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Original Article

Epigallocatechin-3-gallate induces apoptosis and proliferation inhibition of glioma cell through suppressing JAK2/STAT3 signaling pathway

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Abstract: Excess proliferation and apoptosis inhibition are the major pathologic feature of glioma. Epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, has been considered a potential therapeutic and chemo-preventive agent for cancer. However, its effect and mechanism on glioma remain to be elucidated. In this study, the effect of Epigallocatechin-3-gallate (EGCG) on glimas and its mechanism were investigated in cultured U251 cells by the methylthiazoletetrazolium (MTT), flow cytometry and western blotting. The results showed that treatment with EGCG can significantly suppress the U251 cell proliferation and induce the apoptosis of U251 cells. The mechanism of EGCG inducing apoptosis and proliferation inhibition of U251 cell were assocaited with suppressing JAK2/STAT3 signaling activation. Taken together, EGCG contribute to the favorable effects of treatment in U251 cell by suppressing JAK2/STAT3 signaling pathways activation.

Keywords: Glioma, epigallocatechin-3-gallate, proliferation, apoptosis, JAK2/STAT3

Introduction

Gliomas are one of the most widle spread malignant cancers rooted in astroglial or astrocytes cells and can damage the central nervous system [1]. Convential treatment incorporats the surgical resection, radiation, chemotherapy, and biological treatment. However, convential therapies for Gliomas are ineffective or insensitive; Therefore, the survival rate is guite low, less than 1 year after standard treatment [2, 3]. Therefore, it is an urgent need to develop more therapy strategies or seek for safety and effective agents for Gliomas.

Epigallocatechin-3-gallate (EGCG) is the major component of polyphenols in green tea and is widely investigated due to its ability to anti-inflammatory, anti-proliferative, and anti-carcinogenic activity [4-6]; however, its molecular mechanisms of its action in Gliomas is still unknown

Janus-activated kinase-2 (JAK2) Signal transducer and activator of transcription-3 (STAT3) signaling have been demonstrated that play an important role in proliferation, apoptosis of gliomas [7, 8] and EGCG have anti-tumor activation in skin tumors by suppressing JAK2/STAT3 signaling [9].

In this study, we investigated the anti-tumor effects of the EGCG on giliomas and the involvement of JAK2/STAT3 signaling in this process. We demonstrate that inhibition of JAK2/STAT3 signaling by EGCG may contribute to its antitumor action in gliomas.

Materials and methods

Reagents

EGCG was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The annexin V-FITC apoptosis detection kit was from Beckman Coulter (Fullerton, CA). Primary antibodies to survivin, Bcl-2, Bax, p-caspase-3, and p-PARP-poly (ADP-ribosyl) polymerase (PARP), b-actin and secondary antibodies were purchased from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to JAK2, p-JAK2, STAT3, and p-STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Human p-STAT3 small interfering RNA (siRNA) and control siRNA were all purchased from Santa Cruz Biotechnology.

