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ORIGINAL ARTICLE

Characterizing invading glioma cells based on IDH1-R132H and Ki-67 immunofluorescence

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Abstract Glioma, the most common primary brain tumor, is characterized by proliferative-invasive growth. However, the detailed biological characteristics of invading glioma cells remain to be elucidated. A monoclonal antibody (clone HMab-1) that specifically and sensitively recognizes the isocitrate dehydrogenase-1 (IDH1) protein carrying the R132H mutation can identify invading glioma cells by immunostaining. To investigate the degree of invasion in gliomas of distinct grades and the proliferative capacity of the invading cells, immunofluorescent staining was conducted using antibodies against IDH1-R132H and Ki-67 on 11 surgical and autopsy specimens of the tumor core and the invading area. Higher numbers of IDH1-R132H-positive cells in the invading area correlated with a higher tumor grade. Double staining for IDH1-R132H and Ki-67 demonstrated that most invading cells that expressed IDH1-R132H were not stained by the Ki-67 antibody, and the ratio of Ki-67-positive cells among IDH1-R132H-positive cells was significantly lower in the invasion area than in the tumor core in all grades of glioma. These data suggest that higher grade gliomas have a greater invasive potential and that invading cells possess low proliferative capacity.

Keywords Glioma · IDH1 · Invasion · Proliferation · Immunofluorescence

Abbreviations
IDH1 Isocitrate dehydrogenase1
GBM Glioblastoma

Introduction

One of the main characteristics of gliomas is that the tumor cells exhibit infiltrative growth within the existing structure of the brain [1]. This invasive growth is a crucial factor that makes gliomas difficult to treat. Because the border between gliomas and the surrounding normal brain tissue is indistinct, extracting the tumor surgically is extremely challenging. To understand glioma biology, the mechanism of glioma invasion must be elucidated. However, one major obstacle that hinders research on invasion is that invading glioma cells cannot be readily identified among existing glial cells, especially when attempting to differentiate invading cells from reactive glial cells at tumor margins.

Isocitrate dehydrogenase 1 (IDH1) mutation has been identified as early and frequent genetic alterations in low-grade glioma and secondary GBMs [2]. By contrast, IDH1 mutations are rarely found in primary GBMs. The IDH1 mutations are remarkably specific to a single codon in the conserved and functionally important Arg132 residue, which is changed to a histidine [3]. Recently, an anti-IDH1-R132H mouse monoclonal antibody that specifically

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recognizes the IDH1-R132H protein was developed [4, 5], which has facilitated the detection of IDH1 mutation in glioma tissues by routine immunostaining. Immunohistochemical staining with the IDH1-R132H-specific antibody has been shown to help distinguish single infiltrating neoplastic cells from reactive astrocytes [6].

The degree of invasion in gliomas of distinct grades has not been defined to date, and the relationship between the invasive and proliferative capacities of malignant glioma cells is not entirely clear. Experimental evidence indicates that cell motility is inherently and inversely correlated with proliferation in a cellular population [7, 8]. However, to the best of our knowledge, no in vivo experiment has demonstrated an association between invasion and proliferation in gliomas.

In this study, we clarified the degree of invasion in gliomas and analyzed the invasive and proliferative properties of glioma cells by immunostaining for IDH1-R132H and Ki-67 in surgical and autopsy specimens of gliomas of various grades. Our results suggest that invasion is more aggressive in high-grade glioma and that invading glioma cells proliferate poorly.

Materials and methods

Samples

Tumor specimens derived from surgical operations or autopsies conducted between 2000 and 2012 were selected from the stock at the Kanazawa University hospital. Pathological grading was performed according to the World Health Organization (WHO) classification [9]. We selected IDH1-R132H-positive tumor cases whose H&E-stained slides included both tumor cores and bordering areas (Table 1).

Table 1 Summary of patients

Case no.	Age (years)	Sex	Pathological diagnosis	WHO grade
1	51	M	Diffuse astrocytoma	II
2	58	M	Diffuse astrocytoma	II
3	55	M	Diffuse astrocytoma	II
4	39	F	Oligodendroglioma	II
5	40	F	Anaplastic oligodendroglioma	III
6	24	F	Anaplastic astrocytoma	III
7	43	M	Anaplastic astrocytoma	III
8	36	M	Anaplastic astrocytoma	III
9	36	M	Anaplastic oligodendroglioma	III
10	45	M	Secondary glioblastoma	IV
11	55	M	Glioblastoma	IV

