S-Adenosylmethionine - induced cytotoxicity in glioblastoma cells does not affect the Igf-1r methylation

Citotoxicitatea indusă de S-adenosilmetionină în celulele de glioblastom nu afectează metilarea Igf-1r

Roxana Ola[1,2], Iulia D. Scorei[3], Ioana Berindan Neagoe[2,4], Ovidiu Bălăcescu[4,] Mihai Bănicioiu[3], Anica Dricu[3,5*]

1. Bio1. chemistry and Developmental Biology, Institute of Biomedicine, University of Helsinki, Finland
2. Department of Genetics, Babes-Bolyai University, Cluj-Napoca, Romania
3. Department of Biochemistry, University of Medicine and Pharmacy Craiova, Romania
4. Oncological Institute "I. Chiricuta", Cluj Napoca, Romania
5. Department of Oncology-Pathology, Karolinska Institute, Stockholm, Sweden

Abstract

Despite treatment consisting of intensive multimodality therapy, glioblastoma (GB) patients have a very poor prognosis explaining the need of searching for novel therapeutic approaches. Exogenous administration of S-Adenosylmethionine (SAM), a methyl donor agent, was suggested to induce cancer cell death by affecting DNA methylation pattern. In this study, we investigate the cytotoxic effect of SAM on two glioblastoma (GB) cell lines (18 and 38) in vitro. For this purpose, we treated the cells with increasing doses of SAM, and analyzed the cell viability by MTT assay, 7 days after the treatment. We found that SAM treatment induced cytotoxicity in both cell lines, but the 38 cells were more sensitive than 18 cells. This biological methyl donor was demonstrated to inhibit the mitogenic effect of growth factors in cancer cells. Here, we hypothesize that SAM induced igf-1r hypermethylation and subsequently IGF-1R downregulation, would be the cause of the cytotoxicity of the SAM treatment in GB cells. This hypothesis was encouraged by our previous results, showing that IGF-1R function is important for GB cell survival and proliferation. First, we found that in both GB cell lines expressing IGF-1R, the igf-1r was partially methylated. To our knowledge this is the first report describing the methylation status of igf-1r promoter gene in glioblastoma cells. The next set of experiments indicated that SAM treatment did not modify the igf-1r CpG island methylation status or the expression of the IGF-1R, suggesting that SAM - induced cytotoxicity is independent of igf-1r methylation in glioblastoma cell lines.

Keywords: *DNA methylation, glioblastoma, Igf-1r*

Rezumat

În cazul tumorilor gliale de tipul glioblastomului (GB), prognosticul este rezervat, chiar în urma unui tratament multimodal, justificând pe deplin necesitatea descoperirii de noi modalități terapeutice. Studii anterioare au

[*]**Corresponding author:** Anica Dricu, Department of Biochemistry, University of Medicine and Pharmacy Craiova, Romania, Bd 1 Mai, nr 62, 200322, Craiova, Romania.
Tel: +46 707321638, +40 729635161 E-mail address: anicadricu@webmail.umfcv.ro, anica.dricu@live.co.uk

arătat că S-adenozil-metionina (SAM), principalul donor de grupări metil în numeroase procese biologice, ar induce moartea celulelor canceroase prin modificarea profilului de metilare ADN. Alte studii ştiinţifice sugerează, că SAM inhibă efectul mitogen al factorilor de creştere în celulele canceroase. De asemenea, studiile noastre anterioare demonstrează importanţa IGF-1R în supravieţuirea şi proliferarea celulelor de GB. În acest studiu, a fost investigat efectul citotoxic indus de SAM în două linii celulare de GB (18 şi 38). Celulele în cultură au fost expuse timp de 7 zile la diferite concentraţii de SAM, iar viabilitatea celulară a fost analizată prin metoda MTT. Rezultatele obţinute au demonstrat, că SAM induce citotoxicitate în ambele linii celulare studiate, cu un effect mai pronunţat în linia celulară 38. Pentru explicarea citotoxicităţii observate în urma tratamentelor cu diferite doze de SAM, s-a analizat posibilitatea că efectul citotoxic indus de SAM să fie cauzat de hipermetilarea oncogenei igf-1r şi inhibarea expresiei proteinei IGF-1R. Rezultatele obţinute au arătat că gena igf-1r este parţial metilată în cele 2 linii celulare de GB. De asemenea, SAM nu are efect asupra profilului de metilare al genei igf-1r. Toate aceste date sugerează faptul că efectul citotoxic indus de SAM nu este relaţionat cu modificarea profilului de metilare ADN al genei igf-1r.

