ORIGINAL ARTICLE

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The Expression Profiles of Angio-miRs in Glioblastomas Invasion Inhibited by Ruxolitinib

Ruksolitinib ile Engellenen Glioblastoma İnvazyonunda AnjiyomiR'lerin Ekspresyon Profili

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ABSTRACT

Aim: MicroRNAs (miR) have an essential role on the regulated gene expression in the human genome. In recent years, a specific miR group was called to angio-miRs due to their role in the angiogenesis, and recent study showed that they involved in the pathogenesis of gliomas. In this study, we investigated the changes in the expression profiles of angio-miRs in glioblastoma cells and identified relationship between these genes and invasion and tumor growth.

Materials and Methods: In this study, glioblastoma tumor spheroids were obtained using the human glioblastoma cell line U-87 MG. 50 nM, 100 nM and 200 nM ruxolitinib were applied to tumor spheroids for 48 hours by using Matrigell method. Tumor volume and invasion formation relative % tumor growth and relative % invasion area were measured in glioblastoma tumor spheroids after 48 hours of treatment. At the same time, quantitative real-time polymerase chain reaction (qRT-PZR) analysis was performed and miR expression profiles were determined. The most important (importance features) miRNAs selected along with the heatmap and volcano plot analyzes were used to display the pattern of the differentially expressed miRs using normalized miR expression profiles.

Results: When the effect of 50 nM, 100 nM and 200 nM ruxolitinib administration to tumor spheroids on tumor volume and invasion was evaluated, a significant difference was found at each dose applied. However, at the dose of 200 nM ruxolitinib, it was observed that the inhibitory effect of tumor invasion was the highest. When miR expression profiles obtained by qRT-PZR test with 200 nM ruxolitinib adminisration were evaluated, it was determined that the expression profiles of 10 miRs increased and the expression profiles of 4 miRs decreased.

Conclusion: In conclusion, angio-miR expression profiles are important because they enable us to better understand the prognostic process of gliomas. Because of their multiple silencing properties, they may contribute to the clinic with further studies in terms of their use as new therapeutic targets and prognostic biomarkers for glioblastoma.

Keywords: miR, glioblastoma, angiogenesis, invasion

ÖZ

Amaç: MikroRNA'lar (miR), insan genomunda gen ifadesinin düzenlenmesinde önemli rolü olan düzenleyicilerdir. Son yıllarda anjiyogenezde rol oynayan spesifik bir miR grubu tanımlanmış (anjiyo-miR) ve bazı anjiyo-miR'lerin gliomalarda etkin rol oynadıkları ortaya konmuştur. Bu çalışmada, glioblastoma hücrelerindeki anjiyo-miR'lerin ifade değerlerindeki değişiklikleri ve bu değişikliklerin invazyon ve tümör büyümesi ile ilişkisini araştırdık.

Gereç ve Yöntem: Bu çalışmada, insan glioblastoma hücre hattı U-87 MG kullanılarak glioblastoma tümör sferoidleri elde edildi. Matrigel yöntemi ile tümör sferoidlerine 48 saat süresince 50 nM, 100 nM and 200 nM ruksolitinib uygulandı. Kırk sekiz saat tedaviden sonra glioblastoma tümör sferoidlerinde tümör hacmi ve invazyon oluşumu relatif yüzde tümör gelişimi ve relatif yüzde invazyon alanı ölçüldü. Aynı zamanda, niceliksel gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PZR) analizi yapıldı ve miR ifade değerleri belirlendi. Farklı şekillerde ifade edilen miRNA'ların modelini görüntülemek için normalize edilen miRNA ifade değerleri kullanılarak heatmap ve volcano plot analizleri ile seçilen en önemli (importance features) miRNA'lar gösterildi.

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Bulgular: Tümör sferoidlerine 50 nM, 100 nM ve 200 nM ruksolitinib uygulamasının tümör hacmi ve invazyon üzerine etkisi değerlendirildiğinde, uygulanan her dozda anlamlı fark saptandı. Ancak 200 nM ruksolitinib dozunda tümör yayılımını engelleyici etkisinin en yüksek olduğu gözlendi. 200 nM ruksolitinib uygulaması ile qRT-PZR testi ile elde edilen miR ifade değerleri incelendiğinde 10 miR'nin ifade değerlerinin arttığı, 4 tanesinin ifade değerlerinin ise azaldığı belirlendi.

