Can miRNA-based real-time PCR be used to classify medulloblastomas?

Vijay Ramaswamy^{1,2,3}, Nardin Samuel^{4,5,6} & Marc Remke^{*,1,2,3}

Medulloblastoma comprises the most common malignant childhood brain tumor. Recent genome-wide profiling approaches and next-generation sequencing studies have resulted in an improved understanding of the biological heterogeneity within this histopathological entity. Although previously considered one disease entity, on the basis of gene expression profiling, four core medulloblastoma subgroups have been defined: wingless (WNT), sonic hedgehog (SHH), Group 3, and Group 4 [1–7]. Several integrated genomic studies have revealed that these subgroups also correlate with distinct patterns of somatic copy number alterations, recurrent somatic mutations, transcriptional and epigenetic signatures, patient demographics, clinical features and outcomes. Survival rates differ dramatically between subgroups, with excellent outcomes observed in WNT patients and a poor prognosis observed in Group 3 patients [8–11]. Intermediate survival rates are typical of SHH and Group 4 patients, despite notable heterogeneity reported within each of these subgroups [1,4,6,11,12]. Several biomarkers allow for prognostic stratification in a subgroup-specific manner, including

MYC amplifications (Group 3), mutations of *TP53* and *TERT* (SHH), and *FSTL5* immunopositivity (Group 4) [12–14]. Due to the profound clinical differences observed between medulloblastoma subgroups, and an increasing interest to devise subgroup specific treatment approaches, robust and reliable subgrouping methodologies are urgently required in the clinical setting.

Several groups have developed subgrouping strategies suitable for archival formalin-fixed, paraffin-embedded (FFPE) samples, which appear more applicable for routine clinical use compared with techniques that require fresh-frozen material. Prospective tissue collection is currently not feasible in some centers and consequently, these centers would not be able to participate in clinical trials that require patient stratification that would be based on molecular analysis of fresh-frozen tissues. Strategies for subgroup prediction from archival FFPE tissue include immunohistochemistry (IHC)-based methods [4,15], targeted gene expression profiling (i.e., NanoString nCounter® System) [16], and DNA methylation profiling (i.e., Illumina methylation arrays) [8, 17].

“With the advent of next-generation sequencing technologies able to identify miRNA transcript levels, and to detect novel tumor subgroup specific miRNA transcripts, miRNA-based subgrouping of medulloblastomas seems a promising future avenue to biologically stratify this heterogeneous disease.”

¹Division of Neurosurgery, Hospital for Sick Children, Toronto, ON, Canada

²Department of Laboratory Medicine & Pathobiology, University of Toronto, Toronto, ON, Canada

³Division of Paediatric Haematology/Oncology, Department of Paediatrics, Hospital for Sick Children, Toronto, ON, Canada

⁴MD/PhD Program, Faculty of Medicine, University of Toronto

⁵Department of Medical Biophysics, University of Toronto

⁶Ontario Institute for Cancer Research, Toronto ON

*Author for correspondence: marc.remke@sickkids.ca

Recently, Kunder and colleagues proposed a real-time PCR-based molecular classification strategy encompassing gene expression analysis of both protein-coding transcripts (mRNAs) and miRNAs [18]. miRNAs are short (18–22 nucleotides) noncoding RNA molecules that fine-tune transcription and translation of up to several hundred target genes. Due to their relatively short length, miRNAs are typically more stable than mRNAs, which denature at room temperature and undergo fragmentation during the process of formalin fixation. Hence, various studies have shown a superior correlation between miRNA expression levels in frozen and FFPE-fixed tissue obtained from the same tumor compared with mRNAs [19,20]. Therefore, miRNAs constitute highly attractive candidate RNA biomarkers for disease classification in a clinical setting. Kunder and colleagues [18] selected subgroup specific markers based on previously published datasets [6,21,22].

A total of nine miRNAs were identified as consistently differentially expressed between medulloblastoma subgroups: miR-23b, miR-135b, miR-148a, miR-182, miR-193a-3p, miR-204, miR-224, miR-365 and miR-592. Specifically, the authors used the TaqMan real-time miRNA assay (Applied Biosystems) on the ABI 7900HT real-time PCR system to determine miRNA expression levels. The evaluation of a miRNA signature using a Taqman based real-time miRNA assay holds the promise of a relatively cost efficient and customizable technical platform if novel subgroup specific markers are identified in the future. Furthermore, individual samples can be immediately processed with a quick turnaround time. Finally, the authors demonstrated that reliable classification of medulloblastoma subgroups can be achieved using their gene panel with an accuracy of 97% determined by predictive analysis of microarrays statistics.