Cuvinte cheie: *Metilare ADN, glioblastom, Igf-1r*

Introduction

Insulin-like growth factor receptor-1 (IGF-1R) is a receptor tyrosine kinase, encoded by the igf-1r oncogene, located on chromosome 15q25–q26, promoting oncogenic transformation, growth and survival of cancer cells (1). IGF-1R overproduction or enhanced activity has been observed in many types of cancer including breast (2, 3), lung (4, 5), colon (6), prostate (7), melanoma tumor types (8) and glial tumors (9).

In glioblastoma, igf-1r was identified as a potential genetic marker (10) and also represents an activator of downstream signal transduction pathways which enhances therapeutic resistance against radiation and chemotherapy (9, 11, 12).

The igf-1r gene expression levels are determined, to a large extent, at the transcriptional level. Molecular characterization of the igf-1r regulatory region revealed that igf-r1 gene promoter region lacks a TATA box, the gene consists in a single specific transcription initiation site and both the 5′ flanking and 5′untranslated region are highly CG-rich (13, 14).

The molecular mechanism responsible for the increased transcriptional activation of igf-1r gene in cancer, however, remains largely unidentified.

Mutations in the igf-1r gene have been shown to lead to abnormalities in the function or number of IGF-1R and may also delay intrauterine and subsequent growth in humans (15). Amplification of the igf-1r locus at band 15q2 has been found in a small number of breast and melanoma cases (2). DNA methylation is an important epigenetic factor, inversely associated with gene expression (16). This process involves a covalent addition of a methyl group to the Cytosine in the next vicinity to Guanine to 5'position of promotor region (16).

Abnormal methylation patterns have been detected early in cancers, including those of the glioblastoma (16*, 17). These changes consist mainly of global DNA hypomethylation, regional DNA hypermethylation and overexpression of DNA methyltransferase 1 (18).

Despite the fact that the pathway leading to hypomethylation in cancer is unknown, it is clear that hypomethylation is a hallmark of most cancer genomes. It has been proposed that hypomethylation contributes to malignancy by direct activation of several oncogenes including c-myc (19), h-ras (20), hox1 (21) and c-ros (22), by activation of latent retrotransposons (23) and/or by inducing chromosome instability (24).

Several reports have suggested that exogenous administration of a methyl donor agent (SAM), the primary substrate for methyltransferase reaction leads to DNA hypermethylation (25, 26) induced cytotoxicity in cancer cells by silencing genes involved in cell proliferation (27).

In this study, we investigate the cytotoxic effect of SAM on two glioblastoma (GB) cell lines. We also hypothesize that SAM in-

duced igf-1r hypermethylation and subsequently IGF-1R downregulation, would be the cause of the cytotoxicity of the SAM treatment in GB cells.

Materials and methods

Cell lines and reagents

For these studies we used 2 primary glioblastoma cell lines (18, 38) which were established from tumors at the Academic University Hospital in Uppsala according to standard procedures (28) and have been previously characterized (29).

The cell lines were cultured in MEM containing 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotic (100 IU/ml penicillin and 100 IU/ml streptomycin).

The cells were grown in tissue culture flasks and incubated at 37°C, 5% CO2, and 90% relative humidity.

MTT assay

The assay is based upon the cleavage of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by metabolically active cells. $4x10^3$ cells/well were cultured in 0.2 ml medium, seeded in 96-well culture plates and incubated for 7 days in growth medium with increasing concentrations of S'Adenosylmethyonine (SAM) (New England Bio-labs) ranging from 0.1 μM to 200 μM. The medium was renewed every second day with fresh amounts of SAM. After treatment, 10 μl of MTT labeling reagent were added to each well and plates were incubated at 37 C for 4 h. Following MTT incubation, the cultures were solubilized and a cell growth curve analysis on control cells (untreated cells) and cells treated with SAM was performed.