Sonuç: Sonuç olarak anjiyo-miR ifade değerleri gliomaların prognostik sürecini daha iyi anlamamızı sağlayabilmeleri açısından önemlidirler. Çoklu susturma özellikleri sayesinde glioblastomalar için yeni terapötik hedefler ve prognostik biyobelirteçler olarak kullanılabilmesi açısından ileri çalışmalarla kliniğe katkı sağlayabilirler.

Anahtar Kelimeler: miR, glioblastoma, anjiyogenez, invazyon

INTRODUCTION

MicroRNAs (miR) are small, single-stranded RNA sequences of 21-25 nucleotides found in the human body. These non-protein-coding small RNA sequences play a role in physiological and pathological processes by inhibiting gene expression in the post-transcriptional modification step of protein synthesis[1]. These pathological processes include many types of cancer, including glioblastomas[2]. The close relationship of miRs with these pathologies has led to their being proposed as new therapeutic agents[3]. Recent studies reveal that these small RNAs can be used not only in the treatment phase, but also as a biomarker with varying expression values[4]. As a result, in recent years, miRs have become one of the most frequently studied subjects in laboratory and clinical studies, especially for incurable cancer types[1-4].

Glioblastomas are among the brain cancers originating from glial cells, growing fast and still fatal with its aggressive nature despite the developing technological opportunities. Although more than 100 subtypes have been defined with their heterogeneous molecular features, the common feature of all of them is high angiogenetic and invasion capacity[5,6]. Glioblastoma cells invade the surrounding healthy brain tissue and spread by sequential angiogenesis. This aggressive spread is thought to be the main difficulty in the treatment of glioblastomas[7]. From this point of view, targeting cytokines and associated signaling pathways involved in this high angiogenetic process seems to be a rational way to treat the disease. However, phase-2 and phase-3 studies of these treatment interventions have been conducted, and although some positive results have been obtained, a treatment protocol that has been included in routine treatment has not been determined yet[8]. For this, a better understanding of the complex molecular mechanism underlying the high angiogenic and invasive characteristics of gliomas is needed.

In recent years, many miRs, called angio-miRs, closely related to tumor angiogenesis have been identified in the field of cancer biology. Among them, angio-miRs such as miR_7, miR_296, miR_15b and miR_93 have been reported to play a role in glioblastomas[9]. In our laboratory, it was previously reported that ruxolitinib effectively inhibited the invasion of gliomas[10]. In another study, a close relationship of this effect with miR_17 and miR_20a was demonstrated[11]. In this study, the relationship between inhibited invasion characteristics and expression values of 34 angio-miRs in glioblastoma three-dimensional tumor clusters was investigated, again using ruxolitinib.

MATERIALS AND METHODS

Supply of Ruxolitinib and Preparation of Cell Line

Ruxolitinib (CAS 941678-49-5) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and human glioblastoma cell line U-87 MG (ATCC® HTB-14™) from American Type Culture Collection; (Manassas, VA, USA). During the study, cells were grown by using Eagle's Minimum Essential Medium (EMEM; catalog no. 30-2003) which was supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA), 1 mM glutamine, and 1% (final concentration) penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and kept in a humidified incubator at 37 °C and 5% CO_2 throughout the entire study.

Construction of Glioblastoma Tumor Clusters and Matrigel Invasion Assay Protocol

Tumor clusters were created using the hanging drop method, with minor modifications[10,11]. Single-cell suspensions were obtained from trypsinized monolayer cell cultures and diluted to the desired cell density using complete EMEM medium with the addition of 0.5% methyl cellulose. 20 µL of cell suspension was pipetted to the inner surface of the top cover of non-adhesive, sterile polystyrene Petri dishes as 40 drops (final concentration of 750-1.000 cells per drop). The upper lids of the Petri dishes with the tumor droplets were inverted and 2 mL of phosphate-buffered saline was placed in the lower dish. After the cells were incubated for 72 hours, tumor cluster formation was observed using the ZEISS Axio Vert.A1 (Oberkochen, Germany) invert microscope. All tumor spheres were collected in 15 mL Falcon tubes and fresh tumor spheres of the same age were used in all experiments.

