ORIGINAL ARTICLE

Expression of stem cell marker and receptor kinase genes in glioblastoma tissue quantified by real-time RT-PCR

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Abstract Glioblastoma is dependent on a specific signaling pathway to maintain its tumor phenotype. The receptor tyrosine kinase (RTK) family mediates the multiple oncogenic growth factor receptor signaling and contributes to the pathogenesis of glioblastoma. Recently, many studies have shown that the expression of stem cell marker in glioblastoma tissue has prognostic significance, which indicates that the quantification of stem cell markers and RTK genes yields biological information about glioblastoma. In this study, we quantified RNA expression levels of stem cell markers [CD133, Nestin, BMI-1, maternal embryonic leucine zipper kinase (MELK), and Notch1–4] as well as RTKs (EGFR, ErbB4, VEGFR1-3, FGFR1, -2, PDGFRA, and PDGFRB) in 42 clinical samples of glioblastoma by the real-time RT-PCR method. We demonstrated that the expression of MELK is exclusively upregulated in glioblastoma tissue. Notch receptor expression is moderately upregulated and is correlated with that of VEGFR2, VEGFR3, and PDGFRβ. Unsupervised clustering identified one unique sample group that showed high expression of most of the genes analyzed. Our results suggest that quantification of these stem cell markers and RTK genes can stratify patients based on the expression profile, which might provide insight into the glioma biology in each cluster.

Introduction

Glioblastoma is the most common malignant primary brain tumor in adults. Despite surgery, radiation, and chemotherapy, the median survival after diagnosis is approximately 12 months. As shown by the Cancer Genome Atlas pilot project, receptor tyrosine kinase (RTK) signaling pathways such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) are frequently altered and activated in glioblastomas [1]. Drugs targeting these oncogenic signaling pathways have shown clinical activity in patients with glioblastomas, and targeted therapy represents a promising new paradigm for the treatment of glioblastomas [2, 3]. Thus, it is of particular importance to evaluate the activation of each RTK pathway in glioblastomas for the development of effective molecular targeted therapy.

It has been recently reported that the expression of some stem cell markers in glioblastoma has prognostic significance [4–9]. Although Nestin and CD133, which are the most accredited, are useful stem cell markers, they are known to be expressed also in non-stem cell populations and play an important role in gliomagenesis. Notch, maternal embryonic leucine zipper kinase (MELK), and BMI-1 (a polycomb gene) are other signaling molecules implicated in the stem cell signaling pathway [10–12]. However, the expression profiles and functional roles of these molecules in glioblastoma tissue have not been systematically investigated. We hypothesized that exploring the stem cell signaling pathway in conjunction with the RTK pathway in glioblastoma tissues would yield prognostic information for patients in the clinic. Furthermore, it

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may be possible that the stem cell signaling pathway as well as the RTK pathway could be molecular targets for a new treatment strategy.

Recent advancements in microarray technology have enabled us to investigate genome-wide molecular profiling and identify a new molecular target. This high-throughput technology, however, is expensive, and it cannot always be integrated into the clinical setting, where methodology that is easier to use and more cost effective is preferred. Real-time reverse transcription-polymerase chain reaction (RT-PCR) technology is easily integrated in the clinic. In this study, we quantified the expression of stem cell marker genes and RTK genes in glioma tissue using a real-time RT-PCR method, performed unsupervised hierarchal clustering, and evaluated the statistical correlation of these expression data.

Materials and methods

Tumor specimens

We collected 42 case samples of glioblastoma from the Kyushu University Brain Tumor Bank; these samples were obtained from patients during surgery under the approval of the ethics committee of our university. For all tumors, a histological diagnosis of glioblastoma was determined based on World Health Organization criteria by board-certified neuropathologists. All 42 cases were primary glioblastomas; secondary glioblastomas and recurrent cases were excluded from this study. Normal brain reference RNA (NBRR) was purchased from Ambion and used as an internal control.

Target genes and primer design

Concerning stem cell marker genes, we evaluated the expression of CD133 and Nestin, which are currently the most accredited markers for the identification of neural stem cells. In addition, MELK and BMI-1 were included in this study because these genes contribute to stem cell maintenance. Notch receptors (Notch1–4) were also included in this study because the Notch signaling pathway is implicated in the maintenance of neural progenitors and in the generation of glia during development of the brain and is altered in gliomas [13, 14]. Of all the RTK genes, we selected nine genes that have been shown to be involved in tumor growth and proliferation in glioblastomas: EGFR, ErbB4, VEGFR1, VEGFR2, VEGFR3, FGFR1, EGFR2, PDGFRA, and PDGFRB. In total, 17 genes were included in this study. Amplification primer pairs were designed for nonredundant regions on the NCBI Reference Sequence (RefSeq) using *Primer 3* software. The target genes and corresponding RefSeq are listed in Table 1.