Cell treatment

For the experimental purposes 600.000 cells were cultured in 60 mm dishes and treated with increasing concentrations of SAM (New England Bio-labs) ranging from 50 μM to 200 μM added to the regular growth media under sterile conditions for 7 days. Culture media was changed every second day and fresh SAM was added.

DNA extraction

Approximately 2 x106 cells from each cell line were collected, centrifuged and resuspended in 200 μl PBS-1X. DNA was extracted using DNA extraction kit (Viogene, Sweden). The quality and integrity of the extracted DNA was evaluated in terms of A 260/280 ratio.

Bisulphite treatment

One microgram of DNA was modified using the CpGenome DNA modification kit (Chemicon, Sweden) as per the manufacturer's instructions. Bisulphite causes deamination of cytosine that is transformed to uracyl (thymine) unless the cytosine is methylated, in this case it remains as cytosine. All samples were resuspended in 25 μl of TE and 1 μl of this was used for subsequent PCR reactions.

Methylation-specific PCR (MSP)

Methylation status of the samples was investigated by MSP as previously described (30). PCR reactions were performed in 25-μl volumes containing 1x manufacterer's buffer, 1 unit of Platinum Taq DNA Polymerase (Invitrogen, Sweden), 6 mM MgCl2, 10 mM dNTP and 10 pmol/μl of each primer. PCR was performed with one cycle of 94^0C for 10 min, 37 cycles of 92^0C for 15 s, 60^0C for 30 s and 72^0C for 30 s, followed by one cycle of 72^0C for 7 min.

Bisulphite modified DNA was amplified with primers designed using the program "Meth Primer: designing primers for methylation PCRs" (31): unmethylated forward primer: 5'-GGAGGTTGATGATGTTGATAGTTT-3'; unmethylated reverse primer 5'-AAAACAAAAA AAACAATACTCCAA-3' (160-bp), and methylated forward primer 5'-GTCGACGACGTCG ATAGTTC-3' and methylated reverse primer 5'-AAAAACGAAAACAATACTCCG-3' (165-bp).

Universal methylated human male genomic DNA (New England Biolabs) was used as positive control for the methylation reaction.

Genomic DNA purified from peripheral blood of a healthy voluntary donor was used as the control for the unmethylated reaction. A blank control containing all PCR products but no DNA template was also included in all PCR reactions.

Resulted products were separated by agarose gel electrophoresis 2%, stained with ethidium bromide and visualized under UV illumination.

Protein measurement

Protein content of cell lysates was determined by a dye-binding assay with a reagent purchased from BioRad. Bovine serum albumin was used as a standard. Protein was determined according to the Bradford method (32).

Western blot

IGF-1R protein expression in glioblastoma cells was examined by Western blotting. Briefly, 100 mg of total cell lysate protein from untreated and treated cells was electrophoretically separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. After fixing and blocking, a polyclonal rabbit antibody reactive to the β-subunit of IGF-1R and a monoclonal mouse antibody reactive to β-Actin (Santa Cruz Biotechnology) were used to probe the membranes and visualized using the ECL system (Amersham Biosciences AB, Uppsala, Sweden).

Results

The effect of SAM treatment on glioblastoma viability

Exogenous administration of the methyl donor agent, S-Adenosylmethionine (SAM), leads to cancer cell death by affecting DNA methylation pattern (33).

To explore the possibility that SAM induces cytotoxicity in glioblastoma cells, we treated the cells with increasing doses of SAM and analyzed the cell viability by MTT assay. Cells were exposed to increased SAM concentrations (ranging from 0.1 to 200 µM) and incubated for 7 days. The medium was replaced every second day with fresh amounts of SAM. The growth inhibition profiles over 7 days SAM treatment is depicted in *Figure 1*. As illustrated in *Figure 1*, SAM treatment resulted in inducing dose-dependent cytotoxicity in both 18 and 38 GB cell lines. In 18 cell line, the cytotoxic effect induced by SAM treatment varied between 4% to 41%, depending of the concentration of the drug (0.1 to 200 µM) (*Figure 1A*). 38 GB cell line displayed increased sensitivity to SAM treatment than the 18 GB cell line, after 7 days of drug incubation. At the smallest drug concentration used (0.1 µM), the