Immunofluorescence staining

Briefly, 5-μm-thick histologic sections were incubated in 5 % skim milk/TBST at room temperature for 30 min to block nonspecific immunoreactions. Next, sections were incubated overnight at 4 °C with anti-IDH1-R132H mouse monoclonal antibody (clone: HMab-1) 1:300 dilution and then with donkey anti-mouse IgG secondary antibodies conjugated with Alexa Fluor-568 (1:1,000; Molecular Probes, Eugene, OR, USA). After rinsing in PBS, sections were incubated with a rabbit polyclonal Ki-67 antibody (clone: MIB-1; Thermo Fisher Scientific, San Jose, CA, USA) at 1:500 dilution at room temperature for 1 h and then with donkey anti-rabbit IgG secondary antibodies conjugated with Alexa Fluor-488 (1:1,000; Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. Then, sections were mounted using Vectashield mounting medium containing DAPI and photographed using a High Standard All-in-One Fluorescence Microscope (Biorevo BZ-9000; Keyence, Osaka, Japan).

Image analysis and quantification

Cells stained for IDH1-R132H and Ki-67 were examined under a Biorevo BZ-9000 microscope. Positively stained cells in a representative region of the tumor center and invading area were counted by a neuropathologist (H.S.) using the Dynamic Cell Count BZ-H1C software (Keyence, Osaka, Japan). Tumor borders were defined using H&E staining. When specimens had clear borders, the region within 200 μm from the border line was demarcated as the tumor margin. In specimens showing diffuse invasive growth, regions with an ~10 % reduction in cell density were determined to represent the end of the tumor center, and regions 200 μm from the end of tumor center were demarcated as tumor margins. Microscopic fields were analyzed at 20× magnification to identify cells positive for IDH1-R132H. For each cell that stained for IDH1-R132H, the presence or absence of Ki-67 immunoreactivity was recorded. The total number of cells counted per field ranged from 500 to 1600.

Results

Immunofluorescent staining of gliomas with IDH1-R132H and Ki-67 antibodies

In the tumor core, most glioma cells were positive for IDH1-R132H (Fig. 1). In the border areas, invading glioma cells were identified as IDH1-R132H-positive cells in

