Assessment of Nestin and Hypoxia Inducible Factor -1α Expression in Apparently Normal Brain Tissue / Peritumoral Areas Adjacent to Astrocytomas

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Received 20 January 2014; Accepted 3 February 2014

Abstract

The surgical biopsy from astrocytomas is usually obtained from enhanced lesion but sometimes may include fragments of apparently normal brain tissue /peritumoral areas. We hypothesize that the evaluation of these fragments for presence of cancer stem cells may have prognostic and /or diagnostic implication. This work has been planned to 1) verify nestin and hypoxia inducible factor-1α location in apparently normal brain tissue/peritumoral areas and its relation to the presence of neoplastic cells in these fragments and 2) analyze the correlation between nestin and hypoxia inducible factor-1α expression in apparently normal brain tissue/peritumoral areas adjacent to different grades of astrocytomas.

Material and Methods: Paraffin-embedded sections of selected thirty specimens of astrocytomas contain apparently normal brain tissue/peritumoral areas (12 diffuse astrocytomas, 6 anaplastic astrocytomas and 12 glioblastoma multiforme) were stained with nestin and hypoxia inducible factor-1α using standard immunohistochemical approaches. The immunoreactivity for nestin and Hypoxia-inducible factor -1α was evaluated.

Results: Compared to nearby astrocytomas, nestin immunoreactivity was scarcely expressed in apparently normal brain tissue/peritumoral tissue. There was statistically significant (P =0.03) gradual increase of mean percentage of nestin positive cells that present in these fragments with increasing grade of the adjacent astrocytomas from diffuse astrocytoma to anaplastic astrocytoma to glioblastoma multiforme (27.1± 4, 38.3±3 and 47.5±5.9, respectively). There was statistically insignificant (P < 0.1) gradual increase of mean percentage of hypoxia inducible factor-1α positive neoplastic cells that infiltrate peritumoral areas with increasing grade of the adjacent astrocytomas. There was statistically significant gradual increase of counting of microvessels that showed positivity for nestin and hypoxia inducible factor-1α expression with increasing grade of the adjacent astrocytomas (P=0.008, P=0.01, respectively).

Conclusion: Nestin and hypoxia inducible factor-1α was expressed in apparently normal brain tissue/peritumoral tissue adjacent to astrocytomas. Positivity for nestin in apparently normal fragments may indicate premalignant changes or nearby tumor when a biopsy contain only apparently normal brain tissue. Evaluation of nestin expression in peritumoral areas may have prognostic value as it may be an indicator for rapid recurrence and resistance of treatment.

Introduction

Astrocytomas represent 75% of all gliomas. Astrocytomas fall into two distinct categories based on how they interact with their surrounding microenvironment: diffuse and localized astrocytomas. Localized astrocytomas have a circumscribed pattern of growth and restricted invasive potential, whereas diffuse astrocytomas are characterized by their cellular infiltration of the peritumoral margin. According to WHO classification, diffuse astrocytomas are classified into three grades: diffuse astrocytoma (AII, WHO grade II), anaplastic astrocytoma (AA,WHO grade III) and glioblastoma multiforme (GBM, WHO grade IV) [1].
75% of patients with AII die either from tumor recurrence or malignant progression [2]. The overall survival times show significant variation between cases with a median of 6 years. The higher grade astrocytomas (i.e. grades III and IV) are progressively more proliferative and invasive than lower-grade tumors with median survival time of about 2-3 years for AA and <1 year for GBM [3]. Recurrence is mainly due to the highly invasive behavior which allows the active migration of glioma cells from the main tumor mass into the surrounding normal brain tissue. Therefore, invasive glioma cells which are localized to the tumor and peritumoral environment are essentially beyond the reach of current therapies [4].

