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Downregulation of TRPM8 channels induce cell death in human DBTRG glioblastoma cells

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The calcium ion (Ca^{2+}) is among the most important second messengers for cellular metabolic processes. Cytosolic calcium concentration ([Ca^{2+}]c) is regulated by calcium signals and that are driven by different kinds of cation channels including the Transient Receptor Potential (TRP)channels. The TRPM8cation channels are calcium ion (Ca^{2+}) permeable non-selective cation channels belongs TRP superfamily. The TRPM8 is expressed in central and peripheral nervous system components especially in pain and cold sensation related nerve endings. Glioblastoma is the most potent cancer type of the human brain, and patients has less survival time after the detection of disease. Recently, TRPM8 cation channels involved in several types of cancers including glioblastoma because of high expression levels in cancer tissues. Hence, [Ca^{2+}]c may change depend on TRPM8 activations from extracellular liquid to cytosol and it can be targeted for prevention of cancer progression. Therefore, we have investigated effects of downregulation of TRPM8 cation channels by siRNA transfections and pharmacological blockade of the channels with its specific antagonist on apoptosis and cell viability, intracellular reactive oxygen species production (iROS), mitochondrial membrane depolarization levels (MMD), caspase 3 and caspase 9 enzyme activity values in DBTRG glioblastoma cells. We have found that TRPM8 cation channels has crucial role in cancer progression and uncontrolled cell proliferation, however TRPM8 cation channels' pharmacologically blockade, or prevention of the channel expression may induce cell death and cellular apoptotic processes. In conclusion, TRPM8 cation channel blockers or downregulation of the channels by genetic manipulations can be useful and potential therapeutic approach against to glioblastoma progression.

Keywords: Calcium signaling, Cell death, DBTRG cells, Glioblastoma, TRPM8 channels

Calcium (Ca^{2+}) is an important ion for many cellular functions including proliferation, neuronal excitation, neurotransmitter containing vesicles releasing and cellular signal transduction processes as a second messenger. The ratio of extracellular to intracellular concentrations is around 10.000-20.000, and increased elevation of cytosolic Ca^{2+} concentration ([Ca^{2+}]c) from its physiological limits (~100 nM) to µM levels may cause cellular apoptosis *via* activating caspases. The difference of cytosolic to extracellular Ca^{2+} concentrations ([Ca^{2+}]e) is under controlled by Ca^{2+} specific or permeable cation channels including transient receptor potential (TRP) cation channels[1,2]. Even though many channel inhibitors of these channels have been discovered, none of them can fully block the cationic influx through the channels. TRP channels are non-selective cation channels, and they related with several neurological diseases and have significant expression levels in neurological tissues. In neuronal cells, TRP channels have been responsible for many physiological actions from signal transduction, intracellular cation signaling, pain sensation to apoptosis, neurodegeneration, mitochondrial oxidative stress related pathways[3-5]. Former studies have shown that TRP channels are different expression and gating manner in many types of cancers including glioblastoma. In human, TRP channel superfamily has 28 members, mostly Ca^{2+} permeable and non-selective cation channels, that are divided into six different subfamilies according to similarity of amino acid sequences which named; Ankyrin (TRPA; 1), Canonical (TRPC; 1-7), Melastatin (TRPM; 1-8), Mucolipin (TRPML; 1-3),

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Abbreviation: AMTB, N-(3-Aminopropyl)-2-[(3-methylphenyl) methoxy]-N-(2-thienylmethyl)benzamide hydrochloride; [Ca^{2+}]c, Cytosolic calcium concentration; [Ca^{2+}]e, Extracellular calcium concentration; Ca^{2+}, Calcium ion; Caspase, Cysteine dependent aspartic proteases; CPZ, Capsazepine; iROS, Intracellular reactive oxygen species; MMD, Mitochondrial membrane depolarization; TRP, Transient receptor potential channel; TRPM8, Transient receptor potential channel melastatin subtype 8