Twenty-four well plates were used for the Matrigel invasion assay. The plates were covered with an extracellular matrix and the excess of this matrix was removed to form a thin layer. Then, 40 µL of tumor clusters collected from the

previous section were taken and mixed with 100 µL of matrigel matrix (Corning, Corning, NY, USA) and 100 µL of collagen type I (Sigma Aldrich) in pre-chilled Eppendorf tubes. 40 µL (3-5 tumor clusters) of this mixture was put in each well. Afterwards, the plate was left in the incubator for 24 hours, then 1 mL of cell culture medium was added to each well. After 24 hours, tumor clusters were treated with vehicle or 50, 100, or 200 nM ruxolitinib for 48 hours. Five replicates were made for each process. After 48 hours of treatment, cell invasion was recorded for 48 hours using an inverted phase contrast light microscope at 20X magnification (ZEISS Axio Vert.A1) equipped with a digital camera. Tumor volume and tumor invasion were calculated according to our previous studies[10,11].

miR Isolation and Quantitative Real Time-polymerase Chain Reaction (RT-qPCR) Analyses

A group of miRNAs called angio-miRNAs (angio-miRs) is well defined in terms of target genes and expression profile and has been shown to play an important role in glioblastoma multiforme (GBM) pathogenesis. Whole genome-wide microarray studies have revealed that more than 50 miRs are involved in hypoxia-related pro- or anti-angiogenesis signaling[12-15]. In our study, 34 angio-miRs determined in this group were used. Isolation of miRs from tumor spheres 48 hours after the administration of ruxolitinib was performed using the mirVana™ miR Isolation Kit with phenol according to the kit protocol. cDNAs were synthesized using a TaqMan™ Advanced miR cDNA Synthesis Kit according to kit protocols. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed on a Quant studio 5 real time PCR (life technologies) using FastStart TaqMan® Probe Master (life technologies). The TaqMan probes and miR sequences used are given in Table 1. miR expression values were calculated by the $2^{-\Delta\Delta Ct}$ method, and RNU6B (Assay ID: 001093) was used as correction factor and endogenous control[16].

Statistical Analysis

The differences in the invasion rate and tumor volume between the control and experimental groups and the relative fold change in miR expression were compared with one-way analysis of variance and Tukey HSD test. Pearson correlation coefficient method was used for correlation analysis. Statistical analyses were performed using Statistical Package for the Social Sciences 20 software with a significance level of $p<0.05$. Heat map analysis, unsupervised hierarchical clustering analysis, volkano plot and feature importance analysis were performed using MetaboAnalyst 4.0 software to identify potential biomarkers/important miRs in three-dimensional tumor clusters with and without ruxolitinib.

RESULTS

In the study, 50, 100 and 200 nM doses of ruxolitinib were applied to glioblastoma tumor clusters and their effects were evaluated after 48 hours. Although a statistically significant effect was observed at each applied dose, it was observed that the effect reached the highest level at the 200 nM dose and this effect was consistent with our previous studies (Figure 1)[10,11]. Therefore, the relationship between miR expression values obtained by qRT-PCR test and this effect dose was analyzed and it was determined that the expressions of 10 miRs increased significantly, and 4 of them decreased significantly (Table 2). The heatmap showed that 200 nM ruxolitinib administration had the highest effect on miR expressions compared to the control group (Figure 2). In the next step, in order to determine the miRs directly related to ruxolitinib, first volcano plot and then OPLS-discriminant analysis were performed and the most important features were determined here. Nine miRs, which were found to be significantly correlated with ruxolitinib applications, which gave similar statistically significant results in both analyses, are summarized in Table 3. Correlation analyses of these identified miRs were then performed with

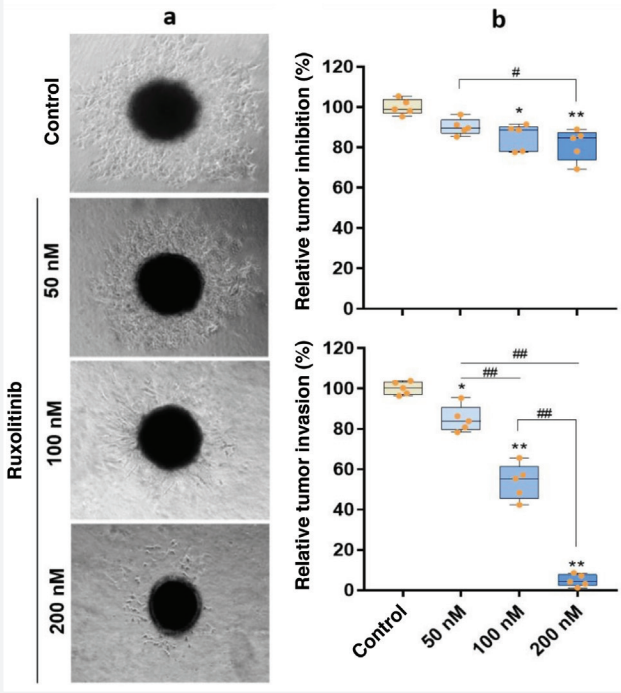

Figure 1. A) GBM tumor spheroids and invasion formation determined by Matrigel method, b) Relative % tumor growth and relative % invasion area in GBM tumor spheres treated with 50 nM, 100 nM and 200 nM ruxolitinib or vehicle for 48 hours. Bars mean±SE, n=5, * statistically different compared to control, #statistically different from other groups, one-way ANOVA, Tukey HSD test, $p\leq0.05$;**, ##$p\leq0.01$

