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Original research article

Apoptosis related microRNAs and MGMT in glioblastoma cell lines submitted to treatments with ionizing radiation and temozolomide

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ABSTRACT

Aim: To evaluate the effect of radiotherapy and temozolomide on the expression of miRNAs apoptotic (miRNAs-21, -221, -222 (anti-apoptotic) and miRNAs-15a, -16 (pro-apoptotic)) and the gene MGMT in glioblastoma cell lines.
Background: The limited knowledge of the molecular biology of malignant gliomas may hinder the development of therapeutic modalities. In this scenario, one of the greatest advances of recent years was the identification of microRNAs. These molecules have an important role in biological processes involving cancer, including glioblastoma.
Materials and methods: Trypan blue was used to verify the cell viability, and real time PCR to quantify the expression of microRNAs and gene 24, 48 and 120 h after exposure to treatments.
Results: There was a statistically significant decrease of expression of miR-15a between 48 and 120 h in line T98 G treated with radiation, increased expression of miR-15a between 24 and 120 h in line U251 treated with radiation and temozolomide, and increased expression of miR-16 between 24 and 120 h in line U251 treated with radiation alone and when combined with temozolomide. There was a decrease in MGMT gene expression, between 24 and 48 h in U343 cells treated with temozolomide.
Conclusions: Ionizing radiation and temozolomide modified the expression of miRNAs studied and MGMT.

1. Background

Diffusely infiltrating astrocytomas account for more than 60% of all primary brain tumors, and Glioblastoma (GBM) is the most common and malignant subtype, besides it is lethal.[1,2] There are several factors that hinder the achievement of better results in the treatment of glioblastomas, they are fast-growing, highly infiltrative and complete surgical removal is very difficult.[3–5] These characteristics and also a phenotypic variability contribute to resistance to radiotherapy and chemotherapy, the main forms of adjuvant treatments to surgery.[6]

The mechanisms of self-initiation, proliferation, angiogenesis, invasion and absence of apoptosis, are some of the most important ability of GBMs. Despite considerable heterogeneity and genetic alterations, some genetic modifications are often dominant, such as: loss of PTEN, amplification of CDK4, amplification factor receptor epidermal growth factor (EGFR), IDH mutation, among others.[7–12]

Although the tumor develops strategies to combat the physiological induction of apoptosis during its growth, in many cases the therapeutic agent maintains its ability to sensitize the cell to the apoptotic cascade. Chemotherapy and radiation induce apoptosis in gliomas and new pro-apoptotic agents are developed.[13]

In the last years, the temozolomide, a chemotherapeutic agent has been used for GBMs. Based on the study by Stupp et al. in which radiotherapy alone was compared with radiotherapy plus temozolomide, there was a statistically significant increase in overall survival among patients with methylation of the MGMT gene promoter, when treated with radiotherapy and chemotherapy, compared with patients undergoing radiotherapy only. In patients

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with no methylation of the MGMT gene promoter, no benefit of the addition of chemotherapy to radiation therapy was observed. These findings led to establishing a standard treatment for glioblastomas and increased interest in the MGMT methylation status, as a marker of response to the temozolomide for the treatment of patients with glioblastoma.[14,15]

The limited knowledge of molecular biology, genetics, causes, and cellular origin of glioblastoma may block the development of therapeutic modalities; however, this scenario is changing rapidly. One of the greatest advances of recent years was the identification of microRNAs (miRNAs), regulators of gene expression. These molecules play an important role in biological processes involving cancer, including glioblastoma.[16–18]

2. Aims

Our goals in this study were to evaluate the effects of radiotherapy and chemotherapy in the expression of microRNAs involved in the apoptotic process, as well as in the expression of the MGMT gene, opening new possibilities for the treatment of patients with GBMs.

3. Materials and methods

3.1. Cell culture

The cells line U251, U343 and T98 G were originally obtained from the American Type Culture Collection (ATCC). Cells were cultured and maintained at 37 °C with 5% carbon dioxide (CO_2) in Dulbelcco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomicin/neomycin and 1% nonessential amino acid (Invitrogen).