Table 1 List of genes analyzed in this study

| Gene	Chromosome map	Reference sequence (Ref Seq)
EGFR	7p12	NM_005228
ERBB4	2q33	NM_001042599
PDGFRA	4q12	NM_006206
PDGFRB	5q31	NM_002609
FGFR1	8p12	NM_015850
FGFR2	10q26	NM_000141
VEGFR1	13q12	NM_002019
VEGFR2	4q11	NM_002253
VEGFR3	5q35	NM_002020.4
Notch1	9q34	NM_017617
Notch2	1p12	NM_024408
Notch3	19p13	NM_000435
Notch4	6p21	NM_004557
Nestin	1q23	NM_006617.1
MELK	9p13	NM_014791
BMI-1	10p13	NM_005180
CD133	4p15	NM_006017

RNA extraction, reverse transcription, and real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from the frozen samples using a commercial *mir*Vana miRNA isolation kit (Ambion, Austin, TX, USA). Reverse transcriptions were performed by random hexamers using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Reverse-transcribed products were amplified by the SYBR method using the ABI PRISM 7500 Fast Real-time PCR System (Applied Biosystems). Briefly, 2 μl cDNA product was used as a template in a 20-μl PCR reaction containing 10 μl Power SYBR Green Master Mix (Applied Biosystems), 200 mM each primer, 1 μl dimethylsulfoxide (DMSO), and 6.2 μl distilled water. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a reference gene. Amplification protocols were as follows: 95°C for 10 min, 40 cycles of 95°C/15 s, and 40 cycles of 60°C/60 s with melting curve analysis. Threshold cycle number (CT) was automatically determined by ABI 7500 Fast System SDS software. All reactions were performed in duplicate.

Data analysis of quantitative RT-PCR and statistical analysis

The average CT value of duplicate runs was determined for each gene, and ΔCT was calculated by subtracting the CT value of the HPRT1 reference gene from that of each gene. The relative expression of each gene was quantified by the $\Delta\Delta$CT method. In this method, $\Delta\Delta$CT was calculated by

Fig. 1 The relative quantity value (*RQ*) normalized by normal brain reference RNA is shown as a log-transformed number for 17 genes. The *bottom* and *top of the box* indicate lower and upper quartiles, respectively. The *band* at the *middle of the box* indicates the median value of all samples. *Upper* and *lower bars* indicate the upper 90th percentile and lower 10th percentile, respectively. For each gene, outliers of this range were not plotted in this figure
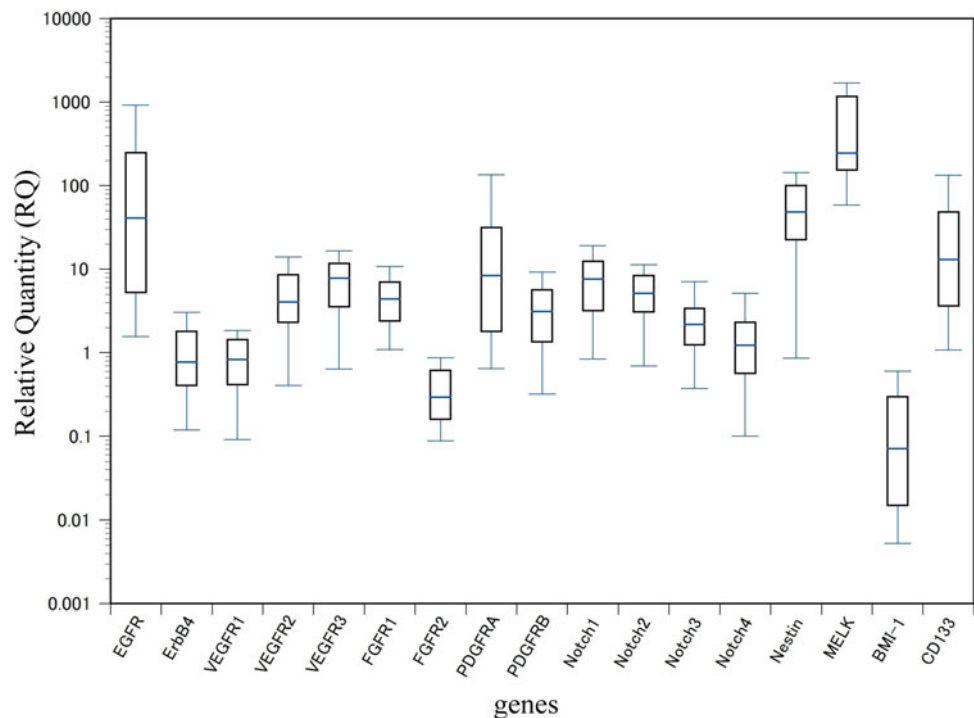
subtracting the ΔCT of the NBRR from that of the target gene, and the relative quantity (RQ) was calculated. To auto-scale the data for clustering, the RQ of each gene was divided by the mean RQ value of all samples, and the data were presented as $\log_{10}$ of this value. Hierarchical clustering using Ward's linkage analysis and Spearman's rank test was performed by JMP 8 statistical software (SAS Institute, Cary, NC, USA).