Astrocytomas may derive from the transformation of neural stem cells or transiently dividing progenitors. Differentiation of mature and immature elements is based on the expression of nestin. Nestin is expressed in stem cells, undifferentiated cells and radial glia during the developing mammalian brain. However, the differentiation of neural stem cells/progenitor cells into postmitotic astrocytic and neural cells is associated by the down-regulation of nestin and the expression of other types of intermediate filaments [5]. Nestin expression is detected in proliferating endothelial progenitor cells, but not in mature endothelial cells. Nestin is also reported in glioblastoma, prostate cancer and colorectal cancer and its expression is more specific for newly formed blood vessels [6].

Subpopulation of cancer cells was called cancer stem cells (CSCs) and also known as tumor initiating or propagating cells [7]. Cancer stem cells arise from cumulative mutation of normal stem cells [8] or originate from dedifferentiation of already differentiated tumor cells [9]. Cancer stem cells are sharing stem cell properties like expression of neural stem cell markers, capacity of self-renewal, and cell differentiation potential. Cancer stem cells have been proposed to be responsible for tumor initiation and progression [10].

Stresses like hypoxia, nutrient starvation, and inflammation can cause CSCs to leave their quiescent state and re-enter the cell cycle [11]. Additionally, hypoxia promotes reprogramming of tumor cells towards CSCs [12]. Gliomas are often characterized by profound hypoxia that leads to upregulation of hypoxia inducible factor-1α (HIF-1α) and overexpression of numerous cytokines and chemokine receptors. Neural stem cells preferentially localize to hypoxic areas in the primary tumor bed and peritumoral areas of glioma xenografts. These data suggest that hypoxia is one of the significant factors in determining stem cell tropism to glioma [13].

Recently, it has been reported that neoplastic cells present not only in enhanced lesion (i.e tumor) but also in peritumor tissue. These neoplastic cells invading into brain tissue are likely to be enriched with CSCs [5] [14] [15] [16]. In our specimens, the surgical biopsy is usually obtained from enhanced lesion but sometimes it may include fragments of apparently normal brain tissue (ANBT). So we hypothesize that the evaluation of these fragments which may be peritumoral areas for presence of CSCs may have prognostic and / or diagnostic implication. To explore our hypothesis, this work has been planned to 1) verify nestin and HIF-1α location in ANBT/peritumoral areas, 2) evaluate nestin and HIF-1α expression in relation to the presence of neoplastic cells in ANBT/peritumoral areas, 3) analyze the correlation between nestin and HIF-1α expression in ANBT/peritumoral areas adjacent to different grades of astrocytomas.

Material and Methods

Selected thirty biopsies contain ANBT/peritumoral areas adjacent to astrocytomas were obtained from the archival material from Surgical Pathology Laboratory, Faculty of Medicine, Assuut University and private laboratories. Tumors were classified according to the WHO 2007 classification [17]. Full clinical data were obtained from the clinicopathological referral sheets. Reevaluation of biopsies revealed the presence of fragments of ANBT/peritumoral areas in 14 diffuse astrocytomas (AII), 6 anaplastic astrocytoma (AA), and 10 glioblastoma multiform (GBM).

Immunohistochemistry

Immunohistochemical analysis was performed on 4 μ-thick sections using the streptavidin-biotin-peroxidase technique. The sections were routinely deparaffinized, rehydrated through graded alcohols to distilled water. Deparaffinized sections were treated with 0.3% hydrogen peroxide for 10 min. Antigen retrieval was carried out by microwaving the slides at 95°C for 12 min and cooling at 25°C for 2 hours. Blocking serum was applied for 10 min. At room temperature, the sections were incubated for 30 min with a rabbit polyclonal antihuman antibody raised against the nestin, diluted 1:100 (Catalog E18610, Spring Bioscience, USA) and with a mouse monoclonal antihuman antibody raised against HIF-1α (Clone H1alpha 67, Catalog_MS-1164-P0, Thermo Scientific, USA), diluted 1:100. The resulting immune complex was detected by a universal staining kit (Anti-polyvalent HRP diaminobenzidine (DAB) Detection System, Catalog. TPD-15, Spring Bioscience, USA), following the instructions attached with the kit. Slides were washed several times in phosphate buffer saline and placed in it for 5 minutes between each step except after peroxidase reagent the washing was by distilled water. Negative and positive controls were included in each staining series.

