Classification of potential pathways affecting the VEGF pathway in glioblastoma multiforme (GBM) recurrent: A bioinformatics study based on differentially expressed- microRNAs

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Abstract

Glioblastoma multiforme (GBM) resistance to anti-angiogenesis drugs results in recurrence of the disease which leads to death. The resistance to anti-angiogenesis drugs that target the VEGF pathway is due to the influence of other pathways. This study aimed to identify and classify the pathways that are related to the VEGF pathway in GBM recurrent. The identification of differentially expressed miRNAs (DEmiRNAs) based on GBM GSE profiles (GSE32466) were carried out using a LIMMA R package and VEGF pathway genes in the KEGG database. Pathways related to DEmiRNAs and VEGF pathway genes were discovered by DIANA-miRPath v3.0, NetworkAnalyst and ToppGene databases, respectively. Inhibitory or activity affecting pathways relating to VEGF pathway were obtained based on XTalkDB database. The classification was determined by the KEGG database. There were 1014 genes that were found to have interaction with VEGF signaling pathway genes. One hundred nineteen pathways were achieved which have overlapping genes with the VEGF pathway genes. The MAPK pathway had the most in common genes with the VEGF pathway (39 genes). A total of 91 pathways were identified in 24 different classes. Several pathways significantly affect the VEGF pathway. Hence, it seems necessary to achieve new targets for combination therapies for GBM.

1 Introduction

Glioblastoma multiforme (GBM), the most aggressive CNS tumor with the poorest prognosis, is caused by extreme and abnormal angiogenesis^[1, 2]. In spite of multiple aggressive management of GBM, the outcome is unpleasant and patients generally die of tumor recurrences^[3]. Angiogenesis is considered one of the main hallmarks of GBM and for this reason, this type of tumor is an attractive candidate for anti-angiogenic therapies, especially during recurrence^[4]. The angiogenesis phenomenon occurs through a variety of modalities mainly vascular endothelial growth factor (VEGF), and other members of the VEGF family play important roles as well. This has signified the basis for the development of anti-VEGF blocking mediators and their use in GBM treatment. Nevertheless, these agents failed to induce significant therapeutic effects^[5]. Bevacizumab (BEV), a monoclonal antibody against VEGF, has only transient effects and GBM recurrence is not avoidable^[6, 7]. Increasing information about GBM-epigenetics help increase our knowledge related to the tumorigenesis, development, and recurrence of GBM^[8]. The interactions between epigenetic factors such as miRNAs and the cell signaling pathways are considered as high-potential targets for new therapeutic approaches^[9]. Several miRNAs can modulate mRNAs, thus generating an extraordinary complex regulatory network involved in many pathways such as drug resistance, angiogenesis, and recurrence^[9–12]. A literature survey of a set of miRNA deregulated in GBM found that they were overexpressed or downmodulated compared to normal brain tissue^[9, 13]. High throughput technologies, including microarrays or next-generation sequencing methods, provided great deal of data that increased our insights into the roles of non-coding RNAs (ncRNAs) and microRNAs^[14].

The purpose of this in silico study was to identify pathways that affect VEGF pathway which may cause anti-angiogenesis drugs resistance during recurrence. As one of the recurrence phenomena is angiogenesis, this database has been used to find indirect pathways affecting GBM angiogenesis. It seems that targeting the VEGF pathway may lead to alternative pathways activation and protection of the main pathway functions. In this systems biology analysis, we tried to identify other pathways that trigger this phenomenon and can affect recurrence via their effect on the VEGF pathway. By categorizing these pathways, we may provide better synergistic therapies with existing drugs for VEGF pathway targeting along with drugs that target other effective pathways. This may help eliminate resistance to anti-angiogenesis therapies in GBM possibly by overcoming its recurrence.

2 Results

2.1 Data collection: Identification of DE miRNAs

The summarized of this in silico analysis is including: One microarray miRNA expression dataset of GSE32466 (GPL10850) was used in this study. The dataset contains 24 samples (miRNA expression profiles between primary and recurrent GBM tissues from the same patients).