GBM: Glioblastoma multiforme

tumor volumes and invasion rates, and as a result, 5 miRs [miR_15b(r:-0,659/-0,861]; miR_19a_3p(r:-0,713/-0,455); miR_31_3p(r:-0,461/-0,533); miR_155_3p(r:-0,572/-0,625); miR_200b_5p(r:-0,673/-0,957) among 9 miRs, which showed a statistically significant correlation with both tumor invasion and tumor growth, were detected (Figure 3).

DISCUSSION

In recent years, with the developments in the field of molecular biology, large volumes of data have begun to be examined, thus it has been demonstrated that miRs are closely related to tumor invasion, and various cancer-specific miRs have been defined[17-19]. It is suggested that inhibition of miRs specific to this cancer type is a new target that can be used in the treatment of cancer[1]. In the present study, miRs associated with U87 glioblastoma invasion, which we blocked using ruxolitinib that we previously used in our laboratory, were determined. In this context, it was determined that there was an increase in 10 miR expression values and a decrease in 4 miR expression values in GBM tumor spheres treated with ruxolitinib for 48 hours. It was determined that miR_15b, miR_18a_5p, miR_19a_3p, miR_21_5p, miR_27a_5p, miR_31_3p, miR_132_5p, miR_155_3p and miR_200b_5p had a statistically significant effect on the invasion and tumorigenesis of U87 GBM spheres.

miR_15b is a miR localized in the 3rd chromosome of the human genome and has been frequently studied in cancer studies

Table 1. Names of mikro-RNAs, Taq-Man probe ID and mature miRNA sequences

micro-RNA	Mature micro-RNA sequence	Taq-Man probe ID
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA	478313_mir
hsa-miR-18a-3p	ACUGCCCUAAGUGCUCCUUCUGG	477944_mir
hsa-miR-18a-5p	UAAGGUGCAUCUAGUGCAGAUAG	478551_mir
hsa-miR-19a-3p	UGUGCAAAUCUAUGCAAAACUGA	479228_mir
hsa-miR-19a-5p	AGUUUUGCAUAGUUGCACUACA	478750_mir
hsa-miR-21-3p	CAACACCAGUCGAUGGGCUGU	477973_mir
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	477975_mir
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC	478532_mir
hsa-miR-31-3p	UGCUAUGCCAACAUAUUGCCAU	478012_mir
hsa-miR-31-5p	AGGCAAGAUGCUGGCAUAGCU	478015_mir
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU	477827_mir
hsa-miR-92a-5p	AGGUUGGGAUCGGUUGCAAUGCU	479205_mir
hsa-miR-101-3p	UACAGUACUGUGAUAACUGAA	477863_mir
hsa-miR-101-5p	CAGUUAUCACAGUGCUGAUGCU	478620_mir
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG	477887_mir
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	477851_mir
hsa-miR-130a-5p	GCUCUUUUCACAUUGUGCUACU	483130_mir
hsa-miR-132-3p	UAACAGUCUACAGCCAUGGUCG	477900_mir
hsa-miR-132-5p	ACCGUGGCUUUCGAUUGUUACU	478705_mir
hsa-miR-155-3p	CUCCUACAUAUUAGCAUUAACA	477926_mir
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGUU	483064_mir
hsa-miR-191-3p	GCUGCGCUUGGAUUUCGUCCCC	477951_mir
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	477952_mir
hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA	477963_mir
hsa-miR-200b-5p	CAUCUUACUGGGCAGCAUUGGA	478753_mir
hsa-miR-210-3p	CUGUGCGUGUGACAGCGGCUGA	477970_mir
hsa-miR-210-5p	AGCCCCUGCCCACCGCACACUG	478765_mir
hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC	477981_mir
hsa-miR-221-5p	ACCUGGCAUACAAUGUAGAUUU	478778_mir
hsa-miR-296-3p	GAGGGUUGGGUGGAGGCUCUCC	478790_mir
hsa-miR-296-5p	AGGGCCCCCCCUCAAUCCUGU	477836_mir
hsa-miR-424-5p	CAGCAGCAAUUCAUGUUUUGAA	478092_mir

miRNA: MicroRNA

in recent years. Xia et al.[20] suggested that miR_15b played a role as a tumor inhibitory agent in cell cycle regulation in glioblastomas. In another study, increased miR_15b expression was reported in analyses performed in serum and cerebrospinal fluid of patients[21,22]. Finally, Chen et al.[23] reported that miR_15b showed its effect on gliomagenesis through sal-like protein 4. In our study, increased expression values of miR_15b were detected and it was thought to play an important role in the molecular process.

