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Glioblastoma Multiforme Formation and EMT: Role of FoxM1 Transcription Factor

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Abstract

Glioblastoma multiforme (GBM) is one of the most malignant cancers in human brain. The prognosis of GBM is extremely poor because it is resistant to radiotherapy and chemotherapy. Improving understanding of the tumor biology brings some new hope to the treatment of GBM. In this review, we discuss the evidence that FoxM1 plays a critical role in the development and progression of GBM by regulating key factors involved in cell proliferation, epithelial to mesenchymal transition (EMT), invasion, angiogenesis and upregulating Wnt/ β -catenin signalling. Our recent experimental findings are also summarized to prove that FoxM1 is a novel therapeutic target against GBM.

Keywords

Glioblastoma; Tumorigenesis; EMT; Invasion; Angiogenesis; FoxM1; Wnt/ β -catenin signalling

1. Introduction

Forkhead box (Fox) proteins are a family of transcription factors that regulate a wide spectrum of metabolic and developmental functions [1, 2]. Characterized by a common, evolutionary conserved, winged helix DNA-binding domain, alteration of Fox protein expression has been linked to cell growth and cycling dysregulation and implicated in tumorigenesis and cancer progression [3, 4]. FoxM1 is a member of the Fox proteins critical for G1-S and G2-M progression. While its expression is turned off in terminally differentiated cells, it is upregulated in a multitude of human solid tumors including breast cancer, non-small cell lung carcinomas, glioblastoma, medulloblastoma, basal cell carcinomas, hepatocellular carcinomas, pancreatic carcinomas, colon cancer and prostate cancer [5–10]. Recent studies have indicated that FoxM1 also mediates a range of downstream events including tumor invasion, angiogenesis and metastasis, quintessential of the malignant phenotypes [11–16]. FoxM1 can also regulate Wnt/ β -catenin signaling

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Conflict of Interest

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pathway through promoting β -catenin nuclear translocation[3, 12]. Such differentiated yet widespread involvement suggests that the expression of FoxM1 is a cardinal event in the development of human cancer and by targeting FoxM1, new effective therapeutics with low toxicity to normal tissue may be possible [17–19].

Glioblastoma multiforme (GBM) is one of the deadliest cancers known to humans. Despite the advances in the surgical and non-surgical treatments of this disease, the average survival of patients with GBM is limited to about 14 months[20, 21]. This harks back to a fundamental lack of understanding of the oncogenic mechanism of glial tumors and underlines the gap between current practice and research in bringing effective treatment to clinical use. In light of this, we sought to investigate whether targeting FoxM1 may open new avenues in the fight against this deadly disease and our data has indicated that FoxM1 is indeed a key mediator, fittingly not only in glioma tumorigenesis but also in invasion, growth, angiogenesis and transformation of glial tumors. In this chapter, we summarized the collective mechanistic evidence on how FoxM1 contributed to GBM formation and progression, both in vitro and in vivo, and offered our perspectives in the development of future therapeutics[6, 19].

2. FoxM1 contributes to the tumorigenesis of GBM

In order to ascertain the role of FoxM1 in the development of GBM, we conducted a series of experiments using human glioma tissues and in-vitro and in-vivo glioma models to characterize the expression of FoxM1 and its alteration in GBM tumorigenesis[11]. First using reverse transcription PCR and immunohistochemical staining for FoxM1, we established that FoxM1 mRNA and protein while not detected in normal brain, were found in low grade astrocytoma, anaplastic astrocytoma and GBM. Moreover, the level of expression in these human glioma specimens correlates directly with tumor grade and inversely with patient survival. In particular, of the 50 GBM patients studied, the median survival was significantly prolonged for those whose tumor did not stain for FoxM1 (26 months) and markedly shortened for those who stained positively (7.5 and 13 months with strongly positive and moderately positive staining of FoxM1 respectively). These results demonstrated a strong correlation between FoxM1 and the development of GBM[11], which was also confirmed by the Cancer Genome Atlas data from GBM specimens[22].

