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Peer Review File

Article information: https://dx.doi.org/10.21037/tp-22-19

First round of external peer review

Reviewer Comments

The authors of the manuscript entitled "A Paediatric Dysembryoplastic Neuroepithelial Tumour (DNET) With Deregulated Stem Cell Markers: A Case Report" made the atteption of analysis of CSC in case of low-grade brain tumour.

General comments:

Comment 1: DNET is rare but not extremely rare entity. The epidemiologic data should be given with appropriate list of references.

Reply 1: We could not find the word "extremely" in the submitted manuscript. In order to provide an effective representation for the current published data for DNET cases, we summarised all the published cases in Supplementary Table 1. This summary included 65 individual references, which would have been difficult to include in the main body of the manuscript, due to the journal's restricted maximum number of references for a case report.

Changes in the text: We removed the word "very", placed on lines 34, 56, and 205.

Comment 2: According to above there is an insufficiency for the reader. The few more cases should be included in such analysis which could be more valuable than.

Reply 2: We recognise the need for more cases to be studied in order to confirm the presence of cancer stem cells as a global phenomenon in all DNET samples. However, this is a case report which presents data for a single patient. The manuscript simply states the presence of these cells in the reported case.

Changes in the text: Line 221, the word "DNET genes" was replaced with "observations", "Further functional large cohort studies are necessary to clarify the diagnostic and prognostic applications of these observations"

Specific comments:

Comment 1: The statements: A) Dysembryoplastic neuroepithelial tumours (DNETs) are very rare intracranial tumours that often have an inconsistent relationship between tissue morphology and disease progression (lines 56-57). B) and Given the complexity and heterogeneity of most cancers, denoting a single gene as the marker for a particular tumour is unlikely (lines 62-64) are quite surprising and should be elucidated. The seeming lack of molecular changes in paediatric LGG is well known problem which is recently explaining mainly through the using of advanced molecular technologies showing for example BRAF and FGFR abnormalities.

Reply 1 A): The statement "Dysembryoplastic neuroepithelial tumours (DNETs) are very rare intracranial tumours that often have an inconsistent relationship between tissue morphology and disease progression (lines 56-57)", was indicated as per the observations seen in reference 1 (Chiang et al., 2019), where they declared in their

findings that "while some pathologic and imaging characteristics of sDNET and cortical DNET overlap, their clinical features, which are largely dictated by anatomic site, demonstrate differences". In addition, in the second quoted reference 2 (Luzzi et al., 2019), where it was mentioned "This is due to the fact that tumor recurrence and malignant transformation have been occasionally reported, especially for “extra-temporal” complex forms (69, 75, 82, 89, 90). Chuang et al (70) reported even a case of untreated classic DNET rapidly transformed into a glioblastoma multiforme. As none of the MRI features have proven to be able to predict the malignancy potential of these rare forms, a detailed molecular analysis involving the stemness markers typical of glioblastoma could become the integral part of the follow-up of DNETs". Thus, perhaps there are subgroups of DNETs which are different in their disease progressions.

Changes in the text: Lines 56-58, we have modified our text to "Dysembryoplastic neuroepithelial tumours (DNETs) are rare intracranial tumours that may have inconsistencies in disease progression (1,2)".

Reply 1 B): The statement "Given the complexity and heterogeneity of most cancers, denoting a single gene as the marker for a particular tumour is unlikely (lines 63-64)", stems from bulk tumour analysis, which indicated the involvement of several genes in many individual cancer types (Bailey et al. Comprehensive Characterization of Cancer Driver Genes and Mutations. Cell. 2018 Apr 5;173(2):371-385.e18). Single cell sequencing, even for low grade meningioma, also indicated the involvement of several oncogenic genes (16). We mentioned the review reference 8 because it indicated the complexity of targeting paediatric brain tumours, which is in part due to the variety of influential gene mutations, and tumour inter and intra heterogeneity.

Changes in the text: Lines 63-65, the statement was changed to "Given the complexity and heterogeneity of most central nervous system tumours (CNSTs), targeting a single gene is unlikely to be highly effective in improving survivorship (8)"

Comment 2 A): The analyzed tumor is not appropriate morphologically and molecularly characterized.

Reply 2 A): In the submitted case report we showed results for MRI, histopathological tests, immunofluorescence staining for consecutively cut tissues and for the corresponding cell line, clonogenic assays and cytotoxic treatments, and whole exome sequencing (WES) for the patient’s tissue and its corresponding cell line. We are open for other methods that are critically needed for this case report.

Comment 2 B): There are to many MRI scans.

Reply 2 B): We think that the MRI scans show the tumour location clearly, a critical result that may be useful for other researchers interested in factors that associate location with progression of DNETs, as indicated by reference 1.

Changes in the text: Figure 1 part G was removed. Numbering was changed in lines 94- 107. Numbering was changed in the Figure 1 legend on lines 332-342.

Comment 2 C): The figures of tumor histology are quite weak (are the authors have the FFPE material?). The Ki-67, S-100, p16, Olig-2, CD34, MAP2, FGFR1, BRAFV600E stainings should be included in the paper.

