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Corresponding Author	FamilyName	**Simone**
	Particle	
	Given Name	**Minasi**
	Suffix	
	Division	Department of Radiological, Oncological and Anatomo-Pathological Sciences
	Organization	“Sapienza” University of Rome
	Address	Viale Regina Elena, 324-00161, Rome, Italy
	Phone	
	Fax	
	Email	simone.minasi@uniroma1.it
	URL	
	ORCID	
Author	FamilyName	**Francesca**
	Particle	
	Given Name	**Gianno**
	Suffix	
	Division	Department of Radiological, Oncological and Anatomo-Pathological Sciences
	Organization	“Sapienza” University of Rome
	Address	Viale Regina Elena, 324-00161, Rome, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Lavinia**
	Particle	
	Given Name	**Bargiacchi**
	Suffix	
	Division	Department of Radiological, Oncological and Anatomo-Pathological Sciences
	Organization	“Sapienza” University of Rome
	Address	Viale Regina Elena, 324-00161, Rome, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Valeria**
	Particle	

	Given Name	**Barresi**
	Suffix	
	Division	Department of Diagnostics and Public Health, Section of Anatomic Pathology
	Organization	University of Verona
	Address	Verona, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Evelina**
	Particle	
	Given Name	**Miele**
	Suffix	
	Division	Department of Oncology/Hematology, Gene and Cell Therapy and Hemopoietic Transplant
	Organization	Bambino Gesù Children’s Hospital, IRCCS
	Address	Rome, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Manila**
	Particle	
	Given Name	**Antonelli**
	Suffix	
	Division	Department of Radiological, Oncological and Anatomo-Pathological Sciences
	Organization	“Sapienza” University of Rome
	Address	Viale Regina Elena, 324-00161, Rome, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Romana**
	Particle	
	Given Name	**Buttarelli Francesca**
	Suffix	
	Division	Department of Radiological, Oncological and Anatomo-Pathological Sciences
	Organization	“Sapienza” University of Rome
	Address	Viale Regina Elena, 324-00161, Rome, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

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Abstract

Medulloblastomas (MDBs) are classified into molecular groups showing peculiar immunohistochemical

and genetic features and distinct DNA methylation profile. Group 3 and group 4 MDBs have the worst prognosis; the former is treated with high-risk protocols and features *MYC* amplification, whereas the latter receives standard-risk protocols and harbors *MYCN* amplification. Herein, we report a unique case of MDB showing histological and immunohistochemical features consistent with non-SHH/non-WNT classic MDB, with both *MYCN* (30% of tumor cells) and *MYC* (5–10% tumor cells) amplification in distinct subclones of neoplastic cells at fluorescence in situ hybridization (FISH), characterized by specific patterns. In spite of *MYC* amplification in only a small percentage of tumor cells, this case had DNA methylation profile consistent with group 3, emphasizing the importance to test both *MYC* and *MYCN* amplifications at a single cell level using highly sensitive methods, such as FISH, for diagnostic and therapeutic purposes.

Keywords (separated by '-')	Medulloblastoma - *MYC* - *MYCN* - Methylation profile - FISH
Footnote Information	Gianno Francesca contributed equally to this work.

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BRIEF REPORT

Case report of a pediatric medulloblastoma with concurrent *MYC* and *MYCN* subclonal amplification in distinct populations of neoplastic cells

Minasi Simone[1] · Gianno Francesca[1] · Bargiacchi Lavinia[1] · Barresi Valeria[2] · Miele Evelina[3] · Antonelli Manila[1] · Buttarelli Francesca Romana[1]

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Abstract

Medulloblastomas (MDBs) are classified into molecular groups showing peculiar immunohistochemical and genetic features and distinct DNA methylation profile. Group 3 and group 4 MDBs have the worst prognosis; the former is treated with high-risk protocols and features *MYC* amplification, whereas the latter receives standard-risk protocols and harbors *MYCN* amplification. Herein, we report a unique case of MDB showing histological and immunohistochemical features consistent with non-SHH/non-WNT classic MDB, with both *MYCN* (30% of tumor cells) and *MYC* (5–10% tumor cells) amplification in distinct subclones of neoplastic cells at fluorescence in situ hybridization (FISH), characterized by specific patterns. In spite of *MYC* amplification in only a small percentage of tumor cells, this case had DNA methylation profile consistent with group 3, emphasizing the importance to test both *MYC* and *MYCN* amplifications at a single cell level using highly sensitive methods, such as FISH, for diagnostic and therapeutic purposes.