Second to evaluate whether a causal relation exists with these observations, we studied the effect of enforcing FoxM1 expression by transfecting FoxM1 expression vector into glioma cell lines with low levels of FoxM1 expression and conversely the effect of silencing FoxM1 by transfecting FoxM1-siRNA into glioblastoma cell lines with a high level of FoxM1 expression. We noted that such genetic manipulation resulted in a predictable increase or decrease in anchorage-independent growth, a hallmark of transformation phenotype, as per whether FoxM1 was upregulated or downregulated respectively. In a similar vein, we evaluated the tumorigenicity of FoxM1 upregulated glioma cells in vivo by direct injection of these cells into nude mice. Under normal circumstances, control anaplastic astrocytoma cells do not form tumour xenografts, but with FoxM1 transfected cells, tumors with histologic features typical of human GBM were detected in the experiment animals. Conversely, the formation of tumor xenografts in nude mice injected with glioblastoma cells

was markedly reduced when FoxM1-siRNA transfected cells were used[11, 13]. The survival of these mice was also significantly prolonged or shortened with respect to whether FoxM1 was transfected or silenced.

Third we examined whether alteration in FoxM1 would also affect cell cycle regulatory genes as predicted by the existing knowledge regarding its functions. By using Western blot analysis, we evaluated the level of Skp2 protein expression and the nuclear level of p27Kip1 protein, the major regulators of G1 to S phase transition, with altered FoxM1 expression in different glioma cell lines. It follows that the level of FoxM1 expression varies directly with Skp2 expression and inversely with p27Kip1 (degraded by Skp2)[11]. In summary, our results have showed that FoxM1 is a key oncogenic factor underpinning the development of GBM and its regulatory function in cell cycle transition plays an important mechanistic role.

3. FoxM1 enhances invasion of glioma cells

One of the hallmarks of GBM is its locally invasive nature and propensity to infiltrate normal brain parenchyma[23]. The cellular mechanisms underlying this process is known to involve tumor-secreted factors, of which matrix metalloproteinases 2 (MMP-2) is considered a key enzyme in the breakdown of the extracellular matrix permitting tumor penetration into normal brain[24].

In an effort to elucidate the mechanistic role of FoxM1 in glioma invasion, we therefore investigated whether FoxM1 expression would alter MMP-2 gene transcription in glioma cell lines. By using MMP-2 promotor reporter plasmids, we evaluated MMP-2 promotor activity in glioma cells following transfection with FoxM1 expression vector or FoxM1-siRNA. It was noted that the luciferase activity as driven by the MMP-2 promotor exhibited a direct variation with FoxM1 expression. To further elucidate whether a direct interaction exists between FoxM1 and the MMP-2 gene, we then studied the binding of oligonucleotides derived from the MMP-2 promotor sequence with FoxM1 protein using electrophoretic mobility shift assay. Notably band shifts consistent with a FoxM1 DNA–protein complex were detected in the assay and such binding was also observed in vivo by using standard chromatin immunoprecipitation (ChIP) assays[15]. These results thus confirm that FoxM1 directly activated the MMP-2 promotor through a FoxM1 binding site. Moreover, by introducing a base-pair change in the FoxM1-binding site, such binding was found to be functional as confirmatory MMP-2 promotor activity attenuation was resulted by this site directed mutation. Finally to obtain corroborative clinical evidence, we analyzed 45 human GBM specimens with immunohistochemical staining for FoxM1 and MMP-2 and fittingly a positive correlation between FoxM1 overexpression and elevated MMP-2 expression was observed.

4. FoxM1 may regulate EMT of glioma cells

MMP-2 also plays an important role in mesenchymal phenotypes in GBM. Some literatures employed the term “glial to mesenchymal transition (GMT)” instead of “EMT”, because glioma cells are derived from glial cells. Several studies have shown that recurrent GBM exhibited mesenchymal subtype in general. The levels of MMP2 are directly correlated with mesenchymal transition in glioma cells. A growing number of findings have linked FoxM1

to EMT in different tumors including pancreatic cancer [25, 26], breast cancer [27], prostate cancer [28], gastric cancer[29], and lung cancer [30]. Interestingly, miR-149, which can block EMT of non-small-cell lung cancer cells, also inhibits GBM proliferation and invasion by reducing the expression of CyclinD1 and MMP-2, two important targets of FoxM1 in GBM [31, 32]. Recent studies also reveal miR-134 as a master suppressive regulator that is inhibited by multiple receptor tyrosine kinases in GBM [33]. miR-134 is known to inhibit epithelial to mesenchymal transition by targeting FOXM1 in lung cancer [34]. These findings suggest a potential role of FoxM1 in contribution to the mesenchymal transition of gliomas that are featured by malignancy and worse outcome. It is tempting to ask whether FoxM1 is directing a mesenchymal transition program in GBM.

