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LABORATORY INVESTIGATION

Single nCounter assay for prediction of *MYCN* amplification and molecular classification of medulloblastomas: a multicentric study

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Abstract

Purpose Medulloblastoma is the most frequent pediatric malignant brain tumor, and is divided into four main subgroups: WNT, SHH, group 3, and group 4. *MYCN* amplification is an important medulloblastoma prognostic biomarker. We aimed to molecular classify and predict *MYCN* amplification in a single assay.

Methods It was included 209 medulloblastomas from 205 patients (Brazil, Argentina, and Portugal), divided into training ($n=50$) and validation ($n=159$) sets. A nCounter assay was carried out using a custom panel for molecular classification, with additional genes, including *MYCN*. nSolver 4.0 software and the R environment were used for profiling and *MYCN* mRNA analysis. *MYCN* amplification by FISH was performed in 64 cases.

Results The 205 medulloblastomas were classified in SHH (44.9%), WNT (15.6%), group 3 (18.1%) and group 4 (21.4%). In the training set, *MYCN* amplification was detected in three SHH medulloblastomas by FISH, which showed significantly higher *MYCN* mRNA counts than non-FISH amplified cases, and a cutoff for MYCN amplification was established ($\overline{X} + 4\sigma = 11{,}124.3$). Applying this threshold value in the validation set, we identified *MYCN* mRNA counts above the cutoff in three cases, which were FISH validated.

Conclusion We successfully stratified medulloblastoma molecular subgroups and predicted *MYCN* amplification using a single nCounter assay without the requirement of additional biological tissue, costs, or bench time.

Keywords Medulloblastoma · NanoString · Molecular subgroups · MYCN amplification · Gene expression

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Abbreviations

ACTB	Actin Beta
CNS	Central nervous system
DAPI	4′,6-Diamidino-2-phenylindole
FFPE	Formalin-Fixed Paraffin-Embedded
FITC	Fluorescein isothiocyanate
FISH	Fluorescence in situ hybridization
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCL	Chloridric acid
LDH	Lactate Dehydrogenase
mRNA	Messenger RNA
MYCN	*MYCN* Proto-Oncogene
SD	Standard deviation
SHH	Sonic hedgehog
SSC	Saline Sodium Citrate
WHO	World health organization
WNT	Wingless
t-SNE	T-distributed stochastic neighbor embedding

Introduction

Medulloblastoma, an embryonal neoplasm of the posterior fossa, is the most frequent malignant brain tumor diagnosed in children, with a peak incidence between 6 and 8 years [1]. It is a biologically heterogeneous central nervous system (CNS) tumor and the World Health Organization (WHO 2016) classified into four molecular subgroups which are associated with different outcomes and therapeutic strategies: WNT-activated, SHH-activated, Group 3 and Group 4 [2]. A more refined medulloblastoma molecular classification have been reported, with several subdivision, such as WNTα, WNTβ, SHHα, SHHβ, SHHγ, SHHδ, Group3α, Group3β, Group3γ, Group4α, Group4β, and Group3γ, which are also associated with distinct clinicopathological and molecular features [3].

The amplification of *MYC* family genes (*MYCC, MYCL*, and *MYCN*) is one of the most common somatic copy-number alterations in cancer [4] being estimated to be present in 28% of cancers [5]. It is a classic mechanism that promotes oncogene hyperactivation [6] and is among the first recurrent genetic alterations described in medulloblastomas [1]. *MYCN* codes an oncogenic transcription factor involved in many biological processes such as cell cycle, apoptosis, the pluripotency of neural stem cells [7], and immune evasion [8], which is essential for the proliferation of cerebellum progenitor cells during both normal and neoplastic cerebellar growth [9, 10]. *MYCN* amplification has been observed in 7% of SHH, 5% of Group 3, 6% of Group 4 medulloblastomas and is associated with dismal outcomes [1]. Therefore, the identification of this genetic alteration could allow more tailored therapeutic strategies [1].

MYCN amplification detection is usually performed by fluorescence in situ hybridization (FISH) [11] with different criteria for its identification, such as the ratio *MYCN*/Control > 2.0; more than eight signals of *MYCN* in more than 10% of tumor cells and/or uncountable tight clusters of *MYCN* signals [12]. The FISH thresholds are not well established [13]. Other techniques such as comparative genomic hybridization (CGH) array [14] and quantitative real-time PCR are also used for *MYCN* amplification analysis [15] but are laborious, time consuming and usually require high amount/quality tumor tissue. Even though the FISH thresholds are not well established and ISH have its caveats, is current a common used method in routine setting [13].

