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DNA methylation and the control of genes involved in malignant processes

PhD thesis (summary)

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Keywords: DNA methylation, Insulin growth factor receptor 1, Non-Small Cell Lung Cancer, Glioblastoma, Carcinogenesis.

Abstract

DNA methylation occurring within the control regions of growth-regulatory genes inactivate their transcription, giving cells selective growth advantages and the possibility to initiate and develop tumors. On the other hand, hypomethylation of proto-oncogenes has been shown to be one of the mechanisms responsible for the abnormal proliferative phenotype through oncogene overproduction.

Non-small-cell lung cancer (NSCLC) and Glioblastomas (GB) are among the most lethal forms of cancer. Cancer patients display a poor prognosis explaining the need for development of new treatment approaches.

Igf-1r oncogene overproduction or enhanced activity has been observed in several tumor localizations including lung and glial tumors. However, the molecular mechanism leading to an increase *Igf-1r* gene expression has not been defined yet. In the first part of this study, it was analyzed the methylation profile of the *Igf-1r* oncogene in NSCLC and GB cell lines, and propose that *Igf-1r* hypomethylation may provide a basis for potential use of hypermethylating agents for cancers treatment. The results showed a hemimethylated *Igf-1r* in all cancer cell lines analyzed. To determine whether increased *Igf-1r* methylation associates with IGF-1R protein downregulation, cancer cells were exposed *in vitro* to a methylating agent,

S-Adenosylmethionine (SAM). NSCLC and GB cell lines showed different levels of sensitivity to SAM treatment.
However, neither *Igf-1r* methylation profile, nor IGF-1R protein expression were affected by SAM treatment, suggesting that SAM-induced cytotoxicity is independent of *Igf-1r* methylation status in these particular cancer cell lines.

Alterations in the *p16*/*Rb1* cell cycle regulatory genes are often associated with cancer initiation and progression. In the second part of the study the methylation profile of *p16* and *Rb1* tumor suppressor genes was further addressed in NSCLC and GB cell lines. We obtained a differential methylation profile of *p16* in NSCLC and GB cell lines. By treatment with a demethylating agent, 5-aza-2'-deoxycytidine (5Aza-dC), P16 protein levels were restored in those cells with methylated *p16* gene, suggesting the use of demethylating agents to reactivate growth-regulatory genes silenced by *de novo* methylation.

A. Introduction

'Epigenetics' respresents the mechanism by which gene expression or cellular phenotypes can be heritably modified in specific lineages and subsequently transmitted during cell division ("epi" in classical Greek means "on top"). In parallel with the increasing understanding of gene function, it became certain that not everything, albeit hereditary, is defined by the DNA sequences in our genes. Even though, the genomic sequence of several complex organisms already exists, the understanding of the complicated network that takes place inside eukaryotic cells is far from complete.

A.1. Epigenetic mechanisms in carcinogenesis

Cancer is a malignant disease in which a group of cells acquire three specific properties: uncontrolled growth, invasion, and very often metastasis.
The word 'cancer' was first used by Hippocrates - the Father of Medicine, which used the Greek words, 'carcinos' and 'carcinoma' to describe tumors. The Greek terms were actually describing a crab, which Hippocrates thought that resembles a tumor.

Cancer development seems to be dependent on both, genetic and epigenetic alterations. Aberrant epigenetic mechanisms are manifested at the level of chromatin packaging but also in localized gene promoter changes that influence the transcription of genes important to cancer development.

A.1.1. DNA methylation

The methylation of cytosines at position 5' of the pyrimidine ring is the only naturally occurring modification in mammalian DNA that predominantly but not exclusively takes place in the sequence CpG.

In mammals, CpG rich regions are named CpG islands. In the human genome at least 60% of the promoters of protein-coding genes are associated with a CpG island. The density of methylation in the promoter region located 5′ to the transcription start site or in the first

exon/intron of a gene is positively or negatively affecting the transcription of that specific gene. In normal cells, CpG islands are unmethylated, whereas the sporadic CpG sites located in the rest of the genome are normally methylated. This methylation pattern undergoes a gradual reversal during aging and leads to sporadic methylation in the CpG islands and a global loss of methylation, but this change is particularly pronounced during carcinogenesis.