After data normalization and quality control between GBM (n = 12) and recurrent GBM samples (n = 12) we identified 79 DE miRNAs: 30 up-regulated miRNAs and 49 down-regulated miRNAs (Supplementary File 1). Then DE miRNAs were used for cluster analysis, the pheatmap package from R software was used for hierarchical cluster analysis of DE miRNAs, as shown in Fig. 1.

2.2 Data collection: VEGF signaling pathway genes

Using KEGG PATHWAY Database (<https://www.genome.jp/kegg/pathway.html>), all genes (n = 59) in the VEGF signaling pathway were collected. As shown in Table 1 gene expression of some of these genes were difference between GBM and normal brain tissue. According to the Expression Atlas site, the expression of 14 genes out of 59 genes in the VEGF signaling pathway showed increased expression, and 37 genes showed decreased expression (adjusted p value ≤ 0.05). For the remaining genes, no changes in expression were observed.

Table 1

VEGF signaling pathway genes: Changes in the expression levels of VEGF pathway genes reported in this table are listed based on the Expression Atlas site (www.ebi.ac.uk/gxa) for GBM Vs. normal. Up: up-regulation, Down: down-regulation, Unknown: There is no report in the Expression Atlas site.

	Symbol	Description	Expression
1	VEGFA	vascular endothelial growth factor A	Up
2	HSPB1	heat shock protein family B (small) member 1	Up
3	SPHK1	sphingosine kinase 1	Up
4	RAC2	Rac family small GTPase 2	Up
5	PXN	paxillin	Up
6	NRAS	NRAS proto-oncogene, GTPase	Up
7	RAF1	Raf-1 proto-oncogene, serine/threonine kinase	Up
8	KDR	kinase insert domain receptor	Up
9	PLA2G4A	phospholipase A2 group IVA	Up
10	SH2D2A	SH2 domain containing 2A	Up
11	MAPKAPK3	MAPK activated protein kinase 3	Up
12	MAPKAPK2	MAPK activated protein kinase 2	Up
13	CDC42	cell division cycle 42	Up
14	NFATC2	nuclear factor of activated T cells 2	Up
15	PRKCA	protein kinase C alpha	Down
16	MAPK11	mitogen-activated protein kinase 11	Down
17	AKT3	AKT serine/threonine kinase 3	Down
18	PIK3CD	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	Down
19	MAP2K2	mitogen-activated protein kinase kinase 2	Down
20	RAC3	Rac family small GTPase 3	Down
21	MAP2K1	mitogen-activated protein kinase kinase 1	Down
22	PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta	Down
23	HRAS	HRas proto-oncogene, GTPase	Down
24	PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	Down

	Symbol	Description	Expression
25	PIK3R3	phosphoinositide-3-kinase regulatory subunit 3	Down
26	NOS3	nitric oxide synthase 3	Down
27	PLCG2	phospholipase C gamma 2	Down
28	MAPK1	mitogen-activated protein kinase 1	Down
29	PRKCG	protein kinase C gamma	Down
30	SPHK2	sphingosine kinase 2	Down
31	PLCG1	phospholipase C gamma 1	Down
32	PPP3CC	protein phosphatase 3 catalytic subunit gamma	Down
33	MAPK12	mitogen-activated protein kinase 12	Down
34	PLA2G4D	phospholipase A2 group IVD	Down
35	AKT2	AKT serine/threonine kinase 2	Down
36	PIK3R2	phosphoinositide-3-kinase regulatory subunit 2	Down
37	PPP3CA	protein phosphatase 3 catalytic subunit alpha	Down
38	PLA2G4C	phospholipase A2 group IVC	Down
39	PLA2G4F	phospholipase A2 group IVF	Down
40	PLA2G4E	phospholipase A2 group IVE	Down
41	SHC2	SHC adaptor protein 2	Down
42	PLA2G4B	phospholipase A2 group IVB	Down
43	JMJD7- PLA2G4B	JMJD7-PLA2G4B readthrough	Down
44	PPP3CB	protein phosphatase 3 catalytic subunit beta	Down
45	PTGS2	prostaglandin-endoperoxide synthase 2	Down
46	PPP3R2	protein phosphatase 3 regulatory subunit B, beta	Down
47	PRKCB	protein kinase C beta	Down
48	PPP3R1	protein phosphatase 3 regulatory subunit B, alpha	Down
49	PTK2	protein tyrosine kinase 2	Down
50	KRAS	KRAS proto-oncogene, GTPase	Down
51	MAPK3	mitogen-activated protein kinase 3	Down