Policystin (TRPP; 2, 3, 5), Vanilloid (TRPV; 1-6)[6]. The TRPM8 cation channels are menthol and cold-sensitive cation channels. TRPM8 channel expression levels very high in neuronal tissues related with pain perception[7]. Menthol or cold-induced activation of TRPM8 channels regulated by their specific (AMTB hydrochloride) and non-specific antagonist (capsazepine)[8,9]. The DBTRG cells are very useful for studying as glioblastoma model *in vitro*[10]. Recent studies have shown that different TRP channels are expressed in this cell line including TRPM8 and its mediated Ca^{2+} signaling is related with migration and proliferation in glioblastoma[11,12] and cell proliferation may reduce by its targeting. In a former study, it was also emphasized that TRPM8 is required for viability and resistance of irradiation in glioblastoma[13]. Moreover, TRPM8 channel expression levels are very high among other TRP channel superfamily in human glioblastoma patient samples[14]. However, we have limited record in literature on how inhibition of TRPM8 mediated Ca^{2+} signaling by downregulation of channel expression affect glioblastoma cell viability or proliferation in glioblastoma cells. Hence, in this study we aimed to investigate that the role of downregulation of TRPM8 channel expression in glioblastoma cells by siRNA transfection on cellular viability, caspases (3, 8 and 9) enzyme activity, apoptosis levels, mitochondrial membrane depolarization and reactive oxygen species production levels in DBTRG cells. Moreover, we also targeted the channels by their specific antagonist treatment to observe similar role on Ca^{2+} signaling.

Materials and methods

Chemicals, cell culture and study design

All reagents, probes and chemicals in the study were purchased from Sigma-Aldrich, USA unless otherwise stated. The TRPM8 siRNA (sc-95009) and Control siRNA (sc-36869) were purchased from Santa Cruz Biotechnology, USA. Sequences of siRNAs were listed in Table 1. The DBTRG cells were purchased from Şap Institute, Ministry of Agriculture and Forestry, Ankara, Turkey. Cells were cultured in RPMI 1640 basal medium mixture containing 10% fetal bovine serum (Cegrogen, Germany) and 1% Penicillin-Streptomycin (Biochrom, Germany) at 37°C in 5% CO_2 humidified incubator (Heal Force HF90, Japan). When cells have reached the ~80% confluence, each flask were trypsinized and split into new flasks or studied in the analyses, and 9-15 number of passages of the cells were used in the experiments. The LipoFectMax™ Transfection Reagent (FP 310) was purchased from ABP Biosciences, USA. The Opti-MEM™ I Reduced Serum Medium for transfections was purchased from TermoFisher Scientific, USA. The transfection process was carried out by following the steps. Briefly, one day before transfection, cells were seeded with growth medium. Around 30-50% confluence of cells transfected in the next day. For each transfection, LipoFectMax™-oligomer complexes were prepared as follows, the siRNA was diluted and mixed gently with an amount of Opti-MEM medium (final concentration of siRNA adjusted to 50 nM) according to the surface area in the vessel containing the cells to be transfected (a). Before each use, LipoFectMax™ stock was vortexed and diluted and mixed gently with the specified amount of Opti-MEM medium based on the surface area in the vessel containing the cells to be transfected. It was kept at room temperature for 5 min (b). Then solutions prepared in a and b were mixed to obtain an oligomer-LipoFectMax™ mixture and it was incubated at room temperature for 20 min. Oligomer-LipoFectMax™ mixture was transferred to culture flasks containing cells after growth medium of the flasks were removed. The flasks were rocked back and forth to facilitate the contact of the mixture with the cells. The cells were incubated with the mixture for 4-6 h, and after that they were replaced with fresh growth medium, and the total incubation time was completed to 48 h. Each control (untransfected) and siRNA transfected DBTRG cells were detached and centrifuged after all incubation time. Cell pellets resuspended in a 1x phosphate buffered saline (PBS), then cells divided into eppendorfs for analyzes.

Table 1 — siRNA sequences of the target TRPM8 channels. A, B and C refer to the different target sequences of each siRNA pool

	TRPM8 siRNA	
siRNA Seq.	Sense	Antisense
A	GGAAUCAGCUAGAGAAGUAtt	UACUUCUCUAGCUGAUUCCtt
B	CGAAUGUUCUCACCUAUUAtt	UAAUAGGUGAGAACAUUCGtt
C	GUGAGACAGUGGUACGUAAtt	UUACGUACCACUGUCUCACtt

