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LETTER TO THE EDITOR

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Refinement of diagnostic criteria for pediatric-type diffuse high-grade glioma, *IDH*- and H3-wildtype, *MYCN*-subtype including histopathology, *TP53*, *MYCN* and *ID2* status

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Diffuse pediatric-type high-grade glioma (pHGG), *IDH*- and H3-wildtype are currently subdivided into three subgroups: pHGG-RTK (Receptor Tyrosine Kinase) 1, pHGG-RTK2, and pHGG-MYCN [2]. Each subgroup is known to present recurrent gene amplifications such as *PDGFRA*, *EGFR* and *MYCN*, respectively for RTK1, RTK2, and MYCN [2] but these amplifications are not specific to these subgroups. Although recurrent histopathological (such as nodules composed of large cells with prominent nucleoli and expression of glial and neuronal markers) [4, 5], and genetic (*TP53* mutations, particularly in a context of Li-Fraumeni syndrome) features [1] have been identified in pHGG-MYCN, the diagnosis of this subgroup is currently confirmed only by DNA-methylation analysis. Because a subset of pHGG-MYCN have been found to harbor an amplification of the *ID2* gene with (12/36, 33%) or without (1/36, 3%) a concomitant *MYCN* amplification (*MYCN+/ID2+*) [2], we have formulated a FISH (Fluorescence in situ hybridization) technique that targets both loci. Taking a monocentric series of 29 pHGG, *IDH*- and H3-wildtype, we studied the status of *MYCN* and *ID2* for each tumor, and correlated the data with histopathological and genetic features (including *TP53* status, somatic and germline), and DNA-methylation profiling.

The results are detailed in Supplementary Fig. 1 and Supplementary Table 1. The integrative histopathological, genetic and epigenetic analyses, including t-Distributed Stochastic Neighbor Embedding analysis (t-SNE) (Supplementary Fig. 2) segregated tumors into: pHGG-RTK1 (n=5), pHGG-RTK2 (n=11), and pHGG-MYCN (n=13). All DNA-methylation proven pHGG-MYCN, except one (#11), harbored an amplification of *MYCN*, and five of them an *ID2* amplification (Fig. 1). A *MYCN* amplification was also observed in 31% (5/16) of pHGG-non MYCN (four RTK2 and one RTK1). However, none of them had an *ID2* amplification. Ten/13 pHGG-MYCN presented the histopathological features previously reported for this subgroup, whereas the three remaining cases showed features typically associated with diffuse astrocytic gliomas. Somatic mutations of *TP53* were present in 12/13 pHGG-MYCN, and four of them (with available data) harbored a germline mutation (Fig. 1). The outsider case (#11) presented the histopathological

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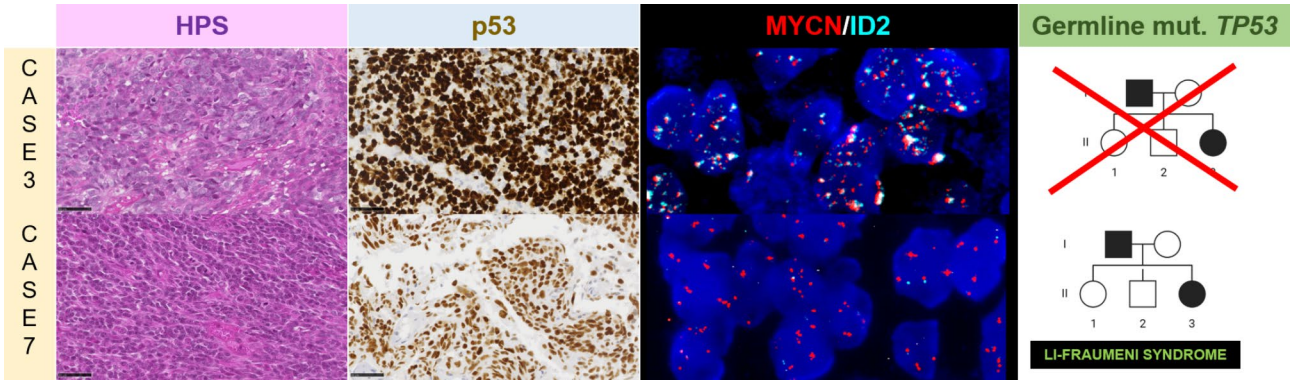