	Symbol	Description	Expression
52	SRC	SRC proto-oncogene, non-receptor tyrosine kinase	Unknown
53	MAPK13	mitogen-activated protein kinase 13	Unknown
54	BAD	BCL2 associated agonist of cell death	Unknown
55	MAPK14	mitogen-activated protein kinase 14	Unknown
56	AKT1	AKT serine/threonine kinase 1	Unknown
57	CASP9	caspase 9	Unknown
58	PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	Unknown
59	RAC1	Rac family small GTPase 1	Unknown

2.3 Pathways related to DEmiRNAs detection

DIANA-miRPath v.3 software was used to find the biological significant pathways of the 79 DEmiRNAs. The DEmiRNAs were differentially expressed between the primary GBM and recurrent GBM. The analysis revealed that 49 groups enriched-pathways were significantly related to 22 up-regulated miRNAs and 29 down-regulated miRNAs. Among up-regulated DEmiRNAs and according to the KEGG pathway maps, hsa-miR-497-5p was present in most pathways (34 pathways), and hsa-miR-204-5p and hsa-miR-551b-3p, in contrast, participated in only one pathway. On the other hand, 57 pathways were related to hsa-miR-17-5p (the most involved pathway) but only 2 pathways were related to hsa-miR-31-3p (the least involved pathway) in down-regulated DEmiRNAs (Supplementary File 2a, 2b).

2.4 Genes and pathways related to the VEGF signaling pathway genes

Interactional genes (1014 genes) were found to have interaction with 59 genes of the VEGF signaling pathway by NetworkAnalyst (Supplementary File 3). In addition, all significant pathways of interactional genes were identified and according to the enrichment analysis 170 significant pathways were recognized for interactional genes by ToppGene. Cancer Pathways had the most interactional genes (180 genes); while, Sphingosine degradation had the fewest interactional genes (3 genes) (Supplementary File 4). Moreover, 119 pathways were achieved by NetworkAnalyst, some of their genes overlapped with the VEGF signaling pathway. The MAPK signaling pathway had the most in common with the VEGF signaling pathway (39 genes). Alpha-Linolenic acid metabolism, Linoleic acid metabolism, Thyroid cancer, Ether lipid metabolism, Adherens junction and IL-17 signaling pathways had the least gene overlapping with VEGF signaling pathway (7 genes) (Supplementary File 5).

2.5 Crosstalk analysis

XTalkDB (<http://www.xtalkdb.org/home>) demonstrated all pathways that inhibited or activated VEGF signaling pathway. Some pathways had inhibition and activation effects (dual effects) on the VEGF signaling pathway, such as Neurotrophin signaling pathway, Notch signaling pathway and Progesterone-mediated oocyte maturation. In contrast, some pathways had only one-way effect (activation or inhibition) on the VEGF signaling pathway. Reciprocally, the VEGF signaling pathway activated or inhibited some pathways. All crosstalks are shown in Fig. 2.

2.6 Common miRs between VEGF pathway and DE-miRNAs

The eight miRNAs obtained (including hsa-miR-204-5p, hsa-miR-17-5p, hsa-miR-195-5p, hsa-miR-497-5p, hsa-let-7a-5p, hsa-miR-221-3p, hsa-miR-26b-5p, hsa-miR-21-5p) were common in both the VEGF signaling pathway and DE-miRNAs in the GSE32466 dataset (supplementary file 7).