In cancer three major events related to DNA methylation occur: **(1)** the global reduction of 5-methylcytosine content in the whole genome – global hypomethylation. Hypomethylation leads to genomic instability, the activation of transposable elements and proto-oncogenes, but also interferes with the expression of the miRNAs, loss of genomic imprinting and X chromosome inactivation.

Proto-oncogenes are known to be highly expressed during rapid cell growth and differentiation, and they are mostly associated with the control mechanism of cellular growth, differentiation and development.

(2) hypermethylation of the CpG islands of many tumor suppressor genes associated with their transcriptional silencing.

(3) everything occurs with an increase in the expression of DNA methyltransferases (DNMTs), the enzymes responsible for maintaining and establishing the methylation profile.

A.2. Igf-1r proto-oncogene

Igf-1r oncogene encodes for the receptor tyrosine kinase Insulin-like growth factor receptor-1 (IGF-1R), which promotes oncogenic transformation, growth and survival of cancer cells. IGF-1R overproduction or enhanced activity has been observed in many types of cancer including breast [1, 2], lung [3, 4], colon [5], prostate [6], melanoma tumor types [7] and glial tumors [8].

Activated IGF-1R downstream signals results in high cell proliferation, malignant transformation, growth and survival of cancer cells.

The *Igf-1r* gene expression levels are determined, to a large extent, at the transcriptional level. Both, the 5′ flanking and 5′ untranslated region are highly CG-rich. Even through a small number of breast and melanoma cases displayed amplification of *Igf-1r* locus at band 15q2 [1], the molecular mechanism responsible for the increased transcriptional activation of *Igf-1r* gene in cancer remains largely obscure.

A.3. p16/Rb1 tumor suppressor genes

p16 is one of the most common tumor suppressor genes affected in many tumor types by the loss of function event; in the same time the control of cell cycling by P16/RB1 pathway is consequently lost in virtually all tumors, either through disrupted *p16* function or mutations of *Rb1*.

p16 (*Cdkn2a*), an inhibitor of the Cyclin D1 dependent protein kinases, is a cell cycle regulator involved in the inhibition of G1 phase progression [9]. Loss of function of *p16* results in higher Cyclin D1 dependent protein kinase activity and thus leads to aberrant phosphorylation

of retinoblastoma (RB1), which accelerates cell growth. Inactivation of *p16* by homozygous deletion or point mutation is one of the most commonly observed aberrations in tumors. However, an alternative mechanism for inactivation of *p16* is the hypermethylation of CpG island extending from the promoter region to the first exon [10, 11]. The frequency of each mechanisms varies with the tumor type.

B. The aims of the study

- to identify the methylation profile of *Igf-1r* in NSCLC and GB cell lines.
- to evaluate the possibility that methylation is the responsible mechanism for IGF-1R overproduction in cancer
- to find appropriate strategies for the inhibition of *Igf-1r* function by using methylating agents as potential therapy agents
- to identify the methylation profile of *p16/Rb1* cell cycle regulatory genes in different subtypes of NSCLC and GB cell lines.
- to evaluate the efficacy of demethylating agents in the reactivation of dormant *p16* tumor suppressor gene and the effect on cancer cell viability.

C. Materials and methods

C.1. Cell Lines and reagents

For these studies 6 NSCLC cell lines: U1810 and U1752 (established at the University of Uppsala [12]) and H157, H125, H23 and A549 (purchased from American Type Culture Collection), and 2 primary GB cell lines: 18, 38 (established at the University of Uppsala [13]) were used. The cell lines were maintained in the recommended conditions by the manufacturer.