Immunoblotting

The TRPM8 cation channels expression levels were studied with western blotting after siRNA transfection procedure. The DBTRG cells were homogenized in 1x RIPA buffer (Biobasic, Canada) centrifuged at 13.000 g for 18 min and supernatants were collected. The amount of total protein of lysates was measured with a Bradford assay at 595 nm by a microplate reader (Tecan Infinite M200 Pro, Austria). For immunoblotting step, equal amounts of proteins (30 µg) were loaded into 8% sodium dodecyl sulfate-polyacrylamide gel, after the gel electrophoresis, gels were transferred to a nitrocellulose membrane. The blots were blocked for 1 h at room temperature with a 5% non-fat dry milk powder (Havancızade, Turkey) solution in Tris Buffered Saline with 0.05% Tween 20. Then, the membrane was incubated with TRPM8 primary antibody (BT-AP09234, BT Lab, China). The β-actin polyclonal antibody and secondary antibody were from GE Healthcare, Amersham, UK. Band signals were visualized using ECL Western HRP Substrate (Millipore Luminate Forte, USA) and images were captured by (G:Box, Syngene, UK) and normalized against the β-actin protein as formerly described[15].

Calcium signaling

Calcium signaling was studied with Fura-2 dye (Invitrogen, Carlsbad, USA). Changes in calcium levels after menthol stimulations were shown by using the Fura-2 340/380 nm fluorescence ratio and were calibrated according to a former method[16]. The AMTB hydrochloride was used for antagonist applications. The TRPM8 channel-mediated cytosolic free calcium concentration ($[Ca^{2+}]c$) was expressed as nM, taking a sample every second as previously described[8].

Wright-giemsa and methylene blue staining

Wright-giemsa and methylene blue staining were performed by using a simple method as previously and detailly described in elsewhere[17].

Apoptosis assay and MTT test

For apoptosis assay, the APOPercentage™ apoptosis assay kit was purchased from Biocolor Ltd, Northern Ireland and was performed according to the manufacturer's procedure. The probe selectively stains only the apoptotic cells because of loss of membrane integrity. Apoptotic cells stain red, so it allows the detection of these cells by using a microplate reader (Tecan Infinite M200 Pro, Austria) as previously described, elsewhere[18]. All data were calculated as fold increase experimental to control. The cell viability was evaluated by MTT test. DBTRG glioblastoma cells were plated in a 96-well plate 1 day before the AMTB incubation. After 24 h incubation with AMTB hydrochloride in different concentrations (from 0 to 200 µM), wells were loaded with MTT 20 µl dye (5 mg/ml) for 4 h at 37°C. After the incubation, medium of wells were discarded and insoluble formazan crystals were dissolved in DMSO and read at 490 nm by using a microplate reader (Tecan Infinite M200 Pro, Austria) as described elsewhere[19].

Enzymaticactivity of caspases

The caspase 3, 8 and 9 enzymatic activities were evaluated by the cleavage of the specific fluorogenic substrates as formerly described[20]. The AC-DEVD-AMC (for caspase 3) and the AC-LEHDAMC (for caspase 9) were provided by Bachem, Switzerland. Enzymatic activity of caspases 3 and 9 were measured by using a microplate reader (Tecan Infinite M200 Pro, Austria) with 360 nm excitation and 460 nm emission wavelengths. The data were calculated as fluorescence units per mg/protein.

Measurement of intracellular ROS production (iROS)

For determination of iROS, dihydrorhodamine-123 (DHR-123, Molecular Probes, USA) probe was used. Cells were loaded with the dye (2 µM) and incubated at 37°C for 30 min. The intensity of fluorescence rhodamine-123 was measured by using a microplate reader (Tecan Infinite M200 Pro, Austria). The 488 nm excitation and 543 nm emission wavelengths were used for the assay[21]. The data were calculated as fold increase experimental to control.

Determinationof mitochondrial membrane depolarization (MMD)

Measurement of MMD was performed by using the JC-1 probe (Santa Cruz Biotechnology, USA). Cells were incubated with the JC-1 dye (1µM) for 30 min at 37°C. Fluorescence changing was analyzed by using a microplate reader (Tecan Infinite M200 Pro, Austria). The data were presented as emission wavelengths' ratio (590/535) and calculated as fold increase experimental to control[22].

Statistical analyses

Acquired data were expressed as mean±standard deviation. The significance of difference among the groups was assessed Man Withney U and one-way ANOVA which is appropriate. Data were analyzed by using the SPSS, version 9.05 (SPSS, Inc., USA). $P<0.05$ was considered as significant.