Some of the limitations mentioned above can be overcome by RNA-based platforms, such as the nCounter (NanoString). The nCounter utilizes a multiplex digital barcode technology which allows the precise and direct automatic counting of up to 800 targets [16]. The technique is based on two probes (35–50 bases pairs each) which bind to a specific RNA sequence. These probes are used as a molecular barcode for measuring gene expression, and an imaging detection system performs an individual count of the numbers of each probe that corresponds to each specific mRNA [17]. One of the most significant advantages of nCounter is that it allows the direct detection of RNA without amplification reactions from degraded clinical specimens such as formalin-fixed, paraffin-embedded (FFPE), which are the main resource of tissue samples in clinical laboratory and research centers [17]. Moreover, it is very objective and highly reproducible, even in samples with a meager amount of RNA. Its gene panels can be easily customized and the semi-automatic system avoids manipulation errors, making it an excellent platform for a routine setting [17].

The present study aimed to improve the detection of *MYCN* amplification in medulloblastomas for a routine setting. In this multicentric study, a training and validation cohort of medulloblastomas constituted 209 cases were molecularly classified using RNA-based technology (nCounter). In the same assay, the *MYCN* mRNA levels were evaluated, and the results were correlated with *MYCN* gene amplification by FISH assays.

Methods

Study design

The present study included 209 medulloblastomas from 205 patients diagnosed between 1995 and 2020, from six different institutions: Barretos Cancer Hospital (Brazil), Federal University of São Paulo (UNIFESP, Brazil), AC Camargo Hospital (Brazil), Ribeirão Preto Medical School (Brazil), Italian Hospital of Buenos

Aires (Argentina) and Hospital São João, Porto (Portugal) (Fig. 1). Experienced neuropathologists reviewed all cases and patients' information and nCounter files were stored at RedCap. The Ethics Committee previously approved the study of Barretos Cancer Hospital (CAAE: 59,979,816.6.1001.5437; nº 4.719.466).

The molecular classification of medulloblastoma was performed using a 34-gene nCounter panel, which was based on the previous panel 22 genes validated and performed at Barretos Cancer Hospital, Brazil [18]. This new gene panel, along with the 22 molecular-signature genes, includes nine extra genes, counting *MYCN* as reported [18, 19]. The study was divided into two parts: training ($n = 50$) and validation ($n = 159$) cohorts (Fig. 1).

Training cohort

In the training cohort, 58 Brazilian medulloblastomas with previously molecular subgroup classification (SHH, Group 3, and Group 4) [18] were selected for *MYCN* mRNA analysis and *MYCN* amplification analyses using nCounter and FISH assays, respectively. Eight cases (13.8%) were excluded due to inconclusive or not detected FISH signals. Finally, data of FISH assays (*MYCN* amplification) from 50 medulloblastomas of subgroups SHH, group 3, and group 4 were matched with mRNA counts (nCounter) to evaluate putative associations and cutoff establishment in the validation set (Fig. 1).

Validation cohort

One-hundred and fifty-nine medulloblastoma samples from 155 patients from Brazil ($n = 109$ from 107 patients), Portugal ($n = 37$), and Argentina ($n = 13$, from 11 patients) were included in the validation set. All cases were molecularly classified, and the *MYCN* mRNA expression was assessed by nCounter®. In one Argentinean patient (ID222), we performed nCounter analyses was performed in three samples: two at diagnosis and one at recurrence. Moreover, 14 medulloblastomas from the validation cohort were also evaluated for *MYCN* amplification by FISH (Fig. 1).

Fluorescence in situ hybridization (FISH)

FISH of the training cohort was performed at the Laboratory of Molecular Diagnosis, Barretos Cancer Hospital. Briefly, 4 µm sections were prepared in microscope slides, and the FISH assay ZytoLight® SPEC MYCN/2q11 Dual Color Probe (ZytoVision, Bremerhaven Germany), which contains green probes for *MYCN* gene (2p24.3) and a red probe for *AFF3* at the pericentromeric region of the long arm of chromosome 2 (2q11.2) were used.

