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Original Article

Virulence estimation by calculation of relative expression of NESTIN in different grades of astrocytoma among Iraqi patients

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Abstract:

Background: Astrocytoma, is heterogeneous tumor of the nervous system and studies on the virulence of these tumors reveal that their behavior is led by small population of cells which are the brain tumor stem cells (BTSCs) that drive the continuous proliferation and self-renewal. From the many markers that annotate BTSCs, are the CD133, and NESTIN.

Objectives: Using CD133, to immunolabel BTSCs niches in paraffin sections of astrocytoma then, extraction of these cells, to calculate fold expression of NESTIN gene across the grades by real time PCR.

Materials and methods: Paraffin blocks of four grades of primary astrocytoma have been selected from three age groups from Iraqi patients. The age groups were stratified into: children (0-17years) adults (18-49years) and older (50-80years). The samples were stained with CD133, then positive areas were extracted to perform relative expression of NESTIN gene by real time PCR

Results: The expression of NESTIN for all grades, increased by tens of folds in relation to calibrator then this fold expression increased dramatically in an ascending manner with increasing of the grade in the same group. The fold expression of NESTIN gene in all grades was the highest in the adulthood (18-49) years group.

Conclusion: Relative expression of NESTIN gene of BTSCs in different grades of astrocytoma can be a useful tool for the assessment of the virulence with ascending the grades. This expression was the highest at age (18-49) years.

Keywords: virulence, astrocytoma, NESTIN, BTSCs, grades, ages

Introduction:

Astrocytoma, the most common primary tumors of the central nervous system (CNS), are derived from astrocyte (1) and are graded, according to WHO-2007 into: grade I, II, III, IV (2). many markers are considered in studies of astrocytoma by immunohistochemistry for the whole bulk tumor (3). Despite laborious treatment of the astrocytoma, most of the tumors develop the ability to resist the treatment and/or the recurrence after surgical removal (4). More recently, researches on solid tumors suggest that the tumor cell population is heterogeneous in behavior regarding to proliferation and differentiation and astrocytic tumors are typically encompassed of morphologically different types of cells that express variable markers of differentiated and undifferentiated cells (5) Accordingly, not all tumor cells have the same ability to proliferate and maintain the growth of the tumor (5,6) Only a relatively small fraction of cells in the tumor, termed brain tumor stem cells (BTSCs), possess this capability to proliferate and self-renew extensively and especially able to differentiate into different "lineages and clones" that generate tumor masses (7,8). While BTSCs have the ability to form a continuously growing tumor (9,10) most of other tumor cells lose the ability to proliferate and self-renew and they differentiate into tumor cells that become the "phenotypic signature of the tumor" (5). Thus, BTSCs are thought to be the driving factor for "intratumoral heterogeneity", cancer virulence, metastasis (11) and the cause for resistance to radiotherapy and/or chemotherapy, and targeting them will lead to "tumor regression" (7,8). Both pediatric and adult bulk tumors are supplied continuously by these "slow cycling stem cells" that harbour the potential of tumour-initiation (12). These distinct compartments demand distinct identification, thereby differential treatments for both BTSCs and bulk tumor cells (13). The BTSCs were solely isolated by expressing the stem cells surface marker "CD133" (14). NESTIN marker also linked to these special virulent populations of brain tumors (15). NESTIN is a class "VI intermediate filament" protein

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that was first described as a Neuroepithelial stem cells marker and it has been shown to be "down-regulated" or completely disappear during Neuroepithelial stem cells differentiation. NESTIN expression has been reported in various tumor cells types originating from the CNS, including astrocytoma. It plays important roles in cellular processes, including migration, and cell cycle regulation (16). From a clinical viewpoint, identification of BTSCs by CD133 and then elucidation of gene expression profile of these BTSCs by NESTIN has substantial implications clinically like virulence prediction of different grades and planning of new therapeutic lines targeting BTSCs utilizing NESTIN expression profile (17). It also allows to estimate the content of stemness inside different grades of the tumors (12).

Thus, specimens of Formalin-Fixed Paraffin-Embedded Tissue astrocytoma have been processed for:

(1) Immunolabelling with CD133, to delineate the BTSCs niches (2) Calculating the relative expression of the gene NESTIN extracted from those CD133 positive BTSCs, by real time PCR, to obtain relative expression in relation to its most basic form grade I , then compared between the grades and ages. We aimed to check the relative expression of NESTIN in between grades and ages of astrocytoma, to reveal if the grading and/or aging has effect upon its expression change.

