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Analysis of Silver Binding Nucleolar Organizer Regions (Agnors) and Mitotic Index in Glioblastomas

Dr. Nitesh Rawat[1], Dr. Sudha Iyengar[2*]
[1](Resident, Department of Pathology Gajra Raja Medical College, Gwalior, M.P India)
[2*](Professor Department of Pathology Gajra Raja Medical College, Gwalior, M.P India)
[2*]Corresponding author: Dr.Sudha Iyengar

Abstract: ***Background****-Staining for Nucleolar organizing regions can be utilized as a modality to grade the glial tumours.The technique of AgNOR has been utilized for prognostic value and in the grading of a tumor and is a well-established method of estimation the proliferative activity of the tumor. Nucleolar organizer regions (NORs), which are loops of DNA (rDNA) encoded for ribosomal RNA (rRNA) production*
Objectives*- Analysis of silver binding nucleolar organizer Regions (AgNORs) and mitotic index in Glioblastomas*
Methods*-100 specimen of glial tumours submitted to the Department of Pathology, Gajra Raja Medical College and J.A Group of Hospitals, Gwalior(India) was studied. Sections were stained with routine H & E stain & with AgNOR stain.All Glial tumours were categorized and graded histologically. The mean AgNOR values (mAgNOR) per nuclei were determined.*
Results*- The average values of mean AgNORs/nucleus (mAgNOR/nucleus) in Glioblastoma Multiforme (GIV)mAgNOR/nucleus ranged from 3.1-4.2 with a group mean of 3.58±0.36 SD.mitotic index per 100 nuclei ranged from 2.5-16.7 with a mean of 15.4*
Conclusion*- The mean number of AgNORs/nucleus (mAgNOR/nucleus) showed a straightforward increase with increasing grade of malignancy. And mitotic index aso showed correlation with the mean AgNORs/nucleus. A wide range of values of AgNOR has been observed, reflecting variations in the biological behavior.*
Keywords*: Glial tumours, Astrocytomas, nucleor organizer region.*

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I. Introduction

Glioblastoma (GBM) is the most aggressive diffuse glioma of astrocytic lineage and is considered a grade IV glioma based on the WHO classification [1]. GBM is the most common malignant primary brain tumor making up 54% of all gliomas and 16% of all primary brain tumors [2]. GBM remains an incurable tumor with a median survival of only 15 months [3]. Treatment is complex, initially consisting of maximally safe surgical resection followed by radiation therapy (RT) and concurrent Temozolomide (TMZ) chemotherapy [4].

The technique of AgNOR has been utilized for prognostic value and in the grading of a tumor and is a well-established method of estimation the proliferative activity of the tumor. Nucleolar organizer regions (NORs), which are loops of DNA (rDNA) encoded for ribosomal RNA (rRNA) production [5,6].

The arrangements studied in this technique are argyrophilic non-histone proteins (AgNORs) whose silver stainability serves as an indicator for transcriptional activity of NORs [7].

A number of studies carried out in different types of tumor demonstrated that malignant cells frequently present a greater amount of AgNOR protein than non-malignant cells [8]. The AgNOR parameter has been proved to represent a reliable tool for defining the clinical outcome of cancer disease, being an independent prognostic factor in many types of tumours [9].

NORs can now be demonstrated relatively easily by routinely processed histological sections, thus the technique is obviously of potential value in diagnostic histopathology

Nevertheless, this method is a valuable aid in routine diagnosis, especially in cases when the biological behavior of a glioma is difficult to determine or when only marginal tumor areas with low cell density or very small specimens are available [11].

Cell population growth occurs as cells pass through interphase and mitosis to complete the cell cycle. Many cells lose the capacity to divide as they mature or divide only rarely. Other cells are capable of rapid cell division. For example, as plant roots grow, cells near the tip of the root, in the apical meristem, divide rapidly to push the root through the soil. The root cap detects the pull of gravity and directs the rapid growth of cells near the tip.One way to quantify cell division is by using the mitotic index: Number of cells in mitosis per 1000 cells.