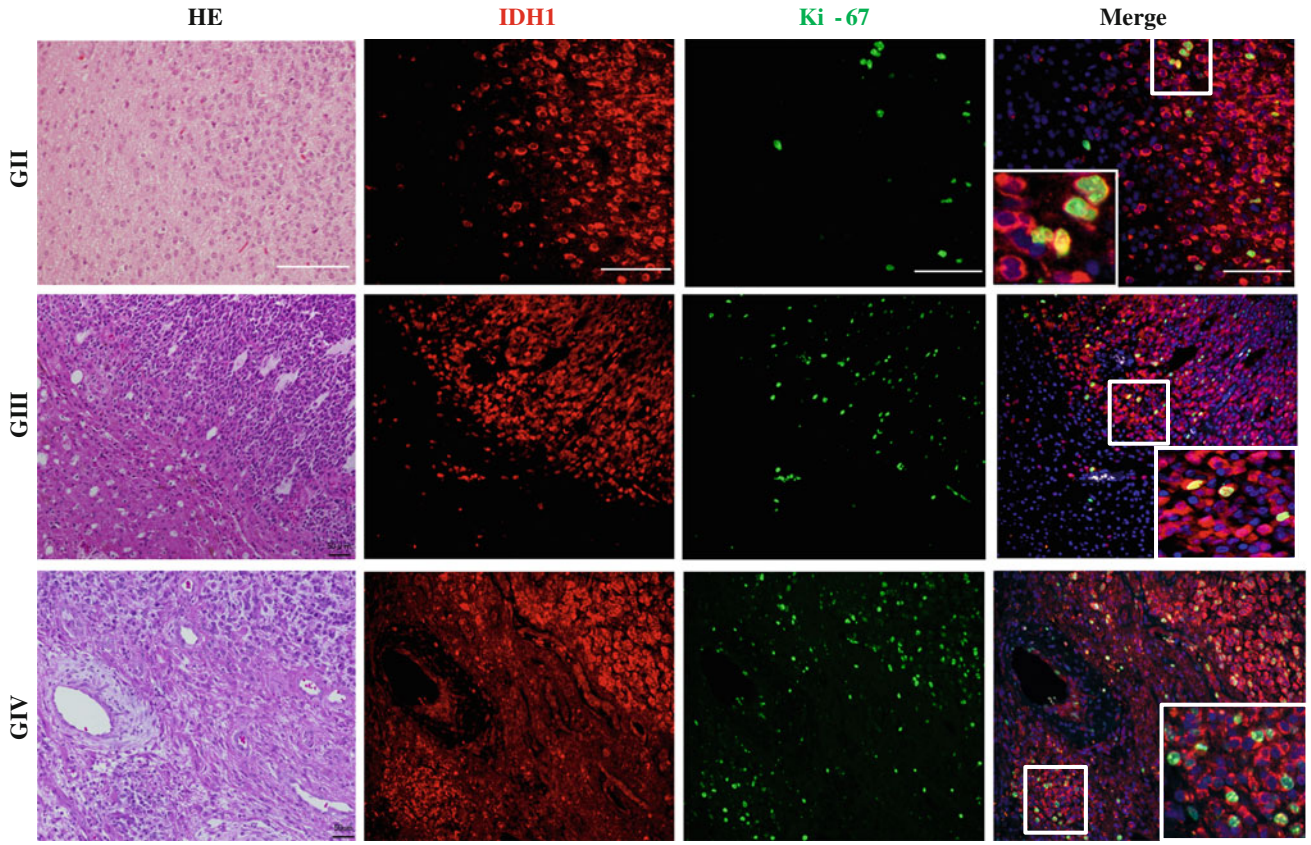

Fig. 1 H&E and double-immunofluorescent stain with IDH1-R132H and Ki-67 antibodies. The *upper right* is the tumor core, and the *lower left* is the invasion area. IDH1-R132H-positive cells (*red*), Ki-67 positive cells (*green*); *inset* showing double-positive cells (*yellow*). *Upper panel*: oligodendroglioma. *Middle panel*: anaplastic oligoastrocytoma. *Lower panel*: primary glioblastoma. The double-positive cell is increasing with upgrading of both the tumor core and invasion area. *Bars* = 50 μm

gliomas of all grades. Furthermore, Ki-67 immunofluorescent staining performed to estimate proliferative activity showed that few Ki-67-positive cells were present in the border areas in all samples compared with the core area. To analyze the proliferative activity of invading cells, double staining was performed for IDH1-R132H and Ki-67 (Fig. 1).

IDH1-R132H-positive cells in the invasion areas

To compare the invasive potential of cells in gliomas of various grades, the cells positive for IDH1-R132H were counted in the invasion areas. The number of invading cells in all cases of grade III gliomas exceeded the maximum number in grade II gliomas, and all cases of grade IV gliomas showed greater numbers of invading cells than all cases of grade II and III gliomas (Fig. 2a). The mean number of cells positive for IDH1-R132H per mm^2 was higher in grade IV gliomas (1554.2 ± 5.67; mean ± SE) than in grade II (504.9 ± 96.67) and III (912.2 ± 66.65) gliomas. These results suggest that higher grade gliomas are more invasive.

Ki-67 immunoreactivity in IDH1-R132H-positive cells

To compare the proliferative potential of glioma cells in the tumor core and the border areas, double immunofluorescent staining was performed for IDH1-R132H and Ki-67, and the cells that were stained for both proteins were counted in each area. As expected, the ratio of Ki-67-positive cells to the total number of cells that were positive for IDH1-R132H in the tumor core was elevated when the degree of malignancy was higher (Fig. 2b). The average Ki-67-positive ratios were 5.98 ± 1.67 % (mean ± SE), 7.8 ± 2.53 % and 13.35 ± 1.27 % in gliomas of grade II, III and IV, respectively. Interestingly, the Ki-67-positive ratio in IDH1-R132H-positive cells was lower in the border areas than in the tumor core in all cases, regardless of the tumor grade, suggesting that invading cells proliferate poorly.

Discussion

In this study, we aimed to determine the degree of invasion in gliomas of distinct grades and the proliferative activity of