3.2. Treatment of cells

The cells cultures were divided into four groups of treatment: an untreated group, named control group; IR group IR treated with ionizing radiation (x-ray 6 mV produced by the Siemens linear accelerator with a dose rate of 2.0 Gy/min at final doses of 14 Gy); TMZ group treated with temozolomide (Temodal®, the compound was diluted to 10 mmol/L using 99% DMSO and stored at −20 °C); and a group treated with the combination of both treatments: IR + TMZ. All groups were analyzed at variable times 24 h, 48 h and 120 h, and all the experiments were performed in triplicate.

3.3. Cell viability assay

To assess the cell viability, we used the exclusion test with Trypan Blue, a dye marker for dead cells. We gathered 50 μL of cells in 50 μL of Trypan Blue (0.4%). Cells underwent Newbauer counting chamber, wherein translucent cells were considered viable, and cells with blue staining were considered dead.

3.4. RNA isolation and real-time polymerase chain reaction

Total RNA was extracted with Trizol reagent (Applied Biosystems, USA) according to the manufacturer's instructions. In preparation of real-time polymerase chain reaction (PCR), reverse transcription of RNA samples was performed using the High-Capacity cDNA kit (Applied Biosystems, USA).

3.5. Real time PCR

The cDNA was amplified with quantitative Real Time Polymerase Chain Reaction (q-PCR) using TaqMan Master Mix (Applied Biosystems) for reaction of microRNAs and SYBR Green (Applied Biosystems) for gene reaction. The sequences of the primers for each gene are shown in Table 1.

Table 1
Sequence of primers.

Gene	Primers forward (-f)	Reverse (-r)
MGMT	CCAGCAAGAGTCGTTCACC	CTCATTGCTCCTCCCACTG
TBP	GAGCTGTGATGTGAAGTTTCC	TCTGGGTTTGATCATTCTGTAG
HPRT1	TGAGGATTTGGAAAGGGTGT	GAGCACACAGAGGGCTACAA

The RNU24 and RNU48 genes were used as an endogenous control for reaction of the microRNAs, and for gene MGMT reaction, TBP and HPRT were used as an endogenous control. All reactions were carried out in duplicate and analyzed with the 7500 Sequence Detection System apparatus (Applied Biosystems). The data were analyzed using the ABI-7500 SDS software.

3.5.1. Statistical analysis

Data are presented as the means ± S.E.M. For the comparison between the groups, at different analysis times, statistical analysis was performed using the Kruskal-Wallis test and Dunn's multiple comparison post-test. P values smaller than 0.05 were considered to be statically significant.

4. Results

4.1. Cell viability versus treatment modality and time analysis

Cell line T98G: Differences were observed between the rates of cell viability of all treated samples compared to samples from the control group at all times, i.e. 24, 48 and 120 h ($p < 0.05$). The viability rates of each treatment group compared to the time of analysis were similar, with differences only between the times of 24 and 120 h ($p < 0.05$), in the IR and TMZ groups (Fig. 1).

Cell line U251: Differences were observed between the rates of cell viability of all treated samples compared to samples from the control group at 24, 48 and 120 h ($p < 0.05$). The viability rates of each treatment group compared to the time of the analysis showed a difference only between the times of 24 and 120 h ($p < 0.05$), groups IR and IR + TMZ (Fig. 1).

Cell line U343: Differences were observed between the rates of cell viability of all treated samples compared to samples from the control group at 24, 48 and 120 h ($p < 0.005$). The viability rates of each treatment group, compared to the time of the analysis showed no differences (Fig. 1).

4.2. MGMT gene expression versus therapeutic modality and time analysis

Cell line T98G: MGMT gene expression, for each time, depending on the type of therapy applied, we observed increased expression of MGMT in the TMZ group (2-fold) and the IR + TMZ group (4-fold) compared and all the other groups, for the time of 24 h. Moreover, we also observed that there was a statistically significantly decreased MGMT gene expression in the IR group when compared to all the the other groups, for the time of 24 h ($p < 0.05$) (Fig. 2).