Results

The TRPM8 channel protein expression levels

Protein expression levels of TRPM8 channels evaluated by western blotting. Mean value of protein bands after β-actin normalization were given in Fig. 1. Relative TRPM8 protein expression levels in control group were very higher than TRPM8 siRNA group given as bar graph in (Fig. 1). It is concluded that downregulation of TRPM8 channels induced by siRNA transfection was decreased to channel protein levels in the groups.

The TRPM8 channel-mediated cytosolic free calcium concentration ($[Ca^{2+}]c$)

Results of TRPM8 channels-mediated elevation of $[Ca^{2+}]c$ was measured by Fura-2 labeled calcium signaling were given in (Fig. 2). Line graph Figure 2A shows real time calcium signaling results (F340/F380) while bar graph (Fig. 2B) shows cytosolic calcium concentration as nM levels. In control group, $[Ca^{2+}]c$ levels were very high compared to TRPM8 siRNA group as being hypothesized. Moreover, AMTB hydrochloride also reduced relative calcium concentration in cytosol by its antagonistic effect. It is concluded that downregulation of TRPM8 channels was also decreased TRPM8 activity mediated $[Ca^{2+}]c$ levels.

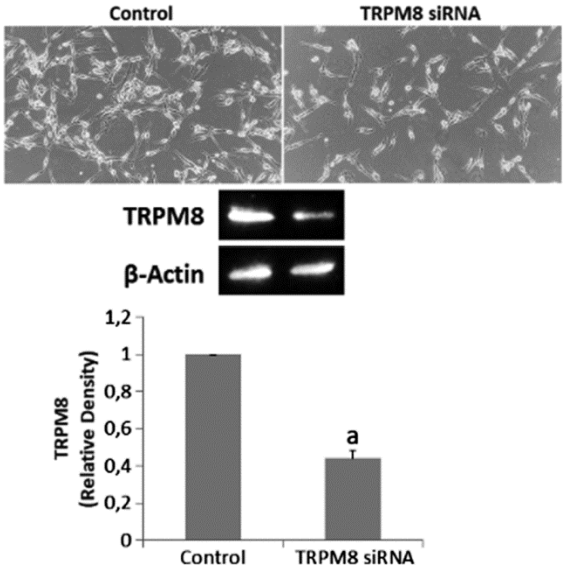

Fig. 1 — Determination of TRPM8 channel expression levels in siRNA-transfected groups. Bright field images (200x) have shown that relative cellular density decreased in transfected group and normal in control group after 48 h transfection period. Relative intensity of western blotting bands of control and TRPM8 siRNA induced groups has shown in bar graph. a: $P< 0.001$ *vs.* the control group

Morphological features of glioblastoma cells after downregulation of TRPM8 channels

The morphological alterations on DBTRG cells were assessed by staining after downregulation of TRPM8 channels by siRNA transfection. Cellular morphological abnormalities with cell confluence were observed with wright-giemsa and methylene blue staining and given in (Fig. 3A-D). It is observed that downregulation of TRPM8 channels decrease cell viability by wright-giemsa modified staining kit (Fig. 3A & C). The morphological change was evaluated by also methylene blue staining and after the transfection counts of adhered viable cells were decreased (Fig. 3B & C).

The caspase 3, 8 and 9 enzymatic activities

Enzymatic activities of caspase 3, 8 and 9 among the groups were given in (Fig. 4A-C). In control group, caspase activities were lower than TRPM8 channel downregulated group, while enzyme activities in downregulated group was very higher than in control group. It is observed that downregulation of TRPM8 channels decreased cell viability and increased apoptosis *via* caspase mediated pathways.