Deparaffinization was performed using xylene, dehydration with ethanol (70 and 100%), and saline sodium citrate (SSC) overnight incubation at 60 °C. The enzymatic digestion was performed using pepsin with 0.01 N HCL at 37 °C for 1 min. The slides dehydration process was performed by sequential washes in ethyl alcohol (70%, 80%, and 100%). The slides were incubated in a humidified box for 22 h for

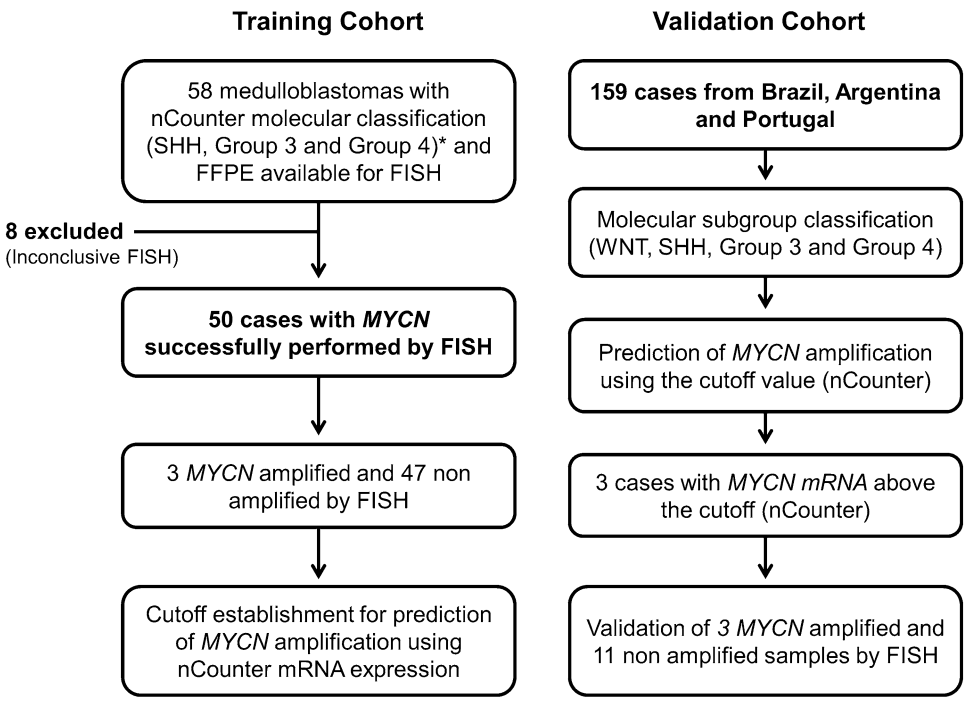

Fig. 1 The study flowchart of medulloblastomas training and validation cohort for molecular subgroups and *MYCN* mRNA counts by nCounter. *Molecular classification previously published [19]

ZytoLight SPEC MYCN/2q11 probes hybridization. Washes with a solution of 2X SSC with 0.3% Igepal (Sigma) and 2X SSC alone were performed at 64 °C and room temperature, respectively, for 4 min. Finally, the nuclei were then visualized with DAPI (blue) (ZytoVision).

The analysis was performed using a fluorescence microscope Nikon Eclipse 50i microscope at × 100 magnification using DAPI, FITC, and Red filters. The image system acquisition was the Applied Spectral Imaging. *MYCN* and control signals were counted in 100 neoplastic cells per sample. Three different criteria for *MYCN* amplification detection were considered: *MYCN*/Control ratio > 2.0; greater than or equal eight signals of *MYCN* in more than 10% of tumor cells and/or uncountable tight clusters of *MYCN* signals [12].

FISH experiments of the validation cohort were performed using three different assays for *MYCN* amplification analysis in 14 medulloblastomas. In one case, FISH assay was performed at Barretos Cancer Hospital, as abovementioned described, four cases at Italian Hospital of Buenos Aires, Argentina (CytoCell FISH probes, REF: LPS 009-S / LPS 009, Cambridge, UK) and nine cases at Hospital São João, Portugal (Kreatech FISH probes MYCN 2p24 / LAF 2q11, Leica Biosystems, USA) following manufactures recommendations.

MYCN mRNA analysis by nCounter

In addition to the 22-gene signature for molecular classification, our customized nCounter custom panel harbors nine medulloblastoma-related genes, including *MYCN* [18, 19]. *MYCN* quality control analysis was performed using the nSolver 4.0 software, and the R statistical environment (version 4.1.0) was used to normalize *MYCN* mRNA of medulloblastomas from training and validation cohorts. Normalization was performed based on the geometric mean of the housekeeping genes *ACTB, GAPDH*, and *LDH*.

