ACTA STEREOL 1993; 12/1: 65-70
ORIGINAL SCIENTIFIC PAPER

STEREOLOGICAL ASPECTS OF PATHOLOGY OF GLIOMAS: VOLUME DENSITY OF NUCLEI AND VOLUME CORRECTED MITOTIC INDEX

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ABSTRACT

There is still need to develop objective and reproducible methods of histopathological assessment of gliomas. In this 2-D study we tried to apply some stereological methods i.e. volume density of nuclei and volume corrected mitotic index in evaluation of gliomas. Volume density of nuclei measured in histological slides of cerebral gliomas seems to be a better estimator of malignancy than the cellularity of the tumor. The morphometric analysis also points on the usefulness of volume corrected mitotic index as a more exact indicator of tumor cell proliferation than usual mitotic index.

Key words: glioma, morphometry, mitotic index

INTRODUCTION

The methods and the role of nucleometry in tumor pathology have been reviewed recently by Whimster (1992). One can expect that gliomas (primary tumors that prevail in the Central Nervous System) are the sort of neoplasm that could also benefit from the use of nuclear morphometry first of all because the morphometry of the whole neoplastic glial cell usually cannot be performed in histological section stained with H&E or similar dyes due to indistinct external cell margins. As the result, all the morphometric investigations on gliomas that have been published so far have been based on the measurements of cell nuclei (Giancaspero et al.1984, Klinken et al.1984, Martin et al.1980, Martin et al.1981, Martin and Voss 1982a, Martin and Voss 1982b, Martin et al. 1984, Scarpelli et al.1987). The stereological approach to pathology of gliomas probably deserves some attention. First, we tried to measure the volume density of nuclei of glioma cells (NVD) in the histopathological slice of tumor tissue and to correlate it with tumor's degree of malignancy according to Kernohan's scale and with the level of the expression of glial fibrillary acidic protein (GFAP) by tumor cells which correlates with the differentiation of glioma but inversely correlates with its malignancy i.e. the more anaplastic and malignant glioma, the less ability to express GFAP it shows (Cras et al. 1988. Duffy et al. 1980, Jacque et al. 1979, Kałuża and Adamek 1990, Luevano et al. 1986, Velasco et al. 1980). Secondly, we attempted to assess the usefulness of the so called volume corrected mitotic index, VCMI (Haapasalo et al.1989) in gliomas. In this case the rationale was that in gliomas, especially in malignant ones a marked part of their volume is necrotic, cystic, edematous occupied by frequently distended blood vessels or massive "glomeruloid" proliferations of endothelia or massive infiltrations of other (non-glial) cells like e.g. leukocytes (Fulling and Garcia 1985, Schiffer et al. 1988). It seems that especially mitoses in proliferating endothelia are able to interfere in proper assessment of tumor cells proliferation what

is of important prognostic value.

MATERIAL AND METHODS

Formalin fixed, paraffin embedded tumor samples taken during surgery in neurosurgical clinic from 29 cases of brain glioma were investigated using microscope and digitizing tablet (Micro-Plan-II Donsannto Corp.). The paraffin blocks were cut in 8μ slices and PAP reaction according to Sternberger (1970) with the polyclonal anti-GFAP antibody was performed. Then the slides were hematoxylin stained (to visualize nuclei). Among investigated tumors 2 were in grade I, 4 in II, 13 in III, 10 in IV, however at the time of morphometric measurements the person performing the analysis (one of us, D.A.) did not know exact histo-pathological diagnosis (except the fact, that all tumors were gliomas). The microscopic picture was optically superimposed on the active field of Micro-Plan-II and the contours of nuclei were traced manually by cursor. Micro-Plan-II counted the area of the contour of nucleus. Data were stored in personal computer connected to digitizing tablet and retrieved using statistical package Statgraphics-Plus. The scheme of the evaluation of the histological slide is shown in Figure 1. Large test field (LTF) with magnification of 500x encompassed the area (aLTF) of 98 000 micrometers^2 of histological slide. The area of its central part, central test field (CTF) was exactly 1/9th of LTF. Tested fields were chosen randomly in the way as follows. The first (and all the next) evaluated LTF had to encompass only tissue of tumor however additionally its central part (CTF) had to be devoid of large or distended blood vessels, necrosis, hemorrhage, cystic formations, massive proliferation of endothelium, intensive infiltration of leukocytes or macrophages, and marked oedema. Fields that partially contained unchanged cerebral tissue were also excluded. In other words one can assume that all the CTFs consisted almost exclusively of the neoplastic cells. After skipping the distance of 2 LTF diameters, next examined LTF was the nearest that fulfilled above mentioned criteria. In fact as a rule, 4 - 5 or even many more LTFs that did not fulfilled the criteria had to be skipped to find the proper one. In the CTF the area of all individual nuclei and their number was measured. Mitotic figures were