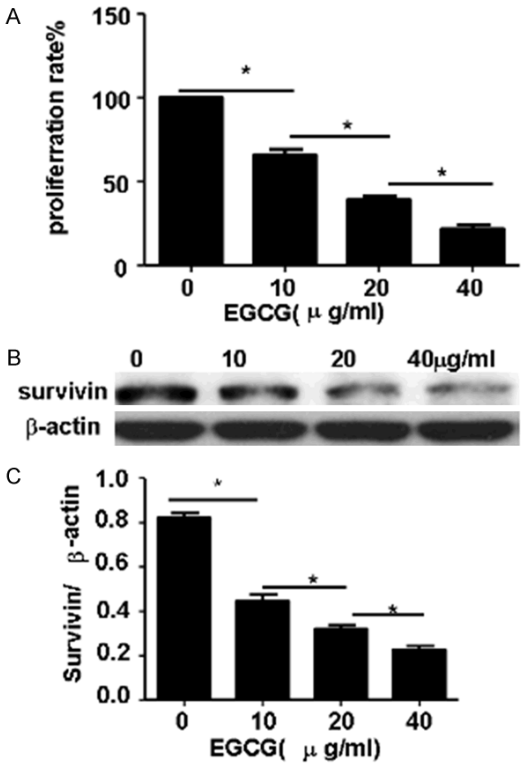

Figure 1. EGCG inhibits U251 cell proliferation. A. Analysis of U251 cell proliferation following treatment of EGCG. U251 cells were treated (72 hours) at increasing doses (0-40 μg/ml). Cell proliferation was evaluated by MTT assay. Columns, mean (n = 3, in triplicate); bars, SD. $^*P < 0.05$; $^{**}P < 0.01$. B. EGCG treatment of U251 cells reduces expression of proliferative proteins. Western blot analyses of U251 cells treated (72 hours) with EGCG to evaluate protein levels of cycl survivin. C. The optical density of surviving protein was quantified by b-actin optical density.

Cells

Human gliomas cell lines U251 was from ATCC and was grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). To obtain the STAT3C-expressing cells, U251 cells were transiently transfected with plasmids containing pRC/CMV-vector and pRC/CMV-STAT3C-Flag using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The murine cell line Renca was also obtained from ATCC and was grown in RPMI 1640 supplemented with 10% FBS.

MTT assay of cell proliferation

Cells, 3×10^3 per well, were seeded in 96-well culture plates the day before EGCG treatment. After the treatment, 20 μl of MTT reagent, 5 mg/ml, was added to each well and incubated for 4 h at 37°C. At the end of incubation, the media were carefully removed by aspiration. One hundred microlitre of DMSO was then added to each well. The plate was gently vortexed for 30 min at room temperature. The absorbance of each well was measured at 490 nm. All experiments were repeated at least three times.

Apoptosis assay

U251 cell were seeded in 60-mm culture dishes in RPMI-1640 with 1% FBS. The following day, cells were treated with indicated concentrations of EGCG for 24-hours. After treatment, floating and attached cells were collected and stained with PI and Annexin V-FITC Apoptosis Detection kit (BD Biosciences) in FACS Wash Buffer (HBSS2/2 containing 2% FBS) according to the manufacturer's instruction. Viable and apoptotic cells were analyzed by flow cytometry (Accuri C6). Data was analyzed using FlowJo software (Treestar).

Western blot

Total protein (20 mg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinyllidene difluoride membrane. Membranes were blocked for 1 hour at ambient room temperature (ART) in 10% non-fat dry milk in TBST (16TBS with 0.1% Tween 20) followed by an overnight incubation at 4uC with primary antibodies in TBST with 5% BSA. Horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were added for 1 hour at ART and detected with Super Signal West Pico substrate (Pierce). Bands were measured as optical density using ImageJ software. The optical density of each band was normalized by b-actin optical density.

Plasmid transfection

U251 cells were transiently transfected with human STAT3 siRNA and control siRNA using LipofectamineTM 2000 (Invitrogen). After 24 hours transfection, cells were treated with EGCG or DMSO control for 24 hours and cell viability was measured siRNA and control siRNA using LipofectamineTM 2000 (Invitrogen).

