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Mini Review

# Molecular markers of glioma initiating cells

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**It is now widely accept that stem cell-like cancer cells, also known as cancer initiating cells (CIC), cancer stem cells or cancer propagating cells exist, in various types of cancers, including malignant glioma. Because it is likely that CICs proliferate indefinitely, express characteristics of tissue-specific stem cells, form tumor and are resistant to chemo- and radio-therapy, it is important to establish their purification methods, characterize them and find therapeutic ways. In this review, I will summarize recent progress about glioma initiating cell that is one of best CIC models.**

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## Introduction

During the last decades, tissue-specific stem cells (TSCs) have been identified in almost all tissues. These cells self-renew and continuously generate the residential differentiated cells that are responsible for tissue functions and homeostasis[1)]. Neural stem cells (NSCs) in the central nervous system (CNS), for example, self-renew and give rise to neurons, astrocytes, and oligodendrocytes throughout life[2)].

The discovery of TSCs made a paradigm shift in cancer research. The use of markers for stem cells and differentiated cells, for instance, revealed that malignant tumors contain both cells that express TSC markers, such as Nestin, Sox2, and Oct4, and cells that express differentiation markers[3-6)], suggesting that these tumors contain stem cell-like cancer cells (also known as cancer stem cells, cancer initiating cells (CICs) or cancer propagating cells).

This idea was also supported by the other findings. For instance, cancer cells as well as TSCs maintain telomerase activity for self-renewal and express various types of detoxifying mechanism that contribute to the drug resistance in cancers, including ATP-binding cassette (ABC) transporters[7-10)] and Aldehyde dehydrogenase (ALDH)[11)], and DNA repair factors that contribute to radioresistance, including Bmi1[12)].

It was demonstrated that CIC-enriched populations can be obtained from cancers and cancer cell lines by exploiting TSC features, including cell surface proteins, CD133,

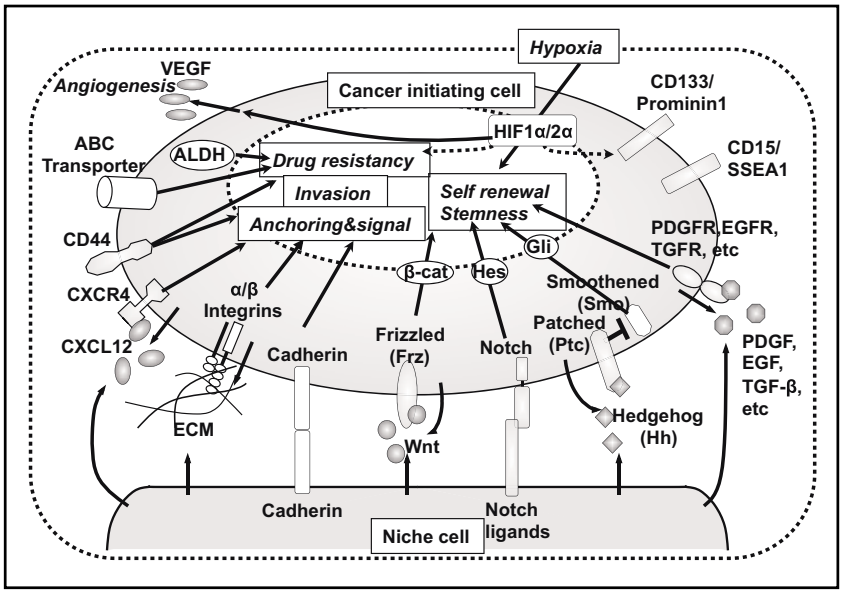


Fig.1 Factors involved in the characterization and maintenance of GIC

GICs as well as TSCs are thought to exist in the special microenvironment "Niche". GICs are anchored in the Niche by adhesion molecules, such as Cadherin and Integrins, and maintained by Niche factors, including Notch, Wnt, Hh, PDGF and EGF. GICs also express various types of factors, such as VEGF, which make and maintain "Niche".