Cell line U251: MGMT gene expression, for each time, depending on the type of therapy applied, we observed increased expression of MGMT (200-fold) in the IR group at 24 h. Moreover, we observed a statistically significant increased MGMT gene expression in the IR + TMZ group when compared to all the other groups ($p < 0.05$), for the time of 120 h. We also observed a statistically significant increased MGMT gene expression in the IR group, between the expressions of 24 and 120 h ($p < 0.05$) (Fig. 3).

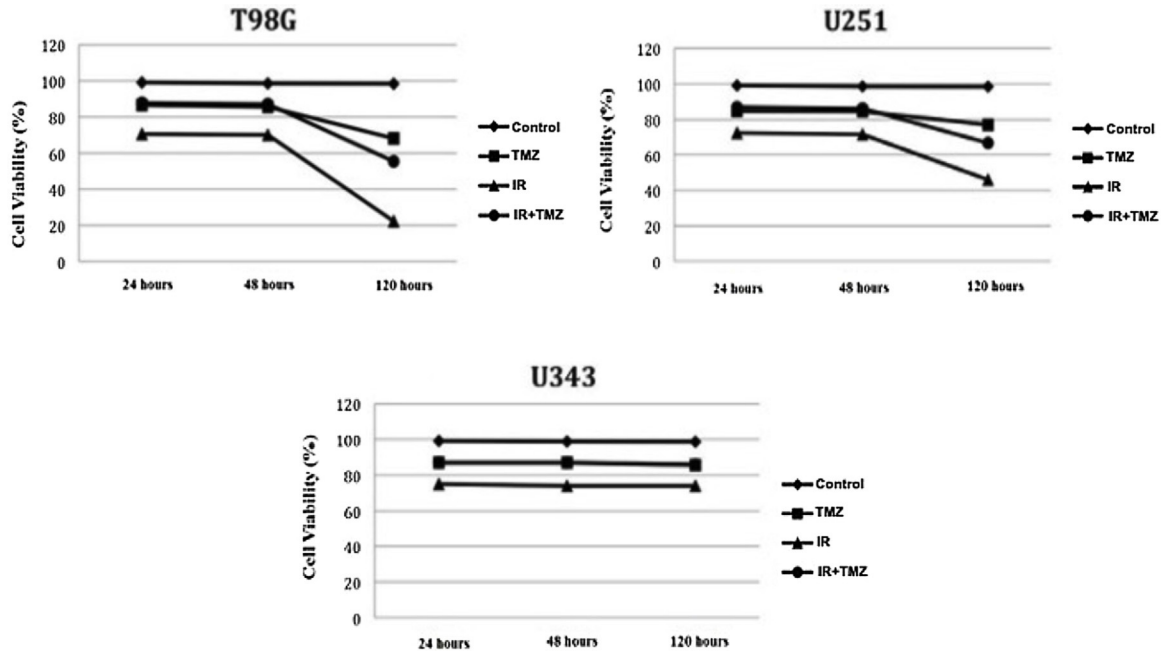

Fig. 1. Cell viability versus treatment modality for T98 G, U251 and U343 glioblastoma cell lines.

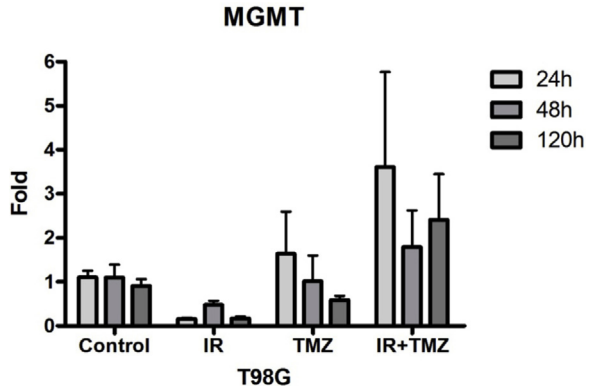

Fig. 2. Expression of the MGMT gene, for each time, according to type of therapy applied for the T-98 G glioblastoma cell line.