Positive control: sections from kidney were used as a positive control for nestin, and nestin positivity was identified as brownish cytoplasmic staining of epithelial tubular cells. Sections from skin were used as positive control for HIF-1α. HIF1α positivity was identified as brownish cytoplasmic staining of basal cell layer of epidermis.

Negative control: additional sections were stained in parallel with omission of the primary antibodies as a negative control.

Immunohistochemical evaluation of nestin and HIF-1α:

The nestin and HIF-1α positive cells in ANBT/peritumoral areas were evaluated according to
Results

From 4 to 10 randomly selected fields in the apparently normal brain tissue fragment in each section were evaluated by two independent observers (Dr. Dalia Elsers and Prof. Dr. Rabab Elgorori) at x400 magnification using a light microscope. The few cases with discrepant scoring were reevaluated jointly and agreement was reached. All immunolabeled cells excluding the endothelial ones were evaluated. The percentage of positive cells was calculated and reported as average ± standard error of men (SEM).

Evaluation of positive microvessels for nestin and HIF-1α: The positive microvessels for nestin and HIF-1α in ANBT/peritumoral areas of each section was calculated according to [18]. Briefly, each section was observed at x100 magnification to identify the microvessels. Positively stained microvessels for nestin and HIF-1α were then counted at x200 magnification. Clusters of endothelial cells which were clearly separated from adjacent astrocytoma cells were recognized as countable microvessels. A total of five randomly selected fields at x200 magnification per slide were evaluated and the mean of positive microvessels for nestin and HIF-1α was calculated.

Statistical analysis

Mean, range and standard error of mean (SEM) were measured. Non – parametric tests and receiver's correlation coefficient with a statistical significance of p < 0.05 were used. (SPSS analysis program, windows 1998).

Results

The studied specimens included AII 14/30 (46.7%), AA 6/30 (20%) and GBM 10/30 (33.3%). The patient's age of the studied astrocytomas ranged from 17 -72 years with mean 45.2 ± 15.4. The mean age of patients with AII, AA, and GBM at the time of diagnosis were 39.1±12.7, 43.1±20, 52±14 years, respectively. The mean percentage of positive cells was calculated and reported as average ± standard error of men (SEM).

Statistical analysis

Non – parametric tests and receiver's correlation coefficient with a statistical significance of p < 0.05 were used. (SPSS analysis program, windows 1998).

Elsers et al. SECI Oncology 2014
DOI: 10.18056/seci2014.2

HIF1-α expression in the studied ANBT/peritumoral areas adjacent to astrocytomas

Hypoxia inducible factor -1α immunoreactivity was localized in the cytoplasm of neoplastic cells infiltrating ANBT/peritumoral areas and it was expressed in endothelial cells of microvessels in ANBT/peritumoral areas.

The expression of HIF-1α in neoplastic cells was noted in ANBT/peritumoral areas (28/30, 93.3%) adjacent to astrocytomas (12/14 AII, 6/6 AA, and 12/12 GBM). There was statistically insignificant (P = 0.1) gradual increase of mean percentage of HIF-1α positive neoplastic cells that infiltrate ANBT/peritumoral areas with increasing grade of the adjacent astrocytomas from AII to AA to GBM (6.4±1.0, 6.7±1.1 and 13±2.9, respectively) as noted in table (1) and Fig (3).

The expression of HIF-1α in endothelial cells lining microvessels was detected in 26/30 (86.7%) of ANBT/peritumoral areas nearby astrocytomas (10/14 AII, 6/6 AA, and 12/12 GBM). There was statistically significant (P = 0.01) gradual increase of counting of microvessels revealed HIF-1α positivity in endothelial cells present in ANBT/peritumoral areas with increasing grade of the adjacent astrocytomas from AII to AA to GBM (2.4±0.5, 3.7±0.6 and 5.5±0.8, respectively). These results are summarized in table (2).
Table (1): Nestin expression in the studied ANBT/peritumoral areas adjacent to astrocytomas

