HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2017.06.011

The relationship between fractional anisotropy value and tumor microarchitecture in late-stage rat glioma

Xiang-Ying Li[1,2], Jian-Qiang Chen[2], Yi-Kai Xu[1✉], Xiang-Jun Han[3]

[1]*Department of Medical Imaging Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, PR China*

[2]*Department of Radiology, Central South University Xiangya School of Medicine Affiliated Haikou Hospital/Haikou People's Hospital, Haikou, Hainan, PR China*

[3]*Department of Radiology, Kangya Hospital, Yiyang, Hunan, PR China*

ARTICLE INFO

Article history:
Received 12 Mar 2017
Received in revised form 17 Apr 2017
Accepted 18 May 2017
Available online 15 Jun 2017

Keywords:
Rat
Glioma
Magnetic resonance diffusion tensor imaging
Fractional anisotropy
Immunohistochemistry

ABSTRACT

Objective: To explore the magnetic resonance diffusion tensor imaging (MR-DTI) features of in the late stage of Wistar rat C6 brain glioma, and the relationship between fractional anisotropy value and tumor microarchitecture.

Methods: The concentration of more than 1.0×10^6/10 μL glioma cells and complete medium were injected stereotactically into the right caudate nucleus of the experimental group (n = 35) and control group (n = 10), respectively. Conventional MRI, DTI, and enhanced T1WI scans were Performed using the GE Signa HD × 3.0T MRI scanner about 3–4 weeks after implantation for the rats. Postprocessing was done using the DTI specific software Function Tool to gain FA image. Many ROIs were drawn avoiding hemorrhage, necrosis areas in tumor parenchyma, the value of FA was recorded. Each surviving rat brain was examined histologically using HE and immunohistochemical staining for VEGF and CD34. Pearson correlation analysis was used to determine the relationships between FA values and VEGF, MVD, cell density, respectively.

Results: A total of 35 tumor-bearing rats were confirmed the tumor formation by the subsequent MRI and pathological examination. The mean FA values of the tumor and the contralateral brain tissue were 0.17 ± 0.03 and 0.31 ± 0.05 respectively, and the difference was statistically significant (t = 12.80, $P < 0.05$). The mean FA value of grade III glioma (n = 12) was 0.16 ± 0.03, and the average FA value of grade IV glioma (n = 23) was about 0.18 ± 0.04. There was no significant difference between the two groups (t = 1.92, $P > 0.05$). FA value in the late stage of Wistar rat C6 brain glioma has significant positive correlation to VEGF, MVD, cell density. The correlation coefficients between FA and VEGF, MVD, and cell density were 0.67, 0.65 and 0.71 ($P < 0.05$), respectively.

Conclusions: The FA value of rat glioma tumor in the late stage can preoperatively provide an accurate, reliable and noninvasive imaging monitoring method to evaluate the microstructure of glioma (cell density, the extent of angiogenesis, fiber bundle integrity and tumor cell infiltration and so on), predict the biological behavior of the tumor and make out surgical plan.

First author: Xiang-Ying Li, Department of Radiology, Central South University Xiangya School of Medicine Affiliated Haikou Hospital/Haikou People's Hospital, Haikou, Hainan, PR China.
Tel: +86 13876959224
Email: lxydoc@126.com
✉Corresponding author: Yi-Kai Xu, Department of Medical Imaging Center, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China.
Tel: +86 13729846668
E-mail: Yikai.xu@163.com

Peer review under responsibility of Hainan Medical University.
Foundation project: This research was supported by the National Natural Science Foundation of China, No. 81360228; the Natural Science Foundation of Hainan Province, No. 813259; the Scientific Research Foundation of Haikou Municipal Science and Technology Information Industry Bureau, No. 2013-72.

