Author's response to reviews

Title: Nestin expression in the cell lines derived from glioblastoma multiforme.

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Author's response to reviews: see over
Dear Editors,

We would like to submit a revised version of our manuscript entitled “Nestin expression in the cell lines derived from glioblastoma multiforme” by R. Veselska, P. Kuglik, P. Cejpek, H. Svachova, J. Neradil, T. Loja and J. Relichova.

In the text below, we explain all changes made according to the reviewer’s suggestions or our comments to their critical remarks. For easier evaluation of the revised version of manuscript, all changes are typed in blue.

Sincerely,

Dr. Renata Veselska

REVIEWER: ROBERT A ROSS

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached):

1. It is not clear whether the authors are attempting to correlate nestin cytoskeletal arrangement, being diffuse or in a traditional filamentous network, with cell morphology and/or transformed phenotype. This needs to be clarified. Along the same lines, the legend to Figure 1C describes “non-tumor cells exhibiting no or very poor signals for nestin”, whereas the text implies it is the tumor cells that differed widely in amount of nestin.

We did not attempt generally to correlate nestin cytoskeletal arrangement with cell morphology and/transformed phenotype. However, the positivity for nestin (in the filamentous network or as a strong diffuse signal) can be used as a marker of transformed phenotype – this observation is in accordance with other published findings using immunohistochemical approaches. In our experiments, a mixture of tumor and non-tumor cells was present in the cell population in primary cultures and during short-time cultivation a proportion of nestin-positive (i.e., transformed) cells was increased. Figure 1 illustrates differences in the nestin expression in the primary cultures and the legend is correct. To clarify the situation, we changed the corresponding part of Results, which was confusing in the previous version of manuscript (page 10):

“In primary cultures, a different positivity for nestin was detected in the same cell population due to a mixture of various cell types (normal and transformed cells of astrocytic origin, endothelial cells) in the same culture. Transformed astrocytes were usually larger in size when compared to normal cells, and in addition to nestin positivity, abnormalities in nucleus morphology were also detectable in these cells (Fig. 1A). Nestin expression and nestin-positive intermediate filament formation were observed also in the smaller cells in a monolayer (Fig. 1B). Nevertheless, nestin expression was very different in the cell population; it varied from none or a poor diffuse signal in cytoplasm of presumably non-tumor cells up to characteristic nestin intermediate filaments as a part of transformed phenotype (Fig. 1C). During short-term cell cultures (between passages 2-4), a proportion of nestin-negative cells was progressively reduced up to complete nestin-positivity in the cell population.”
2. The co-localization studies with nestin and tubulin are not convincing. Whereas nestin does not appear to co-localize with the MTOC (as described by others), the asymmetric distribution of both the microtubular and nestin networks (shown in Fig. 4) do not appear to be random and suggest interaction. Nestin appears to be distributed throughout the cell rather than localized to one area, and it is impossible to determine the position of nucleus and the edge of the cytoplasm to orient oneself. Inclusion of a DAPI stain or of a phase contrast illustration would be helpful. Also, it would be helpful to indicate the nucleoli, mentioned in the text, by arrows on Figure 5.

Interactions between microtubular and nestin networks (via associated proteins) are expected and the whole pattern of cytoskeletal network depends also on the cell type, nucleus position, etc. According to the suggestions by reviewer, figure with DAPI stain was added into Figure 4 (Fig. 3 in the new numbering) and nucleoli were indicated by arrowheads on the Figure 5 (Fig. 4 in the new numbering). Since Reviewer Prof. Takeuchi suggested to reduce Figure 4 (Fig. 3 in the new numbering) (documentation of the same situation in two different cells seems to be redundant), only one set of photos was kept in this figure.

Note:
Due to remarks both of the reviewers concerning redundancy in the Figures 2 and 3, Figure 2 was omitted and the numbering of the following figures was changed.