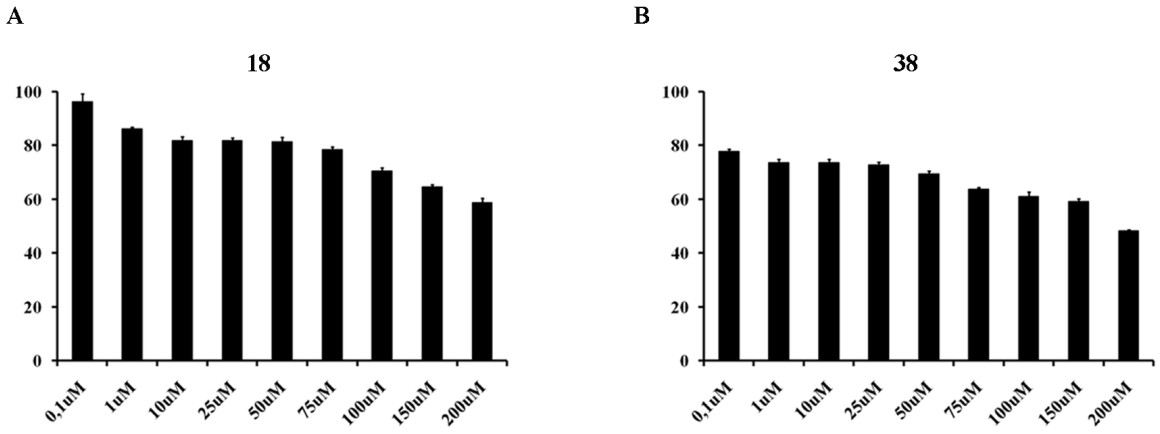

Figure 1. Cell toxicity assay after 7 days of treatment with increasing doses of S-adenosylmethyonine.
All cells were plated at exactly the same density of viable cell per plate in triplicates and were exposed to S-adenosylmethyonine at concentrations ranging from 0.1 to 200 µM in differentiating media for 7days. The medium was refreshed with S-adenosylmethyonine after 3 days. Cell toxicity assay for 18 (A) and 38 (B).

cytotoxicity induced in 38 cell line was 22%, compared to control cells. (*Figure 1B*). At the concentration of 200 µM, cell death induced by SAM treatment was 52% in 38 GB cell line (*Figure 1B)*.

Our results showed that SAM induced cytotoxicity in a concentration-dependent manner in GB cells.

Our results showed that SAM treatment induced an increase in the cytotoxicity in a dose-dependent manner, in both GB cell lines.

Igf-1r methylation status and the protein expression in glioblastoma cell lines

Igf-1r methylation status was investigated by the analysis of the methylation of igf-1r promotor region (49-698 nucleotides corresponding to the first CpG in the igf-1r promoter region) in 2 primary GB cell lines: 18, 38. Extracted DNA was treated with bisulfite and amplified with both methylated and unmethylated primers. Using primers (described above) specific for the unmethylated DNA, the 160 bp unmethylated igf-1r allele was detected in both glioblastoma cell lines (*Figure 1A*). Using primers specific for the methylated DNA, we obtained amplification of 165bp methylated igf-1r allele in the glioblastoma cell lines (*Figure 2A*). As controls for the methylated and unmethylated reaction we used universal methylated human genomic DNA (M-DNA) and human lymphocytes, respectively (*Figure 2A)*. Our data indicate that igf-1r is partially methylated in GB cell lines. To our knowledge this is the first report describing the methylation status of igf-1r promotor gene in GB cells.

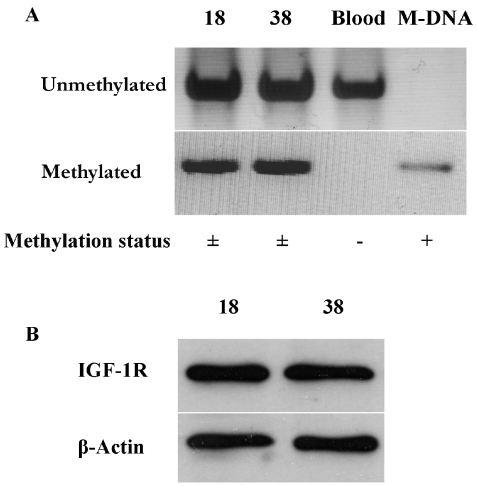

Figure 2. Methylation status of *igf-1r* promoter in glioblastoma cell lines.