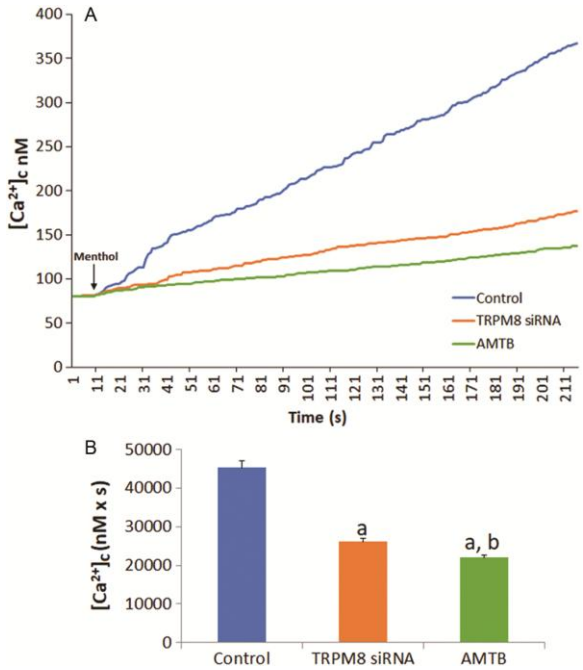

Fig. 2 — Determination of TRPM8 channel-mediated calcium signaling. (A) Time-dependent variation of the TRPM8 cation channel-mediated Fura-2 calcium signaling records; and (B) Findings of TRPM8 cation channel-mediated Fura-2 calcium analysis of control, TRPM8 downregulated and AMTB hydrochloride incubated groups. a: $P< 0.001$ *vs.* the Control group, b: $P< 0.005$ *vs.* the TRPM8 siRNA group

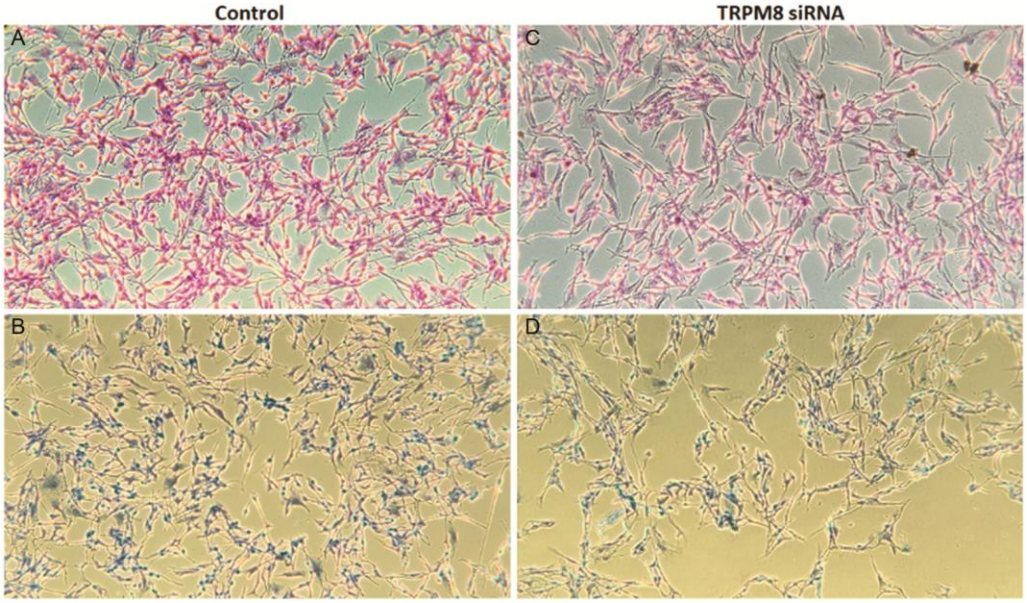

Fig. 3 — Wright-giemsa and methylene blue staining of DBTRG cells after completion of siRNA transfection. When wright-giemsa staining (A & C) aimed to show cell death status, methylene blue staining (B & D) shows morphological changes in DBTRG glioblastoma cells after downregulation of TRPM8 channels. Cells were examined and photographed under the light microscope at 100x magnification

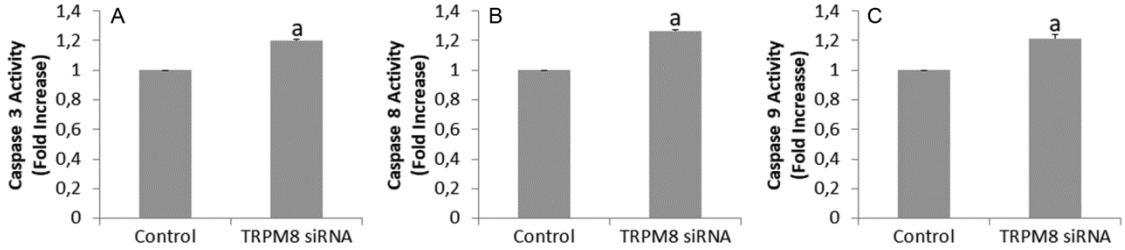

Fig. 4 — The (A) caspase 3; (B) caspase 8; and (C) caspase 9 enzyme activity findings of control and TRPM8 downregulated groups. a: p <0.001 *vs*. the Control group (mean ± SD; n=6)