<table>
<thead>
<tr>
<th>N. of biopsies with ANBT adjacent to astrocytomas</th>
<th>Mean of % of total positive cells</th>
<th>Mean of % of positive NCs</th>
<th>Mean of % of positive RA</th>
<th>Mean of % of positive ANCs</th>
<th>Mean of % of counting MV have positive ECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AII (n=14)</td>
<td>27.9±4</td>
<td>17.5±3.5</td>
<td>6.8±0.9</td>
<td>3.6±1.4</td>
<td>5.6±0.9</td>
</tr>
<tr>
<td>AA (n=6)</td>
<td>39.2±3</td>
<td>21.7±1.7</td>
<td>10±0.0</td>
<td>7.5±1.1</td>
<td>10.8±2.4</td>
</tr>
<tr>
<td>GBM (n=10)</td>
<td>50.8±5.9</td>
<td>32.5±4.7</td>
<td>10±0.5</td>
<td>8.3±3</td>
<td>14±2.6</td>
</tr>
<tr>
<td>Total (n=30)</td>
<td>39.3±3.2</td>
<td>23.9±2.4</td>
<td>8.9±0.5</td>
<td>6.5±0.8</td>
<td>9.5±1.2</td>
</tr>
<tr>
<td>P-value</td>
<td>0.03</td>
<td>0.04</td>
<td>0.01</td>
<td>0.03</td>
<td>0.008</td>
</tr>
</tbody>
</table>


Table (2): HIF-1α expression in the studied ANBT/peritumoral areas adjacent to astrocytomas

<table>
<thead>
<tr>
<th>N. of biopsies with ANBT adjacent to astrocytomas</th>
<th>N. of cases had positive cells</th>
<th>Mean of % of positive NCs</th>
<th>N. of cases had positive VECs</th>
<th>Mean of % of positive ECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AII (n=14)</td>
<td>12</td>
<td>6.4±1.0</td>
<td>10</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td>AA (n=6)</td>
<td>6</td>
<td>6.7±1.1</td>
<td>6</td>
<td>3.7±0.6</td>
</tr>
<tr>
<td>GBM (n=10)</td>
<td>10</td>
<td>13.2±2.9</td>
<td>10</td>
<td>5.5±0.8</td>
</tr>
<tr>
<td>Total (n=30)</td>
<td>28</td>
<td>8.7±1.2</td>
<td>26</td>
<td>3.7±0.5</td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Fig. 1- A & B: Immunohistochemical staining of nestin in ANBT/peritumoral areas adjacent to diffuse astrocytoma. 1-A: Arrow point to ANBT/peritumoral area, x100. 1-B: shows positive neoplastic cells (thick arrow), apparently normal cells (thin arrow) and positive endothelial cells in microvessels in the lower right side, x400. The insert illustrate positive reactive astrocytes are characterized by dendritic morphology and abundant cytoplasm, x1000.

Fig 2 - A & B: Immunohistochemical staining of nestin in ANBT/peritumoral areas adjacent to high grade astrocytoma. 2-A: Apparently normal brain tissue (right side) shows decrease in cell density stained by nestin in comparison to adjacent tumor, x200. 2-B: illustrates more positive neoplastic cells and apparently normal cells, x400 and x1000 (insert).
**Correlations between Nestin and HIF-1α expression in ANBT/peritumoral areas adjacent to the studied astrocytomas:**

Majority of the studied cases (26/30, 78.1%) showed positive staining for both nestin and HIF1-α. All cases of ANBT/peritumoral areas adjacent to high grade astrocytomas were positive for both nestin and HIF-1 α. Two biopsies of AII showed positive staining for nestin and negative expression of HIF-1 α in cells that present in ANBT while four biopsies revealed positive staining for nestin and negative expression of HIF-1 α in endothelial cells lining microvessels.

The mean of percentage of HIF-1α positive neoplastic cells (8.7±1.2) are lower than the mean of percentage of nestin positive cells (23.3±2.4). There is statistically insignificant correlation between expression of nestin and HIF1-α in ANBT/peritumoral areas adjacent to astrocytomas (r = 0.2, P = 0.2).