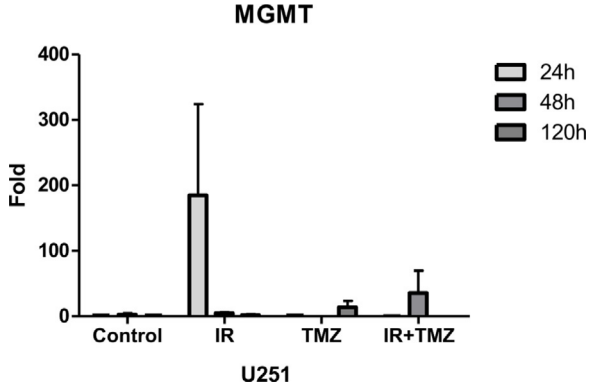

Fig. 3. Expression of the MGMT gene, for each time, according to type of therapy applied for the U251 glioblastoma cell line.

Cell line U343: MGMT gene expression, for each time, depending on the type of therapy applied, we observed in the TMZ group an increased expression of MGMT (300-fold) at 24 h, followed by decreased MGMT expression (10-fold) at 48 h and a new increased expression at 120 h (100-fold). We also observed a statistically significant increased MGMT gene expression in the TMZ group, between the expressions of 24 and 48 h ($p < 0.05$) (Fig. 4).

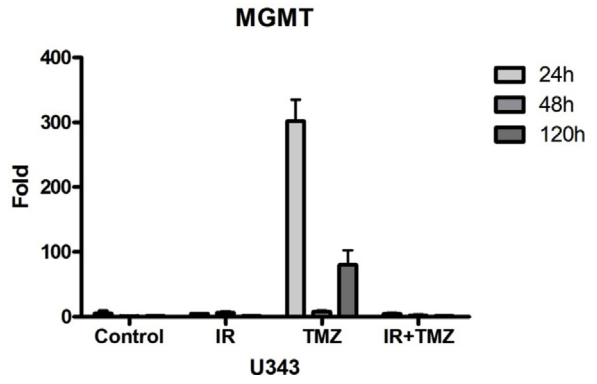

Fig. 4. Expression of the MGMT gene, for each time, according to type of therapy applied for the U343 glioblastoma cell line.

4.3. Expression of miRNAs versus therapeutic modality and time analysis

T98 G cell line: Comparing the expression of miRNAs between treatment modalities at 24, 48 and 120 h, we observed an increased expression of miRNAs miR-222 and miR-16 in the IR + TMZ group, compared to the other groups (with approximately 20-fold) in the time analysis of 24 h. Moreover, at 24 h, there was a statistically significant increased expression of miR-21 in the IR + TMZ group in comparison to the Control and IR groups ($p < 0.05$), there was also a statistically significant increased expression of miR-221 in the IR + TMZ group in comparison to all the others ($p < 0.05$) and in the expression of miR-222 we observed a statistically significant increase in the IR + TMZ group when compared to the Control and IR groups ($p < 0.05$); likewise, a statistically significant increased miR-15 was observed in the IR + TMZ group in comparison to all the other groups ($p < 0.05$); and in the expression of miR-16 we observed a statistically significant increase in the IR + TMZ group