2.7 Identification of classes for final pathways

Class assessment of all finalized pathways was performed by the KEGG PATHWAY database. All 91 finalized pathways were eventually classified into 24 different classes. “Signal transduction” and “Cancer: specific types” are the largest classes in this study. Fifteen pathways were related to “Signal transduction”. Some of these pathways were involved in angiogenesis process such as Hippo, TGF-beta, TNF, mTOR, and othersignaling pathways. Moreover, 20 pathways were involved in “Cancer: specific types” and cancer overview classes. On the other hand, “Cell motility” and “Circulatory system”, and others were the lowest classes determined in this study. These classes were common regulator of actin cytoskeleton and adrenergic signaling in cardiomyocytes pathways. Details of the assigned classes to the final related pathways are shown in Table 2 and Fig. 3. The classification shown in Fig. 3 is based on the frequency of related signaling pathways. Table 2 also shows that the classes we obtained in this study are partly in accord with other studies.

Table 2

The final pathways obtained from the analysis steps based on the KEGG PATHWAY site. Some classes are more diverse in terms of signaling pathways. Based on an evidence, some classes have functions associated with certain phenotypes in GBM or other cancers. For some that are not mentioned in the reference table, there is no relevant reference yet.

No.	Classes	Related signaling pathways	classes or Pathways functions in GBM or cancers	Ref.
1	Signal transduction	<ul style="list-style-type: none"> • MAPK signaling pathway • ErbB signaling pathway • PI3K-Akt signaling pathway • Sphingolipid signaling pathway • FoxO signaling pathway • mTOR signaling pathway • Wnt signaling pathway • HIF-1 signaling pathway • TNF signaling pathway • AMPK signaling pathway • Phosphatidylinositol signaling system • Hippo signaling pathway • TGF-beta signaling pathway • VEGF signaling pathway • NF-kappa B signaling pathway 	Signaling pathways are most affected by mutations and epigenetic changes in cancer cells and are involved in essential GBM hallmarks.	[26, 27, 45]
2	Cancer	<ul style="list-style-type: none"> • specific types: (Prostate cancer, Chronic myeloid leukemia, Glioma, Pancreatic cancer, Renal cell carcinoma, Non-small cell lung cancer, Colorectal cancer, Acute myeloid leukemia, Endometrial cancer, Small cell lung cancer, Melanoma, Bladder cancer, Thyroid cancer) • Overview: (Pathways in cancer, Proteoglycans in cancer, Viral carcinogenesis, Central carbon metabolism in cancer, Choline metabolism in cancer, MicroRNAs in cancer, Transcriptional misregulation in cancer) 	In addition to the unique changes, there are common pathways involved in GBM and other cancers.	[46]

No.	Classes	Related signaling pathways	classes or Pathways functions in GBM or cancers	Ref.
3	Endocrine system	<ul style="list-style-type: none"> • Insulin signaling pathway • Thyroid hormone signaling pathway • Estrogen signaling pathway • Progesterone-mediated oocyte maturation • Oxytocin signaling pathway • Prolactin signaling pathway • Adipocytokine signaling pathway • Thyroid hormone synthesis 	Various studies have observed the roles and effect of the endocrine system on GBM cell phenotypes.	[29–33, 47, 48]
4	Infectious disease	<ul style="list-style-type: none"> • Bacterial (Bacterial invasion of epithelial cells, Shigellosis, Epithelial cell signaling in Helicobacter pylori infection, Salmonella infection, Pathogenic Escherichia coli infection, Legionellosis) • Viral (Hepatitis B, HTLV-I infection, Epstein-Barr virus infection, Hepatitis C, Measles) • Parasitic (Malaria, Chagas disease (American trypanosomiasis)) 	To date, there is no study linking GBM to the causes of infections other than the virus.	[34]
5	Cellular community - eukaryotes	<ul style="list-style-type: none"> • Focal adhesion • Adherens junction • Gap junction • Signaling pathways regulating pluripotency of stem cells 	roles of the cellular community are related to the characteristics of invasion, migration, and apoptosis of GBM cells	[35,49–51]
6	Cell growth and death	<ul style="list-style-type: none"> • Apoptosis • Cell cycle • p53 signaling pathway • Oocyte meiosis 	Important pathways such as cell cycle and apoptosis are involved in the basic hallmarks of GBM that are associated with resistance to present treatment.	[52–55]