3. The authors’ suggestion – that nuclear staining of nestin is seen in Fig. 4 – is difficult to see and harder to interpret. Since these are whole cells, diffuse staining of nestin filaments above or below the nucleus could be perceived as intranuclear. While the authors state that there is nucleolar staining (possibly seen as the punctate green stain in Fig. 5), they are neither clear on this point nor do they illustrate it in their confocal micrographic analysis or their EM photomicrographs.

We agree with the reviewer that is difficult to interpret a diffuse staining in the position of cell nucleus seen in Figure 4 (Fig. 3 in the new numbering) as the nuclear staining. However, to ensure that the stained nestin is localized really in the cell nucleus, we applied the software cross-section throughout the cell using confocal microscopy that confirmed intranuclear and nucleolar staining of nestin as seen in Figure 5C (Fig. 4C in the new numbering). We also modified the formulation concerning nucleolar staining in the manuscript (page 11):

“The results showed that nestin was indeed detectable in the cell nucleus, including the nucleoli (Figure 4A,C-D).”

In the corresponding EM photomicrograph, i.e. Figure 9 (Fig. 8 in the new numbering), the area of nucleolus, in which the signal for nestin is also detectable, is indicated (Nu) and the figure legend is changed.

4. The authors do not propose a function for the nuclear localization of nestin nor do they correlate it with malignancy. Thus, the authors need to speculate about the function of nuclear (and possible nucleolar) nestin.
The part Discussion in the manuscript was enlarged by requested topic (page 14):

„All these findings suggest that nestin expression in tumor cells is closely related to dedifferentiated status and increased malignancy. In addition to the role of cytoskeletal components in cell growth and motility that is associated with metastatic potential, some proteins of intermediate filaments identified in cell nucleus may affect organization of chromatin or they may serve as specific regulators of gene expression [41-42].”

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct):

1. It would appear from the Abstract that this would be a paper detailing the role of intermediate filaments, and especially nestin, in tumor malignancy – a topic not addressed at all in the Results section. Instead, the paper is essentially a description of the organization of nestin in glioma cells, in culture, during interphase and mitosis. Interesting, but not what is expected.

A relevant part of abstract was changed (page 3):

“Our work was focused on detailed study of the nestin cytoskeleton in cell lines derived from glioblastoma multiforme, because re-expression of nestin together with downregulation of GFAP was previously reported in this kind of brain tumors.”

2. Inclusion of cytogenetic analyses is irrelevant to the main thrust of their study and could be omitted. However, if it is to be included, there should be an actual karyotype to allow the reader to judge the quality yielded by these cells. Moreover, according to ISCN 1995, the description should be expressed relative to the closest ploidy (in the case of GM7, to a triploid or tetraploid chromosome number).

Cytogenetic analyses were used for the confirmation of the occurrence of tumor cells in culture, because there is no other intracellular or cell surface marker, which can be used for detection of glioblastoma cells (with the exception of nestin detection), and classification of astrocytic brain tumors is routinely based only on histological criteria (nuclear atypia, mitotic activity, proliferation of endothelia). In addition to the GTG-banding, we used also other molecular cytogenetic methods for positive proof of glioblastoma cells in culture (interphase FISH, CGH and HR-CGH).

However, we agree with the reviewer that the complete results of cytogenetic analyses are irrelevant to the main thrust of the study. For this reason, a part of Results entitled “Cytogenetic analysis” was omitted and a short description of karyotypes was included in the next part entitled “Characterization of cell lines” (page 9):

“Characterization of cell lines:
The tumor character of derived cell lines was verified by GTG-banding during a short-term cultivation, i.e. between passages 2-4. The GM7 cell line was identified as a near-tetraploid with a large number of structural and numerical abnormalities. The GM10 cell line was described as a near-diploid one.
Astrocytic origin of these cell lines was proved by indirect immunofluorescence...”
3. Given the substantial normal diploid population in GM10, what were the percentages of cells that were positive for vimentin and/or GFAP? One long time observation is that growth in culture frequently selects for vimentin-expressing cells, and normal fibroblasts also express vimentin. Why not provide an illustration?