The cutoff establishment for prediction of *MYCN* amplification was determined by the arithmetic mean ($\overline{X}$) *of MYCN* mRNA counts of non-amplified samples multiplied by four times the standard deviation (σ): $\overline{X} + 4\sigma$ of *MYCN* mRNA normalized counts.

The IBM SPSS statistics software (Mann–Whitney test) was applied to evaluate statistical differences in *MYCN* mRNA normalized counts between medulloblastoma molecular subgroups and cases with or without *MYCN* amplification.

Results

Cutoff determination of *MYCN* mRNA counts associated with *MYCN* amplified medulloblastomas

We initially included 58 medulloblastomas with conclusive molecular classification and FFPE samples available. Eight cases were excluded due to pre-analytic conditions, leading to the absence of signals or excessive background, which resulted in inconclusive FISH analysis (Fig. 1).

Therefore, the training cohort included 50 medulloblastomas with conclusive results for molecular classification by nCounter and successfully *MYCN* amplification analysis by FISH (Fig. 1, Supplementary Table S1). From these cases, 27 were SHH (54%), six group 3 (12%) and 17 group 4 (34%) (Fig. 2a). The analysis of *MYCN* normalized mRNA counts by nCounter showed three SHH medulloblastomas with high mRNA counts, while the remaining 47 medulloblastomas showed lower *MYCN* mRNA counts (Fig. 2b).

In the FISH analysis, we observed *MYCN* amplification in 3 out of 50 cases (6%) (Fig. 3b and c), and 47 showed no *MYCN* amplification (Fig. 3a). The three medulloblastomas with *MYCN* amplification by FISH showed significantly higher *MYCN* mRNA normalized counts ($\overline{X}$ = 16,402.1, σ = 3042.4) in comparison with 47 medulloblastomas with

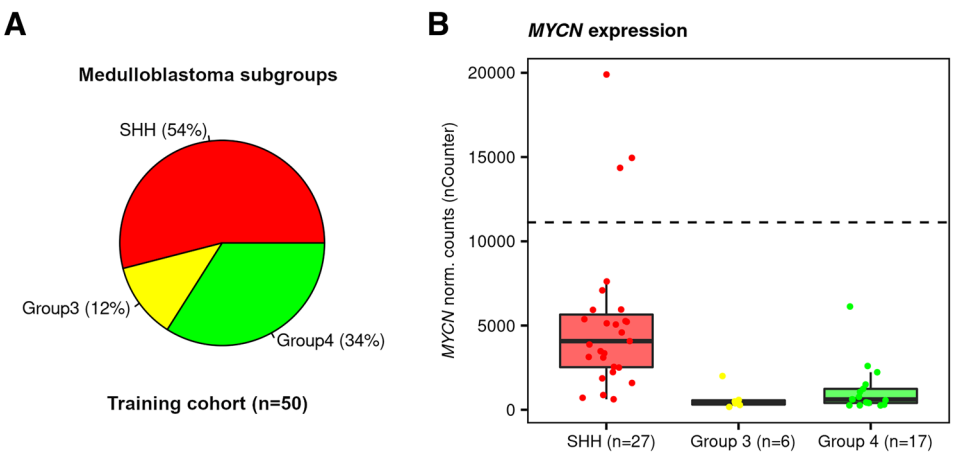

Fig. 2 Training cohort ($n = 50$ medulloblastomas from Brazil). **a** Molecular classification of 50 medulloblastomas included in the training cohort (SHH, Group 3 and 4) from Brazil. **b** *MYCN* mRNA normalized counts in different medulloblastomas subgroups