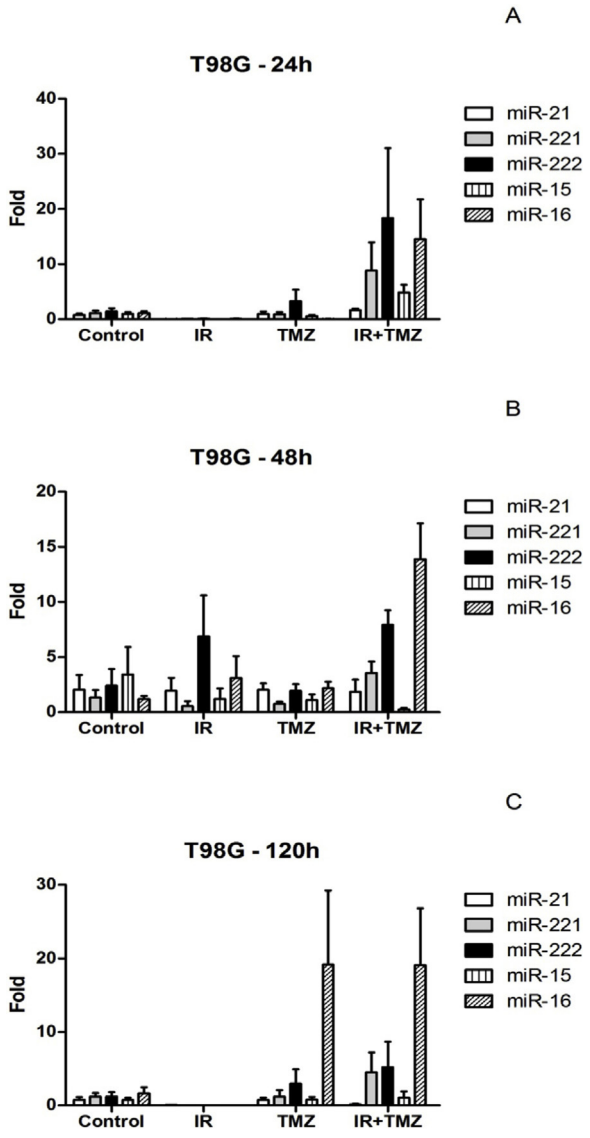

Fig. 5. MicroRNAs expression in T98 G glioblastoma cell line analyzed 24, 48 and 120 h (a, b and c, respectively) after treatment.

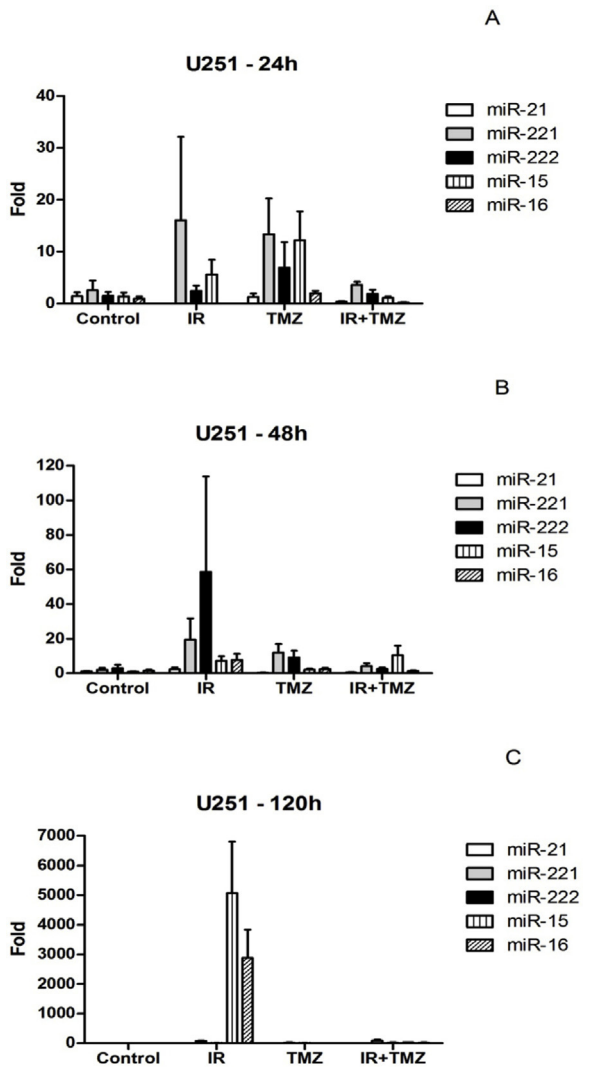

Fig. 6. MicroRNAs expression in U251 glioblastoma cell line analyzed 24, 48 and 120 h (a, b and c, respectively) after treatment.