1. Introduction

The recurrence of glioma is a long-standing clinical problem, especially in high-grade glioma, and preoperative assessment of glioma microstructure (cell density, the extent of angiogenesis, fiber bundle integrity and tumor cell infiltration and so on), to predict the biological behavior of the tumor, the development of surgical programs is of great significance. Magnetic resonance diffusion tensor imaging (MR-DTI) is a noninvasive functional magnetic resonance imaging technique that can reflect the changes of microstructure *in vivo*, such as cell density, degree of vascular proliferation, fiber bundle preservation and destruction and degree of tumor infiltration invasion. It is widely used in the evaluation of microstructure [1–5]. In view of the biological characteristics of rat C6 glioma model similar to human glioma, we established the late stage rat glioma model to study the value of diffusion tensor imaging (DTI) in assessing the microstructure characteristics of the high grade glioma to ensure that the experimental results are more reliable and reproducible.

2. Materials and methods

2.1. Glioma cell inoculation

The animal study was conducted in accordance with the guidelines and approval of the institutional animal care and use committee. Female Wistar 45 rats (220–315 g) were purchased from Dong Chuang Laboratory Animal Technology Services, Kaifu District, Changsha city. Rats were divided into the experimental group (n = 35), and the sham group (n = 10) according to random number tables. C6 glioma cells were bought from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium, supplemented with 10% fetal cattle serum and 1% penicillin streptomycin mixed liquid. During the logarithmic growth phase, the cells were tackled with pancreatic enzymes, and a small sample of cells were removed for calculation using a microscope. The Trypan Blue Exclusion Test was conducted to ensure that cell viability was over 95% prior to inoculation.

Each rat was anesthetized with 1% pentobarbital sodium (40 mg/kg) and placed in a stereotactic apparatus (RWD Life Science and Technology Co., Ltd.). The skin of the head was cleaned and an incision was made to expose the skull to identify the bregma and lateral positions, and a 1 mm hole was drilled using a dentist drill through the skull 1 mm anterior and 3 mm lateral to the bregma on the right side. C6 glioma cells (1×10^6/10 μL) were injected at a depth of 6 mm from the dural surface, and then retracted 1 mm back. The injection was fully completed within 10 min (1 μL/min), a waiting time of 10 min was implemented following injection and then the needle was slowly withdrawn. The bone was sealed off with wax to prevent any cell suspension reflux. As control, five rats were inoculated with complete medium with no C6 cells in the same location.

2.2. MRI examination

MRI experiments were performed using a GE 3.0T Signa HD × medical magnetic resonance scanner (United States General Electrical Medical Group, GE Healthcare), with a gradient field of 50 mT/m, a switch rate of 150 mT/m/ms, and a rat-dedicated RF coil (China Shanghai Chen Guang Company). Each survival rat was anesthetized by intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg). In order to reduce effect of intravenous gadolinium-DTPA on diffusion tensor MR imaging for the evaluation of brain tumors [6,7]. Conventional MRI and DTI were done before contrast medium injection about 3–4 weeks after cells implantation, centered on optic chiasma. These times were based on the theory that it grew rapidly and became more infiltrative after 3–4 weeks after cell implantation [8–10].

Imaging parameters for T1-weighted images were as follows: repetition time (TR) = 350 ms, echo time = 19 ms, a slice thickness = 3.0 mm, interlayer spacing = 0 mm, field of view (FOV) = (8 × 8) cm^2, (192 × 192) matrix. The scanning parameters for T2WI were: TR = 3000 ms and TE = 120 ms, slice thickness, interlayer spacing, matrix, and FOV were the same as those of T1WI. The T2-Flair propeller parameters were imaged using the following parameters: TR = 9000 ms, TE = 155 ms, echo chain length = 36, bandwidth = 31.25 MHz, layer thickness = 3.0 mm, and FOV = (15 × 15) cm^2.

Diffusion tensor data were acquired with a spin echo multi-shot echo planar imaging (EPI) pulse sequence (TR = 2 500 ms, TE = 91 ms, slice thickness = 3 mm, motivate times = 16). The diffusion-weighting gradient schemes with 15 non-collinear directions with a b value of 800 s/mm^2 were used; thereafter, we obtained an additional measurement without diffusion gradient encoding (b value, 0 s/mm^2). This based on the study theory that by applying the diffusion imaging gradients in a minimum of six directions, a diffusion tensor image can be measured [11].