II. Material Methods

- This study was conducted for a period of one and a half years (Oct 2016-March 2018) on 100 specimen of glioblastomas submitted to the Department of Pathology, Gajra Raja Medical College and J.A Group of Hospitals, Gwalior.
- Patients of both sex were taken into consideration.
- History of the patients along with relevant investigations was recorded. Particular stress was given on the age, sex and clinical findings of the patient
- Thin sections were cut and stained with routine hematoxylin and eosin stain. All slides are thoroughly evaluated for histopathological features.
- All glial tumours are categorized histologically according to WHO Criteria.
- The sections are stained with AgNOR stain and The mean AgNOR values (mAgNOR) per nuclei were determined.
- For determination of MI, serial sections were stained with H & E. All counts were performed at a magnification of 400 x. Identical areas of parallel sections were chosen for the calculation of the MI/1000 cells. Regularly MI was also examined by counting all cells in adjacent fields. MI was based on median counts of 1000 cells in glioblastomas.

Principle

Negatively charged carboxyl groups in the nonhistone proteins of nucleolar organizer regions bind initially to Silver ions and cause the reduction of ions to the metal. Submicroscopic nuclei of metallic Silver, then act as foci for the further deposition of Silver drawn from the less electronegative Sulphydryl groups. The-SH groups cause the micro deposition of Silver into the characteristic black dots of Silver grains which are visible at low microscopic magnifications.

AgNOR Staining method

1. Sections were dewaxed in xylene, hydrated through alcohols to water.
2. Then the sections were rinsed with deionized water 3 times.
3. Sections were incubated with working staining solutions (freshly prepared) for 60 minutes under dark room temperature condition.
4. Sections were rinsed in deionized water for 10-15 minutes.
5. Sections were dried cleared in Xylene and mounted in DPX.

III. Results

Table 1: Mean values of AgNORs in tumoral nuclei

Malignancy grade	Average number of AgNORs/nucleus	
	Range	Group (Mean±SD)
Glioblastoma (G IV)	3.1-4.2	3.58 ± 0.36

Table 2: mitotic index per 1000 tumoral nuclei

Malignancy grade	Mitotic index per 1000 cells	
	Range	Mean
Glioblastoma (G IV)	2.5-16.7	15.4

The average values of mean AgNORs/nucleus (mAgNOR/nucleus) in Glioblastoma Multiforme (GIV)mAgNOR/nucleus ranged from 3.1-4.2 with a group mean of 3.58±0.36 SD.mitotic index per 100 nuclei ranged from 2.5-16.7 with a mean of 15.4.