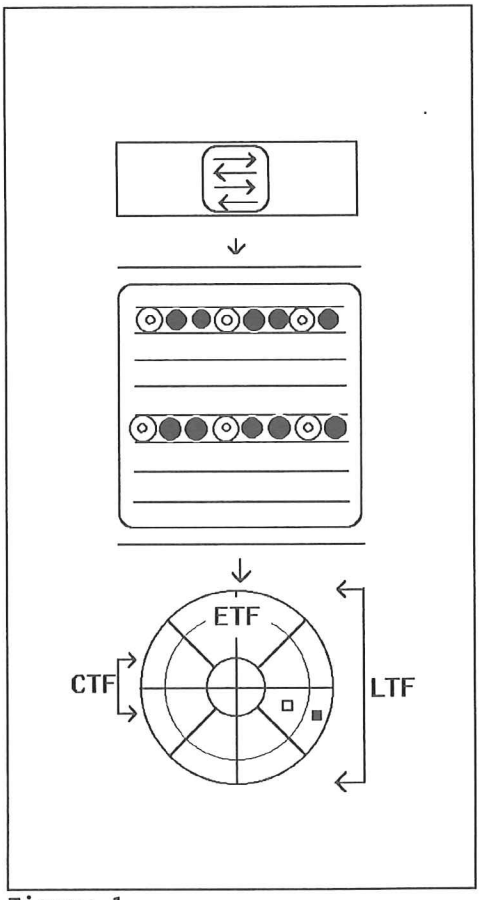

Figure 1
Scheme of the analysis of histological slide. LTF: "large test field". CTF: "central test field" equals 1/9th of LTF. ETF: "external test field" equals 8/9th of LTF.

counted independently in LTFs and CTFs. The examination of the given case ended when either the "path" of consecutive microscopic fields reached the end of the histological slide or the total number of counted nuclei in CTFs surpassed 1000. As it was mentioned above it is impossible to measure nucleus/cell ratio in the individual cell in the histological preparation of glioma however it seems that the criteria adopted here for the CTFs are such that these regions consisted of almost only neoplastic cells and the extracellular space can be regard as negligible, hence nuclear volume density may be good and exact estimator of nucleus/cell volume ratio. The measurement of NVD in each CTF was based on the Delesse's rule namely NVD = ASUM/aCTF where ASUM is sum of areas of all nuclei in CTF and aCTF (an area of CTF) = 10900μ^2 i.e. 1/9th of LTF. Since the thickness of the slide (8 μm) is comparable with the mean size of investigated objects (i.e. nuclei) the correction according to Holmes (Weibel 1973, pp. 277-278) was applied in such a way that ASUM in individual CTF was divided by

$$(1+\frac{3}{2}*\frac{T}{D})$$

where T is thickness of the slide and D is the mean diameter of nucleus in the given field according to formula as follows. (With the assumption that nuclei are of spherical shape.)

$$D = 2\sqrt{\frac{A}{\pi}}$$

where A is the mean area of nuclei counted separately for each individual CTF.

RESULTS

In the 29 cases of glioma 583 microscopic fields chosen accordingly to presented above rules were measured.