CD44, and CD15, activity of specific enzymes such as side population (SP) and ALDH, sphere formation assay, or a combination of these features[3-5]. However, there are opposite evidences showing that such marker-negative cells from tumors and cancer cell lines can also form tumors when transplanted *in vivo*[13, 14]. Therefore, it still remains uncertain whether the existing isolation methods can identify *bona fide* CICs or non-CIC can obtain the characteristics of CIC in the special condition.

Gliomas are the most common brain tumors with characteristics of normal glial cells, astrocytes and oligodendrocytes, and classified into four grades (WHO grade I-IV) by pathological features. Approximately 50% of Glioma is glioblastoma multiforme (GBM), most malignant glioma (WHO grade IV), with a median survival of one year. Despite tremendous efforts to cure GBM, survival time of the patients has not changed over decades. The finding of GBM initiating cells (GICs), which express NSC markers, including Nestin and CD133, and are highly resistant to radio- and chemo-therapies, brought a strong impact to GBM research. Indeed, it has been demonstrated that the inhibition of NSC-maintaining genes, such as Sox2 and hypoxia inducible factors (HIFs), prevents GIC tumorigenesis[15, 16].

Among various types of CICs, it is sure that GIC is one of best models to analyse the fundamental mechanism in CICs, because the experimental procedures for neural lineage cells and GBM are well established. Moreover, it was revealed that most of mutations in GBM assemble in three signaling pathways, p53, retinoblastoma (Rb) and receptor tyrosine kinase pathway[17, 18], all of which are commonly mutated in other types of cancer. Thus, it is likely that both the experimental procedures and results of GIC research are widely applied to other CIC research. In this review, I will summarize GIC markers (Fig.1) and discuss about their potency as therapeutic target.

## Cell surface markers for GIC

Singh et al. has reported their success in separating brain CICs from human medulloblastoma and GBM using an anti-CD133 antibody that recognizes a variety of different stem cells. Here, as few as one hundred $CD133^+$ GBM cells formed tumors in NOD-SCID brain[19]. It was also shown that CD133 induces the expression of an ABC transporter, P-glycoprotein/ABCB1[20]. However, since there is evident that $CD133^-$ GBM cells is also tumorigenic and the expression is induced in hypoxia[21], it is still controversial whether CD133 is a bona fide marker for GICs.

CD15, also known as Stage-Specific Embryonic Antigen 1 (SSEA1) or Lewis X (LeX), was shown to be a general CIC marker on GBM[22]. Since CD15 as well as CD133 is also expressed on NSC and their progenitor cells, it is unlikely that it is a therapeutic target.

It was also shown that a cell surface chemokine receptor CXCR4, which mediates proliferation and invasion, is expressed on GICs and CXCL12, the CXCR4 ligand, activates GIC proliferation[23]. Moreover, it was demonstrated

that CXCR4+ cells from human glioma specimens form tumorspheres in culture and form xenograft tumors, whereas CXCR4- cells did not so[24]. Together, these findings suggest that CXCR4 is a marker for GICs.

Rich and his colleagues have shown that integrin α6, a member of integrin family of extracellular matrix receptors, as a new marker for GICs: they succeeded to enrich GICs using anti-integrin α6 antibody and demonstrated that knockdown of integrin α6 can inhibit GIC tumorigenesis *in vivo*[25].

Cadherins are also involved in GIC tumorigenesis and invasion via the E- to N-cadherin switch. It is of interest that co-localization of N-cadherin and integrin α6 induces activation of extracellular signal-regulated kinase that regulates proliferation and invasion[26]. It was also shown that interaction of connexin 43 with E-cadherin decreases GIC invasiveness[27].

It was demonstrated that A2B5, which is a golden marker for oligodendrocyte precursor cells (OPCs), can be used to separate human GIC[28, 29], suggesting the possibilities that GICs originate from OPCs or acquire OPC characteristics in their transformation. In fact, there is increasing evidence that OPC is a cell-of-origin for GICs[30, 31].