Keywords Medulloblastoma · *MYC* · *MYCN* · Methylation profile · FISH

Introduction

Medulloblastoma (MDB) represents a heterogeneous class of embryonal tumors, arising from stem cells or granule neuron progenitor and frequently growing into the fourth ventricle. It is histologically classified into four subtypes, named classic, desmoplastic/nodular, with extensive nodularity, and large cell/anaplastic [1–3], and into four molecularly defined groups, called wingless (WNT), sonic hedgehog (SHH), and groups 3 and 4. MDBs of different molecular groups arise from different cells-of-origin and show peculiar mutations, copy number variations (CNVs), and transcriptional and methylation profiles [3–5]. The integration of histological and molecular features of MDB allows stratifying patients into different risk categories [4–7]. However, gene expression and DNA methylation profiling demonstrated a high heterogeneity in the MDBs of the same molecular group [4, 8]. Therefore, clinical trials are currently investigating individualized therapeutic strategies [4–8].

The deregulation of proteins belonging to the MYC family (*MYC*, *MYCN*, *MYCL*) characterizes many different tumor types. Indeed, 28% of samples in the Cancer Genome Atlas (9000 samples across 33 different tumor types) harbor the alteration of at least one of the three MYC proteins [9]. The upregulation of C-MYC and N-MYC expression can descend from several genetic alterations, including point mutations, amplification, translocation, or activating mutations [9–11], though it is mainly due to gene amplification.

In pediatric MDB, somatic *MYC* or *MYCN* amplification represents frequent driver genetic events [3–5, 12, 13]. *MYC* amplification is associated with shorter survival in all molecular groups, although it is mostly observed in MDB of molecular Group 3 [3–5, 12–14]. *MYCN* amplification

Gianno Francesca contributed equally to this work.

Minasi Simone
simone.minasi@uniroma1.it

[1] Department of Radiological, Oncological and Anatomo-Pathological Sciences, "Sapienza" University of Rome, Viale Regina Elena, 324-00161 Rome, Italy

[2] Department of Diagnostics and Public Health, Section of Anatomic Pathology, University of Verona, Verona, Italy

[3] Department of Oncology/Hematology, Gene and Cell Therapy and Hemopoietic Transplant, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

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is also associated with poor clinical outcome, though better than that seen in tumors with *MYC* amplification, and it is mainly found in MDB of SHH group and group 4 [3–5, 12–14]. *MYC* and *MYCN* amplifications are considered mutually exclusive in MDB and in other cancers [9, 15, 16].

In this report, we present an unusual case of medulloblastoma, epigenetically classified as group 3, harboring *MYC* and *MYCN* amplifications in different neoplastic subclones.

Case presentation

A 4-year-old male referred to the Azienda Ospidaliera Integrata Verona (Italy) for vomiting, cephalea, and walking deficit. Computerized tomography and magnetic resonance imaging (MRI) disclosed a solid lesion, 3 × 3 × 5 cm in size, in the fourth ventricle. The mass adhered to the cerebellar vermis and had inhomogeneous contrast enhancement (Fig. 1A). There was a leptomeningeal enhancement in the conus medullaris, which was consistent with leptomeningeal dissemination. The patient was submitted to surgical resection of the mass. Post-surgical MRI did not show enhancing areas in the surgical bed. Fifteen days later, the patient started chemotherapy protocol, consisting in a first cycle with methotrexate and vincristine, and a second one with etoposide. One month later, he was administered a third chemotherapy cycle with carboplatin and vincristine.

The FFPE block of the tumor sample was sent to Umberto I° Policlinico in Rome, Italy, as part of the centralization of pediatric brain tumors. Histologically, the tumor was classified as MDB, classic subtype (Fig. 1C), on the basis of morphological evaluation by Hematoxylin–Eosin (H&E) and reticulin staining.

Immunohistochemistry (IHC) was carried out using streptavidin–biotin–immunoperoxidase protocol on an automated immunostainer (Leica Bond III).