Values of intracellular ROS production (iROS) and mitochondrial membrane depolarization (MMD)

The iROS production levels and MMD levels were given in (Fig. 5A & B). In control group, ROS production and MMD levels were lower than TRPM8 channel downregulated group, while enzyme activities in downregulated group was very higher than in control group. It is observed that downregulation of TRPM8 channels increased iROS and MMD values.

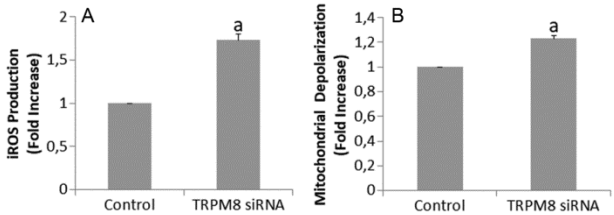

Fig. 5 — Determination of intracellular ROS production (A) and mitochondrial membrane depolarization (B) of study groups. a: *P*< 0.001 *vs.* the Control siRNA group (mean ± SD; n=6)

Findings of apoptosis and cell viability

Apoptosis levels of DBTRG cells were measured after siRNA transfection and the results were given in (Fig. 6A). In control group, apoptosis level was higher than in downregulation induced group. Moreover, incubation of AMTB at different concentrations also reduced cell viability in DBTRG cells were given in (Fig. 6B & C). It is observed that downregulation of TRPM8 channels increased apoptosis, and AMTB results also showed that TRPM8 related calcium trafficking could have positive effect on cell viability in DBTRG glioblastoma cells.

Discussion

Glial cells are second most common cell type in the brain after neurons, there are limited number of studies about TRP channels both on the level of gene expression and channel functionality (15). Glioblastoma multiforme is the most malign and

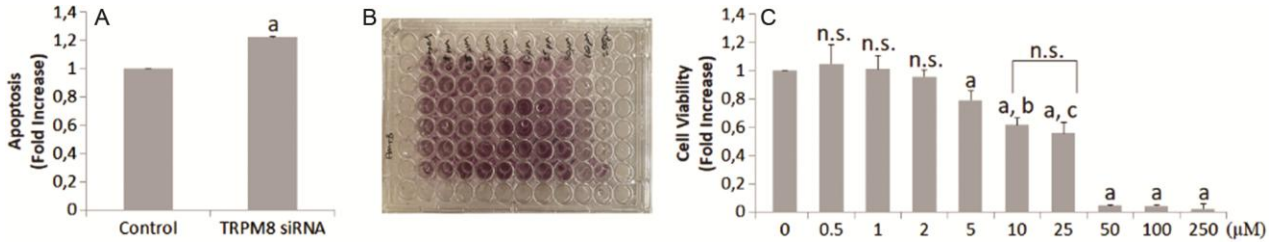

Fig. 6 — Determination of apoptosis and cell viability of DBTRG cells. (A) Apoptosis findings of control and TRPM8 downregulated groups. a: $P< 0.001$ *vs*. the Control group (mean ± SD; n=6); (B) The 96-well plate image of AMTB hydrochloride incubated DBTRG cells; and (C) Effects of different doses of AMTB hydrochloride incubation for 24 h on cell viability parameter in DBTRG cells. n.s.: no significance with Control group. There is also not significance between 10 μM and 25 μM group. a: $P< 0.001$ *vs.* the Control group, b: $P< 0.005$ *vs*. the 5 μM group, c: $P< 0.001$ *vs*. the 5 μM group (mean ± SD; n=8)

