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FUNDAMENTAL STUDY ON THE EFFECTS OF EPHA2 INHIBITATION IN ANGIOGENESIS SIGNALING PATHWAYS OF HUMAN MALIGNANT GLIOMA CELLS

PENYELIDIK

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2018

Progress Summary

Project Progress : 100.00%
Budget Used : 100.00%
Human Capital :100.00%

Current Outcome

Type	Number
Activities	6
Publication	2
Exhibition	0
Intellectual Property	0
Product	0

Milestone

No.	Description	Project Completion Contribution	Expected Completion Date	Completed Percentage	Actual Completion Date	Contributed Progress
1	Completion of establishment of malignant glioma cell culture in vitro.	5	31/01/2014	100	31/07/2014	5.00%
2	Completion of determining the gene expression level of EphA2 in malignant glioma cell line.	5	30/06/2014	100		5.00%
3	Completion of determining the protein expression level of EphA2 in malignant glioma cell line.	15	30/09/2014	100		15.00%
4	Completion of determining the effects of EphA2 gene silencing on the genes of interest involving angiogenesis signaling pathway.	40	31/03/2015	100		40.00%
5	Completion of investigating the effects of EphA2 gene silencing on cell proliferative rate of malignant glioma cells.	20	31/07/2015	100		20.00%
6	Completion of data analysis and writing of report.	15	30/11/2015	100		15.00%
	Overall Progress					100.00%

Research Abstract

An increasing number of studies have shown a correlation between EphA2 overexpression and high activities of angiogenesis in malignant gliomas. To test whether EphA2 regulates angiogenesis via VEGF and it's receptors, VEGFR-1 and VEGFR-2, the genes and protein expression levels were evaluated following gene knockdown with siRNA. Proliferation assay showed reduction of relative cell viability from 24 hour to 48 hour upon siRNA transfection (p=0.02). EphA2 knockdown significantly suppressed the expression of VEGF (p=0.023) and VEGFR-2 (p=0.0299) at gene levels in U-87 cell line. Contrary, the similar knockdown effects was not seen in DBTRG cell line. Gene expression analysis in DBTRG showed a trend of elevated VEGF and downregulated VEGFR-2 with no significant difference. VEGFR-1 gene expression was not detected in both cell lines. At protein level, EphA2 expression before and after siRNA transfection was not detected in both cell lines. The differences seen in genes regulation of U-87 and DBTRG cell lines may be due to the fact that both cell lines are from different origin. Our results suggested possible role of EphA2 in angiogenesis pathway via VEGF and VEGFR-2 regulations in U-87 cells. Activation of VEGF/VEGFR-2 complex may trigger multiple downstream signaling pathways which resulting in endothelial cell survival, proliferation, migration, and differentiation as well as vascular permeability.