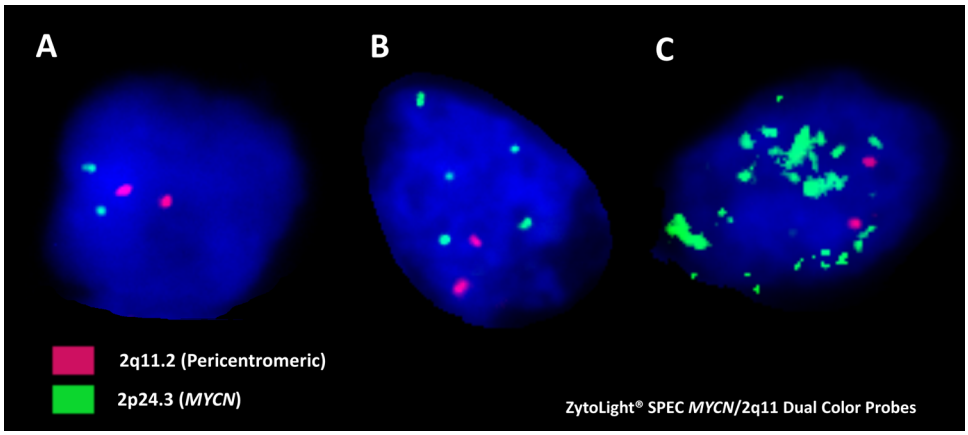

Fig. 3 Representative FISH images of *MYCN* amplification analysis in medulloblastoma samples (ZytoLight® SPEC *MYCN*/2q11 Dual Color Probes). The cancer cell nucleus is colored in blue (DAPI). **a** Non-amplified case: two red nuclear signals for 2q11.2 (control) and two green signals for *MYCN*. **b** *MYCN* amplification determined by ratio *MYCN* (green)/ control (red) signals > 2. **c** *MYCN* amplification is determined by uncountable *MYCN* signals (green)

no *MYCN* amplification ($\overline{X}$ = 2442.6, σ = 2170.4), $p < 0.0001$ (Supplementary Table 1). To establish a cutoff, we used arithmetic mean + 4σ of non-amplified cases, leading to an expression value of 11,124.3 (Fig. 2b).

Validation of *MYCN* mRNA cutoff for *MYCN* amplification prediction in a cohort of 159 medulloblastomas

Using the cutoff value of 11,124.3 established for the prediction of *MYCN* amplification, we validated this mRNA *MYCN* threshold in a cohort of 159 additional cases from 155 patients, from four Brazilian institutions ($n = 109$ from 107 patients), one Portuguese ($n = 37$), and one Argentinean institution ($n = 13$ from 11 patients).

The analysis of the molecular subgroups showed that 65 (41.9%) medulloblastoma were SHH, 32 (20.6%) WNT, 31 (20.0%) group 3, and 27 (17.4%) group 4 (Fig. 4a). Applying the cutoff value of normalized *MYCN* mRNA counts, we detected three cases whose counts were above to the established value, being one case from Brazil (group 4) (Fig. 4b), and two SHH cases from Portugal (Fig. 4c) and Argentina (SHH) (Fig. 4d). The FISH analysis of the Portuguese (ID 269, data not shown) and Argentina (ID 222c) cases were done at the Laboratory of origin and confirmed the presence of *MYCN* amplification. The FISH analysis of the third case (ID123) was performed at Barretos Cancer Hospital and also depicted *MYCN* amplification (Fig. 5). Moreover, in 11 cases with *MYCN* mRNA below the cutoff, the FISH analysis corroborates the absence of *MYCN* amplification (Supplementary Table 2).

Interestingly, in one patient from Argentina (ID 222), we performed the nCounter analysis of molecular subgroup and *MYCN* mRNA counts in three samples, including two at diagnosis (222a and 222b) and one at recurrency (222c). Both nCounter and FISH methodologies showed the presence of *MYCN* amplification only in the recurrence biopsy (Fig. 5, Supplementary Table 2). Patients ID198 and ID211 had two different tumor samples from diagnosis each one. We observed variation in *MYCN* mRNA counts between different tumor samples of the same patient (ID198_A9 = 2132.2, ID198_A10 = 1078.8; ID211_A1 = 4211.2 and 211_A2 = 5766.8). However in both cases the counts were below the establish threshold. The molecular subgroup observed was SHH for both cases and did not change between different tumor samples from each patient. One note, all eight cases that were excluded from the training set due to the inconclusive FISH results were further included in the validation set and exhibited values of *MYCN* mRNA levels below the threshold, suggesting the absence of *MYCN* amplification.

Overall, 209 cases from 205 patients, male/female rate: 1,4, median age of 11 years (0–56), were classified molecularly and assessed for *MYCN* mRNA counts. The t-distributed stochastic neighbor embedding (t-SNE) according to molecular classification ($n = 205$) and the age distribution ($n = 195$, 10 missing) < = 3 (Infant, $n = 22$), > 3–18 (Child, $n = 117$) and > 18 years old (Adult, $n = 56$), is shown in Supplementary Fig. S1.

We found 6/209 cases (2.87%) with *MYCN* mRNA counts above the established cutoff, five from the SHH subgroup and one from group 4 (Supplementary Fig. S2). All six medulloblastomas with *MYCN* mRNA counts above the cutoff showed *MYCN* amplification by FISH.