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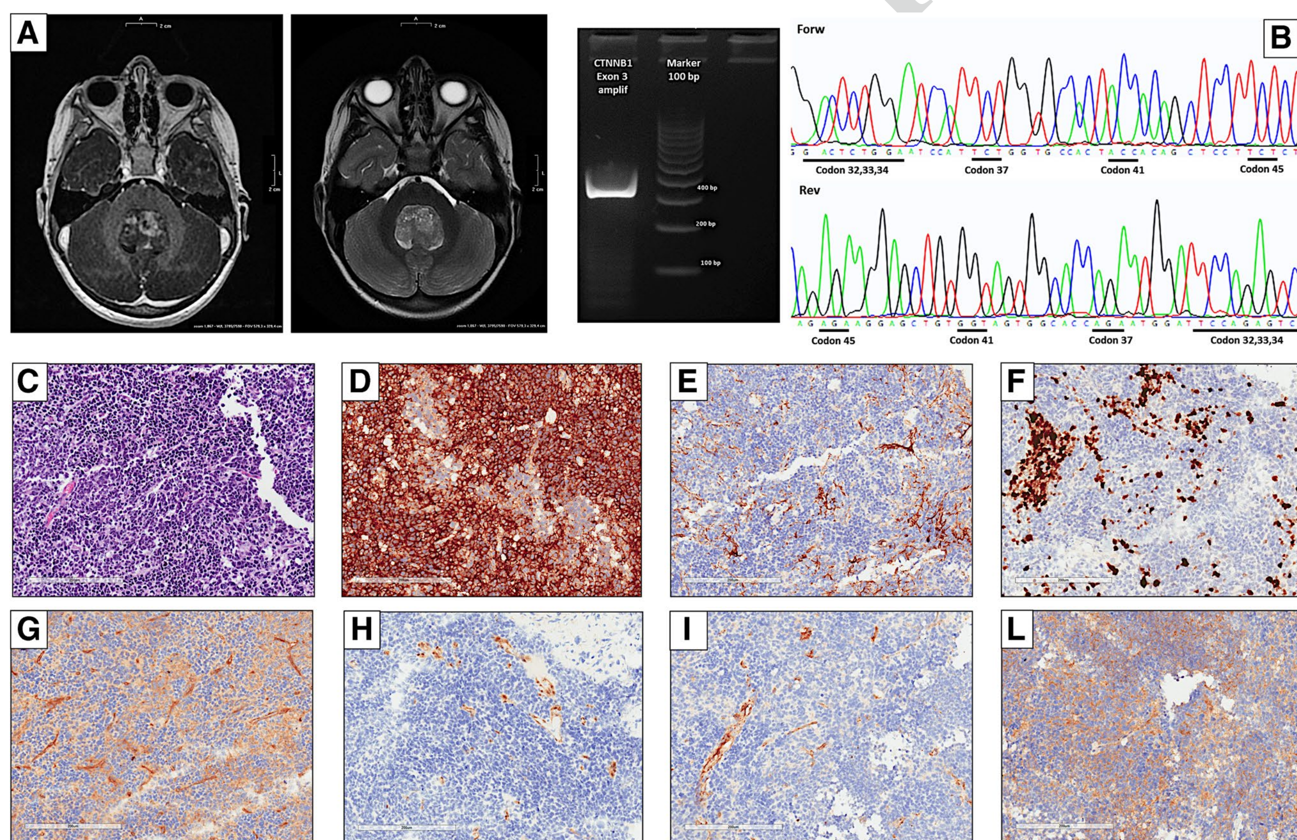

Fig. 1 **A** MRI appearances of a solid lesion of 3 × 3 × 5 cm in size in a 4-year-old child on transverse contrast-enhanced T1-weighted (left) and T2-weighted (right) images, evidencing inhomogeneous contrast enhancement adhered to the cerebellar vermis in the fourth ventricle, typical location of MDB. **B** PCR amplification of the exon 3 of *CTNNB1* and bidirectional Sanger sequence traces do not show mutations; codons most commonly involved in tumorigenesis of MDB as 32, 33, 34, 37, 41, and 45 are highlighted. **C**–**L** Representative H&E shows a classic histology, characterized by small round nuclei without increase cells size, absence of desmoplasia, Homer Wright rosettes, and nodules (**C**). Immunohistochemical panel evidences synaptophysin strong immunopositivity (**D**), GFAP negativity (**E**), Ki67 index of around 30% (**F**), GAB1, YAP1, and Filamin A triple negativity (**G**–**I**), while β-catenin is positive in the cytoplasm and immunonegative in the nucleus (**L**)