C.2. MTT assay – growth inhibitory 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. For the *Igf-1r* study, cells were incubated for 7 days in growth medium with increasing concentrations of SAM (New England Bio-labs) ranging from 0.1-200μM. For the *p16/Rb1* study, all cell lines were incubated for 5 days in growth medium with increasing concentrations of 5Aza-dC ranging from 0.5-10μM. Results were represented as cell growth curve analysis of untreated cells/ treated cells with SAM or 5Aza-dC.

C.3. Cell treatment

For the experimental purposes cells in culture were treated with increasing concentrations of SAM ranging from 50-200μM for the *Igf-1r* study or 5Aza-dC (Sigma-Aldrich) ranging 0.5-5μM for the p16/Rb1 study.

C.4. Methylation-specific PCR (MSP)

MSP was performed as described previously [14]. DNA from each cell line was subject to Bisulphite treatment using CpGenome DNA modification kit (Chemicon, Sweden) followed by PCR. The PCR conditions are described in detail in the thesis.

C.5. Western blot

Protein quantification was done according to Bradford method [15]. Cells were lysed in lysis buffer and subjected to Western Blot. A polyclonal rabbit antibody reactive to the β-subunit of IGF-1R, a polyclonal rabbit antibody reactive to P16 and RB1 (Santa Cruz Biotechnology) and a monoclonal mouse antibody reactive to β-Actin (Santa Cruz Biotechnology) were used as primary antibodies. Anti-rabbit or anti-mouse secondary antibody conjugated to HRP, were used as secondary antibodies. The proteins were visualized using the ECL system (Amersham Biosciences AB, Uppsala, Sweden).

D. Results

D.I. Methylation status of Igf-1r in NSCLC and GB cell lines

1060bp of the 5' flanking region containing the *Igf-1r* promoter were mapped for CpG islands. Using MethPrimer software, two CpG islands were identify in the promoter region, the first CpG corresponding to the 49-278bp and the second CpG located between 711-937bp in the promoter region of *Igf-1r*.

D.I.2. Igf-1r methylation status in NSCLC and GB cell lines

To identify the methylation status of the *Igf-1r* promotor region, primer pairs were designed specifically to recognize the methylated versus unmethylated *Igf-1r* gene. The methylation profile was then analyzed in 6 NSCLC and 2 primary GB cell lines. Following bisulphit treatment, the modified DNA was subjected to PCR.

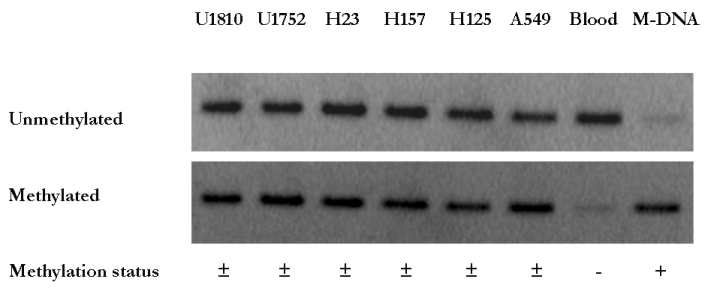

Figure 1. **Methylation status of *Igf-1r* promoter in NSCLC.** The presence of the PCR product in methylated lanes indicates the presence of methylated allele (165bp) *of Igf-1r*, and the unmethylated lanes indicates the presence of unmethylated allele (160 bp) of Igf-1r; M-DNA, universal methylated human genomic DNA, (positive control for the methylated band); blood (positive control for unmethylated band).

PCR amplification with both, methylated versus unmethylated primer pairs was obtained in all NSCLC (Figure 1), and also in the primary GB cell lines (Figure 2) suggesting a hemimethylated state for *Igf-1r* regardless of the cell subtype.