No.	Classes	Related signaling pathways	classes or Pathways functions in GBM or cancers	Ref.
7	Neurodegenerative disease	<ul style="list-style-type: none"> • Parkinson's disease • Huntington's disease • Prion diseases 	There is little evidence between the biomarker of some of these pathways and GBM.	[36]
8	Immune system	<ul style="list-style-type: none"> • Platelet activation • Leukocyte transendothelial migration • B cell receptor signaling pathway 	The immune system of the CNS plays an important role in the biological processes of GBM, such as angiogenesis.	[37, 38]
9	Nervous system	<ul style="list-style-type: none"> • Neurotrophin signaling pathway • Long-term depression 	Neurotrophic factors (NTFs) are the most well-known factor common between the Nervous system and GBM. Stress and lifestyle may also be linked to GBM patients' longevity through this class.	[56–58]
10	Cardiovascular disease	<ul style="list-style-type: none"> • Arrhythmogenic right ventricular cardiomyopathy (ARVC) • Viral myocarditis 	There are no studies on this relationship for humans.	[59]
11	Development and regeneration	<ul style="list-style-type: none"> • Dorso-ventral axis formation • Axon guidance 	The role of the axon guidance pathway has been identified in GBM invasion.	[60]
12	Folding, sorting and degradation	<ul style="list-style-type: none"> • Protein processing in endoplasmic reticulum • Ubiquitin mediated proteolysis 	There is evidence for the role of ubiquitination and the endoplasmic reticulum in GBM progression.	[39, 61]
13	Transport and catabolism	<ul style="list-style-type: none"> • Endocytosis • Phagosome 	There are examples of these two pathways in GBM.	[62, 63]
14	Cell motility	<ul style="list-style-type: none"> • Regulation of actin cytoskeleton 	Lack of regulation of actin cytoskeleton is effective in cell migration and angiogenesis of GMB.	[64, 65]

No.	Classes	Related signaling pathways	classes or Pathways functions in GBM or cancers	Ref.
15	Circulatory system	• Adrenergic signaling in cardiomyocytes	Activation of β -adrenoceptors causes cell proliferation in GBM.	[66]

3 Discussion

In silico studies are essential to finding new pathways and genes involved in biological processes. This study used the GSE32466 data set, which includes both recurrent and primary GBM groups. GBM angiogenesis plays an essential role in its recurrence, and the GSE32466 data set is the best available dataset containing data on GBM recurrence^[3]. Our main goal was to identify classes of indirect pathways that induce VEGF in GBM recurrence. Further understanding of GBM neoangiogenesis may improve our understanding about mechanisms of resistance to anti-angiogenetic drugs. The development of multi-omics approaches has led to an impressive deal of new information on a variety of mechanisms and created the opportunity to target GBM selectively^[9]. In this study, by identifying the miR-DEGs, we found the pathways they target. These pathways seem to be involved in the recurrence of GBM and may also include pathways associated with angiogenesis. The most well-known angiogenic pathway currently treated in cancer therapy is the VEGF signaling pathway. Therefore, we chose the VEGF pathway as our focus hoping that by simultaneously targeting multiple pathways affecting angiogenesis, we may improve the chances of effectively controlling angiogenesis in cancer therapy^[15]. Studies showed that, there are other signaling pathways affecting the function of the VEGF pathway. For example, a study has shown that glioma cells produce TGF- β superfamily ligands which bind TGF- β receptors (TGF- β R). In other study, Sui *et al.* found that VEGF-mediated angiogenesis was activated in the normoxia mode by the TGF- β 1 / β -catenin / TCF3 / LEF1 signaling pathway, and in the hypoxia by the HIF-1 α / β -catenin / TCF3 / LEF1 signaling pathway^[16 17]. Therefore, the TGF- β pathway can be one of the pathways affecting the function of the VEGF pathway.