Moreover, TGF- β promotes EMT of GBM cells by inducing phosphorylated Smad2/3 thereby the increased expression of MMP-2, Snail, and MMP9 in GBM cells [35]. TGF- β -dependent signaling is one of the critical signaling pathways in EMT. Our recent study reported that FoxM1 regulates TGF- β signaling by interacting with Smad3. TGF- β induces its signaling transduction by stimulation of Smad3 phosphorylation. Phosphorylated Smad3 forms a complex with Smad4 and then translocate to nucleus to regulate the expression of downstream target genes. On the other side, the TGF- β signaling pathway is negative regulation by specialized inhibitory factors, including TIF1 γ . TIF1 γ inhibits TGF- β signaling pathway by monoubiquitination of Smad4, which leads to disassembly of the Smad3/Smad4 transcriptional complex and forced exit of Smad3 from the nucleus. FoxM1 interacts with Smad3 and sequesters TIF1 γ from binding to Smad4, thereby inhibiting TIF1 γ -induced monoubiquitination of Smad4 [36]. As a result, FoxM1-Smad3 interaction sustains Smad3 binding to Smad4 and hence retention of Smad3 in the nucleus, which leads to increase the activity of TGF- β 1 signaling. Deficiency of FoxM1 in breast cancer cells impairs migration, invasion and metastasis by inhibition of TGF- β 1 signaling. Deletion of FoxM1 in MEF cells also abolished TGF- β 1-induced Smad3/Smad4 complex formation and transcriptional activity of the TGF- β 1 signaling [36]. Considering that both FoxM1 and Smad3/Smad4 are present in the nucleus, we therefore reasoned that FoxM1 may also play an important role in EMT of glioma cells by regulating TGF- β 1 signaling.

5. FoxM1 enhances glioma angiogenesis

The malignant behavior of glioma is closely related to the expression of angiogenic factors in promoting aberrant growth and invasion[37]. Vascular endothelial growth factor (VEGF) is a key mediator in this process and not surprisingly is overexpressed in many glioma cell lines[38]. However the molecular basis of this overexpression has not been fully elucidated and the role of FoxM1 expression in driving this aberrant constitutive expression in glioma has not been investigated.

In order to evaluate the effect of FoxM1 expressions on VEGF gene expression and the angiogenesis of gliomas, we conducted a series of investigation to ascertain the interaction between FoxM1 protein and VEGF transcription[13]. Using the same methodology above, we determined both in vitro and in vivo that FoxM1 bound specifically to VEGF promoter and caused functional activation of VEGF transcription. Conversely, mutation of FoxM1-binding sites significantly attenuated VEGF promoter activity. To further verify the clinical

relevance of these observations, we used the nude mouse model as mentioned above and confirmed that while the degree of vascularisation and VEGF expression were prominent in the brain tumors formed by controlling glioma cells, considerably lower activities were noted in the brain tumors formed by FoxM1 expression inhibited cells. The phenotypic effect as demonstrated by endothelial cell tube formation assays provided confirmatory evidence that FoxM1 inhibited cells exhibited significantly lowered angiogenic ability than controls. Collectively, our results indicate that FoxM1 overexpression is a key event in driving angiogenesis in glioma formation and its control over VEGF transcription plays an important functional role.

6. FoxM1 promotes astrocyte transformation and GBM formation

One of the key strategies in the development of effective anticancer treatment is to target the root of the neoplastic processes by halting the transformation of normal cells into malignant phenotypes. In GBM, studies into the molecular cascades leading to the transformation of normal astrocytes into malignant glial cells have revealed pRb, p53 and PTEN-PI3K-Akt pathway as some of the key components of this malignant process[39, 40]. However, for normal human astrocytes (NHA), although inactivation of p53 and pRb pathways could lead to the formation of immortalized cell lines mimicking low-grade astrocytoma, such inactivation was not sufficient to induce the malignant phenotypes. It appears that some remaining oncogenic events are required for full transformation to take place[41, 42].