when compared to the IR and TMZ groups ($p < 0.05$) (Fig. 5A). In 48 h, we observed expression patterns similar to microRNAs miR-222 (15-fold) and miR-16 (10-fold) in the IR + TMZ group and an increase in miR-222 (7-fold) in group treated only with IR (Fig. 5B). At 120 h a decreased expression of miRNA-222 was observed in the IR + TMZ group compared to the other groups, and increased expression of miR-16 (20-fold) in the IR + TMZ group and TMZ group (20-fold) compared to the other groups. At 120 h, there was also a statistically significant decreased expression of miR-221 in the IR + TMZ group in comparison to the IR group, mir-16 expression was also decreased in the IR + TMZ group when compared to the IR group ($p < 0.05$) (Fig. 5C).

U251 cell line: Comparing the expression of miRNAs between treatment modalities, at 24, 48 and 120 h, we observed an increased expression of miR-221 (15-fold) in the IR group and TMZ group (13-fold) and also decreased expression of this miRNA in the IR + TMZ group (3-fold). The miR-15a was up-regulated in the TMZ group (12-fold) in comparison to the other groups in the time analysis of 24 h. Moreover, at 24 h, there was a statistically significant increased expression of miR-21 in the Control and TMZ groups in comparison to the IR group ($p < 0.05$). There was also a statistically significant increased expression of miR-16 in the TMZ group when compared to the IR and IR + TMZ groups ($p < 0.05$) (Fig. 6A). At 48 h, we observed increased expression of miR-222 (60-fold), in the IR group in relation to the other groups (Fig. 6B). At 120 h, there was an increase in the expression of miRNAs miR-15a (5000-fold) and miR-16 (3000-fold) in the IR group compared to other groups. At 120 h, there was also a statistically significant increased expression of miR-221 in the IR and IR + TMZ groups in comparison to the Control and TMZ groups ($p < 0.05$), miR-15a expression was increased in the IR group in comparison to all the others groups ($p < 0.05$), miR-16 expression was statistically significant increased in the IR group when compared to all the other groups ($p < 0.05$) (Fig. 6C).

U343 cell line: Comparing the expression of miRNAs between treatment modalities, at 24, 48 and 120 h, we observed an increased expression of miRNAs miR-21 (30-fold) and miR-15a (25-fold) in IR + TMZ group compared to the other groups in the time analysis of 24 h (Fig. 7A). At 48 h, we observed decreased expression of miRNAs miR-21 (1-fold) in the IR + TMZ group and miR-15a remained up-regulated (60-fold) compared to the other groups. The microRNAs miR-221 (30-fold), miR-222 (25-fold) and miR-16 (40-fold) expression also increased at 48 h. Moreover, at 48 h, we also observed that there was a statistically significant increased expression of miR-16 in the IR + TMZ group in comparison to the Control and IR groups ($p < 0.05$) (Fig. 7B). At 120 h, an increased expression of miR-15a