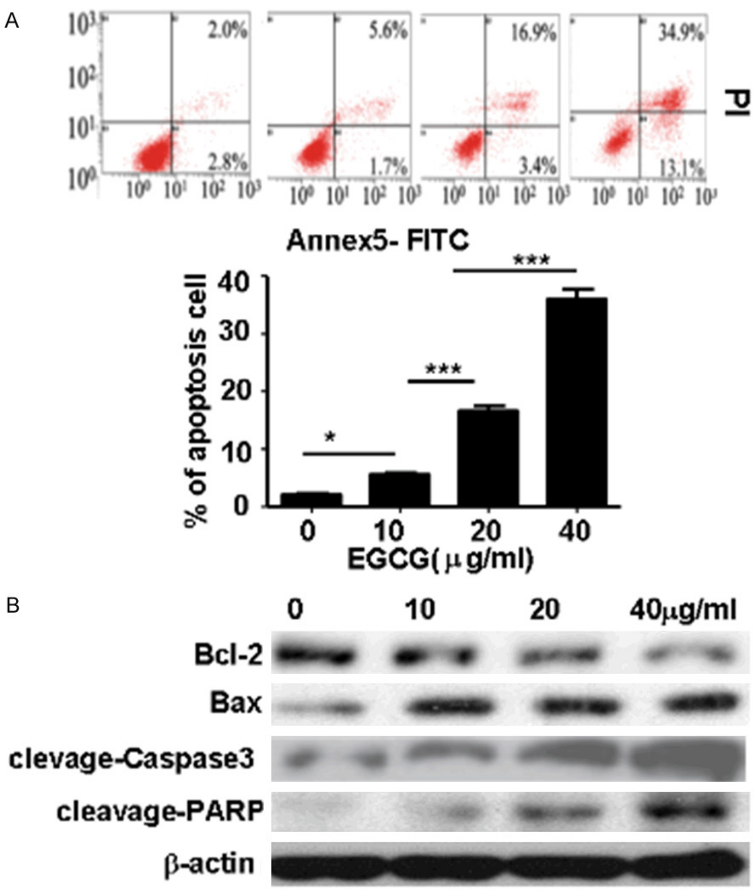

Figure 2. EGCG induces apoptosis in U251 cells. A. Analysis of U251 cell apoptosis following treatment of EGCG. U251 cells were treated (72 hours) at indicated doses, harvested, and stained with Annexin V-FITC and PI. Annexin V-FITC positive apoptotic cells were determined by flow cytometry. Columns, mean (n = 3, in triplicate); bars, SD. B. EGCG treatment of U251 cells regulate the expression of apoptosis related proteins. Western blot analyses of U251 cells treated (72 hours) with EGCG, to evaluate protein levels of Bcl-2, Bax, cleaved PARP and cleaved caspase3.

After 24 hours transfection, cells were treated with EGCG or DMSO control for 24 hours and cell viability was measured

Statistical analysis

All experiments were performed in triplicates. The results are expressed as mean ± SD. For statistical analysis, Student's T-tests were performed using SPSS software. Statistical significance was accepted at the level of $P < 0.05$.

Results

EGCG inhibits the proliferation of U251 cells

To determine whether EGCG has direct anti-tumor effects in gliomas cells, the U251 cells was treated with different concentrations of EGCG by MTT assay. As shown in **Figure 1A**, EGCG showed significant inhibition of cell proliferation in a concentration-dependent manner and proliferation inhibition is over 40% at 10 μg/ml (**Figure 1A**). Western blotting was also performed to determine the downstream factors mediating the effects of EGCG on U251 cells. The results showed that EGCG treatment of U251 can reduce the expression of key pro-proliferative proteins, survivin.

EGCG induce the apoptosis of U251 cells

We next investigated whether EGCG induced the apoptosis of U251 cells. After treatment with EGCG for 72 hours, 50% U251 tumor cells were Annexin-V positive as defined by flow cytometry (**Figure 2A**). To further confirm the activation of EGCG in inducing apoptosis of U251 cells, we detected the expression levels of Bax, Bcl-2, activated caspase-3 and PARP cleavage. EGCG can significantly increase the expression of Bax, cleaved-caspase-3 and cleaved PARP, along with decreasing the expression of Bcl-2 in a concentration-dependent manner (**Figure 2A**). Collectively, these data indicated that EGCG has potent anti-proliferation and pro-apoptotic effects on human U251 cells.