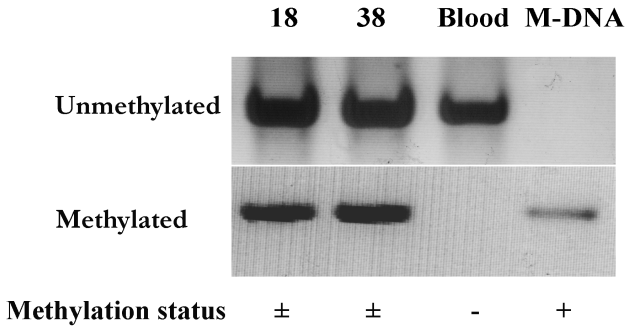

Figure 2. **Methylation status of *Igf-1r* promoter in GB cell lines.** The presence of the unmethylated PCR product of *Igf-1r* (160 bp), and the methylated allele of *Igf-1r* (165bp); blood (positive control for unmethylated band), M-DNA, universal methylated human genomic DNA, (positive control for the methylated band)

To identify any correlation between gene-protein expression, the IGF-1R levels was next assayed by Western Blot and identify the protein at the cell surface in all 6 NSCLC (Figure 3) as well as in primary GB cells (Figure 4).

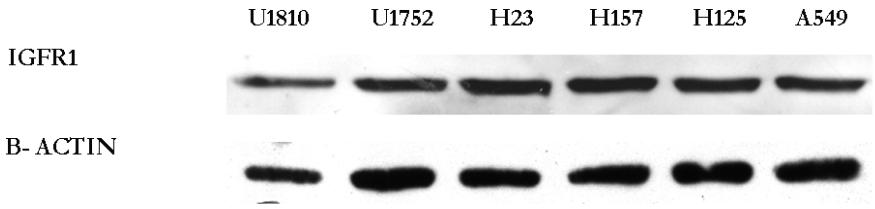

Figure 3. Protein expression levels in U1752, U1810, H23, H157, H125 and A549 NSCLC by Western blot for IGFR1 and β-Actin (loading control protein).

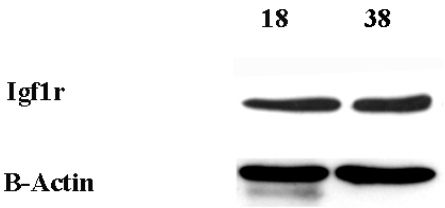

Figure 4. IGF-1R expression levels in 18 and 38 GB cell lines by Western blot for IGF-1R and β-Actin (loading control protein).

D.I.3. The effect of SAM treatment on NSCLC and GB cells viability

SAM is the principal methyl group donor in many biological reactions, including DNA methylation, and *in vitro* studies have been shown that SAM induces cell death by interfering with the DNA methylation patterns. To explore the importance of SAM induced methylation on NSCLC and GB cells survival, cells in culture were treated with increasing doses of SAM ranging from 0.1-200μM for 7 days and cell viability was analyzed by MTT assay (Figure 5). The effect of SAM on cell viability was in general modest in all NSCLC cell lines.

At the highest concentration used (200μM), SAM treatment resulted in an average of 36% reduction in cell growth in U1810 cells (Figure 5A), 18% reduction in U1752 cells (Figure 5B), 15% reduction in H23 cells (Figure 5C), 41% reduction in H157 cells (Figure 5D), 29% reduction in H125 cells (Figure 5E), and 22% reduction in A549 cells (Figure 5F), when compared to non-treated cells.