Materials and methods:

In year September 2018-April 2019, 121 Specimen blocks of primary brain tumors was retrieved from the archives of pathology of department/Specialized Surgery Hospital in Baghdad/Iraq. Astrocytomas were diagnosed and graded according to the "WHO, histological classification". The specimens were categorized equally into the foue grades of astrocytoma and distributed into 3 different age groups, which are (group of childhood (0-17years), group of adulthood and middle age (18-49 years) and group of elderly age group (50-80years). Each subgroup of each grade has 11 samples. The study lasted from September 2018-to April 2019. First of all, staining by CD133 Polyclonal Anti- CD133 (MBS355164) (My Biosource) and Ready-to-use IHC/ICC kit (Biotin free), One-Step HRP Polymer anti-Mouse, Rat & Rabbit IgG with DAB # MBS841593 (My Biosource) was done, to localize region of CD133 positive cells, which are referred to the (BTSCs), then "tissue coring" was performed for isolation of these regions from the blocks to perform RNA extraction. The core biopsy was deparaffinized by adding xylene then ethanol (100%). Deparaffinized Cores was grinded by tissue grinder, and homogenized by pipetting up and down several times, and the pellet was air dried for approximately 15 min before proceeding with RNA extraction. For RNA extraction, Whole RNA extraction was performed using RNeasy FFPE from (Qiagen). Samples were incubated in an optimized lysis buffer, which contains proteinase K, to release RNA from the sections. A short incubation at a higher (80 c°) temperature partially reverses formalin crosslinking of the released nucleic acids, improving RNA yield and quality. This was followed by DNase treatment that had been optimized to eliminate all genomic DNA, Next, the lysate was mixed with Buffer RBC. Ethanol was added to provide appropriate binding conditions for RNA, and the sample was then applied to a RNeasy MinElute spin column, where the total RNA binds to the membrane and contaminants were efficiently washed away. RNA was then eluted in 14 µl of RNase-free water. (manufacturer protocol, Qiagen). Equipment used for measuring the concentration of RNA is Quantus fluorometer (promega). The concentration of the RNA was 6.7 ng/µl. Equipment for measurement of the purity of the RNA was Nanodrop spectrophotometer (thermofischer scientific) at 280/260 wavelength. A ratio of 1.8 was obtained. Reverse transcription is done by "QuantiTect® Reverse Transcription" (Qiagen for use in two-step RT-PCR. It comprises two steps: elimination of genomic DNA and reverse transcription. The purified RNA sample is incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA, then RNA is used directly in reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C and is then inactivated at 95°C. Kit used for Real time PCR was "QuantiTect® SYBR® Green PCR" (Qiagen) for quantitative real-time two-step reverse transcription-PCR using SYBR Green I. Device was TOptical thermocycler (Analytica-Jena, Germany). Primer of real time PCR for amplicon length of 93 was: forward CAAGACTTCCCTCAGCTTTC (sense), reverse AGGACTGGGAGCAAAGAT (antisense). Real time PCR-Reaction setup (Total reaction volume 50 µl), 2x QuantiTect SYBR Green PCR Master Mix 25 µl, Primer A 2.5 µl, Primer B 2.5 µl, cDNA 5 µl, RNase-free water 15 µl. Thermocycler protocol: 1) initial activation step 15 min at 95°C **(**HotStarTaq DNA polymerase activation) 2) Denaturation 15 s at 94°C 3) Annealing 30 s at 60°C 4) Extension 30 s at 72°C. Number of cycles 50. No-template control and no- reverse transcriptase control was included in reactions. To find the fold expression value (algorithms-ΔΔCt) was used. The ΔΔCt method, compares results from experimental samples with both a calibrator (grade 1) and a normalizer housekeeping gene expression (GAPDH). The experimental samples being grade II, III, IV for the group of children and adult and grades III, IV for the older. The calibrator being the grade I as a baseline for the groups of the children and adults and grade II as a baseline for the group of older. Analysis of data was carried out using SPSS-25 (Statistical Packages for Social Sciences- version 25). The significance of difference of different means were tested using Students-t-test for difference between two independent means or ANOVA test for difference

among more than two independent means. Tukey's test used for calculating the significance between pairs of means. Statistical significance was considered whenever the P value was equal or less than 0.05