Despite the compelling arguments for using real-time PCR based miRNA detection to distinguish between medulloblastoma subgroups, there are limitations associated with such an experimental approach. The utility of gene signatures consisting exclusively of miRNAs as subgroup specific markers requires further study. Admittedly, subgroup specific expression of several miRNAs have been identified and validated in independent patient cohorts using distinct detection techniques [6,18,21–24]. Examples of validated subgroup specific miRNA biomarkers

comprise up-regulation of miR-23b, down-regulation of miR-182 in SHH tumors, or up-regulation of miR-592 in Group 4 medulloblastomas [6,18,23,24]. However, there are currently no studies in the literature that have demonstrated that miRNA-based signatures alone will enable the identification of all four core medulloblastoma subgroups. The two studies that achieved acceptable discrimination of medulloblastoma subgroups included protein-coding transcripts in their gene expression signature. Northcott *et al.* only included *MYC* and *MYCN* expression levels, [21] and Kunder *et al.* a set of 12 protein-coding genes. miRNA-based subgroup discrimination appears to be particularly difficult due to the lack of positive subgroup markers for SHH and Group 3 tumors. Bai *et al.* clustered 32 primary medulloblastomas solely based on miRNA expression and demonstrated that over 10% of samples clustered differently as compared with genome-wide expression arrays. Furthermore, unsupervised hierarchical clustering suggested a particularly poor separation of Group 3 from Group 4 tumors [23]. Based on the current knowledge of subgroup specific miRNA expression patterns, it appears that a minor subset of protein-coding mRNAs may still be required to achieve accurate subgroup prediction. Therefore, despite the limitations of mRNA analysis on FFPE tissue, concomitant mRNA transcript expression analysis still remains an important consideration for transcriptionally based disease stratification. Indeed, robust and reproducible mRNA analysis from small amounts of FFPE tissue is feasible using the nanoString nCounter.

Taken together, from a technical perspective, we believe that real-time PCR holds a few disadvantages compared to other subgrouping strategies (i.e., nanoString nCounter technology or DNA methylation arrays) including the number of pipetting steps leading to a relatively long handling time per sample and potentially less reproducible results. None of the currently available assays for medulloblastoma subgrouping have yet reached Clinical Laboratory Improvement Amendments certification. The experimental findings in the Kuder *et al.* study, using a PCR-based approach, are highly encouraging and largely confirmed previously described clinical characteristics associated with the four subgroups. These include the bimodal age distribution of SHH tumors, equal gender distribution in WNT tumors, male preponderance in Group 3 and Group 4 tumors, desmoplastic histology in

SHH medulloblastomas, and large-cell anaplasia in Group 3 tumors. In addition, the authors confirmed previously ascribed prognostic implications of medulloblastoma subgroups (WNT > SHH / Group 4 > Group 3; ordered from good to poor prognosis) [5]. The authors also demonstrated a relatively high proportion of WNT tumors (23%), suggesting potentially distinct ethnicity dependent subgroup distribution.

Overall, the real-time PCR assay described by Kunder *et al.* revealed not only biologically, but also clinically relevant observations and validations in a non-overlapping cohort of primary medulloblastomas. With the advent of next-generation sequencing technologies able to

identify miRNA transcript levels, and to detect novel tumor subgroup specific miRNA transcripts, miRNA-based subgrouping of medulloblastomas seems a promising future avenue to biologically stratify this heterogeneous disease.

Financial & competing interests disclosure

M Remke received a fellowship of the Dr Mildred Scheel Foundation/German Cancer Aid. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