**Discussion**

Astrocytomas are characterized by aggressive invasion into surrounding brain tissue and recur rapidly despite maximal surgical resection. Conventional treatments can not fully eliminate the infiltrated resistant cancer cells. These resistant cancer cells are enriched with CSCs. The discovery of brain tumor stem cells and their chemo- and radioresistance have provided a reasonable explanation for the difficulty in these tumors treatment and the high rate of relapse. A better understanding of the molecular makeup of CSCs and the key differences between CSCs and non- CSCs could lead to powerful novel therapies to treat astrocytomas by preventing recurrences. Stem cell-based gene therapies may be an effective way to target brain tumors [19].

The present study was performed on selected 30 biopsies that contain ANBT/peritumoral areas adjacent to astrocytomas. Histopathological examination of ANBT revealed the presence of infiltrating neoplastic cells and reactive astrocytes. These results are in accordance with previous literatures [5] [16]. Aggressive invasion of glioma cells into brain tissue often prevents complete surgical resection and leads to tumor recurrence [14]. So, adjacent apparently normal tissue infiltrated by neoplastic cells are frequently present in biopsies of astrocytomas.

In this series, all ANBT adjacent to astrocytomas showed positive expression for nestin. Nestin positivity was detected in neoplastic cells that infiltrate the ANBT/peritumoral areas, in reactive astrocytes and in apparently normal astrocytes. These results are in agreement with the results of the literature [5]. Nestin may be considered a maker for neural stem cells in the adult mammalian brain and distinguishes less differentiated from differentiated cells in astrocytomas [20]. So, expression of nestin in neoplastic cells that infiltrate the adjacent normal brain tissue in studied astrocytomas point to stem cells or undifferentiated nature. Cancer stem cells or undifferentiated cells that expressing nestin are able to migrate from the primary tumor and invade into normal surrounding brain forming microsatellites of malignant cells that can evade a surgical resection [5] [19]. Astrocytoma cells generate considerable amounts of glutamate which can lead to excitotoxity of neurons in the brain parenchyma surrounding the astrocytoma and thus promoting the invasive and growth of cells [21]. Nestin is an intermediate filament protein (IF) that is considered one of crucial modulators of cell movement through interactions with other IF. Nestin downregulation inhibited the invasion into ECM components. Restoring nestin expression in nestin-downregulated cells restored the original capacity of these cells to migrate and invade [22] [23].

Following various CNS injuries and diseases, astrocytes are activated and described as “reactive astrocytes” [24] [25]. Pronounced changes in astrocytes morphology noted in studied ANBT/ peritumoral areas adjacent to the astrocytomas like abundant cytoplasm and large eccentric nucleus are accompanied by nestin...
expression. These results are concomitant with data in the literatures [21] [26]. Nestin expression is downregulated in precursor cells upon their differentiation along their respective neural or glial cell types. Re-expression of nestin has been shown in reactive astrocytes following brain injuries [27] [26]. Nestin is involved in cell division, structural integrity and mobility of cells. Thus, nestin induction plays a role in the change of normal resting astrocytes to reactive astrocytes allowing for the structural remodeling of these cells in response to various injuries [28].

Nestin immunoreactivity of apparently normal cells in studied ANBT/peritumoral area may support the idea of an induced premalignant state, which may lead to a full transformation or may represent the initiation of independent foci of transformed cells [5].

The aforementioned data guide clarification of the gradual increase of nestin expression in neoplastic cells and apparently normal cells in studied ANBT/peritumoral areas with increasing grade of the adjacent astrocytomas from AII to AA to GBM. These results are in line with previous studies [29] [30]. They noted that nestin was gradually upregulated with advance grading of the astrocytomas. Accordingly, the upregulation of nestin in ANBT/peritumoral areas is imitating its expression in astrocytomas. These results are discordant with results of Idoate et al [14] who noted that nestin expression is strong and constant in the tissue around the tumor, but is mostly a glial reaction, not specific of the neoplasm. This discrepancy may be attributable to methodological issues as they studied only peritumoral tissues in GBM.