Results:

Table 1: Mean NEATIN gene expression by tumor grade and age group

Astrocytoma Grade			Folds e 2-(ddct)
	Children (<18y)	Adults (18-49)	older (=>50)
Grade I	1.83±0.51 (1.27-2.50)	1.32±0.23 (1.07-1.53)	-
Grade II	9.49±5.25 (6.40-17.30)	12.59±4.58 (9.91-17.87)	1.49±0.16 (1.40-1.67)
Grade III	19.28±9.17 (8.45-30.27)	44.05±21.05 (29.04-68.11)	11.08±6.84 (6.19-18.89)
Grade IV	29.28±19.08 (9.06-54.19)	73.98±16.82 (57.68-91.27)	47.85±34.19 (21.55-86.49)
P value	0.021*	0.001*	0.066

-Data were presented as Mean±SD (Range)

*Significant difference

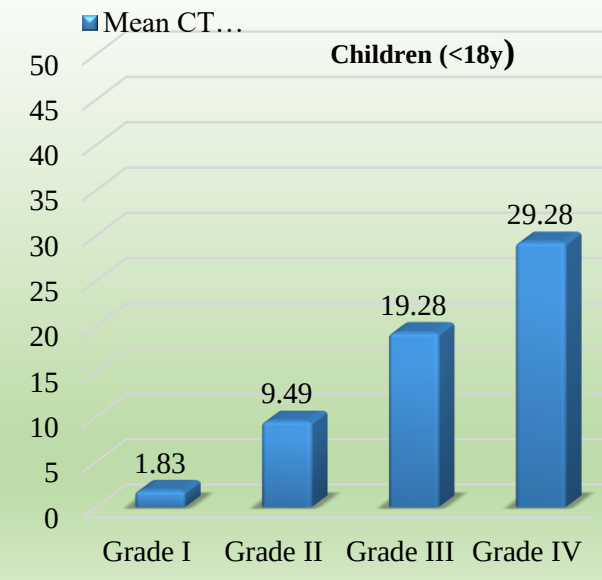

Figure 1: NESTIN gene expression by tumor grade in children

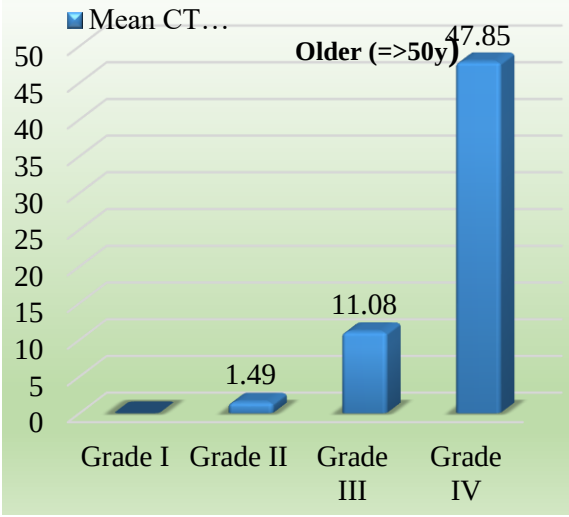

Figure 2: NESTIN gene expression by tumor grade in adults

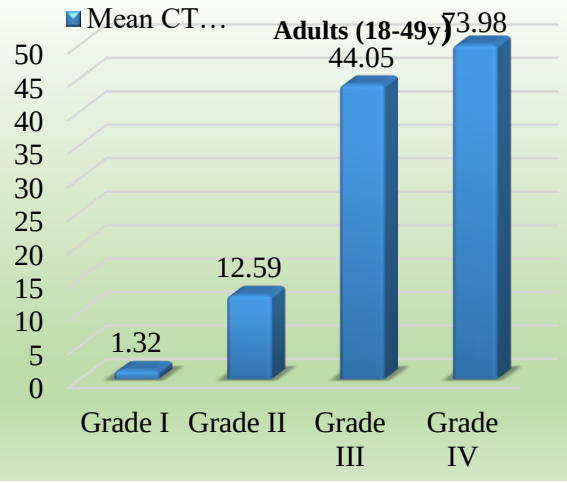

Figure 3: NESTIN gene expression by tumor grade in older adults

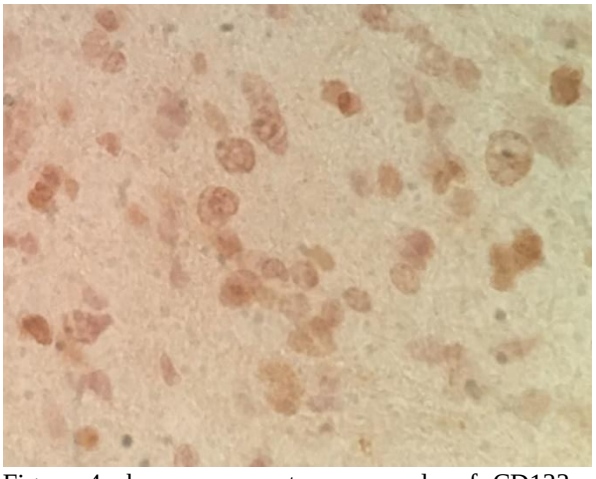

Figure 4 shows represent one sample of CD133 immunostaining of grade IV astrocytoma. Stained with rabbit CD133 polyclonal antibody and followed o-use IHC/ICC kit (Biotin free), One-Step HRP Polymer anti-Mouse, Rat & Rabbit IgG with DAB. x1000 oil immersion by Ready -t.

Discussion

The study was designed to compare NESTIN relative expression of BTSCs in astrocytoma between different grades in three age groups among Iraqi patients, by real time PCR, hoping that the outcome ultimately can be taken in consideration in the future management, prognosis and decision according to grade and patient's age. We calibrated the different grades in relation to grade I as the grade I is the least neoplastic and according to WHO is regarded as non-infiltrating and usually curable (18). Because of lack of similarity between the stem cells of the two tissue (19,20), we wanted to assess the expression in low and high grades astrocytoma in relation to its most benign grade. We wanted to be more specific and selective on the study in taking the population of BTSCs with tumorigenic potential, that is positive for CD133 (21), and exploring them for gene expression of NESTIN to analyze their genetic behavior in accordance with increasing grade of the tumor. NESTIN is a marker of immaturity as it represents the immature content of neuronal tissue (22), which is represented by BTSCs family that still has the ability to proliferate and self-renew (23). Our results revealed that for the experimental grades (grade II, III, IV) in groups of children and adults; and grade (III, IV) for the older group; NESTIN expression increased dramatically by tens of folds for each experimental grade in relation to its calibrator. This fold expression of experimental grades has furthermore incremented dramatically in an ascending manner with increasing of the grade from lower to higher, within the same group. This increment in NESTIN expression agreed with the results of (Ehrmann et al., 2005) (22). However, the latter conducted the study by immunohistochemistry without age grouping in addition to include different glioma tumors other than astrocytoma. Our results were consistent also with (Lin et al., 2015) (23), who found a correlation between NESTIN immunohistochemistry and astrocytoma tumor grade, while Lin 's study was conducted by flowcytomerty and immunohistochemistry, our study used gene expression profile by real time PCR, instead of the immunohistochemistry. Our study were