Results

Quantification of stem cell markers and RTK genes

Amplified products were successfully obtained for all genes. The CT value of NBRR was less than 35 in all genes except MELK, for which the CT value was 38.4, indicating that MELK expression is very low or absent in healthy brain tissue. The RQ value was box-plotted as log-transformed numbers (Fig. 1). For each gene, outlying cases in the upper and lower 10% of expression were not plotted in this figure. In glioblastoma tissues, EGFR, PDGFRA, Nestin, MELK, and CD133 are strongly expressed compared to normal brain tissue. Notably, MELK expression is exclusively upregulated in glioblastoma tissue without quantifiable expression in normal brain tissue. The expression of VEGFR2, VEGFR3, FGFR1, PDGFRB, and Notch1–3 are slightly higher than that in normal brain tissue. Notably, the stem cell marker BMI-1 showed decreased expression in glioblastoma tissues. In total, the median RQ value of ErbB4, VEGFR1, FGFR2, and BMI-1 was less than 1, which showed that the expression level of each of these genes is less than or equal to that in normal brain tissue.

Hierarchical clustering categorized glioma tissues into three clusters

To compare the expression profiles of the different samples and detect groups of samples with similar expression profiles, we performed hierarchical clustering of the expression data of the 13 genes (excluding ErbB4, VEGFR1, FGFR2, and BMI-1) with higher expression compared with normal brain tissue. The dendrogram of this clustering demonstrated that all 42 glioma tissues could be categorized into three clusters (Fig. 2). Although we could not conclude the expression profile of samples within cluster 1, cluster 2 showed a high level of expression of almost all genes including stem cell markers and RTKs, and cluster 3 showed a low level of expression of all genes.

Spearman's rank test of genes with strong correlation of expression

To evaluate the correlation of the expression patterns among these 13 genes, we performed pairwise Spearman's correlation tests. Strong correlation was detected among Notch1–4, PDGFRB, VEGFR2, and VEGFR3 (Table 2. There were no significant correlations among stem cell markers.
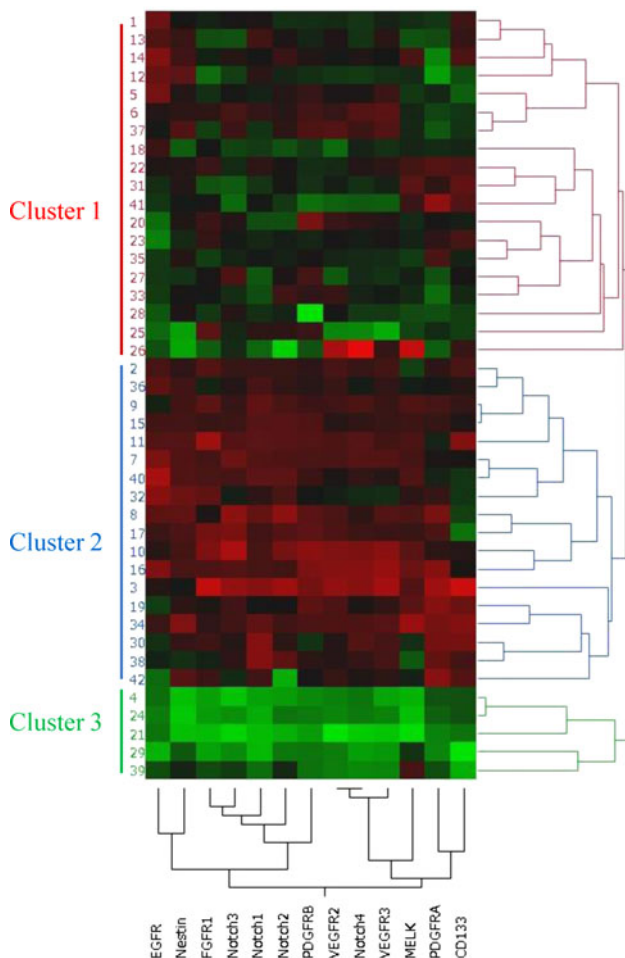
Fig. 2 Hierarchical clustering analysis demonstrated that all the samples could be classified into three clusters based on the analysis of 13 genes. In *cluster 2*, samples show high expression of most of the genes. By contrast, expression of all the genes is low in *cluster 3*

Discussion

Our results demonstrated that the expression of MELK, an atypical member of the snfl/AMPK family of serine-threonine kinases, which are key regulators of the proliferation and maintenance of glioma stem cells, is exclusively upregulated in glioblastoma tissues in contrast to Nestin, CD133, and Notch, expression of which is also detected in normal brain tissue. The RQ of MELK expression is the same or higher than that of EGFR, which has been known to be overexpressed in glioblastoma tissue. It has been also reported that MELK expression is positively increased according to tumor grade [15]. Our results therefore imply that the MELK signaling pathway can be a therapeutic target for glioblastomas.