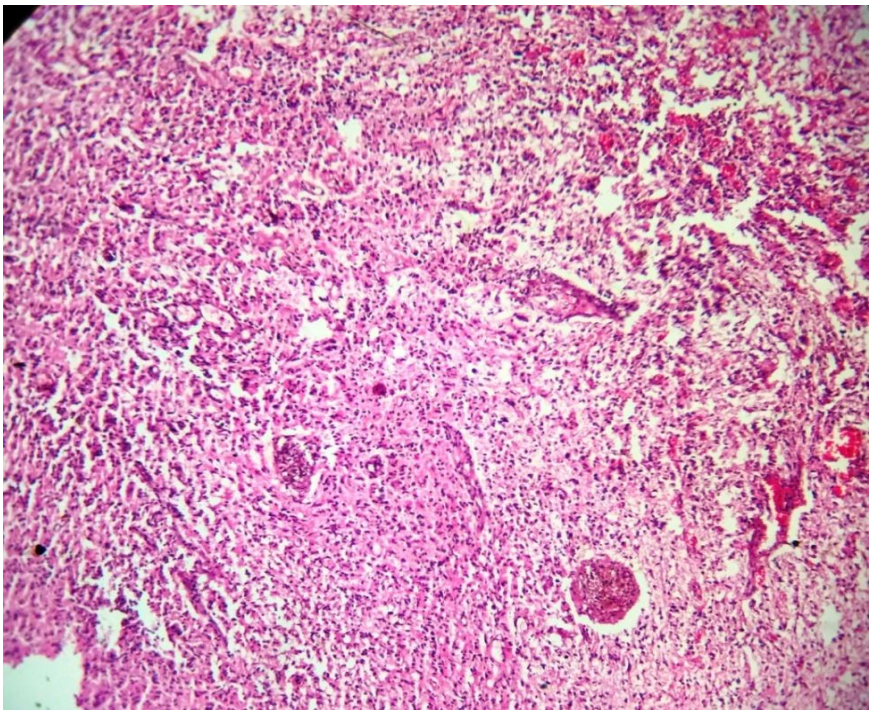

Figure 1 : Glioblastoma Multiforme (Endothelial cell proliferation) (40X,H&E)

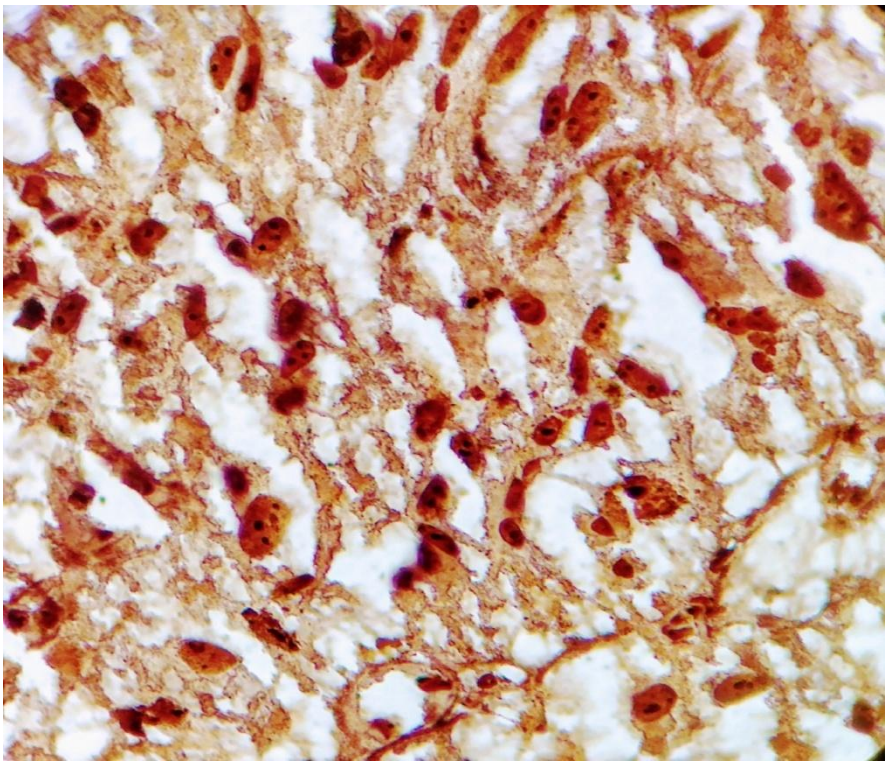

Figure 2: AgNOR staining of Glioblastomaindicating black dots in tumoral nuclei (silver impregnation method, magnification x100).

IV. Discussion

This was a prospective study done by critical analysis of the information obtained from the patient's requisition forms and record files of pathology department Gajra Raja Medical College, Gwalior (Madhya Pradesh) from 1st December 2016 to 30th May 2018.

The requisite information was collected from the patient's requisition forms which were sent from Bhagwat Sahay Hospital Department of neurosurgery Jayarogya group of hospital Gwalior (Madhya Pradesh).

Analysis of the AgNOR's mean number is a widely accepted method for diagnosis of a wide range of tumors, both on cytological and histological preparations.