common type of glial cancer in the brain[23]. Until the date, although conventional approaches were adopted to anti-cancer activity on tumor cells based on phytochemical treatments[24-26], but novel methodologies including downregulation of target proteins has become more applicable against to proliferation of cancer cells[27]. To a recent hypothesis, TRP channels may have role also in cancer development, progression, and malignancy because of their effective role in Ca^{2+} signaling and TRP targeted strategies can be useful tool to achieve effective solutions to cancer development[28-31]. Latest years, different types of TRP channels have been investigated in cancer research and some of them are founded to be associated with the inhibition of cancer cell proliferation. When TRPV1 channels modulated pharmacologically metastatic properties decreased by the TRPV1 channel activation in cancer cell lines. Former studies have shown that TRPV1 targeted chemotherapeutic treatments can be more effective to reduce to cell viability of cancer cells[32,33]. In a previous study, Lepannetier *et al*. have investigated the effects of TRPC1 mediated calcium influx in U251 glioblastoma cells and they have founded that downregulation of TRPC1 reduces the chemotactic behavior in cells[11]. However, we have limited reports on TRPM8 cation channels which has found highest expression pattern among other type of TRP channels from human glioblastoma tissue samples[14]. In the study, researchers have investigated gene expression levels of 8 different TRP channel subtypes by using qRT-PCR in patients with glioblastoma. It was also concluded that since TRP channel expression levels may be related with the survival time of patients at more or less than 12 months after detection of the disease patients suffered from glioblastoma multiforme. TRPM8 channels are thought that potential biomarker in some cancer types[34]. There are also some reports how TRPM8 channels contribute to cell invasion in glioblastoma. In the study, researchers have been revealed that TRPM8 channel agonist menthol evoke cell migration in glioblastoma. Inhibition of TRPM8 by its non-specific (capsazepine or anthranilic acid) antagonist also decrease the migration capacity of glioblastoma cells. In a previous study, it has been suggested that menthol may have role in glioblastoma cell migration and proliferation and researchers have suggested that TRPM8 cation channels mediated calcium signaling could be necessary for glioblastoma cell migration[35]. In another research, authors concluded that TRPM8 channels are expressed in DBTRG glioblastoma, are required for cell survival and may be a therapeutic target against to glioblastoma[27]. Therefore, in this study we have investigated that how downregulation of TRPM8 channel expression regulate TRPM8 mediated calcium signaling, apoptosis, cellular morphology, iROS and MMD related processes in human DBTRG glioblastoma cells. We also used channel specific antagonist to evaluate how pharmacologically inhibition of TRPM8 channel activity regulates cellular viability of glioblastoma cells. Alike a former study, we have observed that TRPM8 channels may contribute to cell survival of glioblastoma cells and incubation with different concentrations of AMTB hydrochloride decrease to cell viability in DBTRG cells[36]. Moreover, downregulation of TRPM8 channels was another inhibitor factor on cell viability and it is also confirmed to a former study. It is reported that low TRPM8 channel expression levels increase patient survival with Kaplan-Meier plotter survival analysis, and they have also concluded that downregulation of TRPM8 cation channel expression by using short hairpin RNA (shRNA) increase glioblastoma cell death *via* mitogen-activated protein kinase mediated pathway in U251 glioblastoma cells[36]. Similarly, we

also found that downregulation of TRPM8 channels by siRNA transfections, appreciably decreased the cell spread intensity in cell culture vessels. To draw a frame for cell death mechanism, we also performed that caspase 3, 8 and 9 enzyme activities and apoptosis assay. We found that caspase enzymes activity raised by the downregulation of the channel protein and apoptosis levels increased. Morphological changes and the cell death were proved also by methylene blue and wright-giemsa staining. Calcium signaling is very important for each cell type and also for cancer cell progression. Several studies revealed that inhibition of calcium influx could be increase of apoptosis in cancer cells[37-40]. Until the date, it has been elucidated that TRP channels are mostly located on neuronal cell membranes and may have a role in neuronal apoptosis by excessive increase of intracellular calcium concentrations *via* TRP channel related cellular activities[41,42]. TRPM8 related calcium influx analyzed with Fura-2 calcium signaling assay and we found that downregulation of the channels decreases channel-mediated calcium release. Moreover, pharmacologically inhibition of TRPM8 channels by using AMTB much more decreased calcium flow from extracellular media, and it is significantly observed that both mechanisms of channel inhibition increase cell death and decrease viability by calcium-dependent pathway. These findings inversely correlated to a former study that channel gating induced by menthol increased migration potential of glioblastoma[12]. In this context, we conclude that TRPM8 channels could be a potential therapeutic target for glioblastoma and similar type of cancers.

Conflicts of interest

The authors declare no conflicts of interest.

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