In the present study, The HIF-1α positivity in ANBT/peritumoral areas was noted adjacent to most (93.3%) of the studied astrocytomas but with diminished values of mean percentage of HIF-1α positive cells (8.7±1.2) in comparison to mean percentage of nestin positive cells (36.2±3.2). The HIF-1α was only detected in neoplastic cells. There was irrelevant increase with increasing astrocytomas grading. The environment in ANBT/peritumoral areas adjacent to studied astrocytoma is more or less normoxic. The stability and activation of HIF-1α in cancer cells may be regulated by many factors rather than hypoxia like sustained stimulation of different growth factors and its receptors, cytokine pathways and oxidative stress under normoxic and hypoxic conditions. Also, inactivation of tumour suppressor proteins such as von Hippel lindeau (VHL) and p53 may impair HIF degradation and lead to HIF-1α protein accumulation [31].

Hypoxia inducible factor-1α positivity could not be detected in reactive astrocytes in studied ANBT/peritumoral tissue nearby astrocytomas because astrocytes are very resistant to hypoxia due to several factors. First, astrocytes have large glycogen stores that can be metabolized to glucose and supply energy during ischaemia and glucose deprivation. Secondly, astrocytes have a low energy demand. Thirdly, astrocytes have a higher level of reduced glutathione, an important antioxidant [32]. Positivity of astrocytes for HIF-1α means that they were exposed to severe hypoxia so mild hypoxic condition in peritumoral areas was not sufficient to cause any significant changes in HIF-1α expression [33].

Nestin immunoreactivity was detected in endothelial cells lining the microvessels in the studied ANBT/peritumoral areas bordering the astrocytomas in agreement with previous studies [5] [29]. Nestin was detected in proliferating endothelial cells either in adult tissues that replenish by angiogenesis or in cancers and was considered as a marker for neovascularization [34] [23] [6]. In the studied ANBT/peritumoral areas, the mean counting of microvessels stained with nestin was increased with advance grading of the adjacent astrocytomas. Also nestin positive cells increased in the studied ANBT/peritumoral areas with increased adjacent astrocytoma grade and these cells may represent CSCs that augment angiogenesis by expressing elevated levels of vascular endothelial factor (VEGF) [7]. So angiogenesis is enhanced in upgrading mode and hence nestin positive microvessels.

Hypoxia inducible factor-1α expression that detected in endothelial cells lined microvessels in ANBT/peritumoral areas in studied astrocytoma and increased with astrocytomas grading may be resulting from cyclic hypoxia in endothelial cells. Cyclic hypoxia (i.e. several cycles of hypoxia / reoxygenation) induce MicroRNA transcription. Micro RNA regulates HIF-1α isoforms specifically in endothelial cells by stabilizing HIF-1α isoforms through impairing its degradation [35] [36].

In the present work, the correlation between the expression of nestin and HIF-1α was irrelevant in peritumoral areas nearby astrocytomas. Profound hypoxia that characterizes astrocytomas leads to upregulation of HIF-1 α and plays an important role in trafficking of stem cells to astrocytoma [37]. However, the expression of HIF-1α in the studied ANBT/peritumoral areas may be related to many factors other than hypoxia like aberrant expression of many genes like wild-type p53 and VHL [31].

Conclusion

As CSC are the usual mechanism for tumor initiation and resistance to adjuvant therapy, so evaluation of ANBT/peritumoral areas for nestin and HIF-1α positive neoplastic cells may be an indicator for rapid recurrence and treatment resistance. Positivity for nestin in apparently normal fragment may indicate premalignant changes or nearby tumor so evaluation of ANBT may have a diagnostic role as it suggest the presence of tumor when the biopsy contain only ANBT. Further study for evaluation of CSCs presence in ANBT/peritumoral areas (non enhanced area) in context of more specific marker for CSC is recommended as nestin expression appears to define dedifferentiation state of cells.

References

[1] Hoelzinger DB, Demuth T, Berens ME: Autocrine factors that sustain glioma invasion and paracrine biology in the brain