The miR_17-92 family is a polycistronic miR family that is involved in the coding of more than one protein and has

Table 2. Relative miR expression levels determined in GBM tumor spheres in the groups administered 200 nM ruxolitinib, compared to the control groups without treatment, and the results of the related statistical analysis

				95% CI	
miRNA	**Mean value**	**Standard deviation**	**p value**	**Decrease**	**Increase**
MiRs with significantly increased expression					
miR_15b	12.567	2.988	0.041	-22.321	-0.520
miR_18a_5p	154.640	18.073	0.000	-218.814	-87.006
miR_19a_3p	44.298	4.543	0.000	-59.818	-26.688
miR_21_3p	2.559	0.389	0.047	-2.900	-0.019
miR_27a_5p	6.543	1.927	0.120	-12.505	1.553
miR_31_3p	226.034	46.217	0.012	-391.855	-54.810
miR_130a_5p	4.144	0.802	0.046	-5.920	-0.064
miR_132_3p	1.824	0.135	0.002	-1.316	-0.325
miR_155_3p	2.657	0.418	0.038	-3.204	-0.101
miR_200b_5p	33.275	5.785	0.005	-52.840	-10.623
MiRs with significantly decreased expression					
miR_21_5p	0.370	0.093	0.002	0.308	1.259
miR_210_3p	0.546	0.103	0.019	0.097	0.974
miR_221_3p	0.619	0.060	0.003	0.159	0.673
miR_424_5p	0.537	0.129	0.036	0.053	1.393
miRs without significant change					
miR_18a_3p	1.508	0.351	0.984	-1.501	1.472
miR_19a_5p	3.132	0.934	0.232	-5.443	1.390
miR_23a_3p	0.627	0.125	0.074	-0.049	0.996
miR_27a_3p	0.667	0.114	0.109	-0.081	0.757
miR_31_5p	1.038	0.087	0.932	-0.346	0.376
miR_92a_5p	1.114	0.373	0.885	-1.458	1.266
miR_92a_3p	0.906	0.108	0.614	-0.297	0.491
miR_101_3p	2.072	1.446	0.683	-6.324	4.221
miR_101_5p	1.403	0.226	0.347	-1.215	0.445
miR_130a_3p	6.394	1.568	0.064	-11.106	0.331
miR_126_3p	0.881	0.081	0.335	-0.174	0.490
miR_132_5p	13.145	7.780	0.384	-40.506	16.229
miR_155_5p	3.413	1.562	0.663	-7.154	4.641
miR_191_3p	1.190	0.603	0.861	-2.389	2.012
miR_191_5p	0.873	0.074	0.229	-0.146	0.580
miR_200b_3p	2.555	0.799	0.295	-4.428	1.408
miR_210_5p	0.728	0.113	0.159	-0.128	0.735
miR_221_5p	2.629	1.243	0.823	-5.289	4.247
miR_296_3p	1.054	0.218	0.958	-0.802	0.845
miR_296_5p	1.843	0.281	0.278	-1.717	0.518

CI: Confidence interval, miRNA: MicroRNA

6 members[24]. In our study, we detected an increase in the expression values of miR_18a and miR_19a in this family (Table 2). It has been reported that the expression of mir_18a is increased in human prostate, breast and colorectal cancers, especially with its close relationship with apoptosis[19,25]. They reported a correlation between the increased tumor grade of