1. Nuclear volume density.

The analysis that was performed disclosed highly significant relation between mean volume density of nuclei in glioma tissue and the degree of malignancy of tumor according to Kernohan's

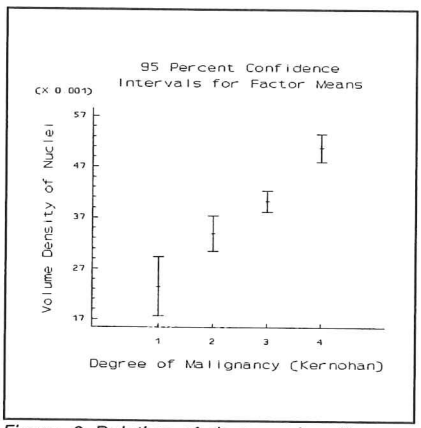

Figure 2 Relation of degree of malignancy (Kernohan's scale) and NVD. Degree 1 refers to benign glioma, degree 4 to most malignant one.

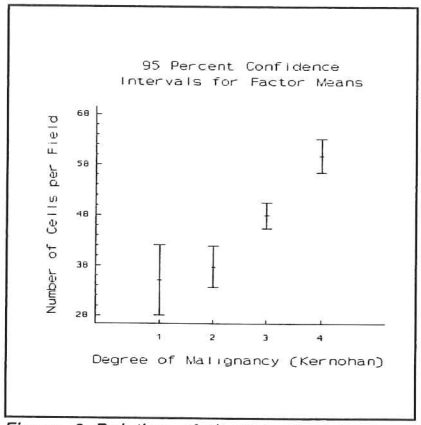

Figure 3 Relation of degree of malignancy (Kernohan's scale) and cellularity of glioma. Degree 1 refers to benign glioma, degree 4 to most malignant one.

scale i.e. the more malignant glioma the higher NVD was noted (Figure 2).

It is important to stress here that according to the method that was applied, NVD can be regard as denoting directly the volume nuclear/cell ratio of glioma cells. We tried also to compare the relation of cell profile density ("cellularity") of tumor and its malignancy because the cellularity is known to be highly correlated with the worsening of prognosis in glioma (Schiffer et al.1988). For this purpose the number of cells in each CTF was used [1]. As it is shown in Figure 3 the cellularity (counted in the same CTFs as NVD) was, although, significantly higher in more malignant gliomas, did not differentiate tumors of grade 1 and 2. We tried also to check relation of NVD to local (in every CTF) level of the expression of GFAP in tumor cells. For this purpose the ratio of GFAP-positive cells (POSRAT) to all (neoplastic) cells in each CTF was counted. Figure 4 presents the result of regression analysis of NVD on POSRAT. Coefficient of linear regression is (-)0.257. In other words, decreased expression of GFAP coincides with the higher NVD in a given CTF. This rule is especially significant for the fields with the highest NVD however a group of some fields with high POSRAT and relatively low NVD in right part of Figure 4 can be noted. It seems to be the result of the fact that mean size of nuclei in GFAP-positive cells turned out to be significantly larger than that of GFAP-negative ones. We measured (using Micro-Plan-II) that average area of contour of nuclei of GFAP-positive glioma cell was 36.8μ^2 and it was significantly higher than that of GFAP-negative cell (28.8μ^2 $p<0.001$).

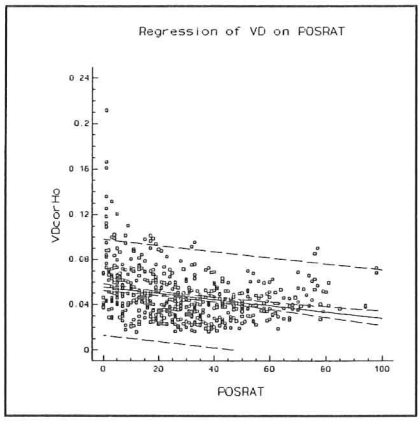

Figure 4
Regression of NVD corrected acc. to Holmes, VDcorHo (Weibel, 1973) on POSRAT.

2. Volume corrected mitotic index.

As it was explained above, one can assume that the CTFs encompass purely glial (and mostly neoplastic) tissue hence the number of mitotic figures counted in these fields is in fact identical with volume corrected mitotic index. In contrast to CTF, the LTF consisted of all possible "ingredients" of tumor mass (not only of glia-derived cells but e.g. blood vessels,

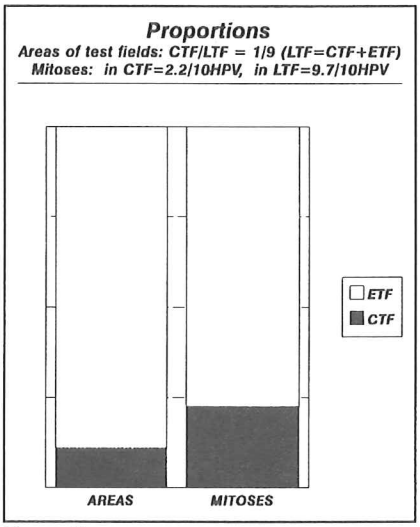

Figure 5
Mutual proportions of the areas of test fields and mean number of mitoses.