in contradiction to those of (Hatanpaa et al., 2014) (24), who found the NESTIN expression to be similar for grade II and III and only increased in grade IV, However, that study was conducted by cell culture and flowcytometry which may have caused such contrast. In addition, other study (Seifert et al., 2015) (25) found grade I to be the same as grade IV by gene expression, however the gene expression they analyzed was not the NESTIN, it was gene of cytokine receptor pathway not NESTIN. Other studies, (Ishiwata et al., 2011and Lin et al., 2015) (26,23) explored NESTIN in glioma and reported that expression of NESTIN was associated with "cell growth, migration, invasion and adhesion to extracellular matrices" in low- and high-grade gliomas. Studies also have shown high immunoreactivity of NESTIN in astrocytoma associated with poor overall survival (27,28). When we compared the experimental grades in different age groups: we see the fold expression of NESTIN gene in successive grades is the highest in the adulthood (19-49) years for all the grades, other study (Seifert et al., 2015) (25) found that many genes were more upgraded in the elderly group, however, our study analyze NESTIN gene and this inconsistency might be attributed to difference in the gene. The fact that higher grade tumors show the most fold expression of NESTIN gene expression, suggests that immature content of higher grades astrocytoma dominate over the lower grades and this may explain the bad prognosis of higher grades of astrocytoma. This upgrading of NESTIN may be quite important to BTSCs for maintenance of immaturity state of BTSCs and thus, in conserving the character of stem cells. This was consistent with (Chen et al., 2007) (29), who demonstrated that preservation of stem cells identity is done by overexpression of multiple genes. All potential outcomes, attributed to BTSCs like invasion, resistance and recurrence and NESTIN may be required by the BTSCs to execute several functions related to the virulence of the tumor as it is regarded as cytoskeletal scaffolds in the nucleus and cytoplasm (30), and its main role contributing to cell motility and growth (31, 23, 32) thus we may consider NESTIN increment in higher grades of astrocytoma, responsible for cellular migration and metastatic potential and thus virulence of high grade tumor. As such, we suggest that NESTIN can be considered as a good candidate for reflection of the prognosis values and prediction of virulence across the grades in different ages. This is also suggested by with (Hlobilkova et al.,2009) (27), who stated that expression of NESTIN has been correlated with poor prognosis, however, it is in disaggreement with Chinnaiyan et al.,2008 (33), who did not support NESTIN relationship to prognosis. Nevertheless, the latter study analyzed the prognostic value on glioblastoma multiform patients only via immunohistochemistry and without comparison with lower grades. We suggest NESTIN expression across astrocytoma grades can be considered as new biomarker for integration with corresponding histological diagnosis in the prediction of virulence and also help in planning of therapy based on NESTIN target. The highest fold expression of NESTIN in all grades, was in the adult group, which suggest that virulence estimation reflected by NESTIN is most prominent in this age group, in comparison to others.