T1WI in the transverse plane and T1-3D-Bravo (IR FSPGR, inversion recovery fast spoiled gradient echo) sequences were performed after the administration of 2.5–3.0 mL of gadolinium diethylenetriamine-pentaacid (Magnevist; Schering AG, Germany) per kilogram of body weight with T1-3D-Bravo parameters: a slice thickness = 0.8 mm, FOV = (8 × 8) cm^2, (256 × 256) matrix, flip angle = 15°, bandwidth = 31.25 MHZ, prep time = 380 ms.

2.3. MRI image processing

Post-processing of the original images via Function Tool after DTI canning was required to avoid non-brain regions like CSF and the ventricle [12]. Combining T2WI, T2-FLAIR and T1WI with T1-3D-Bravo enhanced, we were able to locate the tumor. Under the guidance of experienced neurological surgeons and neuro-imaging experts, the fractional anisotropy (FA) values of the corresponding parts were obtained by selecting multiple isometrical ROIs of the tumor, the contralateral brain tissue and the control group.

2.4. Histological examination

2.4.1. HE staining

After scanning, the live rats were anesthetized with an overdose of 1% pentobarbital sodium, and then perfused with 4% paraformaldehyde liquid via the left ventricular to fix the whole brain, and then we proceeded to check the organs for metastasis. Following this, we put the specimens into 4% paraformaldehyde liquid for 24 h.

Based on the location of optic chiasma, the brain tissue was continuously sectioned. We randomly selected one section from

each sample to carry HE staining and observed the C6 glioma differentiation (WHO classification) under a light microscopic.

2.4.2. Immunohistochemistry examination (SP method)

Immunohistochemical staining of GFAP, S-100β, vascular endothelial growth factor (VRGF) and CD34 was detected by streptomycin biotin-protein (SP) method according to the instructions, with normal primary antibody animal serum and buffer instead of primary antibody as negative control. VEGF was predominantly expressed in brown or brown granules in the cytoplasm of the cells, and CD34 was stained with vascular endothelial cells as brown granules. MVD count with reference to the domestic scholar Liu Ying *et al* method [13], first, the entire tissue slice was scanned in the low magnification (40×) field to avoid bleeding and necrotic areas and to find positive cells with clear staining and low background staining (MVD expression). Any of the stained endothelial cells or vascular endothelial cells in the tumor, as long as they were separated from adjacent microvascular and tumor cells, could be used as a microvessel, the number of microvessels in the 5 visual field was calculated at 400 magnification, and the mean value was taken as the MVD value of the tumor. The percentage of VEGF positive cells was identical to that of MVD. With reference to Xiao-Yi Wang *et al* method [14], cell density analysis was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). The color pathology was converted to black and white photographs, and the tumor nuclei showed low density. The area of the tumor nucleus divided by the total area of the picture was the tumor cell density (calculated as a percentage). Each tumor cell density took the average of 5 photos.

2.4.3. Statistical analysis

All the results were expressed as mean ± SD, and were analyzed by IBM SPSS Statistics 24.0, and $P < 0.05$ showed evidence of significant difference. If the data were consistent with normal distribution and variance homogeneity, the two-sample *t*-test was used to analyze the difference of FA values of different grades of gliomas, Otherwise, non-parametric test (Kruskal–Wallis) was used. Pearson correlation analysis was used to calculate the correlation between the FA value of all tumor-bearing rats and the percentage of VEGF positive cells, MVD and cell density.

3. Results

3.1. MRI results

In the experimental group, 35 rats were examined by MRI and were confirmed to have tumor formation in subsequent pathological examination. The glioma-bearing rats showed long T1 and T2 non-uniform signals about 3–4 weeks after implantation of C6 glioma, The glioma was strengthened uniformly or ring-shape enhanced by contrast enhancement. The MRI results of the 10 control rats did not show an exceptional signal. The mean FA values of the tumor and the contralateral brain tissue were 0.17 ± 0.03 and 0.31 ± 0.05 respectively, and the difference was statistically significant ($t = 12.80$, $P < 0.05$) by two independent samples. The mean FA value of grade III glioma was 0.16 ± 0.03, and the average FA value of grade IV glioma was about 0.18 ± 0.04. There was no significant difference between the two groups ($t = 1.92$, $P > 0.05$).