The presence of the visible PCR product in unmethylated lanes indicates the presence of unmethylated allele (160 bp) of igf-1r, and the methylated lanes indicates the presence of methylated allele (165bp) of igf-1r; blood (positive control for unmethylated band), M-DNA, universal methylated human genomic DNA, (positive control for the methylated band); (A). IGF-1R expression in 18 and 38; β-actin (loading control protein) (B).

Next, we determined the IGF-1R protein expression levels in the glioblastoma cell lines. After membrane fractionation, the levels of the IGF-1R protein were assayed by western blotting. Our results show that both glioblastoma cell lines express IGF-1R protein at the cell surface (*Figure 2B*). β-Actin was used as a loading control.

The effect of the SAM treatment on IGF-1R expression pattern

The possibility that DNA methylation is involved in the regulation of igf-1r transcription levels and IGF-1R protein expression was investigated in 18 and 38 cell lines.

SAM is the principal methyl group donor in many biological methylation reactions, including DNA methylation.

To investigate if SAM induced cytotoxicity in glioblastoma cells interfered with igf-1r promoter methylation status, cells were treated with drug *concentrations ranging* from 50 to 200 µM of SAM for 7 days, and the igf-1r gene methylation and the protein expression were assessed as described above.

Surprisingly, we found that SAM treatment modified neither the igf-1r CpG island methylation status (*Figure 3*), nor the expression level of the IGF-1R protein in any of the glioblastoma cell lines analyzed (*Figure 4)*.

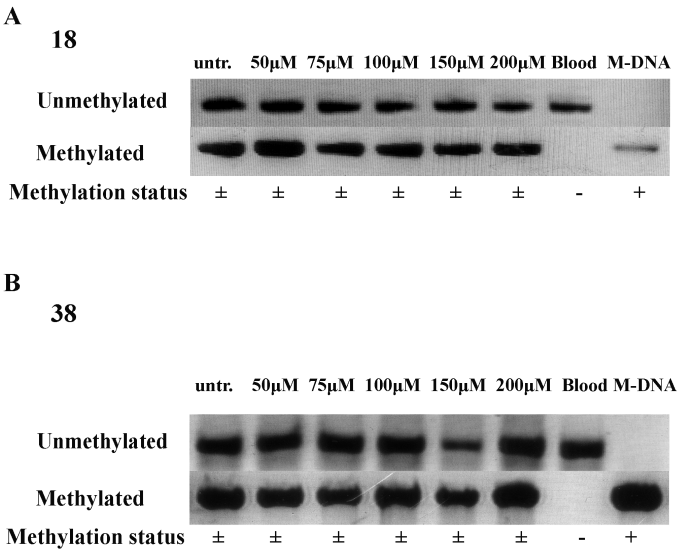

Figure 3. Methylation status of igf-1r promoter gene after SAM treatment.

All cells lines were treated with increased doses of SAM for 7 days and the MSP was performed on the extracted DNA from the treated cells in comparison with the untreated cells. The partial methylation status of igf-1r was maintained after the SAM treatment in both glioblastoma cell lines: 18 (A) and 38 (B).

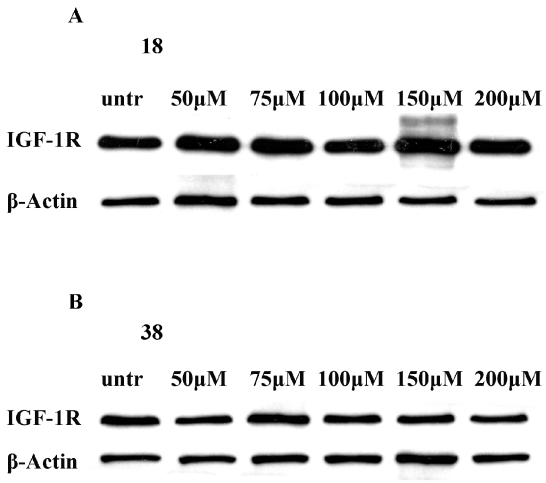

Figure 4. IGF-1R expression after SAM treatment in glioblastoma cell lines.