Conclusion:

We conclude that the grade of the tumor its self, affect the expression of NESTIN gene and its relative expression prominently increased by tens of folds in each grade in relation to calibrator and this fold expression is furthermore increased in ascending manner from lower grades to high-grade astrocytoma in the same age group. This proportional grade-expression relationship took its maximum at the

adulthood group between age 19 and 49 years. We propose that the relative expression of NESTIN may be a useful tool for the assessment of the virulence of successive grades of astrocytoma which is reflected more at the adulthood group between age 19 and 49 years.

Conflict of interest

There is no conflict of interest to be declared.

Ethical clearance

All subjects included in this study are according to the Local Ethical Committee of the Ministry of Health/ Iraq.

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Contribution of authors:

The first author is PHD student and the two other authors are her supervisors

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تقدير العدوانية بواسطة حساب التعبير النسبي للموروثة NESTIN في الدرجات المختلفة للورم النجمي للمرضى العراقيين

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أ.د هدى مهدي الخطيب
أ.د. ختام الخفاجي

الخلاصة: الورم النجمي ورم متباين التكوين والراسات حول عدائية الأورام تكشف ان هذا التصرف وراءه مجموعة خلايا جذعية خاصة بالورم والتي تسبب التضاعف المستمر والتجدد الذاتي. من المعلمات التي تحدد الخلايا الجذعية الورمية هو ال CD133 وكذلك ال NESTIN
الأهداف: باستخدام CD133 لتعليم الخلايا الجذعية الورمية في النماذج الشمعية للاورام النجمية وبعدها استخلاص هذه الخلايا لحساب التعبير الجيني الضعفي لل NESTIN عبر الدرجات المختلفة بواسطة REAL TIME PCR
المواد والطرائق: نماذج شمعية من اربع درجات من الورم النجمي الابتدائي اختيرت من ثلاث مجاميع عمرية من المرضى العراقيين. المجاميع العمرية صنفت الى اطفتا (0-17) بالغين (18-49) وكبار (50-80). النماذج صبغت بCD133 بعدها تم استخلاص المناطق الإيجابية لانجاز التعبير الجيني النسبي لNESTIN
النتائج: التعبير الجيني للnestin لكل الدرجات ازداد بعشرات الاضعاف بالنسبة للقياس البدائي لها وكذلك هذ التعبير الضعفي قد تزايد بشكل ملحوظ وتصاعدي مع زيادة الدرجة. التعبير الضعفي لل netin كان الأعلى في مجموعة البالغين
الاستنتاج: التعبير النسبي ل nestin للخلايا الجذعية الورمية في مختلف الدرجات للورم النجمي هو وسيلة مفيدة لتقييم العدوانية للورم مع تزايد الدرجة الورمية وهذا التعبير كان الأعلى في مجموعة البالغين (18-49)
مفتاح الكلمات : العدوانية، الورم النجمي ، NESTIN، درجات، اعمار