EGCG inhibits JAK2/STAT3 signaling in U251 cells

To explore the underlying mechanisms of EGCG anti-tumor effect on U251 cells, we selectd the JAK2/STAT3 signalling. STAT3 is constitutively activated in diverse cancers, including U251, we assessed whether EGCG inducing the apoptosis of U251 was associated with STAT3 inhibition. Theses results showed that EGCG had no effects on total STAT3 protein levels in U251

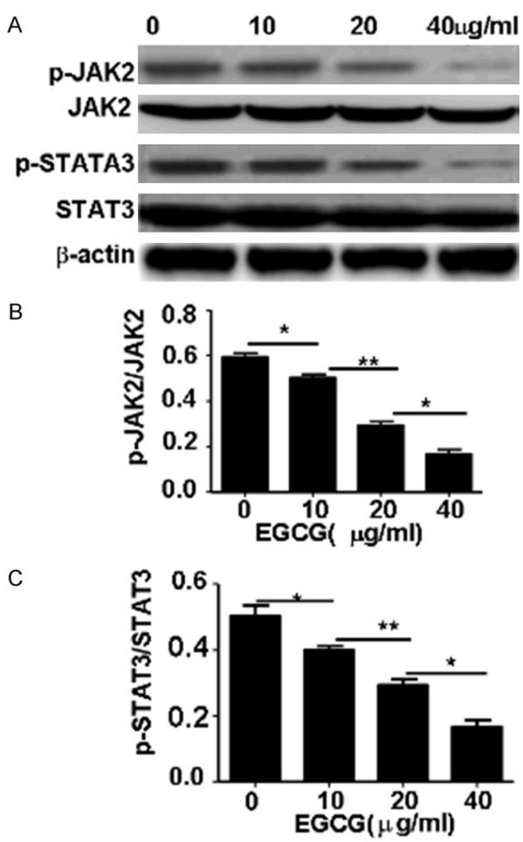

Figure 3. Effect of EGCG on the expression of JAK2 and STAT3t. A. A representative result for Western blot analysis the expression of JAK2 and STAT3; B. Semi-quantitative analysis of U251 cell studied in each group. The relative amount of JAK2 in each group was normalized by β-actin, $^{*}P < 0.05$; C. Semi-quantitative analysis of U251 cell studied in each group. The relative amount of STAT3 and p-STAT3 in each group was normalized by β-actin and presented as a ratio between STAT3 and p-STAT3.

cells, but it can inhibite the expression of activated STAT3(p-STAT3) at 72 h after EGCG treatment (**Figure 3A**). We further assessed the potential effects of EGCG on the expression of JAK2, which is also widley activated in cancer cells. U251 tumor cells showed the the high expression of activated JAK2 (p-JAK2), which was decreased by EGCG in a concentration-dependent manner (**Figure 3A**). The results suggest that EGCG could inhibit the activation of JAK2/STAT3 signaling pathway n in a - concentration dependent manner.

EGCG-inducing apoptosis is regulated by STAT3 signaling in U251 cells

To further investigate whether STAT3 activity directly influences the biological effects of EGCG in U251 cells, an expression vector encoding a constitutively-active STAT3 mutant, STAT3C [10] or an empty control vector (vector) were transfected into U251 cells. Transfected cells were confirmed by Western blot analysis (**Figure 4A** Left). Expression of constitutively-active STAT3 in U251 cell promoted resistance to the anti-proliferative and pro-apoptotic effects of EGCG (**Figure 4A-C** Right). Our initial results (**Figure 1B**) showed that EGCG treatment inhibited several STAT3-regulated proteins important for tumor cell survival and proliferation. In agreement with this finding, siRNA-mediated knockdown of STAT3 in U251 cells significantly reduced the expression of known STAT3 downstream genes, survive (**Figure 4B**). We further demonstrated that siRNA-mediated knockdown of STAT3 sensitized U251 cells to the anti-proliferative and inducing-apoptosis effects of EGCG (**Figure 4D-F**).