Other studies have shown that the HGF/c-MET signaling pathway plays an important role in angiogenesis and tumor growth. Hence, the function of this signaling pathway is synergistic with the VEGF/VEGFR signaling pathway, for instance they have a greater stimulating effect on angiogenesis and tumor growth when treated simultaneously^[18, 19]. Accordingly, the purpose of our study was in silico identification of the pathways with the potential to target the VEGF signaling pathway in recurrent GBM indirectly. In this study, the pathways with overlapping genes or pathways that indirectly affect the VEGF signaling pathway affecting GBM recurrence, were investigated. The VEGF/R targeting therapies are designed to treat GBM angiogenesis; however, the clinical effect of these therapies is limited due to the emergence of drug resistance during treatment^[4]. The mechanisms underlying cancer drug resistance is still not fully understood. Therefore, it is critical to discover the mechanisms of drug resistance to increase the success rate of GBM treatment, which is a recurrent disease^[20, 21]. In recurrent GBM,

chromosomal instability (CIN) is considered an important mechanism leading to tumor heterogeneity. In addition, high heterogeneity affects the dysregulation of microRNA levels and, consequently, their related functions [9]. In this study, we reached the effective pathways through DEmiRNAs in recurring GBM. Based on our findings, among 79 significant DEmiRNAs, eight miRNAs overlapped with the VEGF pathway. Therefore, it seems that if other microRNAs play any role in angiogenesis, they may also indirectly affect the VEGF pathway. Indirect effects may occur in the form of connection or overlap, both of which were examined using NetworkAnalyst. Zhang *et al.* found that, the anticancer drug convallatoxin induces apoptosis in colon cancer cells by cross-linking the JAK2/STAT3 and mTOR/STAT3 signaling pathways and inhibiting angiogenesis [22]. Bo Zhang *et al.* showed that increasing LncRNA ANRIL expression caused the overexpression of the VEGF gene and induced angiogenesis by activating the NF- κ B signaling pathway in a mouse model of diabetes mellitus with stroke [23]. Another study found that activation of the Jagged1/Notch1 signaling pathway by 17 β -estradiol is associated with angiogenic factors that may turn tumor cells into angiogenic phenotypes [24]. In the study by Nabors *et al.*, it was shown that, extracellular signals such as hypoxia and cytokines induced over-expression of angiogenic factors in glioma cells [25].

GBM cells are involved in many mutations that significantly affect cancerous processes. These mutations lead to low survival rates by increasing the complexity of treatment and resistance to therapy in patients. Different pathways in the intracellular signaling network are dys-regulated due to mutations [26, 27]. Our study and others also showed that signal transduction pathways are the main pathways that affect angiogenesis. In a functional study in a breast cancer cell line, Zhang *et al.* showed that the MAPK signaling pathway plays a role in regulating angiogenesis. In this pathway, HGF/SF, together with its receptor (c-MET), causes angiogenesis by increasing VEGF gene expression [28]. Twelve percent of the pathways identified and examined in our study affect other cancers as well. In our study, various endocrine systems were identified as pivotal pathways indirectly related to the VEGF pathway. Various studies have emphasized the role and impact of these pathways on the patients, survival, cancer development, and migration of cancer cells and the recurrence of GBM, thus affecting GBM patient survival rates [29, 30]. Consistent with this study, the role of steroid hormones, prolactin (PRL) and its receptor (PRLR), and leptin-related signaling in GBM has been identified in some functional studies, making them potential therapeutic targets for future studies [31–33]. The role of oncoviruses in various cancers has also been identified. There are not many studies on the role of viruses in GBM, but McFaline-Figueroa *et al.*, has pointed to the presence of cytomegalovirus in GBM [34]. Another class of pathway identified in our in silico analysis is the eukaryotic community. This class includes signaling pathways mostly associated with invasion and apoptosis in cancers, including those in GBM [35].