GM10 cell line was maintained only until passage 10. Because high-grade astrocytomas are generally positive for vimentin, there is impossible to use the vimentin positivity as a main criterion for detection of glioblastoma cells. Practically all cells in the culture were positive for vimentin, and minimum of them (approximately 5% on average) was simultaneously positive for GFAP (page 9 of the manuscript). To be sure about the fraction of tumor cells in the culture, we carried out at passage 6 also molecular cytogenetic analysis using interphase FISH with the probe against centromere of chromosome 7: approximately 35% of cells with polysomy 7 were detected in the culture.

4. It would be helpful to the reader for the photomicrographs of nuclear staining images (Fig. 7 C, D and Fig. 8C) to be in the same figure, separate from those with cytoplasmic staining. Also unclear is what the authors mean by “short fibers” when referring to nuclear staining. Is this referring to the linear arrangement of immunogold stain, which could be binding to strands of DNA, and not filamentous nestin? Why is the negative nucleolar stain included in Figure 8 rather than 9?

Sets of EM photomicrographs were rearranged according to suggestions by Reviewer. Short fibers seen in Figure 9B-C (Fig.8B-C in the new numbering) are indicated by arrows now. We suppose that these fibers are not caused by direct binding of immunogold to strands of DNA, because on the control specimens, which were treated only by secondary antibody conjugated with gold particles, similar arrangement of grains is not detectable and immunogold signal is generally very sporadic.

5. There is a typographical error in the legend to Figure 9 “(B-X)” should be “(B-C)”.

This error was corrected:

“...several larger nestin aggregates (B–C) were also observed...”

REVIEWER: TOSHIYUKI TAKEUCHI

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. Page 9, Cytogenetic analysis in the Results: Although the Authors analyzed the karyotypes of the two cell lines, GM7 as a hyperdiploid and GM10 as a pseudodiploid, this analysis is not related with the following nestin filament localization study. Since the Author describes, “Changes in the intermediate filament proteins in the brain tumors are associated with tumor malignancy and invasiveness” (Abstract), this Reviewer
expects that one of the cell lines is more malignant and invasive than the other, depending on their karyotypes. However the karyotype analysis is only descriptive and no comparison data on the two cell lines are available in malignancy and invasiveness, such as growth rate, and invasiveness using a Boyden chamber.

Considering similar remarks by Prof. Ross (Minor Essential Revisions No. 1 and 2), author’s comments are given above as well as changes made in the manuscript.

The sentence from Abstract was referred a common feature of astrocytic tumors: more malignant and invasive high-grade astrocytomas (i.e. anaplastic astrocytoma and especially glioblastoma multiforme) exhibit downregulation of GFAP together with re-expression of nestin, whereas low-grade astrocytomas expressed GFAP and nestin is downregulated similarly as in non-tumor cells of astrocytic origin.

Due to the fact that our study was not aimed at comparison of genetic changes and malignancy/invasiveness of these cell lines, we did not performed experiments with Boyden chamber. Population doubling time of GM7 cell line was measured to characterize growth parameters of these cells for other experiments, but the results are not related to the study on nestin expression.

Quoted sentence from Abstract was replaced by more suitable formulation. Results of cytogenetic analysis were minimized and included into part of Results entitled “Characterization of cell lines” in the revised version of manuscript (see above; page 9 of the manuscript).

2. The Authors emphasize that apparently, most important finding of our study is the discovery of nestin in the nucleus (page 14, last paragraph), and refer to Ref 36 that using indirect immunofluorescence, a similar diffuse signal for nestin in the cell nucleus was identified also in primary cultures of glioblastoma cells [36]. However the Ref [36] shows the figure of nestin staining strongly in the nucleus, weakly in the cytoplasm (Figs, 2 C and E), the Ref [36] does not mention to the nuclear localization of nestin in the nucleus in the text. Furthermore, the Authors refer to Ref [41] that another study ---- nestin binds to the nuclear DNA in cell lines with N-myc amplification [41]. In this Ref [41], DNA binding of nuclear nestin is observed only in the presence of cisplatin in N-myc amplified neuroblastoma cells. Is nuclear nestin detected without cisplatin in both GM7 and GM10? The Ref [41] stated that nestin may be one mediator of N-myc-associated tumor aggressiveness of human neuroblastoma. In GM7 and GM10, what is an aggressiveness marker corresponding to amplified N-myc? The karyotype can be utilized for this marker?