Discussion

In this study, we evaluated the therapeutic potential of EGCG against gliomas and its potential mechanism of action. In line with previous study that EGCG has the anti-tumor action in the gliomas by suppressing the proliferation and inducing apoptosis of U251 cell [11, 12]. It is well know that activated STAT3 promotes tumorigenesis by inducing apoptosis and inhibiting proliferation [13, 14]. In various cancer types, including leukemias and solid cancers of the colon, prostate and pancreas, aberrant activation of STAT3 crucially contributes to cancer progression [15]. STAT3 is constitutively activated in human gliomas and is an independent prognostic indicator which indicated STAT3 represents a promising therapeutic target for the treatment of gliomas [16-18]. Several study showed several small molecule inhibitors of STAT3 could decrease the proliferation and induce apoptosis in tumor cell. But whether this inhibitor required STAT3 for its anti-tumor effects was not directly assessed. EGCG, a novel a major polyphenol in green tea, has been recently reported with anticancer effects, inhibiting growth of breast cancer, skin cancer and chronic myeloid leukemia cells [13-15]. Our results demonstrate that EGCG could suppress STAT3 activation, in part through inactivation of upstream JAK2 in U251 cell lines. JAK2 phosphorylate leads to the recruitment and activation of the STAT3, which then leads to STAT3-mediated transcriptional regulation. In our