3.2. Pathological results

HE staining showed tumor cell density, scattered in varying degrees of necrosis and hemorrhage. According to the World Health Organization (WHO) 2016 brain tumor classification and grading standards, in this study, 35 tumor-bearing tumors were grade III–IV, Including 12 grade III and 23 grade IV.

3.3. Correlation between FA value and the percentage of VEGF positive cells, MVD and cell density

The mean FA value, percentage of VEGF positive cells, MVD and cell density of the glioma tumor was 0.17 ± 0.03, (44.31 ± 6.84) %, 6.46 ± 2.17 (bar/high magnification) and (30.70 ± 5.37) % respectively. The FA values of tumor-bearing rats were positively correlated with the percentage of VEGF positive cells, MVD and cell density, and the *r* values were 0.67, 0.65 and 0.71, respectively ($P < 0.05$).

4. Discussion

Glioma is the most common intracranial malignant tumors, of which high-grade gliomas accounted for about 70% of gliomas, the current treatment is surgical resection, but the recurrence rate is high, which seriously affect tumor therapeutic effect. The biological characteristics of rat C6 glioma were close to that of human glioma, the degree of malignancy in the late stage is more than grade III, and experimental results have good repeatability is important to study the microstructure of high-grade glioma tumor. In this study, rat C6 glioma model was established. After 3–4 weeks of inoculation, MRI scan was performed. The results showed that the model was stable and reliable, and the growth rate was high, the rate of inoculation was 100%. According to WHO classification criteria, the pathology showed that the gliomas of the rats were grade III–IV, which was consistent with the majority of scholars [15–17], which provided some reference for establishing the high grade glioma model by selecting the time window.

DTI, which has been well-developed in recent years, can describe the movement characteristics of water molecules *in vivo* and reflect the microstructure change of the organization. FA, the ratio of anisotropic composition in diffusion tensor to the entire diffusion tensor, is the most common parameter. FA has a range of 0–1, where FA = 0 for entirely isotropic organizations and FA = 1 for fully anisotropic organizations. In theory the diffusion of anisotropy is obvious in the fiber bundles of brain white matter. DTI can indirectly reflect the integrity of the organization through the FA value, especially for the evaluation of the advantages of white matter microstructure becomes more pronounced. In order to ensure the accuracy of FA value, we propose the following methods for this experiment: 1) the diffusion gradient field is applied in the 15 directions, because the more the scanning direction of the gradient field applied, the more the ellipsoidal surface of the selected point, the sampling error is smaller, the more accurate the measurement of the anisotropy [18–20]. It is possible to obtain a more accurate FA value by applying 15 directions of diffuse gradient field to the brain tissue of rats. 2) In order to avoid the increase of FA value of the contrast agent Gd-DTPA, the DTI scan was completed before the contrast agent was injected to eliminate the effect of the contrast agent on the FA value [6]. 3) In the selection of the region of interest, this study combined multiple sequences of T2WI, T2-FLAIR, T1WI

enhanced examination and T1-3D-bravo volume enhancement to accurately determine the tumor. Especially the application of volume enhancement sequence, not only the rat brain tissue with good T1 contrast, but also to complete the sub-millimeter-level isotropic volume imaging, is helpful to avoid bleeding and necrosis and other areas that affect the measurement results, and visually display of tumor location, size and surrounding anatomical characteristics, also accurately evaluate the internal microstructure information of the tumor. 4) When expressing the results, multiple ROIs were selected as the FA value of the tumor to ensure that the measurement results were more accurate.

In this study, we found that the average FA value (0.18 ± 0.04) of grade IV glioma tumor was higher than that of grade III tumor (0.16 ± 0.03), but there was no significant difference between the two groups. It is speculated that grade III and IV gliomas belong to high grade gliomas with little difference in internal microstructures, resulting in no difference in FA values between the two groups, which is similar to that of Won Yi *et al* [21]. In addition FA values are affected by a variety of factors such as tumor cell density, extracellular smell, white matter myelin sheath integrity, and therefore the role of FA in glioma grading is still controversial, but experts at home and abroad have the same point of view that the higher the glioma level is, the greater the FA value is [21,22].