After 7 days of SAM treatment, cell lysates from untreated and treated cells were electrophoretically separated on a 10% SDS-PAGE gel and IGF-1R expression levels were detected in comparison with the untreated cells in glioblatoma cell lines: 18 (A) and 38 (B). β –actin was used as a loading control.

Discussions

Glioblastomas (GB) remain one of the most lethal forms of cancer. Despite, the multimodality treatment including: craniotomy, gamma knife radiosurgery, chemoradiotherapy and targeted therapy, GB patients have a very poor prognosis explaining the need of searching for new approaches in the treatment of this disease.

Recent study showed that SAM (S-Adenosyl methionine) is an attractive agent for treatment of liver cancer (34). More importantly, it has been demonstrated that SAM inhibits apoptosis in normal hepatocytes, but it was found to have a pro-apoptotic effect in liver cancer cells (34).

In the present study, we found that SAM, induced cytotoxicity in two GB (18 and 38) cell lines, in vitro. We found that both GB cell lines were sensitive to SAM treatment, cell lines displayed cytotoxic responses to SAM treatment in a dose dependent manner but differences between the two cell lines were evident in antiproliferative responses.

Our data showed that 38 GB cells were more sensitive to drug treatment in comparison with 18 cells, especially at the smallest drug concentration used in this study (0.1 µM SAM). At the concentration of 0.1 µM SAM, the cytotoxicity induced by drug treatment was 4% in the 18 cell line and 22% in the 38 cell line. At the highest concentration of drug (200 µM SAM), the difference between the cell cytotoxicity induced by treatment was much smaller, 200 µM SAM treatment inducing 41% in the 18kj40 cell line and 52% in the 38 cell line.

It is well known that growth factor signaling pathways are often upregulated in GB and contribute to oncogenic initiation and further progression (35). We have previously shown that in GB cells, IGF-1R together with PDGFR inhibits proliferation in high-grade glioma cells (12, 35).

The interference of SAM-induced cytotoxicity in GB cells with igf1r methylation could, therefore, not be excluded. This hypothesis was encouraged by other reports that identified DNA hypomethylation as the molecular mechanism responsible for the upregulation of several oncogenes like members of Eph family of receptor tyrosine kinases (RTK) (36), the c-fms oncogene that encodes for CSF-1R (37), the erbB2/neu (38) and other oncogenes such as c-myc (19), hox11 (21) h-ras (20) and c-ros (22). Thus, in the present study we address the possibility that the igf-1r hypermethylation by SAM could be the responsible mechanism for SAM-induced cell death by down-regulation and impairing IGF-1R in GB cells.

First, we investigated the igf-1r methylation pattern in GB cells. Igf-1r promoter was mapped for CpG islands by using MethPrimer software. We identified 2 CpG islands consistent in high CpG dinucleotides. Amplification of the CpG islands region using specific primers for the methylated versus unmethylated igf-1r alleles revealed surprisingly a partial methylation status of the igf-1r promoter region. As expected, the GB cells expressed IGF-1R at the cell surface and the level of protein was similar in both GB cell lines analyzed. The functional significance of the partial methylation status of a gene is not fully understood.

Since SAM induced cell death in the GB cell lines analyzed, we next investigated whether SAM-induced igf-1r CpG island hypermethylation could mediate the drug cytotoxicity in the cells. Surprisingly, neither changes in the igf-1r CpG island methylation, nor in the level of IGF-1R protein expression was seen in GB cells, after SAM. The lack of change in igf-1r gene methylation profile and in the expression of IGF-1R following SAM treatment indicates that the antitumor effect of SAM administration was independent of igf-1r gene methylation status in GB cell lines investigated.

In this study, we have shown that igf-1r gene is partially methylated in glioblastoma cell lines. To our knowledge, no study thus far has examined the igf-1r methylation profile in GB cells. We also demonstrated that SAM-induced cytotoxicity is independent of igf-1r methylation status in GB cells.

Further studies are warranted to determine the generality and the biological importance of igf-1r partial methylation in cancer cells.