Overall, 64 samples were evaluated by both nCounter and FISH assays, six *MYCN* amplified and 58 non-amplified medulloblastomas. All samples with *MYCN* amplification showed *MYCN* mRNA counts above the cutoff, and every case without amplification had values below the cutoff, leading to a positive predictive value of 100% of *MYCN* amplification by nCounter.

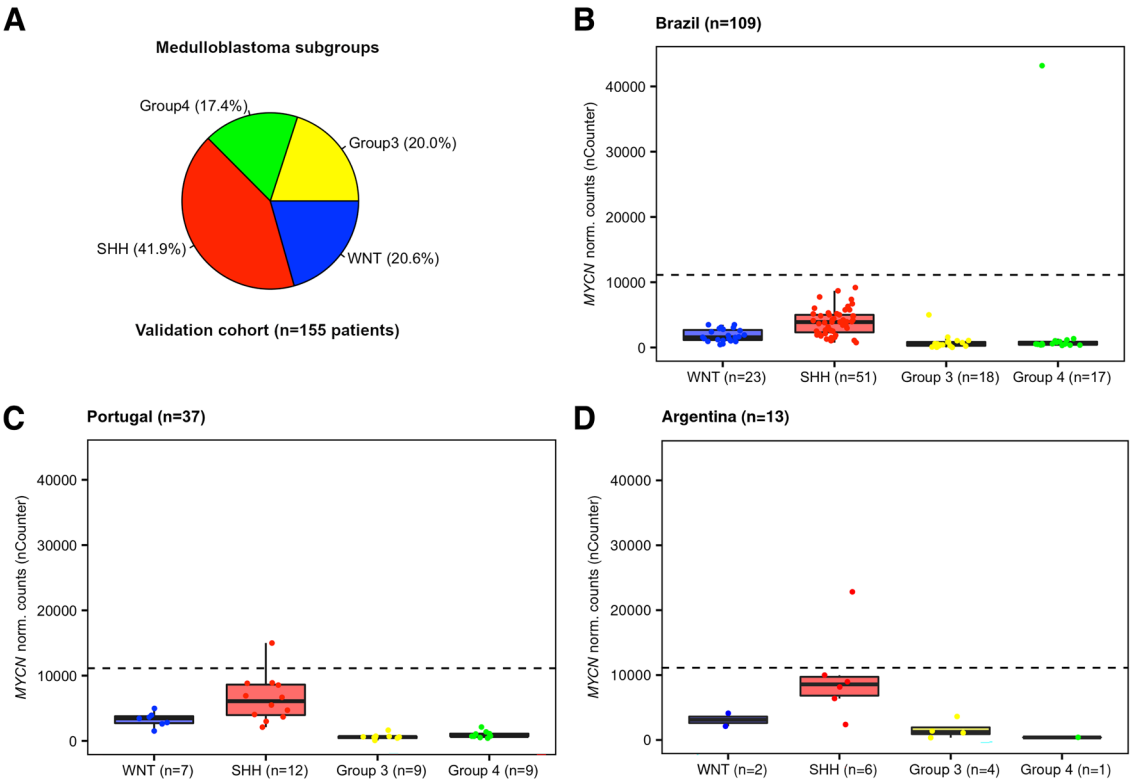

Fig. 4 Validation cohort ($n=159$ medulloblastomas/155 patients from Brazil, Portugal, and Argentina). **a** Molecular classification of 155 medulloblastoma patients included in the validation cohort (WNT, SHH, Group 3 and 4). **b** *MYCN* mRNA normalized counts in medulloblastomas samples from Brazil ($n=110$). **c** Portugal ($n=39$), and **d** Argentina ($n=13$)