The study also found that the FA value of the brain glioma tumor and VEGF positive cells percentage, MVD, cell density were positively correlated. Similarly, foreign scholars such as Beppu *et al* [23] found that the mean FA value of high grade gliomas was lower than that of normal brain tissue by performing DTI in 19 patients with glioblastoma and measuring the FA values of tumor and normal brain regions, that positive correlation was observed between FA value and cell density ($r = 0.73$, $P < 0.05$) and between FA value and MIB-1 index ($r = 0.80$, $P < 0.05$), and suggest that the FA value of glioblastoma as determined by DTI prior to surgery is a good predictor of cell density and, consequently, proliferation activity, the damage of the fiber bundle caused the FA value to decrease, and the two together determine the FA value of the glioma. Domestic scholar Liu Ying *et al* [13], in the study of the relationship between fractional anisotropy value and tumor microarchitecture found that there was a positive correlation between FA and micro vessel density ($r = 0.668$, $P = 0.009$), cell density ($r = 0.625$, $P = 0.017$) and positive rate of VEGF ($r = 0.748$, $P = 0.002$), and believe the FA value of glioma tumor is related to microstructure, and the internal structure is very different in different stages of the same tumor or at different levels of tumor. Thus, by measuring the FA value of the glioma tumor, the microstructure characteristics of the glioma tissue can be evaluated and the degree of malignancy of the tumor can be predicted.

In conclusion, the relationship between FA value and microstructure was studied by establishing the late stage model of C6 glioma in rats, can ensure that the experimental results have good reproducibility, also provides a good experimental basis for the preoperative understanding of microstructure characteristics of glioma (cells Density, the degree of vascular proliferation, *etc*.), presumptive glioma biological behavior and preoperative classification, to develop a surgical program.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Wang S, Zhou J. Diffusion tensor magnetic resonance imaging of rat glioma models: a correlation study of MR imaging and histology. *J Comput Assist Tomogr* 2012; **36**(6): 739-744.

[2] Hashim E, Caverzasi E, Papinutto N, Lewis CE, Jing R, Charles O, et al. Investigating microstructural abnormalities and neurocognition in sub-acute and chronic traumatic brain injury patients with normal-appearing white matter: a preliminary diffusion tensor imaging study. *Front Neurol* 2017; **8**: 97.

[3] Timmers I, Roebroeck A, Bastiani M, Jansma B, Rubio-Gozalbo E, Zhang H. Assessing microstructural substrates of white matter abnormalities: a comparative study using DTI and NODDI. *PLoS One* 2016; **11**(12): e0167884; http://dx.doi.org/10.1371/journal.pone.0167884. eCollection 2016.

[4] Kinoshita M, Arita H, Okita Y, Kagawa N, Kishima H, Hashimoto N, et al. Comparison of diffusion tensor imaging and 11C-methionine positron emission tomography for reliable prediction of tumor cell density in gliomas. *J Neurosurg* 2016; **125**(5): 1136-1142.

[5] Schmidt C, Wiener E, Ludemann L, Kunte H, Kreutz KM, Becker N, et al. Does IIH alter brain microstructures? – a DTI-based approach. *Headache* 2017; **57**(5): 746-755.

[6] Zolal A, Sames M, Burian M, Nováková M, Malucelli A, Hejčl A, et al. The effect of a gadolinium-based contrast agent on diffusion tensor imaging. *Eur J Radiol* 2012; **81**(8): 1877-1882.

[7] Chiang GC, Pinto S, Comunale JP, Gauthier SA. Gadolinium-enhancing lesions lead to decreases in white matter tract fractional anisotropy in multiple sclerosis. *J Neuroimaging* 2016; **26**(3): 289-295.

[8] Bryukhovetskiy I, Manzhulo I, Mischenko P, Milkina E, Dyuizen I, Bryukhovetskiy A, et al. Cancer stem cells and microglia in the processes of glioblastoma multiforme invasive growth. *Oncol Lett* 2016; **12**(3): 1721-1728.