Summary of Research Findings

Angiogenesis is an important process for tumor growth beyond a microscopic size. It involves a sequence of coordinated events that is initiated by the expression of more than 25 different growth factors and cytokines. EphA2 plays vital role in regulating angiogenesis and known to be over expressed in GBM tumors. The roles of EphA2 in angiogenesis signaling pathway was investigated by discovering its relation to most common key player of angiogenesis; Vascular Endothelial Growth Factor (VEGF) and its receptors; VEGFR-1 and VEGFR-2.
After transfection of EphA2 gene with small interfering RNA (siRNA), its expression difference before and after the inhibition was calculated along with tumor angiogenesis regulator; VEGF, VEGFR-1 and VEGF-R2. VEGF play roles in promoting tumorigenesis via angiogenesis. It was proven when mouse brains were injected with VEGF-expressing cancer stem cell that resulted in vascular-rich GBM, tumor-associated hemorrhage, and high morbidity in malignant brain tumors (Oka et al., 2007). Meanwhile, it receptors, VEGFR-1 contributes to vascular sprouting and metastasis (Wong, Prawira, Kaye, & Hovens, 2009) and VEGFR-2 acts as receptor for mediating the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. It involves in all aspects of normal and pathological vascular-endothelial-cell biology (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006).
Based on this study, the effects of EphA2 knockdown in U-87 cells lead to significant downregulation of VEGF and VEGFR-2 by p=0.023 and p=0.0299 respectively. In contrast to U-87 cells, DBTRG relative gene expression level of EphA2 in siRNA-EphA2 treated group compared to untreated group showed a significant knockdown of EphA2 gene expression by p=0.013. It led to elevated expression of VEGF dR1.38 yet lowering VEGFR-2 expression dR0.72. However, both VEGF and VEGFR-2 expression before and after EpHA2 inhibition are not statistically different in DBTRG cell line.
Another tumor angiogenesis marker; VEGFR-1, both cell lines U-87 and DBTRG expression was almost undetectable VEGFR-1. No amplification was detected for VEGFR-1 in U87 and late Ct Value (>40) for VEGFR-1 in DBTRG. Interestingly, our protein analysis results show that with significant knockdown of EphA2 protein in cells treated with EphA2 siRNA, no difference of protein expression was detected in both U87 and DBTRG cells. Similarly, no different of VEGFR2 protein expression was observed in U87 cells. Nevertheless, a trend of reduced VEGFR2 expression was seen in DBTRG cells treated with EphA2 siRNA. From the 2 subunit of proteins, both shown reduced protein intensity.

The results indicated that U-87 did not express VEGFR-1 gene which in accordance to Mesti et.al that showed undetectable VEGFR-1 in the similar cell line (2014). Meanwhile, late Ct Value (>40) for VEGFR-1 in DBTRG indicated low expression level of the gene. Ct values in this range have poor precision, low-fold changes and more difficult to accurately quantify (ABI Guidelines in RT PCR).
Based on those observation, undetectable VEGFR-1 expression and higher VEGFR-2 expression in GBM indicates VEGF receptors expression are independent to each other even the biological effects of VEGF are mediated by this two tyrosine kinase receptors; Flk-1 (VEGFR-1) and Flt-1/KDR (VEGFR-2). This is in accordance to (Ferrara, 2004) that signaling and biological properties of these two receptors are strikingly different. VEGFR-1 has the highest affinity for recombinant human VEGF with dissociation constant (Kd)of 10–20 pM. Conversely, VEGFR-2 has a lower affinity for VEGF, with a Kd of 75–125 pM. Here, VEGFR-2 is the key signaling receptor while VEGFR-1 functions as a 'decoy' and may not be primarily a signaling receptor receptor that able to regulate the activity of VEGF on the vascular endothelium in a negative fashion by sequestering and rendering (Ferrara, 2004). Binding to VEGFR-1 may prevent VEGF from interacting with VEGFR-2 (Wong et al., 2009).
Upon knockdown of EphA2 gene by siRNA, expression of angiogenesis related markers (VEGF and VEGFR2) were subsequently downregulated in U-87 cell. We demonstrated that there was a relation between EphA2 expression with VEGF and VEGFR-2 in U-87 cells. In this regard, VEGF may act as a regulator to VEGFR-2. This is in line to a research that found a direct measure of VEGFR-2 activity following VEGF stimulation in human medulloblastomas which deduced VEGF can act as an autocrine mitogen/survival factor for the tumor cells themselves (Slongo et al., 2007). Moreover, activation of VEGF/VEGFR-2 complex triggers multiple downstream signaling pathways which results in endothelial cell survival, proliferation, migration, and differentiation as well as vascular permeability (Hicklin & Ellis, 2005). Generally, it is agreed that VEGFR-2 is the major receptor mediating the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A.
By knowing that there are close relation between VEGF and VEGFR-2, we investigated how are they related to EphA2 expression. Miao et al. (2014) reported EphA2 expression in human brain microvascular endothelial cells (HBMEC) was upregulated by VEGF through its binding to VEGFR-2 and subsequently activating the intracellular PI3K/Akt and ERK1/2 signaling pathways, which contribute to an increase in paracellular permeability. Chen et. al (2006) suggested that there's specific