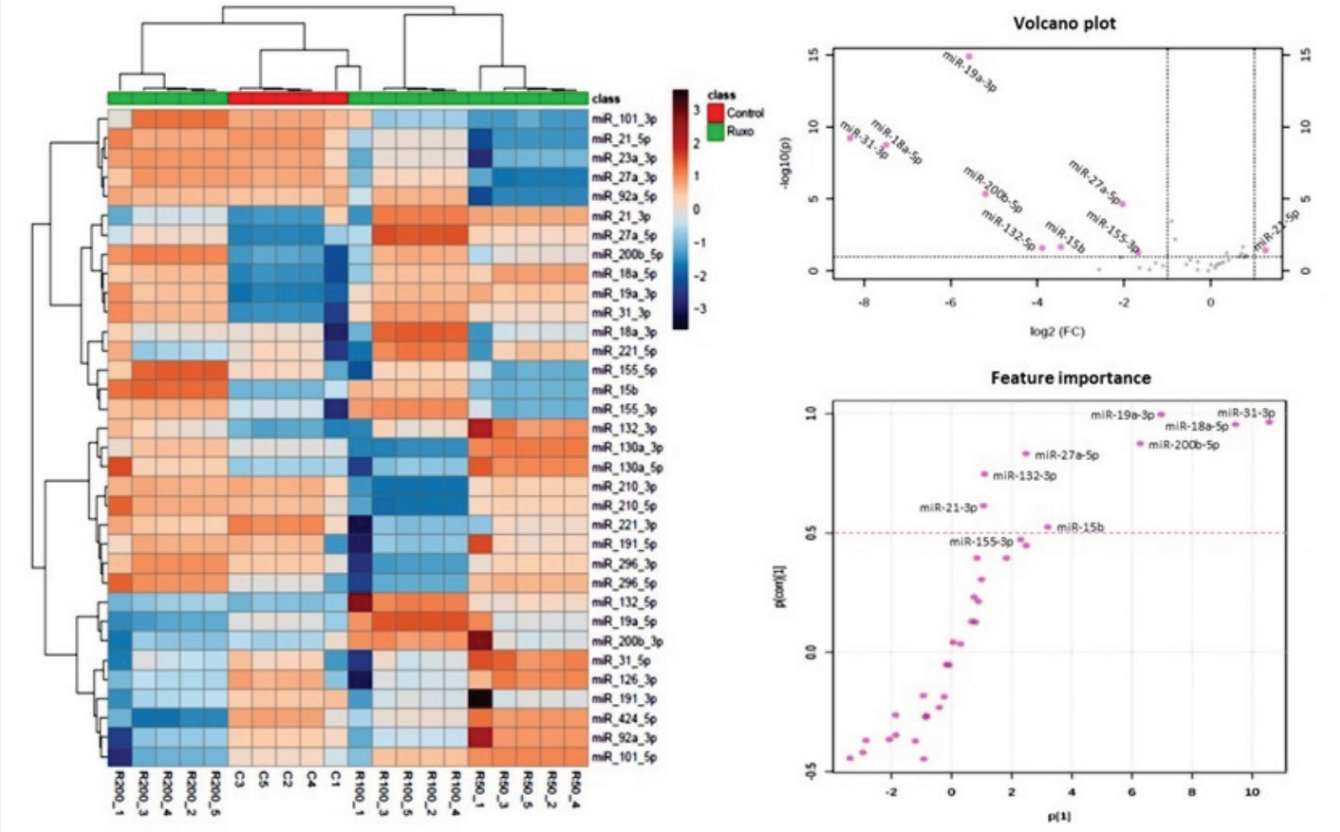

Figure 2. a) Heatmap analysis using normalized miR expression values (distance measurement parameter: euclidian, cluster algorithm: ward), b) The most important miRNAs selected with fold change threshold (x) 2 in Volcano plot analysis and t-test significance level threshold (y) 0.1. The red dashed line shows miRs significantly above the threshold (both fold increase and p values were undergone logarithmic transformation), c) S-graph combining covariance correlation [p (corr)] loading profile, showing variable significance obtained by OPLS-Discriminant analysis. All biomarker analyses were performed between 200 nM ruxolitinib-treated and untreated control groups

miRNA: MicroRNA

Table 3. The most important features of miRs determined by Volcano plot and Discriminant Analysis of Orthogonal-Orthogonal Projections to Latent Structures

	Volcano plot				Important feature	
miRNA	**FC**	**log2(FC)**	**p value**	**-log10(p)**	**p[1]**	**p(corr)[1]**
miR_15b	0.0910	-34.5750	0.0216	16.6640	320.8360	0.5251
miR_18a_5p	0.0056	-74.8870	1.74E-05	87.5930	944.6110	0.9546
miR_19a_3p	0.0209	-55.7790	1.31E-11	14.8820	69.7660	0.9965
miR_21_5p	23.9670	12.6110	0.0372	14.2940	106.7570	0.6144
miR_27a_5p	0.2433	-20.3910	2.27E-02	46.4320	247.7050	0.8329
miR_31_3p	0.0031	-83.1770	5.95E-06	92.2570	105.6860	0.9647
miR_132_5p	0.0674	-38.9060	0.0253	15.9630	109.7980	0.7473
miR_155_3p	0.3118	-16.8130	0.0528	1.2770	230.0530	0.4726
miR_200b_5p	0.0272	-51.9990	4.46E-03	53.5050	628.0550	0.8749