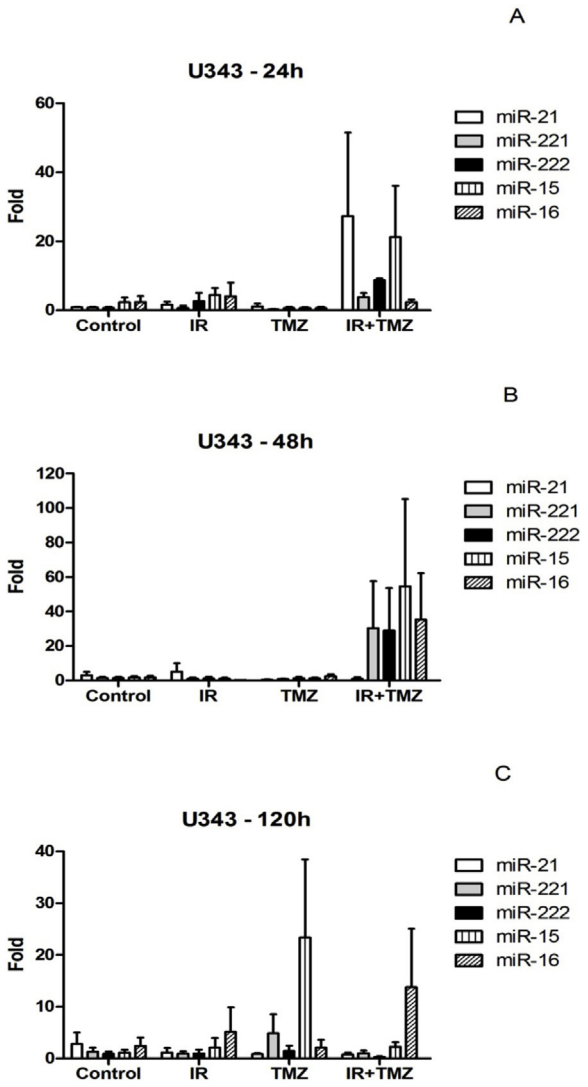

Fig. 7. MicroRNAs expression in U343 glioblastoma cell line analyzed 24, 48 and 120 h (a, b and c, respectively) after treatment.

(25-fold) was observed in the TMZ group, compared to the other groups, as well as an increased expression of miR-16 (15-fold) in the IR + TMZ group, compared to the other groups (Fig. 7C).

5. Discussion

The possibility of ionizing radiation and temozolomide altering the expression of MGMT gene and the expression of miRNAs was one of the hypotheses of our study. And, since the histopathological analysis of tumors alone seems insufficient, molecular understanding of tumors may bring us information about new possibilities of treatment.[7]

In our study, we compared the expression of miRNAs and the MGMT gene after exposure to standard therapy available for glioblastoma, and we found a significant increase in MGMT expression after 24 h of treatment followed by a decrease at 48 h and an increase again at 120 h in U343 cells treated with temozolomide. We also observed higher values of MGMT gene expression in T98 G cells treated only with temozolomide and when combined chemotherapy and radiotherapy (in relation to other groups) at 24 h. In line U251, MGMT showed higher expression in cells subjected to radiation alone after 24 h of treatment.

The silencing of the MGMT gene has been considered an important predictor of response to chemotherapy with alkylating agents in the treatment of patients with glioblastoma and anaplastic astrocytoma, the determination of a ˇstatusfrom the methylation of MGMT gene promoter is a ˇsensoröf chemo-sensibility.[19–22] The study of Neto et al. (2019), among others, showed that the ionizing radiation and temozolomide reduced the viability of cancer cells from GBM patients, moreover, this treatment was able to modify the MGMT gene.[23] However, not only information on the methylation of the promoter of the gene, but also its degree of expression may have an effect on the prognosis of patients, the lower the gene expression, the higher survival free of disease progression, and the better the response therapy and increased overall survival in both univariate and multivariate analysis. These are the findings of a study involving the analysis of tumor samples from 63 patients with malignant gliomas.[19]

The role of microRNAs as modulators to anti-cancer responses was reported in many studies as that conducted by Niemoeller et al. and Ondracek et al., in which the authors found several microRNAs involved in resistance to response to treatment with ionizing radiation. However, little is known about the behavior of the combination treatment microRNAs chemotherapy and radiotherapy (temozolomide and ionizing radiation), another aspect unexplored are different doses of ionizing radiation, as well as the effect of different doses of radiation in the period after treatment.[24,25]