In 1986, Ploton et al.[11] have used and improved the silver impregnation method brought in histopathological practice by Goodpasture and Bloom in 1975[12]. Ploton et al. [13] suggested that the number of AgNORs/cell correlates with the proliferative activity of a cell and may be an indicator of malignancy, because larger the number of AgNORs more active is the proliferation, and thus a more malignant cell.

AgNORs have been found to be markers of proliferation in brain Gliomas because from 1990 onwards some articles have shown linear correlations between the number of AgNORs, binding index of Ki-67, Proliferating Cell Nuclear Antigen (PCNA), or histological grade and the mitotic rate[21].

Hara et al. 1991 [14] calculated mAgNOR/vascular nucleus of Astrocytomas grade II, III and IV (1.80±0.13, 2.87±0.50, and 3.13±1.13) and found it significantly higher than that of the vascular nucleus of the normal brain. It must be noted that in the present study we did not calculate mAgNOR/vascular nucleus. Hara et al. used 1979 WHO classification of CNS neoplasms and we used 2016 WHO classification of CNS neoplasms. All these results showed that proliferative activity, both in vascular tumoral cells and in tumoral cells of the gliomas, increased with increasing grade of malignancy and therefore evaluation of AgNORs is a useful marker in deciding the grade of malignancy.

Janczukowicz (2003)[15]found a significant relationship between the number of AgNOR and histological grade in astrocytic tumors, but the main stress was on the presence of a small overlapping of extreme values between GII and GIII Astrocytomas and between GIII and GIV[17]

Gabriela – Florenta Dumitrescu et al. (2010)[16]concluded that the presence of an increased mean number of mAgNOR/vascular nucleus in Anaplastic Astrocytomas (GIII) and, especially,in Glioblastomas Multiforme (GIV) suggest that the proliferative processes of vascular cells and tumor cells are interconnected.

Hoshino and Wilson [17] found Tc of 36-152 h without differences between their glioblastomas and malignant astrocytomas. Tc of these two oncotypes computed from our data are also only slightly different and are within the same order as the data of Hoshino and Wilson. Nevertheless, the authors did not give data for grade II gliomas. The prolongation of Tc in comparison to glioblastomas, however, was shown in an earlier study of Hoshino et al. by a [3H]/[14C]thymidine doublelabeling technique; 58-230 h for four glioblastomas, 154-316 h for three malignant astrocytomas and 1154 h for one differentiated astrocytoma. This is in very good agreement with our calculation using a different technique, but which is statistically valid due to a large number of cases. If this is correct, the proliferation activity of a glioma would depend on GF and Tc in the same way. Therefore, the knowledge of the exact size of Tc could be important for the planning of adjuvant postoperative therapy.

R. Schroder, K. Bien, R. Kott, I. Meyers, and R. Viissing et al.[18] studied The relationship between Ki-67 labeling and mitotic index in gliomas and meningiomas: demonstration of the variability of the intermitotic cycle time and found that On serial frozen sections Ki-67 LI and MI were determined in nearly identical areas of 32 glioblastomas, 20 grade III astrocytomas, 21 grade II astrocytomas and 20 selected cases of meningioma. The data not only clearly showed different median values of LI and MI for the various malignancy grades, but also similar regression coefficients for each glioma type. A non-linear relationship between the two indices was found for all glioma cases with high significance and high correlation coefficient; (LI) = 5.6 (MI).

V. Conclusion

The present study comprised of the prospective histopathological study of 100 cases of glial tumors from 1st December 2016 to 30th may 2018 in the department of pathology, Gajra Raja Medical College, Gwalior(M.P.).

The mean number of AgNORs/nucleus (mAgNOR/nucleus) showed a straightforward increase with increasing grade of malignancy. And mitotic index aso showed correlation with the mean AgNORs/nucleus. A wide range of values of AgNOR has been observed, reflecting variations in the biological behavior.

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