Acknowledgments

Grant support: European Commission Grants QLGA-CT-2000-60005, CNMP 41-063 Bucharest.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Yu, H. and Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst, 2000. 92(18): p. 1472-89.
2. Almeida A, Muleris M, Dutrillaux B, Malfoy B. The insulin-like growth factor I receptor gene is the target for the 15q26 amplicon in breast cancer. Genes Chromosomes Cancer, 1994. 11(1): p. 63-5.
3. Dricu A, Kanter L, Wang M, Nilsson G, Hjertman M, Wejde J et al. Expression of the insulin-like growth factor 1 receptor (IGF-1R) in breast cancer cells: evidence for a regulatory role of dolichyl phosphate in the transition from an intracellular to an extracellular IGF-1 pathway. Glycobiology, 1999. 9(6): p. 571-9.
4. Cosaceanu D, Carapancea M, Alexandru O, Budiu R, Martinsson HS, Starborg M et al. Comparison of three approaches for inhibiting insulin-like growth factor I receptor and their effects on NSCLC cell lines in vitro. Growth Factors, 2007. 25(1): p. 1-8.
5. Kiaris H, Schally AV, Varga JL, Groot K, Armatis P. Growth hormone-releasing hormone: an autocrine growth factor for small cell lung carcinoma. Proc Natl Acad Sci U S A, 1999. 96(26): p. 14894-8.

6. Xiao XY, Zhou XY, Yan G, Sun MH, Du X. Chromosomal alteration in Chinese sporadic colorectal carcinomas detected by comparative genomic hybridization. Diagn Mol Pathol, 2007. 16(2): p. 96-103.
7. Figueroa JA, Lee AV, Jackson JG, Yee D. Proliferation of cultured human prostate cancer cells is inhibited by insulin-like growth factor (IGF) binding protein-1: evidence for an IGF-II autocrine growth loop. J Clin Endocrinol Metab, 1995. 80(12): p. 3476-82.
8. Xie Y, Skytting B, Nilsson G, Brodin B, Larsson O. Expression of insulin-like growth factor-1 receptor in synovial sarcoma: association with an aggressive phenotype. Cancer Res, 1999. 59(15): p. 3588-91.
9. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res, 2002. 62(1): p. 200-7.
10. Hui AB, Lo KW, Yin XL, Poon WS, Ng HK. Detection of multiple gene amplifications in glioblastoma multiforme using array-based comparative genomic hybridization. Lab Invest, 2001. 81(5): p. 717-23.
11. Carapancea M, Alexandru O, Fetea AS, Dragutescu L, Castro J, Georgescu A et al. Growth factor receptors signaling in glioblastoma cells: therapeutic implications. J Neurooncol, 2009. 92(2): p. 137-47.
12. Carapancea M, Cosaceanu D, Budiu R, Kwiecinska A, Tataranu L, Ciubotaru V et al., Dual targeting of IGF-1R and PDGFR inhibits proliferation in high-grade gliomas cells and induces radiosensitivity in JNK-1 expressing cells. J Neurooncol, 2007. 85(3): p. 245-54.
13. Cooke DW, Bankert LA, Roberts CT, Jr., LeRoith D, Casella SJ. Analysis of the human type I insulin-like growth factor receptor promoter region. Biochem Biophys Res Commun, 1991. 177(3): p. 1113-20.
14. Werner H, Bach MA, Stannard B, Roberts CT, Jr., LeRoith D. Structural and functional analysis of the insulin-like growth factor I receptor gene promoter. Mol Endocrinol, 1992. 6(10): p. 1545-58.
15. Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E et al. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med, 2003. 349(23): p. 2211-22.
16. Laird, P.W. and R. Jaenisch. The role of DNA methylation in cancer genetic and epigenetics. Annu Rev Genet, 1996. 30: p. 441-64.
16* Ola, R., Neagoe Berindan, I., Balacescu, O., Mougsen A, Dricu A. Methylation of p16/rb1 in NSCLC and Glioblatoma cancer cells. Biology and therapy of cancer cell. Vol1, 1.oct 2009.
17. Martinez R, Martin-Subero JI, Rohde V, Kirsch M, Alaminos M, Fernandez AF et al. A microarray-based DNA methylation study of glioblastoma multiforme. Epigenetics, 2009. 4(4): p. 255-64.
18. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res, 1998. 72: p. 141-96.
19. Bianchi NO, Bianchi MS, Alitalo K, de la Chapelle A. The methylation pattern of normal and truncated amplified human c-myc oncogenes. Biochim Biophys Acta, 1989. 1007(3): p. 350-5.
20. Vachtenheim, J., I. Horakova, and H. Novotna. Hypomethylation of CCGG sites in the 3' region of H-ras protooncogene is frequent and is associated with H-ras allele loss in non-small cell lung cancer. Cancer Res, 1994. 54(5): p. 1145-8.
21. Watt, P.M., R. Kumar, and U.R. Kees. Promoter demethylation accompanies reactivation of the HOX11 proto-oncogene in leukemia. Genes Chromosomes Cancer, 2000. 29(4): p.371-7.
22. Jun HJ, Woolfenden S, Coven S, Lane K, Bronson R, Housman D et al. Epigenetic regulation of c-ROS receptor tyrosine kinase expression in malignant gliomas. Cancer Res, 2009. 69(6): p. 2180-4.
23. Bestor, T.H. and B. Tycko. Creation of genomic methylation patterns. Nat Genet, 1996. 12(4): p. 363-7.
24. Tuck-Muller CM, Narayan A, Tsien F, Smeets DF, Sawyer J, Fiala ES et al..DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. Cytogenet Cell Genet, 2000. 89(1-2): p. 121-8.
25. Detich N, Hamm S, Just G, Knox JD, Szyf M. The methyl donor S-Adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-Adenosylmethionine. J Biol Chem, 2003. 278(23): p. 20812-20.
26. Fuso A, Cavallaro RA, Orru L, Buttarelli FR, Scarpa S. Gene silencing by S-adenosylmethionine in muscle differentiation. FEBS Lett, 2001. 508(3): p. 337-40.
27. Shukeir N, Pakneshan P, Chen G, Szyf M, Rabbani SA. Alteration of the methylation status of tumor-promoting genes decreases prostate cancer cell invasiveness and tumorigenesis in vitro and in vivo. Cancer Res, 2006. 66(18): p. 9202-10.
28. Ponten, J. and B. Westermark. Properties of human malignant glioma cells in vitro. Med Biol, 1978. 56(4): p. 184-93.
29. Hagerstrand D, Hesselager G, Achterberg S, Wickenberg Bolin U, Kowanetz M, Kastemar M et al. Characterization of an imatinib-sensitive subset of high-grade human glioma cultures. Oncogene, 2006. 25(35): p. 4913-22.
30. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A, 1996. 93(18): p. 9821-6.
31. Li, L.C. and R. Dahiya. MethPrimer: designing primers for methylation PCRs. Bioinformatics, 2002. 18(11): p. 1427-31.
32. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 1976.