Reply 2 C): The histopathological images were taking by a qualified neuropathologist, who carefully examined the tissue and declared its nature prior to any questions related to the presented work. In addition, we immunofluorescence stained fresh

frozen tissues with 11 markers including Ki-67 and Olig-2. Furthermore, we specifically screened FGFR1 and BRAFV600E for any COSMIC mutations using exome sequencing for both the tissue and the corresponding cell line.

Comment 2 D): The precise molecular/IHC characterization should be made for BRAF and FGFR genes (CNV, mutations, fusions).
Reply 2 D): We have mentioned on lines 205-206 that "In our sample, no COSMIC variations were found in the tissue's DNA for *FGFR1, BRAF or NF1* genes". The answer as to why our sample does not have mutations in these genes is mentioned in the introduction, lines 59-62 " Out of all 283 published DNET cases, 143 were paediatric patients (age: 0–14; Supplementary Table 1). Out of 63 DNA-examined paediatric DNET cases, 33 samples were shown to have fibroblast growth factor receptor 1 (FGFR1) variants". The literature research clearly shows that not all histopathologically identified paediatric DENTs have mutations in these genes.
Changes in the text: Added to lines 206-208 "which is consistent with the aforementioned information mentioned in the introduction and the Supplementary Table 1, as not all histopathologically identified paediatric DNETs have mutations in these genes"

Comment 2 E): The phrase: In our sample, no COSMIC variations were found in the tissue's DNA for FGFR1, BRAF or NF1 genes. However, several variants were detected in other genes (e.g. IGF2BP3) that most likely have redundant functions in pathways associated with the aforementioned genes, which may have a parallel effect to FGFR1 mutations should be explained more throughly.
Reply 2 E): The notion that tumour genes are organised in pathways is clearly agreed on by oncologists following Hanahan and Weinberg collections of publications for the hallmarks of cancer. In the discussion we suggested that since both FGFR1 and IGF2BP3 influence the phosphatidylinositol 3-kinase/mitogen-activated protein kinase (PI3K/MAPK) pathway, that perhaps mutations in these genes may have redundant effects on this pathway, which may prove to be important in DNETs.
Changes in the text:

- A Supplementary Table 6 showing the selected rare COSMIC variants with damaging coding consequences present in both the tissue and the corresponding cell line Jed99_DNET, but not in the blood (TCnB), was added. This table shows the details of somatic related variants including the IGF2BP3 variant.
- The text was added to lines 160-164 "Supplementary Table 6 shows selected rare COSMIC variants with damaging coding consequences present in both the tissue and the corresponding cell line Jed99_DNET, but not in the blood (TCnB). This table shows details for somatic related variants including a variant in the Insulin growth factor-2 binding protein 3 (*IGF2BP3)* gene".
- The text was added in lines 211-214: "For example, a TCnB COSMIC variant was detected in the *IGF2BP3* gene. Both FGFR1 and IGF2BP3 influence the phosphatidylinositol 3-kinase/mitogen-activated protein kinase (PI3K/MAPK) pathway (29, 30), therefore perhaps mutations in these genes may have redundant effects on this pathway, which may prove to be important in DNETs."

Comment 3 A): The presence and proper identification of cancer stem cells in brain tumor is still problematic. There is no universal CSC in brain tumors and there are not

universal markers.

Reply 3 A): Single-cell analysis of different CNS cancers has shown clearly the existence and the diversity of CNS Cancer Stem Cells (CSCs). Targeting the diverse types of CNS CSCs has been shown to be synergistic (9-18).

Changes in the text: Line 67-69 added more references 9-13 and we rephrased the sentence "CSCs are cancer cells that utilise stem cell pathways, enhance tumorigenesis and contribute to drug resistance, and their associated markers have been identified in several CNSTs (9-18)".

Comment 3 B): The authors should explained how the selection of plausible indicators was made.

Reply 3 B): This information is clearly available in the Supplementary Methods File 1, with associated references provided. The method was added as supplementary file because of the structure requirement of a case report format.

Comment 3 C): In the section DNET fresh frozen tissue is highly positive for numerous CSC markers there are listed together such markers as GFAP, Ki67, OLIG2, VIM, TUBB3, Nestin, CD133 and Sox11. Are they really the CSC markers? The potential CSC ones were negative.

Reply 3 C): The tumour was also positive for CTNNB1, NUDCD3, OLIG2, and the WNT-signalling-related protein FZD9, which are all stem cell related proteins. In addition, it is the presence of multiple differentiation markers associated with distinct paths (GFAP, VIM, and TUBB3), in adjacent regions of the consecutively cut tissue that imply the presence of thwarted differentiation and CSCs presence.

Comment 4: What is the origin of Jed99_DNET cell line. What is their characterization?

Reply 4: The method for retrieving the cell line is available in the Supplementary Methods File 1, with the associated references provided. Figure 3 shows clear characterisation of the primary corresponding cell line, and all critical COSMIC variants detected in the cell line are shown in Supplementary File 4.