FC: Fold change, log: Logarithm, corr: Correlation, p: Significance level

glioblastomas and increased miR_18a expression values[26,27]. On the other hand, Jiang et al.[28] targeted miR_18a as responsible for the low expression values of the receptor-related orphan receptor A (RORA) protein, which they associated with a good prognosis, in high-grade glioblastomas. It has been suggested that the other member of the family, miR_19a, is expressed in many types of cancer and can be used as a biomarker of cancer progression[29]. Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor gene and is the target protein of miR_19a. It has been reported that with the inhibition of miR_19a, the activation of PTEN is increased and the invasion of glioma cells is prevented in this way[30]. In the study of Malzkorn et al.[31], they reported that there was an increase in miR_19a expression values in tissues obtained from people with a diagnosis of glioblastoma, and that miR_19a inhibition prevented cell proliferation of glioblastoma cells. In the present study, a statistically significant increase was detected in the expression values of miR_19a (Figure 3). These results suggest that the effect of ruxolitinib on tumor invasion may be related to miR_19a rather than miR_18a.

miR_31 is a miR known for its anti-cancer properties for many types of cancer, but also reported to inhibit metastasis in breast cancer[32,33]. Its oncogenic properties have been reported in lung and colon cancers[34,35]. Its decreased expression has been reported in glioblastomas compared to normal brain tissue[36]. The target protein of miR_31 is radixin[30]. Radixin is a member of the ezrin/radixin/moesin protein family, which is responsible for binding between the cell membrane and the cytoskeleton[37]. Wang et al.[38] reported that decreased miR_31a and high radixin expression were associated with poor Karnofsky performance and poor survival in patients with glioblastoma. In another study, it was reported that miR_31 inhibits nuclear factor-kappa B signaling, which is known to be highly effective in glioblastomas[39]. In our study, increased expression of miR_31a was detected with ruxolitinib administration (Table 2). This increased expression was also found to correlate with inhibition of invasion (Figure 3). Ruxolitinib is a janus kinase (JAK) inhibitor. It is thought that the target protein of miR_31, radixin, is also included in the structure of JAK[40]. These data suggest that there is a molecular link between JAK/STAT signaling and miR_31 in the invasion inhibitory effect of ruxolitinib, but this needs to be confirmed by further studies.

miR_155 is a well-known oncogenic miR with 147 target genes identified in the literature. The presence of many target genes is met with increasing interest in their clinical significance[41]. Elevated expression values have been reported in many types of cancer, including glioblastoma, lung cancer, breast cancer, Burkitt's lymphoma, and leukemia[41-43]. In the study by D'Urso et al.[44], they found an increase in the expression of miR_155 in both primary and secondary glioblastoma patients and suggested that it showed its oncogenic effect in glioblastomas through the target gene of miR_155, γ-*aminobutyric acid A receptor 1* (GABRA 1)[44]. GABRA 1 is the receptor for gamma acetyl amunobutyric acid (GABA), whose role in brain functions is well known. GABA is one of the main inhibitory neurotransmitters in the human brain. With its increased expression, it is known to increase tumor cell proliferation in glioblastomas[45]. In the present study, increased miR_155 expression was detected in U87

a

Variable		Tumour volume	Invasion rate
1. Tumour volume	Pearson's r	—	
	p-value	—	
2. Invasion rate	Pearson's r	0.707***	—
	p-value	< .001	—
3. miR_15b	Pearson's r	−0.659**	−0.861***
	p-value	0.002	< .001
4. miR_18a_5p	Pearson's r	−0.543*	−0.357
	p-value	0.013	0.123
5. miR_19a_3p	Pearson's r	−0.713***	−0.455*
	p-value	< .001	0.044
6. miR_21_5p	Pearson's r	0.138	−0.079
	p-value	0.561	0.741
7. miR_27a_5p	Pearson's r	−0.413	−0.136
	p-value	0.070	0.567
8. miR_31_3p	Pearson's r	−0.461*	−0.533*
	p-value	0.041	0.015
9. miR_132_5p	Pearson's r	−0.144	−0.058
	p-value	0.544	0.807
10. miR_155_3p	Pearson's r	−0.572**	−0.625**
	p-value	0.008	0.003
11. miR_200b_5p	Pearson's r	−0.673**	−0.957***
	p-value	0.001	< .001