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Tumor cells showed diffuse positivity for synaptophysin (Fig. 1D) and only focal positivity for GFAP (Fig. 1E). The medium Ki67 labeling index was 30% (Fig. 1F), though multiple areas showed a higher index. Expression of p53 was absent. Negativity for GAB1, YAP1, and Filamin A (Fig. 1G–I) excluded SHH subgroup, whereas the lack of nuclear positivity for β-catenin (Fig. 1L) or *CTNNB1* mutations at exon 3 on Sanger sequencing (Fig. 1B) excluded WNT subgroup. Based on GAB1, YAP1, Filamin A, and β-catenin expression, the tumor was diagnosed as non-SHH/non-WNT classic medulloblastoma.

MYC and *MYCN* copy number variations were analyzed using fluorescence in situ hybridization (FISH) with *MYC* (8q24.21) orange/CEP8 green and *MYCN* (2p24.3) orange/CEP2 green probes, according to protocols and manufacturer instructions (Empire Genomics). Signals were counted with Axio Imager M1 microscope (Carl Zeiss) in 200 nuclei for each sample. Locus-specific/CEP signal ratio between 1 and 2 was considered as gain, a signal ratio > 2 was considered as amplification; in particular, > 10 orange signals vs. 2 green signals per diploid genome were scored as a high copy number amplification.

MYC was amplified in around 5–10% of cells (close to cut-off of 10%) and *MYCN* in around 30%. However, the two genes displayed different amplification pattern. *MYC* amplification was present in single and dispersed neoplastic cells and was characterized by innumerable orange signals (> 20) throughout the interphase nucleus, often distributed in microclusters, compared with 2/4 signals of reporter probe (Fig. 2A). On the other hand, *MYCN* amplification was present in large neoplastic regions, characterized by homogeneous high-level amplification with around 10 orange signals in average compared with 2/4 signals of reporter probe (Fig. 2B). To simultaneously analyze the two genes, we combined the two probes with four different fluorophores via multicolor-FISH: MYC red/CEP8 gold and MYCN green/CEP2 aqua (Empire Genomics). As shown in Fig. 2C, *MYC* and *MYCN* are never amplified in the same cells but rather in distinct subclones.