## Signaling pathways in GIC

It is well known that loss of p53 function promotes the accelerated cell proliferation and malignant transformation[32]. Indeed, over 65% of human glioma was shown to contain TP53 gene deletion and mutation[33]. Moreover, additional evidences indicated that other p53 signaling factors, including Murin-double-minute 2 (MDM2) that binds to, destabilizes, and inactivates p53, and the Chromodomain helicase DNA binding domain 5 (Chd5) that regulates cell proliferation, cellular senescence, apoptosis, and tumorigenesis, are also mutated in malignant glioma[32-35]. In total, it was revealed that about 90% of human glioma have mutations in p53 signaling pathway[17, 18] (Fig.2). Although the effector molecule of p53 pathway is the p21 cyclin-dependent kinase (Cdk) inhibitor that regulates progression of cells through the G1 cell-cycle phase, it has not been demonstrated that p21 gene itself is an oncogenic target in human cancers.

Retinoblastoma (Rb) is another essential tumor suppressor protein that regulates the G1 checkpoint[36]. Hypophosphorylated form of Rb sequesters E2F transcription factor and arrest cells at the G1 checkpoint. Once Rb is hyper-

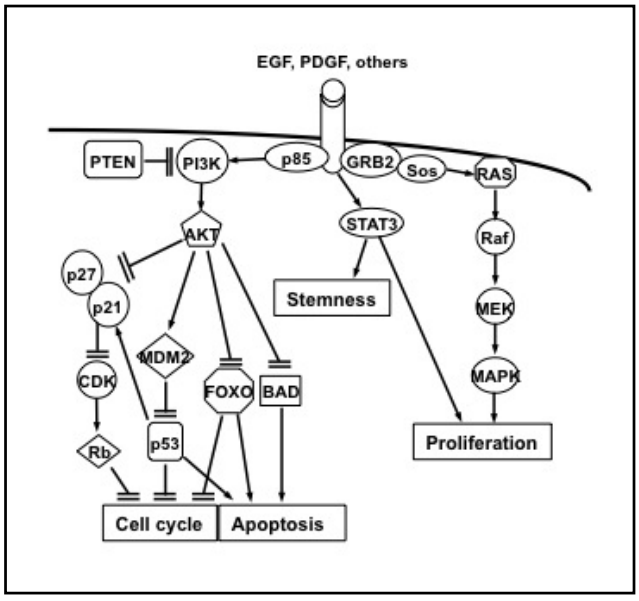


Fig.2 Signaling pathway involved in GICs

Various kinds of growth factors, including EGF, PDGF and LIF, activate PI3K/AKT, Ras/Raf/MAPK, STAT3 and other pathways, which regulate cell cycle, apoptosis, differentiation, and cell proliferation.

phosphorylated by Cyclin D and Cdk4/6 complex, phosphorylated Rb releases E2F, E2F induces the expression of cell cycle regulators, and then the cells enter S phase. In contrast, p16/Ink4a Cdk inhibitor binds to Cdk4/6, prevents the complex formation of Cdk4/6 and Cyclin D, and maintains Rb hypophosphorylation. Mutations in Rb pathway have been frequently identified in many types of malignant tumors. For example, mutations in Rb signaling pathway, including Cdk4 amplification and p16/Ink4a deletion, was found in about 80% of GBM[17, 18, 37].

Signaling pathways (Ras/Raf/MAPK and PTEN/AKT pathways) of receptor tyrosine kinases (RTKs), including the Platelet derived growth factor receptor, the Epidermal growth factor receptor, the Fibroblast growth factor receptor, the Insuline-like growth factor receptor and the Leukemia inhibitory factor receptor that play a role for the maintenance of TSCs and amplifying precursor cells, are frequently mutated in tumors[38]. For instance, activation of RTK pathway was found in about 90% of GBM[17, 18]. In particular, it has been shown that small GTP protein Ras, one of essential oncogenes, and its negative regulator, type1 Neurofibromas gene (NF1), are mutated in many kinds of human cancers and that the phosphatase tensin homolog (PTEN), which inhibits function of the phosphoinositol tri-

phosphate kinase (PI3K) that activates Akt, is frequently inactivated in malignant tumors[39].