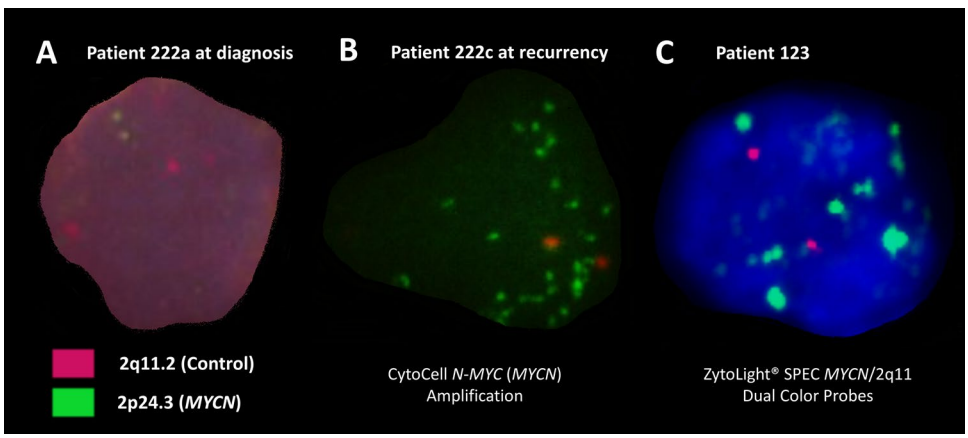

Fig. 5 FISH analysis of medulloblastomas from validation cohort. **a** No *MYCN* amplification in patient 222 at surgery (222a Argentina). **b** *MYCN* amplification in patient 222 at recurrence (222c Argentina). **c** *MYCN* amplification was confirmed in Patient 123 (AC Camargo, Brazil)

Discussion

In the present multicenter study, we showed that an RNA-based technology (nCounter) could be easily applied to simultaneously undertaking the molecular classification of medulloblastoma and predict *MYCN* amplification. Overall, the 205 medulloblastomas were classified in WNT (20.6%), SHH (41.9%), group 3 (20.0%), and group 4 (17.4%). These frequencies are in line with the frequencies reported in the literature [20].

Along with molecular classification, we analyzed the presence of *MYCN* amplification by FISH and *MYCN* mRNA levels by nCounter assays. We identified a significant association between *MYCN* mRNA counts and gene amplification. The cutoff value to predict *MYCN* amplification was initially established in the training cohort of 50 cases, using the arithmetic mean of *MYCN* mRNA counts of non-amplified

samples plus four standard deviations, which led to a value of 11,124.3. This cutoff was further applied in a validation cohort of 159 medulloblastomas of different countries. In the training and validation medulloblastoma cohorts from Brazil, Argentina, and Portugal, we have found *MYCN* mRNA counts above the cutoff in 5.4% (5/92) of SHH and 2.3% (1/44) group 4 medulloblastomas. All six cases also showed *MYCN* amplification by FISH assay. In addition, in 61 cases with below the cutoff value, the FISH also corroborates the absence of *MYCN* amplification, leading to a concordance of 100% among these two methodologies.

A recent study of our group described a failure rate of 5.6% of nCounter technology in RNA analysis from FFPE biopsies [21]. On the other hand, we observed a FISH failure rate of 14% (8/58—training cohort) in the analysis of FFPE tumor specimens. This caveat of FISH is well reported, and the use of the nCounter technology in FFPE tissue can overcome several FISH pitfalls and allow the determination of *MYCN* status in a higher number of routine cases, as it was observed in these eight cases, which were inconclusive by FISH assay. Moreover, the FISH assay is rather laborious, not automatized, and a time-consuming methodology that has other disadvantages such as tissue sample variability, signal fading, cytologic artifacts, limited detection of large alterations, and discrepancies between different observers are reported [22, 23]. Therefore, this nCounter approach has significant advantages compared with FISH for *MYCN* evaluation.

An association of gene amplification with high mRNA counts by nCounter technology was recently described in lung cancer [24]. The authors showed that 92% of patients with very-high *MET* mRNA also exhibited gene amplification by FISH and/or next-generation sequencing (NGS), suggesting that nCounter can be more feasible to patient selection for target therapy [24].

In the present study, we observed concordance of *MYCN* amplification by FISH and higher mRNA by nCounter in all medulloblastomas evaluated. Interestingly, one case from Argentina showed *MYCN* amplification and high mRNA at tumor recurrence, but not at diagnosis three years earlier. As expected, the molecular subgroup SHH did not change at the recurrence. This result follows a study that reported two cases in which *MYCN* amplification was not present in the diagnosis but was detected only at tumor recurrence in 5 and 7.5 years after diagnosis [25]. Recently a study evaluating paired medulloblastomas samples from both diagnosis and relapse, described *MYCN* amplification as an important biomarker of tumor relapse [26].

Our findings of *MYCN* amplification frequencies are in accordance with the literature data, which have been reported *MYCN* amplification in 7% of SHH cases, 5% in group 3, and 6% in group 4 medulloblastomas [1]. More recently, *MYCN* amplification was reported to be associated with SHHα and group4α medulloblastoma subgroups, each associated with worse outcomes [3].