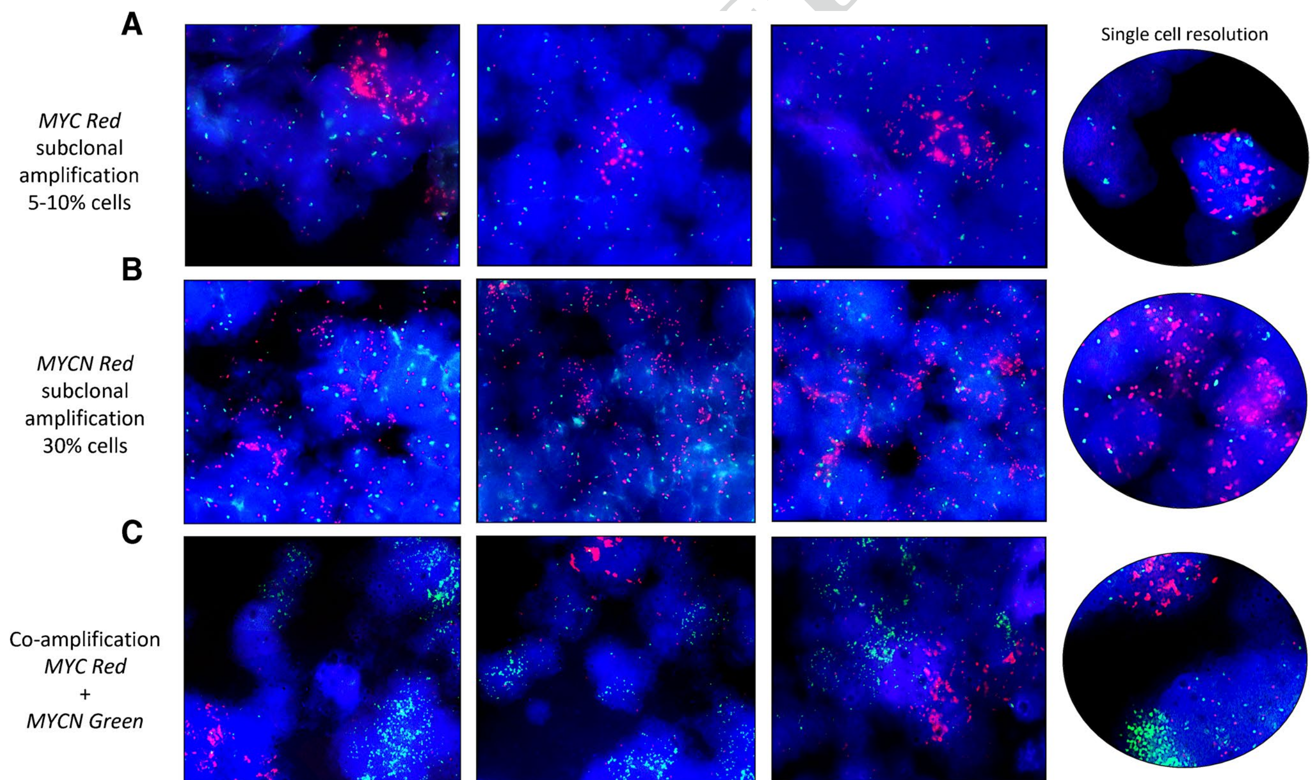

Fig. 2 **A** FISH panel of *MYC* with dual-color probe (Empire MYC/CEP8 spectrum O/G) shows subclonal amplification in single neoplastic cells (5–10% of cells in total), with an innumerable dispersed signals often distributed in microclusters. **B** FISH panel of *MYCN* with dual-color probes (Empire MYCN/CEP2 Spectrum O/G) shows subclonal amplification in large neoplastic regions (30% of cells in total) characterized by homogeneous high-level *MYCN* amplification with around 10 orange signals per cell in average. **C** Multicolor-FISH panel of *MYC* (Empire MYC/CEP8 spectrum R/Go) and *MYCN* (Empire MYCN/CEP2 spectrum Gr/Aq) allows to analyze simultaneously the presence of amplification. The images show the presence of different cells with *MYC* amplified in red and *MYCN* amplified in green; CEP probes are not visualized, but they show normal patterns in all cells

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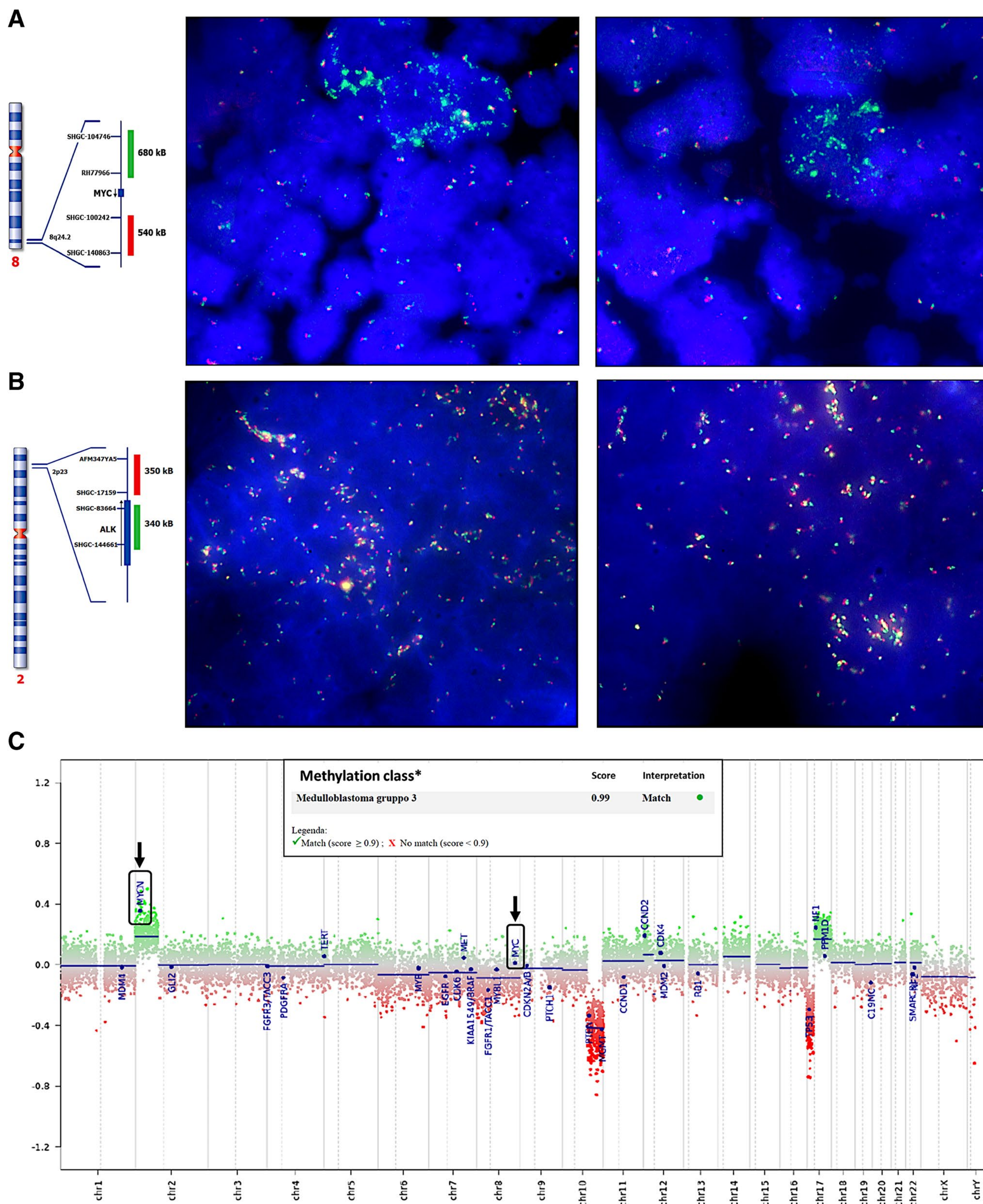

