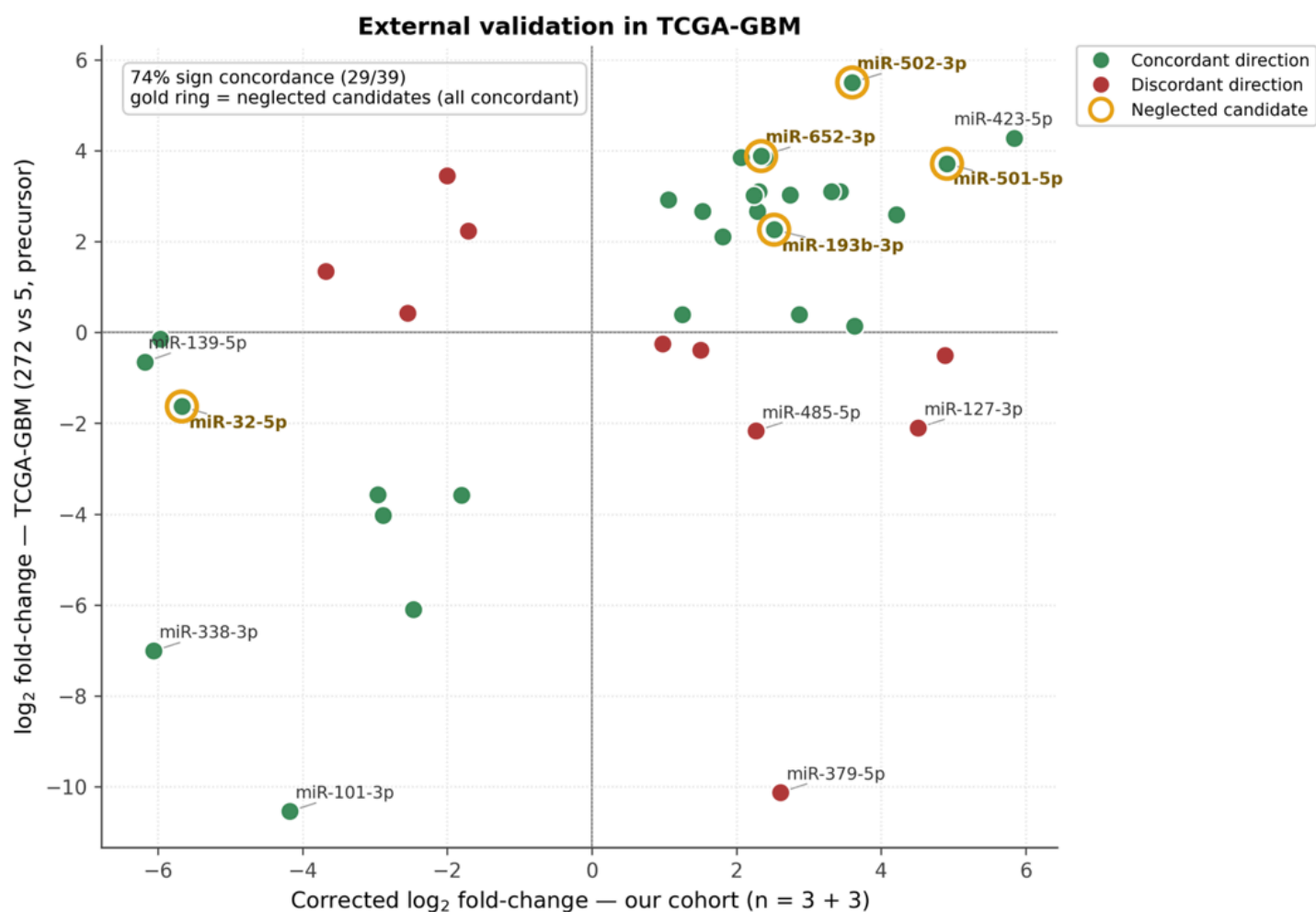


Figure 4

External validation in TCGA-GBM (272 tumours vs 5 normal, precursor level). Our corrected $\log_2$ fold-change (x-axis) versus the TCGA fold-change (y-axis): green = concordant direction, red = discordant; gold-ringed points are the neglected candidates, all concordant. Overall sign concordance 74%.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1.docx](#)