Herein, we have found that *MYCN* mRNA expression was significantly higher in the SHH subgroup than WNT, Group 3 and 4, which is in accordance with previous studies from our [19]and other groups [27]. *MYCN* amplification [11, 27, 28], and *MYCN* overexpression are reported to be associated with adverse prognosis in medulloblastomas [10]. The present study did not analyze the impact of *MYCN* expression on prognostication, which will be performed in subsequent studies with a higher number of cases.

In addition to medulloblastoma, the evaluation of *MYCN* amplification is clinically relevant since it is often associated with adverse patient outcomes in other pediatric brain tumors such as high-grade gliomas, pineal region tumors, atypical teratoid rhabdoid tumors, ependymomas [28]. In fact and direct or indirect *MYCN* target therapy is a promising anticancer strategy which is under investigation [28]. Further studies should be performed to explore the potential of *MYCN* mRNA evaluation by nCounter to predict *MYCN* amplification in other tumor types as well as to investigate the putative associations of mRNA counts with other recurrent gene amplifications found in medulloblastomas such as *GLI1, YAP1* present in SHHα subgroup, *MYCC* amplification in Group 3γ and *CDK6* amplification observed in Group 4α [3].

The introduction of nCounter technology in the clinical practice for molecular oncology provides a wide range of advantages since it allows a rapid, reliable, and reproducible assessment of gene expression from a variety of biological samples, such as FFPE, serum, plasma, liquid base cytology [29–32]. Moreover, the nCounter is a multiplex assay that allows the assessment of up to 800 targets using a low amount of RNA, usually 50–300 ng [33]. The use of nCounter should also offer a cost-effective alternative to evaluate many targets simultaneously [17]. Nevertheless, the nCounter technologies have some challenges for clinical and routine application. One challenge is the data normalization and analysis, which requires trained staff for this purpose [17]. Furthermore, nCounter is an emerging technology available in a few oncologic hospitals and research centers, especially in underdeveloped countries where this technology is expensive and often inaccessible.

In conclusion, incorporating *MYCN* mRNA analysis in routine will provide a practical method to predict *MYCN* amplification in FFPE medulloblastoma samples without additional costs or necessity of biological material to improve the patient risk stratification and provide more molecular data for future tailored therapies.

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Author contributions DAM (ORCID 0000–0002-0764-447X): Data collection, experimental procedures, data analysis, manuscript writing. LSdS (ORCID 0000–0002-7470–4655): Data analysis, prognostic results discussion, manuscript review. MFZ: FISH experiments, data analysis, and manuscript review. MB: nCounter experiments, manuscript review. FEdP: nCounter experiments, manuscript review. IVVS: Pathological review of tumor samples, results, discussion, manuscript review. GRT: Pathological review of tumor samples, results, discussion, and manuscript review. MdMM: Pathological review of tumor samples, manuscript review. FS: Pathological review of tumor samples, results, discussion, and manuscript review. LNS: Pathological review of tumor samples from Ribeirão Preto Medical School, Ribeirão Preto, São Paulo, Brazil. JNS: Pathological review of tumor samples, results, discussion, and manuscript review. SMFM: Clinical review of cases, results, discussion, and manuscript review. ML: Clinical review of cases, results, discussion, and manuscript review. GNMH: Clinical review of cases, results, discussion, and manuscript review. HG-R (ORCID 0000–0003-2056-464X): Pathological review of tumor samples, results, discussion, and manuscript review. SC: Clinical review of cases, results, discussion, and manuscript review. MJGdC: Clinical review of cases, results, discussion, and manuscript review. SN: Clinical review of cases, results, discussion, and manuscript review. MJS: FISH experiments, data analysis, and manuscript review. JP: Pathological review of tumor samples, results, discussion, and manuscript review. CAJ: Clinical review of cases, results, discussion, and manuscript review. BMM: Clinical review of cases, results, discussion, and manuscript review. RMR (ORCID 0000–0002-9639–7940): Supervisor and project coordinator, results, discussion, manuscript writing, and review.

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

Declarations

Conflict of interests The authors of the present study declare that they have no conflicts of interests**.**

Ethical approval The institutional review board approved this retrospective study from Barretos Cancer Hospital (CAAE: 59979816.6.1001.5437 Number: 4.719.466).

Consent to participate Not applicable.

Consent to publication Not applicable.

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