[1]. *The cellularity was counted in such a way that from the number of nuclei in a given (every) CTF, the number of "additional" nuclei in evidently bi- or multinucleated cells was subtracted. In the same fields where the contours of nuclei were traced, the expression of GFAP was evaluated as a proportion of a number of GFAP+ cells to a number of all cells.*

leukocytes etc.) and frequently a significant proportion of their area was occupied by severe necrosis. Mitoses counted in LTFs reflect the "usual" or "typical" mitotic index.
The mean of mitotic figures counted in LTFs was 9.7 per 10 LTFs and corresponding mean mitotic index counted in CTFs was 2.2 per 10 CTFs. In other words the proportion of mitotic index in CTFs to that of LTFs was like 1 to 4.4 what is markedly different than corresponding proportion of areas of CTF and LTF (aCTF/aLTF = 1/9). This discrepancy (twofold difference) seems to confirm the importance and the usefulness of volume corrected mitotic index in the more objective assessment of proliferation activity of gliomas since the "normal" mitotic index can lower even twofold the real mitotic activity of glioma cells.

DISCUSSION
Ad.1.
On the base of what was presented above it seems that NVD measured in random microscopic fields according to the described method may be an objective and useful tool in histopathological evaluation of gliomas. Not only it seems to be able to differentiate different levels of malignancy of glioma but also negatively correlates with the expression of GFAP in tumor cells and it is well established that the ability to express the GFAP decreases in glial tumors with increasing malignancy (Cras et al. 1988, Duffy et al. 1980, Luevano et al.1986, Schiffer et al.1986). The cellularity of is also a marker of malignancy (Schiffer et al.1988). The NVD and cellularity (number of cells per CTF) are positively correlated however the coefficient of correlation is not high (0.45) and it may be interesting that the NVD seems to be better than the evaluation of cellularity of glioma in differentiation especially between benign and low-malignant glioma (Figure 3 and Figure 2). A question can be raised whether the assumption of spherical shape of neoplastic cell nuclei is justifiable. In our opinion, et least in the case of glioma, it is rather the size of nuclei (and of cells) varying from very minute to monstrous that differentiates them than their shape (that in vast majority is near-to-spherical or oval). Therefore the separate counting of the mean nuclear size in each CTF has been done.
Ad.2.
Prognostic value of proliferation activity of glioma cells expressed by mitotic index (MI) is well established (Fulling and Garcia 1985, Schiffer et al.1988) however there exists an important problem of reproducibility of mitoses counting in glial tumors (Montironi et al.1988). Volume corrected mitotic index seems to be helpful in elevation of reproducibility of MI counting in gliomas since in these tumors, especially malignant ones, massive proliferations of endothelium, necroses and foci of hemorrhages are almost constantly concomitant and they may occupy marked part of tumor tissue hence influencing MI counting. MI counting adopted in our work (mitoses in CTFs) fulfills the criteria and the idea of volume corrected mitotic index (Haapasalo et al.1989) and at the same time it seems to reassure the randomness of choosing the microscopic fields that are evaluated. Moreover it can be easily joined with e.g. (also random) assessment of tumor cellularity through cell counting. We cannot totally exclude the possibility that some of the nuclei in CTFs did not belong to neoplastic glioma cells and surely probably the very singular of them may have belonged to non-neoplastic (reactive) glial cells or even to cells of non-glial origin et all. The second possibility is higher because the admixture of reactive glia usually exists in marginal zone of tumor and according to the adopted method, any LTF even partially encompassing either the border of tumor or the marginal "zona incerta" was excluded from the evaluation.

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Received: 1993-07-13
Accepted: 1993-09-23