In order to figure out whether *MYC* and *MYCN* subclonal amplifications were gene-limited or included neighboring regions on 8q24 and 2p24, respectively, we used *MYC* dual-color break probe (Kreatech), which includes a green probe complementary to 5' proximal region (≈ 150 Kb upstream) and an orange probe complementary to 3' distal region of *MYC* (≈ 450 Kb downstream) and *ALK* dual-color break probe on 2p23 (Kreatech). As shown in the figures, the

Fig. 3 **A** FISH using dual-color break probe (MYC 8q24 Break O/G, KBI-XL006, Kreatech) shows subclonal amplification of the 5′ proximal region in single and dispersed neoplastic cells (5–10%) characterized by innumerable green signals (> 20) often distributed in microclusters (similar to *MYC* locus-specific probe), compared with normal pattern of 3′ distal region with 2 orange signals per cell. **B** FISH using dual-color break probe (ALK 2p23 Break O/G, KBI-10747, Kreatech) shows subclonal amplification in large neoplastic regions (30%) characterized by homogeneous high level of amplification with around 10 orange/green "fused" signals in average, a pattern similar to *MYCN* locus-specific probe. **C** Methylation classification and CNVs profile obtained via 850,000 Infinium Methylation EPIC-Bead-Chip. Results show our MDB classified as group 3 with a score of 99% on the basis of methylation profile. The CNVs profile evidences the presence of 2p chromosome amplification, with *MYCN*. *MYC* amplification is not found. *PTEN* loss on 10q23.31 and isochromosome 17q are also found. *MYCN* (2p24) and *MYC* (8q24) are highlighted by boxes

133 amplification included the 5′ proximal region in 5–10% cells
134 (> 20 green signals) but not the 3′ distal region (2 orange
135 signals) downstream of *MYC* gene (Fig. 3A); *ALK* was
136 amplified in 30% cells (≈ 10 orange/green signals) showing
137 a pattern similar to *MYCN* (Fig. 3B).
138 Finally, extracted gDNA (0.5 µg) was bisulfite converted
139 (DNA methylation-gold, Zymo Research), and methylation
140 analysis was performed using Human Infinium Methylation
141 EPIC-Bead-Chip (Illumina) with 850,000 genome-wide
142 methylation sites, as previously described [4–6, 17, 18].
143 Our case was classified as group 3 on the basis of its
144 methylation profile with a score of 0.99 (Fig. 3C). CNVs
145 were also obtained from the array, confirming the presence
146 of amplification in a large region on chromosome 2p (includ-
147 ing *MYCN* and *ALK*), while *MYC* amplification was not
148 found (Fig. 3C). In addition, CNVs profile revealed *PTEN*
149 (10q23.31) hemizygous loss and loss-17p/gain-17q (isoch-
150 romosome 17q).