72: p. 248-54.
33. Pakneshan P, Szyf M, Farias-Eisner R, Rabbani SA. Reversal of the hypomethylation status of urokinase (uPA) promoter blocks breast cancer growth and metastasis. J Biol Chem, 2004. 279(30): p. 31735-44.
34. Lu, S.C. and J.M. Mato. S-Adenosylmethionine in cell growth, apoptosis and liver cancer. J Gastroenterol Hepatol, 2008. 23 Suppl 1: p. S73-7.
35. 35. Palanichamy, K., M. Erkkinen, and A. Chakravarti. Predictive and prognostic markers in human glioblastomas. Curr Treat Options Oncol, 2006. 7(6): p. 490-504.
36. Dottori M, Down M, Huttmann A, Fitzpatrick DR, Boyd AW. Cloning and characterization of EphA3 (Hek) gene promoter: DNA methylation regulates expression in hematopoietic tumor cells. Blood, 1999. 94(7): p. 2477-86.
37. Cui J, Yang DH, Bi XJ, Fan ZR. Methylation status of c-fms oncogene in HCC and its relationship with clinical pathology. World J Gastroenterol, 2001. 7(1): p. 136-9.
38. Zhou H, Chen WD, Qin X, Lee K, Liu L, Markowitz SD et al. MMTV promoter hypomethylation is linked to spontaneous and MNU associated c-neu expression and mammary carcinogenesis in MMTV c-neu transgenic mice. Oncogene, 2001. 20(42): p. 6009-17.