In light of this, we designed a series of experiments to evaluate whether FoxM1 may represent the missing piece of the puzzle in the transformation process in GBM. By using a retrovirus based technique to induce FoxM1 expression, we found that when immortalized NHA (by inactivation of p53 and pRb) were infected with FoxM1, these cells exhibit malignant phenotypes typical of GBM. This was demonstrated both in vitro on soft agar plates and in vivo by using the xenograft nude mouse model in which brain tumors with histological and immunohistochemical features typical of GBM were found only in mice injected with FoxM1 infected cells. To elucidate the molecular mechanisms underlying this transformation, we then evaluated whether the Akt pathway was implicated by examining the level of Akt activation and PTEN expression in FoxM1 infected cells with Western blotting and immunohistochemical staining and also the effect on immortalized NHA transformation when Akt was inactivated. The results are consistent in that FoxM1 infected cells exhibited significantly higher Akt activation and reduced PTEN expression correspondingly and FoxM1 dependent transformation in vitro was attenuated when Akt was inhibited[16]. Moreover, we found that such alterations are mediated at a transcription level through FoxM1 binding to the promoter of NEDD4-1 that promotes PTEN ubiquitination, as demonstrated on ChIP assays. The resultant upregulation of PTEN degradation and Akt activation was demonstrated on Western blotting. Taken together, our data has provided the evidence for the first time that FoxM1 overexpression leads to transformation of glial tissue into malignant tumors and its effect on NEDD4-1 upregulation underpins the molecular mechanism of this process[16].

7. FoxM1 promotes β -catenin nuclear translocation and tumorigenicity in glioma

It's well known that Wnt signaling regulates many processes central to cancers, including GBM[12, 14, 43, 44]. β -catenin is a key molecule of Wnt signaling and Wnt activation increases β -catenin protein stability[45, 46]. Wnt/ β -catenin signaling has been found to be involved in the regulation of migration, invasion, and proliferation of malignant glioma cells[47–50].

Moreover, FoxM1, which is overexpressed in many cancers, plays critical roles in Wnt/ β -catenin signaling pathway[12, 51]. Our studies demonstrated that FoxM1 is a downstream target gene of Wnt signalling[12]. FoxM1 could regulate tumorigenicity of GBM through controlling β -catenin nuclear localization and Wnt target-gene expression. We found the direct FoxM1 and β -catenin interaction and their colocalization both in the cytoplasm and nucleus by immunoprecipitation and immunofluorescent staining analysis. When treated with Wnt3a, the protein level of FoxM1 and β -catenin increased obviously, and the Wnt activation promoted their translocation to the nucleus[12]. The nuclear accumulation of FoxM1 and β -catenin could be increased by Wnt3a in a time-dependent manner. Overexpression of Flag-FoxM1 also increased the nuclear accumulation of β -catenin. When knocking down FoxM1 via a FoxM1-siRNA in glioma cells, the levels of nuclear but not cytoplasmic β -catenin decreased, we confirmed it through immunofluorescent staining and immunoblotting analysis. Thus, our data demonstrated that FoxM1 directly mediates nuclear translocation of β -catenin in glioma cells. We also found that overexpression of FoxM1 upregulates the expression level of Wnt/ β -catenin target genes Axin2, c-Myc, cyclin D1 and promoted GBM cells to form brain tumors in nude mice, while knocking down FoxM1 or β -catenin by sh-RNA in glioma cells significantly inhibited tumor formation of the cells in nude mice[12]. All these findings strongly suggest that Wnt-FoxM1- β -catenin plays a pivotal role in the development and progression of GBM.

8. Conclusions

FoxM1 expression contributes to the growth of GBM by promoting uncontrolled cell proliferation, invasion, and angiogenesis through regulating the expression of p27, Skp2, MMP-2 and VEGF[13, 15, 16]. Moreover, FoxM1 plays a critical role in the development of glioma by up-regulating the expression of NEDD4-1, an E3 ligase of PTEN hence down-regulating the level of PTEN[16]. Furthermore, our Cancer Cell paper has shown that FoxM1 plays critical roles in regulating tumorigenicity of GBM through controlling β -catenin nuclear localization and Wnt target-gene expression[12]. Knocking down FoxM1 successfully eliminated the tumorigenicity of GBM. Targeting FoxM1 might be an effective therapeutic strategy to cure the patients of this fatal disease.

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