151 ## Discussion

152 In pediatric MDBs, *MYC* and *MYCN* amplifications repre-
153 sent driver genetic events correlated with poor prognosis [7,
AQ5 9, 11–15, 18], [24–26]. They are considered to be mutually
155 exclusive in MDB and all other solid tumors [3, 7, 18], [28,
156 29].
157 Herein, we report the case of a 4-year-old male with a
158 MDB showing leptomeningeal dissemination at the time of
159 diagnosis. Histologically, the tumor was classified as classic
160 subtype and, using IHC, as non-SHH/non-WNT. Intrigu-
161 ingly, the tumor harbored both *MYC* and *MYCN* amplifica-
162 tion via FISH analysis; *MYC* was amplified in approximately
163 5–10% of neoplastic cells, and *MYCN* amplification was
164 found in approximately 30% of neoplastic cells.
165 Because of the rarity of this findings, we performed 850 k
166 methylation array in order to define the molecular group and
167 CNVs [7, 9, 11], [32–34]. According to methylation pattern,
168 this MDB was classified as Group 3, and the CNVs profile
169 revealed the presence of 2p amplification (where *MYCN* is
170 set), *PTEN* loss, and isochromosome 17q, all of which typi-
171 cal cytogenetic features of high aggressive group 3 MDB
172 [3–7, 9–12].
173 In contrast to FISH analysis, which allows detection at
174 a single-cell level even in the presence of very high cel-
175 lular heterogeneity, methylation array did not detect *MYC*
176 amplification, likely because the low percentage (5–10%) of
177 cells harboring this genetic alteration was below its detec-
178 tion limit. Indeed, low content of tumor cells within a large
179 population of normal cells or low amounts of neoplastic cells
180 harboring a specific CNV (< 10%) are acknowledged limits
181 of the 850 k methylation array in CNVs analysis [19].
AQ6 Using multicolor FISH, we found that *MYC/MYCN*
183 amplification occurred in different neoplastic subclones, in
184 accordance with the previously described intratumoral heter-
185 ogeneity of MDB [4, 8], implying a subclonal origin of can-
186 cer cells harboring the two different genetic amplifications.
187 The previous therapeutic protocols for MDB patients
188 (SIOP PNET5 2014) considered either *MYC* or *MYCN*
189 amplification as independent high-risk (HR) factors [20].
190 However, in the current protocols (SIOP HR-MB 2021),
191 *MYCN* amplification is considered as a negative prognostic
192 factor only in SHH tumors, whereas *MYCN*-amplified group
193 4 MDBs are not classified as high risk, but rather treated as
194 standard risk (SR) [20]. Therefore, *MYC*-amplified MDBs
195 may be treated differently from *MYCN* in ongoing clinical
196 trials.
197 The present case emphasizes the importance of iden-
198 tifying and reporting even subclonal amplification of
199 *MYC/MYCN* in small amounts of cells (< 10%), as these
200 could be clinically relevant in the choice of therapeutic
201 protocol (standard vs. high risk) and in the prediction of
202 response to treatments.
203 In conclusion, we report a unique case of pediatric MDB
204 with previously undescribed concurrent *MYC* and *MYCN*
205 amplification. Moreover, the classification of this case as
206 group 3 at methylation array underlines that *MYC* amplifi-
207 cation should be considered as diagnostic relevant even if
208 present in a low percentage of neoplastic cells.
209

Author contribution M.S. and G.F. analyzed the data and prepared the manuscript. B.L. and G.F. performed the histopathological examinations. M.S. performed FISH analyses. B.V. provided MRI imaging and clinical data. M.E. carried out the methylation analysis. A.M. was responsible for the histopathological diagnosis. B.F.R. was responsible for molecular diagnosis and revised the manuscript. M.S. and B.F.R. were responsible for conceptualization. 210–216

Data availability The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions. 217–219

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220 ## Declarations

221 **Ethics approval and informed consent** Ethics approval not required.
222 The written informed consent was obtained from the father of the
223 patient.

224 **Conflict of interest** The authors declare no competing interests.

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