Comment 5: The detailed culture conditions should be given especially when CSC are analyzed.

Reply 5: This information is clearly available in the Supplementary Methods File 1, with associated references provided. The method was added as supplementary file because of the structure requirement of a case report format.

Comment 6 A): In the discussion the authors wrote: Our case report elucidated the combined deregulated expression of several CSC markers in a paediatric DNET. Such statement is overestimation after weak analysis of one tumor.

Reply 6 A): We clearly showed that both the tissue and corresponding cell line were positive for CTNNB1, NUDCD3, OLIG2, and the WNT-signalling-related protein FZD9, which are all stem cell related proteins. In addition, it is the presence of multiple differentiation markers associated with distinct paths (GFAP, VIM, and TUBB3), in adjacent regions of the consecutively cut tissue that imply the presence of thwarted differentiation and CSCs presence. The statements are clear that these observations are for the single presented case.

Comment 6 B): Moreover the authors noticed: Thus, in this case, the triple positivity more likely reflects the progenitor-like state of cancercells (line 172-173). There are no such data in the manuscript. In the Fig. 2 only single staining were showed with no sure that the tumor cells were analyzed.
Reply 6 B): Figure 2 shows the staining of a consecutively cut tissue with 4µm width of each section. Using this same approach has previously shown that the analysis of a single marker throughout the consecutive sections along a depth of 32µm, indicated a strong correlation of expression for both adjacent and distal sections of meningioma tissues (referenced 12). Basic analysis locating CSC niches across consecutive sections has also been attempted previously in breast cancer tissues (Gerdes MJ, Gökmen-Polar Y, Sui Y, expression for consecutive regions. In addition, in Figure 2, FZD9 and CTNNB1 antibodies were used to co-stain the same section. Since these sections are closely related in their expressions, the collective positivity is also indicative of an active CSCs programme in the stained region. Furthermore, Supplementary Figure 1 shows positivity for all of these markers in the tissue, albeit the sections were not consecutive in that figure. Critically, Figure 3 shows, whenever possible, the co-expression of CSCs markers in the corresponding cell line at an early passage of 15, which must have had an inherited ability to express these proteins from the mother tumour tissue.
Changes in the text: An addition was made on line 178 " an observation previously seen in meningioma tissues (12)"

Comment 7: The following overestimated statement is: Both CDC27 and CTBP2 may prove to be critical diagnostic markers for aggressive DNETs. It is not the conclusion after single analysis.
Changes in the text: Lines 201-203, the statement was changed to " Further functional large cohort studies are necessary to clarify whether either CDC27 or CTBP2 are critical markers for aggressive DNETs."

Comment 8: Supplementary figure 1 is in bad resolution and there is no data what tumor/cell line undergone this analysis.
Reply 8: Supplementary Figure 1 shows the immunofluorescence images showing the highest expression seen for each tested marker in the tissue, as mentioned on line 113.
Changes in the text: The figure legend for this figure was transferred from the figure to the supplementary appendix.

Comment 9: Supplementary table 1 is in wrong format/orientation and is unreadable. Moreover this data are probably unnecessary.
Reply 9: There are no structured formats for supplementary tables. We strongly think it is important to include this table. All the published cases were summarised in Supplementary Table 1. This summary included 65 individual references, which would have been difficult to include in the main body of the manuscript, due to the journal's requirement of a maximum number of references for a case report.

Comment 10: The gene names should correspond to the approved symbols and always should be indicated in italics.
Reply 10: We apologise for any grammatical errors. The manuscript was reviewed and corrected.
Changes in the text: Line 198-199 Added: nicotinamide adenine dinucleotide (NAD) +

hydrogen (H) (NADH).

Second round of external peer review

Below are listed the notes that should be considered by the authors again

1. Reply for specific comment 1 (Reply 1A)

Please do not select the literature for the hypothesis. The hypothesis should be rather validated by existed research and scientific data.

Our replay: We mentioned that "CSCs are cancer cells that utilise stem cell pathways, enhance tumorigenesis and contribute to drug resistance, and their associated markers have been identified in several CNSTs (9-18). Data on the status of CSCs in DNETs have not been published" and thus we think it was worth addressing the question of: "we investigated the status of cancer stem cell (CSC) genes associated with resilience and drug resistance in a paediatric DNETs".

2. Reply for specific comment 2 (Reply 2C)

The authors state that the tumor was carefully examined with 11 markers. Please give the illustration of Ki-67, Nestin, Map-2, S-100 stainings. There is still no answer is the diagnosis was made without the FFPE tissue sample?

Our replay: Several publications indicate that using immunofluorescence on frozen tissues is a powerful diagnostic technique. Please see reference Tan et al., 2020 cancer communications 40;4:135-153.

3. Reply for specific comment 3 (Reply 3C)

The question still remains which of the listed markers the authors consider to be CSC markers for such type brain tumor.

Our replay: We totally agree with the aforementioned statement, and this is why our lab's our intention has been to bring some clarity on the identity of CSCs in individual CNS tumors.