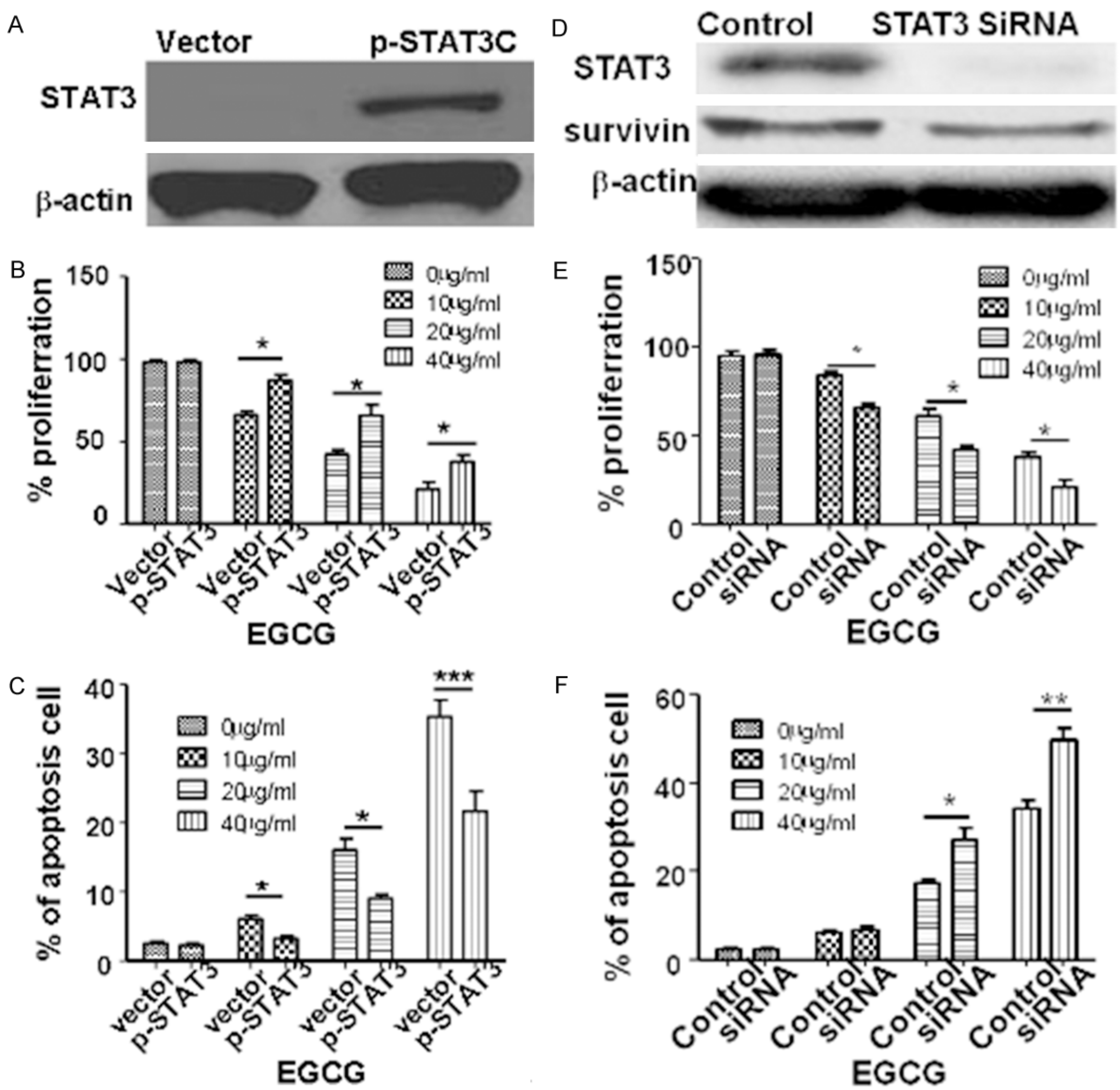

Figure 4. Levels of STAT3 activity affects the direct antitumor effects of EGCG. A. Over expression of a constitutively activated STAT3 (STAT3C) rescues U251 cells from apoptosis induced by EGCG. Pooled U251 tumor cells containing a control vector, pRC-vector, or the pRC-STAT3C expression vector were treated (72 hours) with EGCG at different concentrations (0, 10, 20, 40 μg/ml). B. Cell proliferation was analyzed by MTT assay. C. Tumor cells positive for both Annexin Vand PI, as determined by flow cytometry, were considered apoptotic. Columns, mean (n = 3, in triplicate); bars, SD. $^{*}P < 0.05$; $^{**}P < 0.01$. D. STAT3 inhibition reduces expression of genes important for proliferation. U251 tumor cells were transfected with STAT3 or control siRNAs and total cell lysates were collected 72 hours after transfection. Western blot analyses of lysates with indicated antibodies. E. Knockdown of STAT3 enhances the effects of EGCG on U251 tumor cell growth arrest. U251 tumor cells transfected with either control or STAT3 siRNA followed by treatment (72 hours). With EGCG at indicated doses. Cell proliferation was analyzed by MTT assay Columns, mean (n = 3, in triplicate); bars, SD. $^{*}P < 0.05$; $^{**}P < 0.01$. F. Tumor cells positive for both Annexin V and PI, as determined by flow cytometry, were considered apoptotic. Columns, mean (n = 3, in triplicate); bars, SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

study, we found that the U251 cell is constitutive activation of STAT3 and EGCG treatment dramatically inhibited STAT3 activity, associated with upstream JAK2 inhibition. We further demonstrated that the anti-proliferative and pro-apoptotic effect of EGCG in U251 cells was mediated, in part, by inhibition of STAT3 activation. Activated STAT3 has been shown to protect tumor cells from apoptosis by inducing proliferation/survival genes and blunting pro-apoptotic genes [19].

Conclusions

In summary, we demonstrated convincing evidence that EGCG had the effects of inducing apoptosis and inhibiting proliferation in U251 cells in vitro through inhibiting JAK2/STAT3 signaling activation. All together, our data supports that EGCG could be a good alternative therapy for treatment of gliomas in the clinical practice.

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Disclosure of conflict of interest

None.

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References

[1] Jha MK, Suk K. Pyruvate Dehydrogenase Kinase as a Potential Therapeutic Target for Malignant Gliomas. Brain Tumor Res Treat 2013; 1: 57-63.

[2] Farina P, Lombardi G, Bergo E, Roma A, Zagonel V. Treatment of Malignant Gliomas in Elderly Patients: A Concise Overview of the Literature. Biomed Res Int 2014; 2014: 734281.

[3] Nagane M. [Anti-angiogenic therapy for malignant glioma]. Gan To Kagaku Ryoho 2014; 41: 141-147.

[4] Cai J, Jing D, Shi M, Liu Y, Lin T, Xie Z, Zhu Y, Zhao H, Shi X, Du F, Zhao G. Epigallocatechin gallate (EGCG) attenuates infrasound-induced neuronal impairment by inhibiting microglia-mediated inflammation. J Nutr Biochem 2014; 25: 716-725.

[5] Sakamoto Y, Terashita N, Muraguchi T, Fukusato T, Kubota S. Effects of epigallocatechin-3-gallate (EGCG) on A549 lung cancer tumor growth and angiogenesis. Biosci Biotechnol Biochem 2013; 77: 1799-1803.