Fig. 1 Histopathological and molecular features of pediatric high-grade gliomas, MYCN subgroup. The case #3 presented classical features of HGG-MYCN such as a dense proliferation composed of large cells with prominent nucleoli (HPS, magnification x400) with overexpression of p53 (magnification x400). The FISH analysis evidenced an amplification of both *MYCN* (orange signals) and *ID2* (blue signals) loci (magnification x800). There was no Li-Fraumeni syndrome in this case. The case #7 presented classical features of HGG-MYCN such as a dense proliferation composed of large cells with prominent nucleoli and numerous mitoses (HPS, magnification x400) with overexpression of p53 (magnification x400). The FISH analysis evidenced an amplification of *MYCN* locus without amplification of *ID2* gene (magnification x800). There was a context of Li-Fraumeni syndrome. FISH: Fluorescence in situ hybridization; HGG: high-grade glioma; HPS: Hematoxylin Phloxin Saffron; mut.: mutation. Black scale bars represent 50 µm

features of pHGG-MYCN, a *TP53* somatic mutation and a *MYC* amplification (Supplementary Fig. 3).

As previously reported [2], a subset of pHGG-MYCN presented a co-amplification of *MYCN* and *ID2* (*MYCN+/ID2+*), which was specific for this subgroup of pHGG, and another subset of pHGG harbored an amplification of *MYCN* without *ID2* amplification (*MYCN+/ID2-*). The current series showed that all pHGG-MYCN

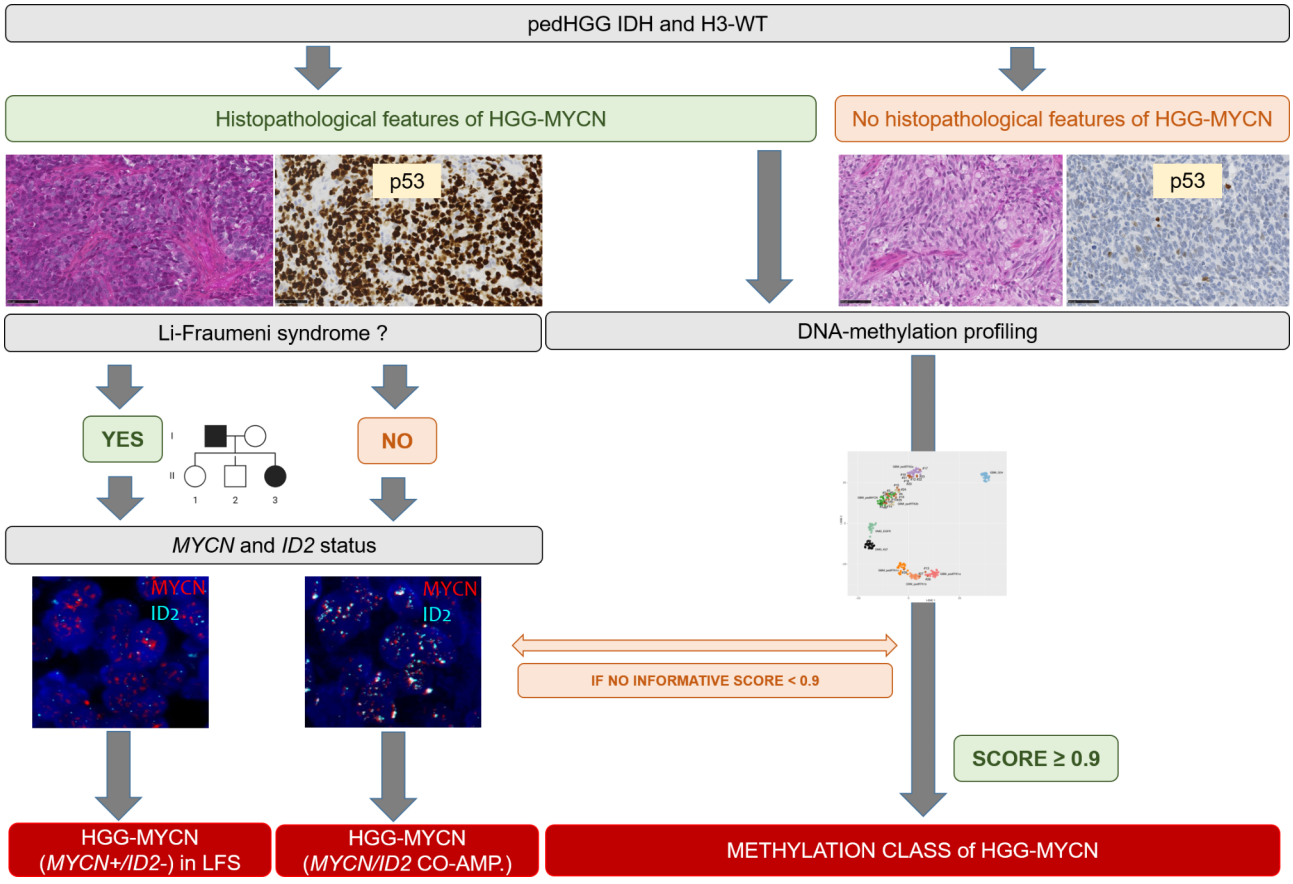

Fig. 2 Diagnostic approach for pediatric high-grade glioma, with *MYCN*-amplification. Co-amp.: co-amplification; HGG: high-grade glioma; LFS: Li-Fraumeni syndrome; ped: pediatric; Wt: wildtype. Black scale bars represent 50 µm