Interestingly, some of the final pathways we identified are shared between GBM and some diseases and processes not related to cancer. Some of these pathways are associated with neurodegenerative disease, cardiovascular disease, endocrine and metabolic disease, and the digestive system. Perhaps the reason for their apparent irrelevance is the absence of studies investigating the correlation or the common factors between them and GBM. However, in some neurodegenerative diseases, such as prions, a link

between the prion protein (PrPC) and GBM has been observed ^[36]. The immune system is another class identified in our study. GBM has mechanisms that can help it escape the central nervous system (CNS) immune system and promote some of its own processes, such as angiogenesis ^[37, 38]. Some treatments are also being considered in the relapse phase; folding, sorting, degradation, and transport are processes that have not received much attention in cancer studies. However, one study has suggested the role of UBE3C in ANXA7 ubiquitination in the progression of glioma ^[39]. Other critical pathways requiring further laboratory studies are those associated with beta-adrenergic, cytoskeletal, endocrine, and metabolic diseases. The extracellular matrix (ECM) in GBM is a unique environment that plays an essential role in creating the invasion phenotype and GBM recurrence ^[40]. Some lipid metabolism is related to some properties of GBM, including those involved in the maintenance of cancer stem cells (CSCs) ^[41]. The pathways identified in our analysis may be related to both angiogenesis and the recurrence of GBM.

In GBM, 51 genes are altered in the VEGF signaling pathway (Table 1). However, according to our in silico results, there are other signaling pathways that can indirectly affect VEGF signaling pathway genes expression levels.

4 Materials And Methods

4.1 Study design

There were 6 main steps based on differentially expressed miRNAs (DEmiRNAs) to find classes of pathways related to the VEGF signaling pathway genes as outlined in the pipeline: data collection including identification of DEmiRNAs based on an expression dataset, finding VEGF signaling pathway genes, detection of associated genes and pathways to VEGF signaling pathway genes, crosstalk between pathways, detection of final related pathways, and class assessment for pathways.

4.2 Data collection sources

Data collection was done in two separate sections: finding DEmiRNAs between primary and recurrent GBM patients, and identifying miRNAs involved significantly in the VEGF signaling pathway.

4.3 Identification of DEmiRNAs

The miRNA expression datasets of GBM were searched using the keywords: 'GBM', 'recurrent' 'Homo sapiens' [porgn: txid9606], and 'Expression profiling by array' against the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>). After a systematic review, one GSE profile (GSE32466) was selected and analyzed. GSE32466 related to miRNA expression was based on GPL10850 (Agilent-021827 Human miRNA Microarray (V3) (miRBase release 12.0 miRNA ID version)).

To identify DEmiRNAs, the GSE32466 dataset was analyzed by Limma R package in Bioconductor that was utilized to mine statistically significant DEmiRNAs based on the difference in their expression values

between samples of the GBM and recurrent GBM. Significant differential expression was determined as a $\log_2$ fold change $\geq |1|$ and the adjusted p-value threshold of 0.05 [42].

4.4 Definition of VEGF signaling pathway genes

In the other section, all genes involved in the VEGF signaling pathway come from The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (<https://www.genome.jp/kegg/pathway.html>). The KEGG is an information base for the networks of genes and metabolic compounds. The expression levels of these genes in GBM were obtained using the Expression Atlas site (www.ebi.ac.uk/gxa).

4.5 Pathway detection for DE miRNAs

DE miRNAs are essential regulators in many biological pathways. To show the roles of each DE miRNAs and learn more about their functions, DIANA-miRPath v3.0 (<http://www.microrna.gr/miRPathv3>) was used. DIANA-miRPath is an online tool that integrates miRNAs with KEGG pathways to provide a deeper understanding of the process by which biological pathways are regulated [43]. The threshold p-value of 0.05 was considered significant.