The relative quantification of RTK genes in this study is consistent with previous studies [16–19]. EGFR is the most highly expressed gene, followed by PDGFRA. The expression of PDGFRB is lower than that of PDGFRA, and

Table 2 List of gene combinations with strong correlation of gene expression

Genes	Correlation coefficient
Notch4 versus *VEGFR3*	0.92
Notch4 versus *VEGFR2*	0.88
VEGFR3 versus *VEGFR2*	0.88
Notch3 versus *PDGFRB*	0.84
Notch2 versus *Notch1*	0.79
Notch3 versus *Notch2*	0.79
Notch3 versus *VEGFR3*	0.78
Notch3 versus *Notch4*	0.78
Notch3 versus *VEGFR2*	0.74
Notch1 versus *VEGFR3*	0.73
PDGFRB versus *VEGFR2*	0.73
Notch2 versus *PDGFRB*	0.72
PDGFRB versus *VEGFR3*	0.72

Correlation coefficient was obtained by Spearman's rank test. All these correlation coefficients were statistically significant ($P < 0.0001$)

VEGFR2 and -3 are more highly expressed than VEGFR1. Regarding other stem cell markers, Nestin and CD133 [20], the two most accredited stem cell markers, are expressed in the same range, although their expression is not tumor specific, which suggests that the biological significance of the expression of these markers should be thoroughly investigated. The Notch pathway is a conserved ligand–receptor signaling mechanism that modulates cell fate and differentiation and plays an important role in the maintenance of stem cell self-renewal. In mammals, there are four Notch receptors (Notch1–4) and five ligands (Jagged1, -2; Delta-like1, -3, -4) [14]. Although the key components of this Notch signaling are reported to be aberrantly activated in gliomas and are considered to be implicated in gliomagenesis, the quantification of these four Notch receptors in gliomas has been inadequately investigated [21]. Our results demonstrated that Notch1 and Notch2 are upregulated by 5- to 20 fold compared with normal tissue, whereas the expression of Notch3 and Notch4 is threefold higher on average, which might suggest that Notch1 and Notch2 signaling are more important than that of Notch3 and Notch4. Conversely, the results of BMI-1 expression in our study showed a discrepancy with previous studies. Although it has been reported that BMI-1 inhibits activation of the tumor suppressor pathway and its expression is highly upregulated in glioblastomas [22], our results revealed that BMI-1 expression is not upregulated compared with normal tissue. Further study is needed to address this discrepancy.

Our clustering data demonstrated that 42 glioma tissues could be classified into three groups based on the

expression of 13 stem cell markers and RTK genes. The demonstrative feature of this clustering is that gliomas in cluster 2 show relatively high expression of all genes, whereas the expression of all genes is relatively low in cluster 3. Further studies are needed to evaluate whether this subclassification can be applied to larger sample sets and whether this clustering has prognostic significance, because we have not included prognostic information in this study. Furthermore, because a recent study has reported a glioblastoma molecular stratification defined as proneural, neural, classical, and mesenchymal based on genomic alteration [23–25], we need to evaluate how each unique cluster falls into these categories.

The clustering data also give us a general description of how the selected genes co-varied with respect to their expression level, and some of the correlation is confirmed by statistical analysis. Pairwise Spearman's correlation analysis on all genes revealed high correlation coefficients in some pairs (see Table 2). Notably, genes listed in Table 2 are all implicated in the angiogenesis of normal and tumor tissue. We did not see strong statistical correlations among stem cell markers MELK, Nestin, CD133, and Notch receptors. In addition, there is a possibility that gene pairs with moderate correlation may have been missed with these criteria, because we have only listed the gene pairs for which the correlation coefficient is more than 0.7. In fact, the correlation coefficient of EGFR and Nestin, and of PDGFRA and CD133, both of which are in the same gene tree in the dendrogram, was 0.68 ($P < 0.0001$) and 0.55 ($P = 0.0002$), respectively.

In summary, we demonstrated that the expression of MELK is exclusively upregulated in glioblastoma tissue. Although Notch receptor expression is moderately upregulated in glioblastoma tissue, its expression showed a strong correlation with angiogenesis-related genes VEGFR2, VEGFR3, and PDGFRB. We need to address the biological and prognostic features of glioblastomas grouped within cluster 2 because this group showed the unique findings that all the genes are highly expressed.

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