We agree with the reviewer that in text of Ref [36] is not mentioned the localization of nestin in cell nucleus. For this reason, we only said in our manuscript that “using indirect immunofluorescence, a similar diffuse signal for nestin in the cell nucleus was identified also in primary cultures of glioblastoma cells [36]” as a comment of Figs 2C and 2E in Ref [36]. According to the remark by Reviewer Prof. Ross (Major compulsory Revision No.3), it is difficult to interpret similar figures unequivocally as a staining of nestin in cell nucleus. Therefore, we used software cross-sections and especially electron microscopy to assure that signal for nestin is localized reliably in cell nucleus and not in the cytoplasm below or above the nucleus. However, we changed in our manuscript the sentence in question (page 14):

“Using indirect immunofluorescence, a similar diffuse signal for nestin in the position of cell nucleus was identified also in primary cultures of glioblastoma cells [36].”
Our experiments with immunogold detection of nestin in the cell nucleus were performed without cisplatin. In Ref [41], cisplatin was used for methodological reasons to investigate possible interactions of nestin and DNA by Western blotting. As mentioned above, possible role of nestin in cell nucleus of glioblastoma cells remains still unclear, because it is difficult to identify one aggressiveness marker like N-myc in neuroblastomas. Glioblastomas are generally the most aggressive astrocytic tumors, but high level of heterogeneity in genetic changes as well as in histomorphology was reported. Briefly, one third of glioblastomas show amplification of EGFR gene (primary glioblastomas), another third show p53 mutation (secondary glioblastomas), and the remaining third show no of these markers. However, it is impossible to distinct these three types of glioblastomas on the histopathological criteria and they all show similar, i.e. the highest aggressiveness. GM7 cell line was identified as near-tetraploid with large number of chromosomal abnormalities: karyotype was described in the previous version of our manuscript, 21 abnormalities (12 gains, 9 losses) was identified using CGH. In GM10 cell line, amplification of EGFR gene was identified using interphase FISH.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

3. Some figures are redundant. In Fig. 2, A and B look similar. In Fig. 4, what is the difference between the pair A and B and the pair C and D?

According this remark as well as suggestions by Reviewer Prof. Ross, Figure 2 was omitted and Figure 4 (Fig. 3 in the new numbering) was reduced (see above).

4. Nestin filaments in Fig. 4 A and C look different from those in Fig 1, 2, and 3. Is this due to the difference in cell line, GM7 or GM10?

The difference in cell line is one of the possible reasons; another may be differences in applied method (double labeling of cytoskeleton) and in the resolution micrographs in Figure 4 (micrographs were taken using CCD camera).

5. In Fig. 5, the Authors stated that the results showed that nestin was indeed detectable in the cell nucleus, especially in the nucleoli (Figure 5 A-B). This Reviewer cannot identify nucleoli in Fig. 5 A-B. Indicate the nucleoli by arrows in the picture.

According similar remark by Reviewer Prof. Ross, nucleoli were indicated by arrowheads in Figure 5 (Fig. 4 in the new numbering) and the formulation concerning nucleolar staining was corrected in the manuscript (see above).

6. Nuclear nestin: Is nuclear localization of nestin seen in Figs. 7C and D detectable in Fig. 9A? The Authors stated that very short fibers were also noticeable in these aggregates (Figure 9B,C). Indicate very short fibers by arrows. In Fig. 9A, are nucleoli abundant with nestin-immunostained grains?
Figures 7C and D (Fig. 8D-E in the new numbering) are not the details of Figure 9A (Fig.8A in the new numbering). Very short fibers seen in Figure 9B-C (Fig.8B-C in the new numbering) are indicated by arrows now. Occurrence of nestin-immunostained grains in nucleolus (indicated by „Nu“) in Figure 9A (Fig.8A in the new numbering) is slightly reduced in comparison with other parts of nucleus.