[6] Ellis LZ, Liu W, Luo Y, Okamoto M, Qu D, Dunn JH, Fujita M. Green tea polyphenol epigallocatechin-3-gallate suppresses melanoma growth by inhibiting inflammasome and IL-1beta secretion. Biochem Biophys Res Commun 2011 28; 414: 551-556.

[7] Stechishin OD, Luchman HA, Ruan Y, Blough MD, Nguyen SA, Kelly JJ, Cairncross JG, Weiss S. On-target JAK2/STAT3 inhibition slows disease progression in orthotopic xenografts of human glioblastoma brain tumor stem cells. Neuro Oncol 2013; 15: 198-207.

[8] van Cruijsen H, Oosterhoff D, Lindenberg JJ, Lougheed SM, Fehres C, Weijers K, van Boerdonk R, Giaccone G, Scheper RJ, Hoekman K, de Gruijl TD. Glioblastoma-induced inhibition of Langerhans cell differentiation from CD34(+) precursors is mediated by IL-6 but unaffected by JAK2/STAT3 inhibition. Immunotherapy 2011; 3: 1051-1061.

[9] Park G, Yoon BS, Moon JH, Kim B, Jun EK, Oh S, Kim H, Song HJ, Noh JY, Oh C, You S. Green tea polyphenol epigallocatechin-3-gallate suppresses collagen production and proliferation in keloid fibroblasts via inhibition of the STAT3-signaling pathway. J Invest Dermatol 2008; 128: 2429-2441.

[10] Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. Cell 1999; 98: 295-303.

[11] Salem MM, Davidorf FH, Abdel-Rahman MH. In vitro anti-uveal melanoma activity of phenolic compounds from the Egyptian medicinal plant Acacia nilotica. Fitoterapia 2011; 82: 1279-1284.

[12] Chen TC, Wang W, Golden EB, Thomas S, Sivakumar W, Hofman FM, Louie SG, Schönthal AH. Green tea epigallocatechin gallate enhances therapeutic efficacy of temozolomide in orthotopic mouse glioblastoma models. Cancer Lett 2011; 302: 100-8.

[13] Wang Y, Ning H, Ren F, Zhang Y, Rong Y, Su F, Cai C, Jin Z, Li Z, Gong X, Zhai Y, Wang D, Jia B, Qiu Y, Tomita Y, Sung JJ, Yu J, Irwin DM, Yang X, Fu X, Chin YE, Chang Z. GdX/UBL4A specifically stabilizes the TC45/STAT3 association and promotes dephosphorylation of STAT3 to repress tumorigenesis. Mol Cell 2014; 53: 752-765.

[14] Lin S, YuJun L, XiaoMing X, WenWen R. Expression and significance of leptin receptor, p-STAT3 and p-AKT in diffuse large B-cell lymphoma. Acta Histochemica 2014; 116: 126-30.

[15] Yu H, Jove R. The STATs of cancer--new molecular targets come of age. Nat Rev Cancer 2004; 4: 97-105.

[16] Shang D, Yang P, Liu Y, Song J, Zhang F, Tian Y. Interferon-alpha induces G1 cell-cycle arrest in renal cell carcinoma cells via activation of Jak-Stat signaling. Cancer Invest 2011; 29: 347-52.

[17] Shang D, Liu Y, Ito N, Kamoto T, Ogawa O. Defective Jak-Stat activation in renal cell carcinoma is associated with interferon-alpha resistance. Cancer Sci 2007; 98: 1259-1264.

[18] El-Hashemite N, Kwiatkowski DJ. Interferon-gamma-Jak-Stat signaling in pulmonary lymphangioleiomyomatosis and renal angiomyoli-

poma: a potential therapeutic target. Am J Respir Cell Mol Biol 2005; 33: 227-230.
[19] Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernández-Luna JL, Nuñez G, Dalton WS, Jove R. *Constitutive activation* of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999; 10: 105-115.