[9] Li XY, Wei X, Liu H, Guo ZY, Zhang YA, Cao P, et al. The establishment of late stage of Wistar rat C6 brain glioma model and MRI imaging. *J Pract Radio* 2012; **28**(3): 135-138.

[10] Huhndorf M, Moussavi A, Kramann N, Hattermann K, Stadelmann C, Jansen O, et al. Alterations of the blood-brain barrier and regional perfusion in tumor development: MRI insights from a rat C6 glioma model. *PLoS One* 2016; **11**(12): e0168174; http://dx.doi.org/10.1371/journal.pone.0168174.

[11] Chen Y, Tymofiyeva O, Hess CP, Xu D. Effects of rejecting diffusion directions on tensor-derived parameters. *Neuroimage* 2015; **109**: 160-170.

[12] Holly KS, Barker BJ, Murcia D, Bennett R, Kalakoti P, Ledbetter C, et al. High-grade gliomas exhibit higher peritumoral fractional anisotropy and lower mean diffusivity than intracranial metastases. *Front Surg* 2017; **4**: 18.

[13] Liu Y, Li CF, Zhang K, Hou JW, Wang Q, Cong PX, et al. The correlation between fractional anisotropy value and tumor microarchitecture in astrocytomas with different grade. *Chin J Radiol* 2007; **41**(4): 355-357.

[14] Wang XY, Deng LM, Liao WH, Chen CQ, Han ZD, Liu F, et al. Apparent diffusion coefficients in the evaluation of the cerebral astrocytomas. *J Clin Radiol* 2006; **25**(3): 201-205.

[15] Connolly NP, Stokum JA, Schneider CS, Ozawa T, Xu S, Galisteo R, et al. Genetically engineered rat gliomas: PDGF-driven tumor initiation and progression in *tv-α* transgenic rats recreate key features of human brain cancer. *PLoS One* 2017; **12**(3): e0174557; http://dx.doi.org/10.1371/journal.pone.0174557.

[16] Perez-Carro R, Cauli O, Lopez-Larrubia P. Multiparametric magnetic resonance in the assessment of the gender differences in a high-grade glioma rat model. *EJNMMI Res* 2014; **4**: 44.

[17] Li XY, Wei X, Chen J, Zhang YA, Yuan Y, Zhan YF, et al. Magnetic resonance imaging and diffusion tensor imaging studies in the late stage of Wistar rat C6 brain glioma. *Chin J Mod Med* 2013; **23**(11): 27-30.

[18] Yao X, Yu T, Liang B, Huang Q, Zhuang S. Effect of increasing diffusion gradient direction number on diffusion tensor imaging

fiber tracking in the human brain. *Korean J Radiol* 2015; **16**(2): 410-418.

[19] Chuck NC, Steidle G, Blume I, Fischer MA, Nanz D, Boss A. Diffusion tensor imaging of the kidneys: influence of b-value and number of encoding directions on image quality and diffusion tensor parameters. *J Clin Imaging Sci* 2013; **3**: 53.

[20] Kim SJ, Choi CG, Kim JK, Yun SC, Jahng GH, Jeong HK, et al. Effects of MR parameter changes on the quantification of diffusion anisotropy and apparent diffusion coefficient in diffusion tensor imaging: evaluation using a diffusional anisotropic phantom. *Korean J Radiol* 2015; **16**(2): 297-303.

[21] Won YI, Chung CK, Kim CH, Park CK, Koo BB, Lee JM, et al. White matter change revealed by diffusion tensor imaging in gliomas. *Brain Tumor Res Treat* 2016; **4**(2): 100-106.

[22] Miloushev VZ, Chow DS, Filippi CG. Meta-analysis of diffusion metrics for the prediction of tumor grade in gliomas. *AJNR Am J Neuroradiol* 2015; **36**(2): 302-308.

[23] Beppu T, Inoue T, Shibata Y, Yamada N, Kurose A, Ogasawara K, et al. Fractional anisotropy value by diffusion tensor magnetic resonance imaging as a predictor of cell density and proliferation activity of glioblastomas. *Surg Neurol* 2005; **63**(1): 56-61.