Notch receptors (Notch1-4 in mammals) are involved in a number of biological functions, including TSC maintenance and tumorigenesis[40, 41]. Following the activation of Notch by ligands (Delta-like-ligand (Dll) 1, 3, and 4, and Jagged 1 and 2), the receptor is cleaved by metalloproteases and $\gamma$-secretase complex, releasing the Notch intracellular domain (NICD). The NICD then activates a number of target genes, including the differentiation inhibitors, hairy and enhancer-of-split (Hes), and cell cycle regulator, Cyclin D1. In gliomagenesis, it has been demonstrated that Notch pathway is crucial for the maintenance of GICs and their tumorigenesis[42, 43]. These findings suggest that Notch signaling inhibitors can be used for GBM therapy. In fact, a number of Notch inhibitors, including RO4929097, MRK003 and MK0752, are now under (pre-) clinical trial.

The Wnt family of secreted proteins regulate diverse developmental processes, including cell proliferation, fate decisions and tumorigenesis[44-46]. When Wnt (20 members in mammals) binds to the receptor (called Frizzled, Frz), activated Frz inhibits degradation of $\beta$-catenin ($\beta$-cat), which is a key transcription factor in the canonical signaling, and induces the expression of target genes, including *c-myc* and *cyclin D1*. It has been shown that Wnt1 and 3a, Frz5 and 8, and $\beta$ -cat are expressed in the developing ventricular and subventricular zones (VZ/SVZ)[47-49], where NSCs exist, and that inactivation of Wnt1, Wnt3a, or $\beta$-cat causes developmental brain defects[46, 50]. Many lines of evidences suggest that Wnt signaling pathway is activated in GBM. For instance, it was shown that Acaete-scute homolog 1, which is an essential transcription factor for the maintenance and tumorigenesis of GICs, activates Wnt signaling by repressing a Wnt signal inhibitor Dickkopf1[51] and that Pleiomorphic adenoma gene like 2, a transcription regulator, induces self-renewal of GICs and inhibits their differentiation by activating Wnt/$\beta$-cat signaling[52]. Thus, hyper-activation of Wnt signaling is likely crucial for GBM development.

Secreted protein Hedgehog (3 members, Sonic, Desert, and Indian, in mammals) is also involved in TSC proliferation and tumorigenesis[53, 54]. Binding of hedgehog (Hh) to the transmembrane receptor Patched1 activates the zinc-finger transcription factor Gli (Gli1-3) and induces the expression of target genes, including *wnt, insulin-growth factor 2* (*igf2*), and *pdgf receptor a*. There are many evidences showing that Hh signaling pathway is essential for NSC maintenance and gliomagenesis[55, 56]: All of Hh signaling components, Gli, Ptc1, and Smo, are expressed in the VZ/SVZ and enforced expression of dominant-negative form of Gli2 (dnGli2), which blocks functions of all Gli members, inhibited proliferation of NSCs and expression of NSC markers, including Sox2 and Prominin1[57]. In gliomagenesis, it was shown that Gli1 is highly activated in GBM as well as medulloblastoma and primitive neuroectodermal tumors[58]. It was shown that Cyclopamine, one of specific inhibitors of Hh signal, blocks the growth of several primary gliomas, medulloblastomas, glioma cell lines, and human GICs[59-61]. Moreover, it was demonstrated that overexpression of dnGli2 prevents tumorigenesis of human GICs[61]. Taken together, these findings suggest that Hh signaling plays an important role in GICs.

Signal transducer and activator of transcription 3 (STAT3), a member of STAT transcription factors, is well-known to be involved in the tumorigenesis of various types of cancer as well as the maintenance of embryonic stem cells and NSCs[62-64]. Activation of STAT3 signaling pathway is also found in malignant glioma and is associated with poor prognosis[65]. It was shown that inhibition of STAT3 not only induces the expression of differentiation markers in GICs but also prevents their proliferation and tumorigenesis[66-68]. Thus, STAT3 is likely a crucial target for therapy.