cases, except one, harbored a somatic *TP53* mutation and a subset of them presented a germline mutation for *TP53*, as previously reported [1]. Interestingly, our results seem to show that pHGG-MYCN *MYCN+/ID2+* are not associated with *TP53* germline mutations, whereas Li-Fraumeni syndrome is present in the subgroup of *MYCN+/ID2-*. Moreover, the current series described for the first time a pHGG-MYCN without amplifications of *MYCN* and *ID2* but harboring a *MYC* amplification. This example may illustrate the phenomenon previously described where a spinal ependymoma classified as "MYCN amplified" by DNA-methylation profiling but harbored a *MYC* amplification [3]. DNA-methylation analysis is still only limited to a small number of centres worldwide or may not be contributive (8/29 cases from this cohort presented a low calibrated score for a methylation class), so alternative methods for routine practice need to be validated. Therefore, histopathology (including p53 overexpression correlated to *TP53* mutation), and FISH *MYCN/ID2* may help diagnose pHGG-MYCN, using a simple algorithm approach (Fig. 2).

While a subset of pHGG-MYCN is found in the tumoral spectrum of Li-Fraumeni syndrome, for exclusion purposes, the co-amplification *MYCN/ID2* seems not to be associated with this genetic predisposition. The association of histopathological and genetic features may potentially represent alternative diagnostic criteria for pHGG-MYCN, particularly if DNA-methylation profiling is not available or not conclusive. FISH analyses of *MYCN/ID2* genes may constitute an interesting diagnostic tool for routine neuropathological practice.

Supplementary Information

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Supplementary Material 1: Figure S1. Clinical, histopathological, genetic and epigenetic characteristics of the cohort.

Supplementary Material 2: Figure S2. t-distributed stochastic neighbor embedding (t-SNE) analysis of DNA methylation profiles of the investigated tumors alongside selected reference samples. Reference DNA methylation classes: diffuse midline glioma H3 K27M mutant (DMG_K27); diffuse midline glioma EGFR-altered (DMG_EGFR); diffuse high-grade glioma, H3.3 G34 mutant (GBM_ G34); pediatric glioblastoma, IDH wildtype, subclass MYCN (GBM_pedMYCN); pediatric glioblastoma, IDH wildtype, subclass RTK1a (GBM_pedRTK1a); pediatric glioblastoma, IDH wildtype, subclass RTK1b (GBM_pedRTK1b); pediatric glioblastoma, IDH wildtype, subclass RTK1c (GBM_pedRTK1c); pediatric glioblastoma, IDH wildtype, subclass RTK2a (GBM_pedRTK2a); pediatric glioblastoma, IDH wildtype, subclass RTK2b (GBM_pedRTK2b).

Supplementary Material 3: Figure S3. Histopathological and molecular features of the case #11. The case #11 presented classical features of HGG-MYCN such as a dense proliferation composed of large cells with prominent nucleoli (HPS, magnification x400) with overexpression of p53 (magnification x400). The FISH analysis failed to reveal any amplification of *MYCN* and *ID2* loci, but there was an amplification of *MYC* gene (green signals, orange signals: centromere of chromosome 8) (magnification x800). There was no germline mutation of *TP53*. FISH: Fluorescence in situ hybridization; HGG: high-grade glioma; HPS: Hematoxylin Phloxin Saffron; mut.: mutation. Black scale bars represent 50 µm.

Supplementary Material 4: Detailed clinical, histopathological, genetic and epigenetic characteristics of the cohort.

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Authors' contributions
ATE, YN and EUC participated in conception, design, collection and assembly of data. ATE, FC, OA, AME and PV conducted the neuropathological examinations. PS, AVD and FS conducted the molecular analyses. ATE drafted the manuscript. All authors reviewed the manuscript and approved the final version.

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Declarations

Competing interests
The authors declare that they have no conflicts of interest directly related to the topic of this article.

Ethics approval
This study was approved by the local ethical committees from GHU Paris Psychiatry and Neurosciences, Sainte-Anne Hospital, and Necker Enfants Malades Hospital. Informed consent was obtained specifically from each patient/family for the constitutional genetic study.

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