We observed increased expression of microRNAs miR-21 and miR-15a in the group of combined treatment (TMZ + RT) after 24 h, followed by decreased levels of expression of miR-21 at 48 h, however, the expression levels of miR-15a remained high in this period. Interestingly, 120 h after the treatment, the miR-15a showed increased levels of expression only in the group treated with TMZ alone. These results may suggest a possible competition between pro- and anti-apoptotic microRNAs in the 24 -h period, followed by the prevalence of inductors apoptosis only after 48 h. The persistent increase in levels of expression of miR-15a after 120 h in the group treated only with TMZ also suggests a relationship between the findings of microRNAs and MGMT gene since, as mentioned previously, an increased in expression of MGMT was observed after 24 h followed by reduction after 48 h and new increase with 120 h after treatment in cell line U-343, which was the most resistant in this study. In 2010, Chaudhry et al. evaluated the expression of several microRNAs in cell culture of malignant glioma treated with radiation ionizing. The samples were irradiated with 3 Gy and miRNA expression was analyzed after 4, 8, 12 and 24 h of exposure to radiation. Among other results, it an increased expression of miRNAs miR-15a, miR-16, miR-143, miR-155 and miR-21 was observed. The authors concluded that the response of glioblastoma to ionizing radiation involves modulation of several miRNAs and that differences between the expression of several miRNAs may be the basis of the sensitivity of cells to treatment.[26] In another study, Li et al. (2011) demonstrated that miR-21 was up-regulated in response to treatment with ionizing radiation and an inhibitor of miR-21 caused a decrease in cell growth and increased apoptosis.[27] Shi et al. (2010) showed that the high expression of miR-21 reduces apoptosis induced by temozolomide.[28]

We observed an increased expression of microRNAs miR-222 and miR-16 in the group with combined treatment (TMZ + RT) in cell line T98 G at 24 h and 48 h, followed by decreased levels of expression of the miR-222 at 120 h. We note that apart from the levels of expression of miR-16 remaining high in the combined treatment group (TMZ + RT) at 120 h, this microRNA also increased in the group treated with temozolomide (TMZ) only. These results may also suggest, as well as in cell line U-343, a possible competition between pro-and anti microRNAs apoptotics, but in both the 24 h and 48 h period. Another finding which agrees with line U-343 is the persistent increase in the levels of expression of an apop-

totic microRNA (miR-16) in the group treated with TMZ, which may also suggest the relationship of microRNAs and the findings of the MGMT gene, as we observed an increased expression of MGMT at 48 h (in the TMZ alone group) and 120 h (TMZ + RT group). Interestingly, the T98 G cell line which was the most sensitive in this study, had the highest rates of death just after 48 h of treatment, with a peak of deaths in the period of 120 h. Other studies have highlighted the role of microRNAs in resistance to the treatment of glioblastomas. Ujifuku et al. (2010) showed in a cell culture assay that the inhibition of microRNAs miR-455−3p and miR-10a can reverse the resistance to treatment with temozolomide.[29]

In cell line U-251, the most interesting findings have been related to the group treated with radiotherapy (RT), since this group has been observed to show increased expression of miR-221 (anti-apoptotic) at 24 h and miR-222 (anti-apoptotic) at 48 h. In the period of 120 h after treatment, the microRNAs miR-15a and miR-16 both pro-apoptotic were up-regulated; inversely, the miR-221 and miR-222 at this time were down-regulated. These findings also allow us to suggest, as well as in other cell line studies, a possible relationship between the findings of the microRNAs miR-15a and miR-16 and the MGMT gene, since the gene expression profile of pro-apoptotic microRNAs and MGMT here studied showed significant changes in the group treated only with radiotherapy. The cell line U-251 also shows high rates of death at 120 h after treatment. In the search to elucidate the molecular mechanisms of action of ionizing radiation Chen et al. (2010) evaluated by technique of microarray the expression of microRNAs before and after radiation. The miR-181a that targets Bcl-2 was down-regulated in malignant glioma cells. These results showed the involvement of apoptosis in resistance at treatment with ionizing radiation.[30]

6. Conclusion

In this study we found evidence suggesting that a new factor may interfere with the analysis of MGMT, namely, the ability of tumor cells to increase or keep the level of expression when subjected to DNA damage, by radiotherapy or chemotherapy, although data in cell cultures and our data suggest that this response may be individualized for each tumor. Hopefully, from the data given, the modulation of molecular markers, including miRNAs, will encourage the emergence of new therapeutic possibilities for patients with gliomas.

Conflict of interest

None declared.

Financial disclosure

None declared.

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