- Remke M, Hielscher T, Korshunov A *et al.* Fstl5 is a marker of poor prognosis in non-wnt/non-shh medulloblastoma. *J. Clin. Oncol.* 29, 3852–3861 (2011).
- Remke M, Hielscher T, Northcott PA *et al.* Adult medulloblastoma comprises three major molecular variants. *J. Clin. Oncol.* 29, 2717–2723 (2011).
- Northcott PA, Jones DT, Kool M *et al.* Medulloblastomics: the end of the beginning. *Nat. Rev. Cancer* 12, 818–834 (2012).
- Northcott PA, Korshunov A, Witt H *et al.* Medulloblastoma comprises four distinct molecular variants. *J. Clin. Oncol.* 29, 1408–1414 (2011).
- Ramaswamy V, Northcott PA, Taylor MD *et al.* Fish and chips: the recipe for improved prognostication and outcomes for children with medulloblastoma. *Cancer Genet.* 204, 577–588 (2011).
- Cho Y-J, Tsherniak A, Tamayo P *et al.* Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. *J. Clin. Oncol.* 29, 1424–1430 (2011).
- Taylor MD, Northcott PA, Korshunov A *et al.* Molecular subgroups of medulloblastoma: the current consensus. *Acta Neuropathol.* 123, 465–472 (2012).
- Hovestadt V, Remke M, Kool M *et al.* Robust molecular subgrouping and copy-number profiling of medulloblastoma from small amounts of archival tumour material using high-density DNA methylation arrays. *Acta Neuropathol.* 125, 913–916 (2013).
- Northcott PA, Shih DJ, Peacock J *et al.* Subgroup-specific structural variation across 1,000 medulloblastoma genomes. *Nature* 488, 49–56 (2012).
- Ramaswamy V, Remke M, Bouffet E *et al.* Recurrence patterns across medulloblastoma subgroups: an integrated clinical and molecular analysis. *Lancet Oncol.* 14, 1200–1207 (2013).
- Kool M, Korshunov A, Remke M *et al.* Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of wnt, shh, group 3, and group 4 medulloblastomas. *Acta Neuropathol.* 123, 473–484 (2012).
- Shih DJ, Northcott PA, Remke M *et al.* Cytogenetic prognostication within medulloblastoma subgroups. *J. Clin. Oncol.* 32(9), 886–896 (2014).
- Remke M, Ramaswamy V, Peacock J *et al.* Tert promoter mutations are highly recurrent in shh subgroup medulloblastoma. *Acta Neuropathol.* 126, 917–929 (2013).
- Zhukova N, Ramaswamy V, Remke M *et al.* Subgroup specific prognostic implications of tp53 mutation in medulloblastoma. *J. Clin. Oncol.* 31(23), 2927–2935 (2013).
- Ellison DW, Kocak M, Dalton J *et al.* Definition of disease-risk stratification groups in childhood medulloblastoma using combined clinical, pathologic, and molecular variables. *J. Clin. Oncol.* 29, 1400–1407 (2011).
- Northcott PA, Shih DJ, Remke M *et al.* Rapid, reliable, and reproducible molecular sub-grouping of clinical medulloblastoma samples. *Acta Neuropathol.* 123, 615–626 (2012).
- Schwalbe EC, Williamson D, Lindsey JC *et al.* DNA methylation profiling of medulloblastoma allows robust subclassification and improved outcome prediction using formalin-fixed biopsies. *Acta Neuropathol.* 125, 359–371 (2013).
- Kunder R, Jalali R, Sridhar E *et al.* Real-time PCR assay based on the differential expression of microRNAs and protein-coding genes for molecular classification of formalin-fixed paraffin embedded medulloblastomas. *Neuro Oncol.* 15, 1644–1651 (2013).
- Xi Y, Nakajima G, Gavin E *et al.* Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA* 13, 1668–1674 (2007).
- Liu A, Tetzlaff MT, Vanbelle P *et al.* MicroRNA expression profiling outperforms mRNA expression profiling in formalin-fixed paraffin-embedded tissues. *Int. J. Clin. Exp. Pathol.* 2, 519–527 (2009).
- Northcott PA, Fernandez-L A, Hagan JP *et al.* The mir-17/92 polycistron is up-regulated in sonic hedgehog-driven medulloblastomas and induced by n-myc in sonic hedgehog-treated cerebellar neural precursors. *Cancer Res.* 69, 3249–3255 (2009).
- Gokhale A, Kunder R, Goel A *et al.* Distinctive microRNA signature of medulloblastomas associated with the wnt signaling pathway. *J. Cancer Res. Ther.* 6, 521–529 (2010).
- Bai AHC, Milde T, Remke M *et al.* MicroRNA-182 promotes leptomeningeal spread of non-sonic hedgehog-medulloblastoma. *Acta Neuropathol.* 123, 529–538 (2011).
- Weeraratne SD, Amani V, Teider N *et al.* Pleiotropic effects of mir-183-96-182 converge to regulate cell survival, proliferation and migration in medulloblastoma. *Acta Neuropathol.* 123, 539–552 (2012).