4.6 Identification of genes and pathways related to VEGF signaling pathway

The genes which had genetic interaction with the VEGF signaling pathway genes were obtained by NetworkAnalyst (<https://www.networkanalyst.ca/>) based on STRING interactome. They showed medium (400) to high (1000) confidence score. The confidence score cutoff was set at 900 and required experimental evidence. Then, pathways detection was performed for interactional genes using the ToppGene tool (<https://toppgene.cchmc.org/>), and the adjusted p-value cutoff of 0.05 was considered significant. Moreover, by NetworkAnalyst, all pathways that shared part of the VEGF signaling pathway genes were collected with an adjusted p-value ≤ 0.05 . The obtained pathways in the above steps were based on the KEGG.

Furthermore, the Expression Atlas database was used (<https://www.ebi.ac.uk/gxa/home>) to find dysregulated gene expression of the VEGF signaling pathway genes in GBM.

4.7 Crosstalk between Pathways

XTalkDB (<http://www.xtalkdb.org>) was used for detection of crosstalk between specific pairs of signaling pathways; crosstalk information has been illustrated based on scientific literature [44]. “VEGF signaling pathway” name was inserted into the XTalkDB to find crosstalking pathway pairs. Finally, we got a list of pathways that have inhibitory or activating effects (or both) on the VEGF signaling pathway. On the other hand, the inhibitory or activating effects of the VEGF signaling pathway on other pathways were also obtained.

4.8 Determination of common miRNAs between VEGF pathway and DE-miRNAs

MiRNAs related to the VEGF signaling pathway were extracted from the miRWalk3.0 database (<http://mirwalk.umm.uni-heidelberg.de/>). These miRNAs were compared by the Venny 2.1 free online tool (<https://bioinfogp.cnb.csic.es/tools/venny/>). This tool was used to find the same miRNAs between DE miRNAs obtained from the Limma R package and those which involved in the VEGF signaling pathway.

4.9 Detection of final pathways and class assessment

To deduce commonalities across the collected pathways, the Venny 2.1 free online tool (<https://bioinfogp.cnb.csic.es/tools/venny/>) was used. At last, classes of final pathways were determined by the KEGG database.

5 Conclusion

Due to the complexity of the biological processes involved in cancers, multiple targeting is very effective in preventing and controlling cancer. Although the VEGF pathway is the most important pathway in angiogenesis process, there are complicated processes in GBM which prevent effective GBM therapy using only VEGF targeting. Our study showed that several pathways or classes might significantly affect the VEGF pathway. Hence, It seems necessary to achieve new targets for combination therapies as well as personal therapies for GBM.

Abbreviations

VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; CNS, central nervous system; IL, Interleukin; TGF-beta, Transforming growth factor beta; TNF, tumor necrosis factor; mTOR, mechanistic target of rapamycin; PI3K, Phosphoinositide 3-kinases; FOXO, forkhead box O; HIF, Hypoxia-inducible factors; AMPK, AMP-activated protein kinase; NF- κ B, Nuclear factor kappa B; HTLV-1, Human T lymphotropic virus type 1; TCF3, Transcription Factor 3; LEF-1, Lymphoid Enhancer Binding Factor 1; HGF, Hepatocyte Growth Factor; c-MET, mesenchymal-epithelial transition factor; JAK2, Janus kinase 2; STAT3, Signal transducer and activator of transcription 3; UBE3C, Ubiquitin-protein ligase E3C

Declarations

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Authors contributions

S.G., designed the concept. M.H., analyzed the data. M.H. S.G., and R.S. wrote the main manuscript text. T.L., and M.R.M., have guided and edited the manuscript. M.S., prepared supplementary files. All authors reviewed the manuscript.

Conflict of interest

The authors declare no potential conflicts of interest to disclose.

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Figures

Figure 2

Crosstalk between VEGF signaling pathways and other pathways: Inhibition, activation and dual effects have been represented by red, green and black edges respectively.

Figure 3

Classification of all finalized pathways: This percentage is sorted by the number of pathways associated with each class. The percentages here are related to the variety of pathways in each class.

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