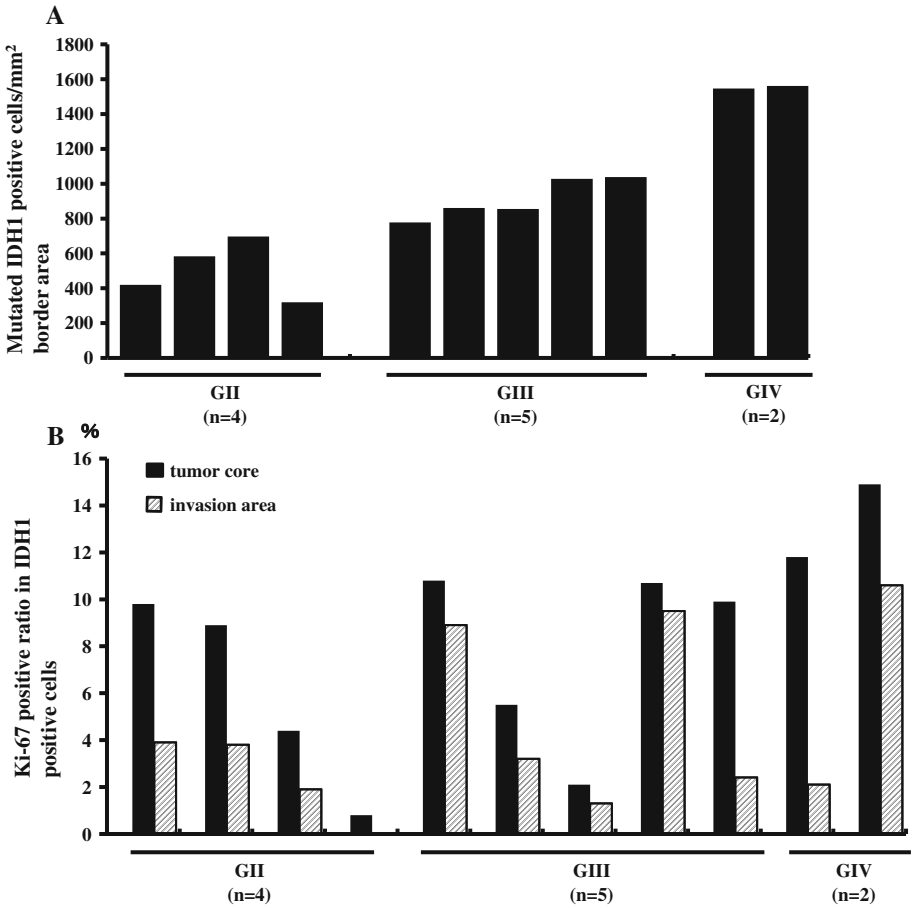

Fig. 2 **a** The number of IDH1-R132H-positive cells per 1 mm^2 in the border area in grade II (*GII*), grade III (*GIII*) and grade IV (*GIV*) gliomas. GIV gliomas showed greater numbers of invading cells than all cases of grade II and III gliomas. Graph shows the data of individual cases. **b** The positive rate of Ki-67-positive cells in IDH1-R132H-positive cells in the tumor core and invasion area. It is lower in the border areas than in the tumor core in all cases. The data of individual cases are shown

invading glioma cells. Colocalization of IDH1-R132H and Ki-67 detected in the cells by using double immunofluorescent staining enabled us to identify proliferating invasive cells. Our results showed that the number of invading cells was greater when the tumor grade was higher and that invading cells were rarely proliferative in vivo.

Accumulated evidence including our results demonstrates that proliferative activity in gliomas is greater in higher grade gliomas [10–12]. By contrast, whether invasiveness is also greater in higher grade tumors in vivo has not, to our knowledge, been shown before. This is because methods have not yet been developed to distinguish invading cells from reactive glial cells, and few studies have quantified glioma cell invasion in clinical samples. Invasion-promoting genes such as matrix metalloproteinases are upregulated in higher grade gliomas, suggesting that higher grade gliomas are more invasive [13–15]. However, based on analyzing advanced MRI data, Deng et al. [16] reported that higher grade gliomas do not always exhibit greater invasiveness. Because IDH1-R132H was reported to be a marker of invading glioma cells [6, 17–19], we used immunofluorescent staining for IDH1-R132H in the border areas of surgical specimens to identify invading cells and demonstrated quantitatively for the first time that higher grades gliomas exhibit greater invasive activity than lower grade gliomas.

Previous cDNA-microarray data indicated that stimulated migration of cells is accompanied by a downregulation of genes responsible for proliferation in vitro [8]. Moreover, extracellular matrix proteins that activate the motility of glioma cells also lower the growth rate of glioma cells [7, 20]. Analysis of proliferation markers in cells obtained from the trajectories of stereotactic glioma biopsies has indicated that invasive cells show a lower proliferation rate [21, 22]. These experimental findings indicate an inverse correlation between motility and proliferation in glioma cells. To demonstrate this inverse relationship in vivo, we conducted double immunofluorescent staining for IDH1-R132H and Ki-67 in glioma surgical specimens. Our data showed that the Ki-67-staining ratio was lower in invading glioma cells than in cells at the tumor core, suggesting that invading cells have a lower proliferative potential than the cells at the tumor core. According to our results presented here, we are currently analyzing the molecules related to invasion and proliferation signaling in invading glioma cells.

In conclusion, using clinical samples, we have demonstrated a negative correlation between invasion and

proliferation in glioma cells, although the results presented here must be validated using a larger number of cases. The novel framework provided by this quantitative and comparative analysis should facilitate future studies examining the histopathology of invading glioma cells and the biology of glioma invasion.

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