Transforming Growth Factor-beta (TGF-$\beta$) is a well-known tumor suppressor, however, there are opposite evidences that it plays an essential role in GBM. For instance, TGF-$\beta$ was shown to induce proliferation of GICs through the activation of PDGF-B and LIF signaling pathway[69, 70]. It was also demonstrated that TGF-$\beta$ activates Sox4-Sox2 axis and Bmi1, both of which are essential regulators in TSC, to maintain GIC tumorigenesis[71, 72]. Together with another evidence that TGF-$\beta$ is an inducer of regulatory T cell that inhibits the activation of immune system, these suggest that TGF-$\beta$ is a central player for gliomagenesis.

## Resistance of GIC to cancer therapies

Cancer cells as well as many kinds of TSCs express a number of ABC transporters. Breast cancer resistance protein 1, also known as ABCG2, excludes the fluorescent dye Hoechst 33342, identifying a SP[9], in which various types of TSCs are enriched, although some research has shown that TSCs exist in both SP and non-SP and that SP

cells do not express stem cell markers[13, 73]. A number of research groups have found that some established cancer cell lines, which have been maintained in culture for decades, and tumors, such as glioma, AML, neuroblastoma, nasopharyngeal carcinoma, and ovarian cancer, contain a small SP. These studies have demonstrated that SP cells — but not non-SP cells — self-renew in culture, are resistant to anti-cancer drugs including Mitoxantrone, and form tumors when transplanted *in vivo*[74]. However, it has been shown that many cancer cell lines do not contain any SP fraction and that non-SP cells in some cancer cell lines generate SP fraction during culture.

ALDH is another detoxifying enzyme oxidizing intracellular aldehydes to carboxylic acids and blocking alkylating agents. Finding of the increased activation of ALDH in TSCs made it possible to identify and purify many types of TSCs, including hematopoietic stem cells and NSCs, using fluorescent substrates of this enzyme and flow cytometry[75]. ALDH activity is now used to separate many types of CICs, including GICs, from tumors and cancer cell lines[76, 77].

Hypoxia inducible factors (HIF) are the oxygen-sensitive transcription factors consisting of HIF-1 ($\alpha$ and $\beta$), -2 ($\alpha$ and $\beta$) and -3 ($\alpha$ and $\beta$). Since it has been shown that HIFs regulate many genes involving in angiogenesis, metabolism, proliferation and survival in hypoxia, they become essential targets for cancer therapy. Indeed, it has been shown that inhibition of HIF-1$\alpha$ and -2$\alpha$ prevents GIC tumorigenesis[16].

## Other characteristics

An increasing evidence points to the fact that CICs as well as TSCs, such as NSCs and mammary gland stem cells, can form floating aggregates (tumor spheres) and be enriched in the spheres when cultured in serum-free medium with proper mitogens, such as bFGF and EGF. However, because all of CICs in spheres are not exposed to the mitogens, monolayer culture method might be better to expand and characterize CICs. Indeed, Pollard et al have demonstrated that monolayer-cultured GICs are homogenous and can be used to identify therapeutic targets[78].

One characteristic of malignant tumor cells is to invade into normal tissue and to metastasize into other tissues. Some of the infiltrating cancer cells cannot be removed by surgical operation and causes recurrence, suggesting that CICs retain high invasion activity. In fact, it has demonstrated that CD133-positive cancer cells highly express CD44 and chemokine receptor CXCR4, both of which mediate cell migration[23, 24].

Clement et al. have demonstrated that the combination of a distinct morphology with a very high nuclear/cytoplasmic ratio and intrinsic autofluorescence (520nm emission upon laser excitation at 488nm) can be used to prepare GICs, called FL1(+) cells that express NSC markers and are malignant[79]. It is of interest to know the molecular mechanism relating the morphology and autofluorescence in GICs.

## Conclusion

A number of new stem cell markers and techniques have been utilized to identify and purify CICs during last decade. However, it is not yet known whether or not such CICs consist of homogenous population, as such marker-negative cells as well as positive cells contain tumorigenic cells. Therefore it is still needed to establish experimental strategies, including the single cell analysis, to identify bona fide GICs and to characterize them, leading to the discovery of novel therapeutic targets and methods.

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### Conflict of Interest Statement

None declared.

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