* p < .05, ** p < .01, *** p < .001

b

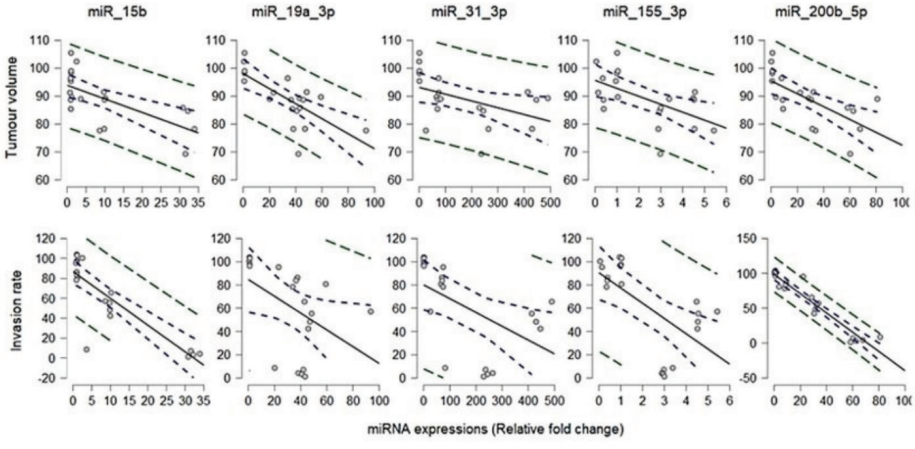

Figure 3. Tumor size, invasion rate, and the most significant angio-miR expression relationship a) Correlation matrix generated according to the Pearson correlation coefficient b) Scattered points, 95% confidence interval (blue line) and 95% predictive interval (green line)

invasion, which was strongly inhibited by ruxolitinib. This result suggests that ruxolitinib does not exert its inhibitory invasion effect on miR_155, which is known for its oncogenic effect.

miR_200b is a member of the miR_200 family and has been reported to be involved in many types of cancer, including glioblastomas[46]. It has been reported to inhibit tumor growth in malignant glioblastoma cell lines and human tissues with low expression values in cell lines[47]. It has been reported that it exerts its inhibitory effect on glioblastomas through its target gene, element-binding protein 1 (*CREB1*)[47]. Liu et al.[48] associated decreased expression of miR_200b with poor prognosis, and they suggested that this effect occurred on another target gene of miR_200b, *RAB* gene family[48]. Chang et al.[49] have reported that increased expression of RAB3C, a member of the RAN family, is associated with high grade and poor prognosis in colorectal cancers, and that the expression of this gene decreases with ruxolitinib administration and prevents cancer cell movement. In our study, a strong correlation was found between U87 cell growth and invasion, which was significantly inhibited by ruxolitinib, in miR_200b expression. Considering the close relationship of ruxolitinib with the RAB family, which is the target gene of this miR, we think that miR_200b may be closely related to the effect of ruxolitinib.

Study Limitations

Although the presented study reveals results that we think are important, it has some limitations. The most important limitation is that the study is an *in vitro* study. In addition, the validation of the obtained data through human tumor tissues can make the results even more reliable. Similarly, investigation of changes in protein expression along with changes in gene expression may contribute to the elucidation of the molecular mechanism more. In addition, we think that our data reflect some important potential clinical scenarios for ruxolitinib-angio-miR association in GBM patients.

CONCLUSION

We demonstrated that GBM growth and invasion modeled in tumor spheroids was significantly inhibited specifically by 200 nM ruxolitinib treatment. We also identified a strong interaction between ruxolitinib and angio-miRs in the ruxolitinib-treated groups. Our results revealed that miR_15b, miR_19a, miR_31_3p, miR_155_3p and miR_200b among 34 angio-miRs that we investigated were statistically significantly changed by ruxolitinib treatment, and all of them were associated with tumor growth and invasion. Our results suggest that ruxolitinib is an effective anti-tumor therapeutic in glioblastoma tumor spheroids, possibly by altering the expression profile of angio-miRs and thereby inhibiting angiogenesis-related signals. However, it is thought that more studies are needed to validate the data of our study and make it clinically usable.

Ethics

Ethics Committee Approval: Commercial cells were used in our study. Ethics committee approval is not required for such studies.

Informed Consent: There is no need.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.D., O.D., Concept: E.D., O.D., Design: E.D., O.D., Data Collection or Processing: E.D., O.D., Analysis or Interpretation: E.D., O.D., Literature Search: E.D., O.D., Writing: E.D., O.D.

Conflict of Interest: No conflict of interest was declared by the authors.

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