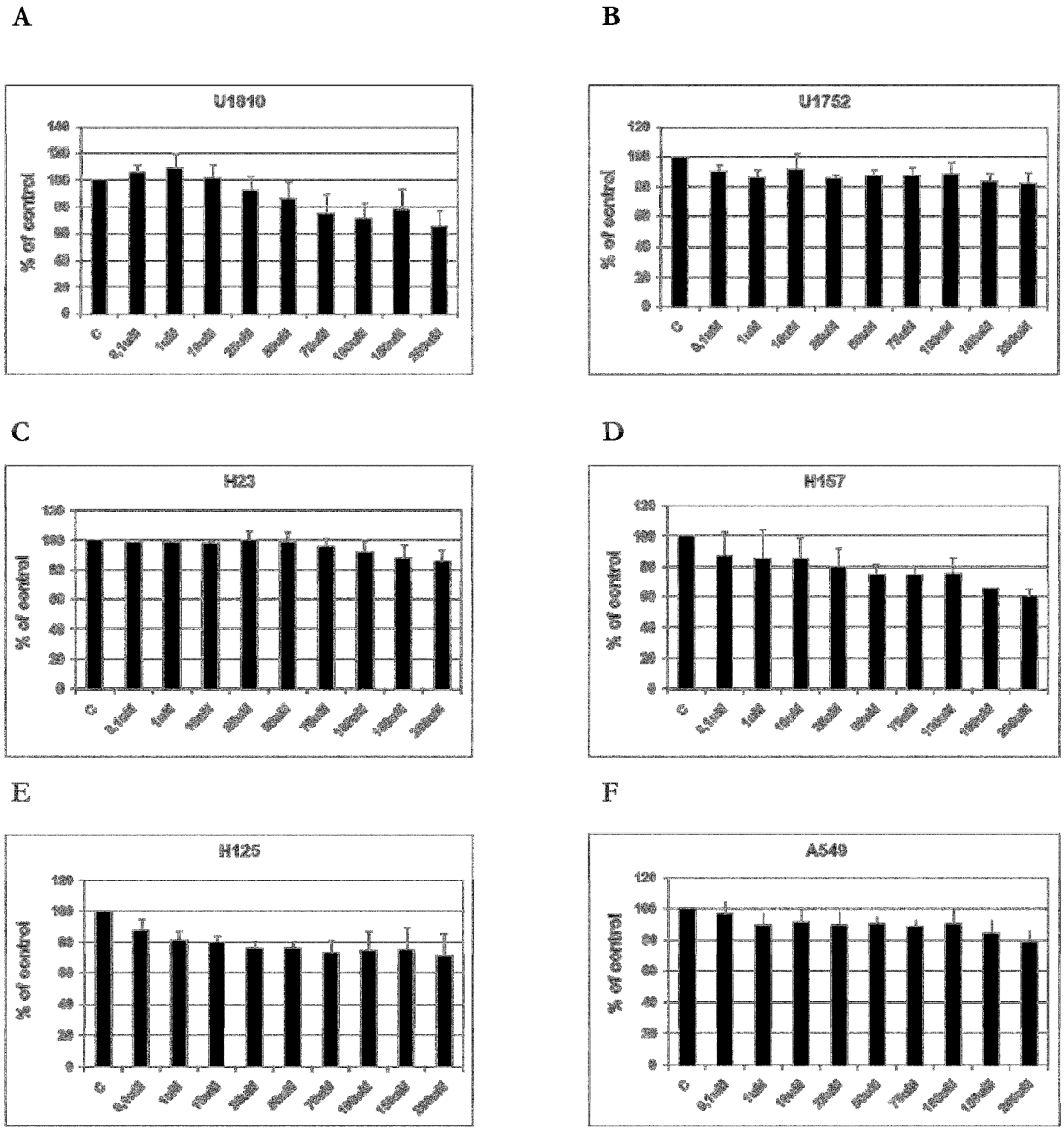

Figure 5. Cell toxicity assay after 7 days of treatment with increasing doses of SAM in NSCLC. All cells, were plated at exactly the same density of viable cell per well in triplicates and were exposed to SAM at concentrations ranging from 0.1 to 200µM in differentiating media for 6 days, the medium was refreshed with SAM every second day. Cell toxicity assay for U1810 (A), U1752 (B), H23 (C), H157 (D), H125 (E), A549 (F).

In GB cell lines, SAM treatment resulted in dose-dependent cytotoxicity.However, 38 GB cell line displayed more sensitivity to SAM treatment than 18 GB cell line, at the highest

concentration used (200µM), SAM induced cell death in an average of 52% in 38 GB cell line (Figue 6B) in comparison with 41% in 18 cells (Figure 6A).

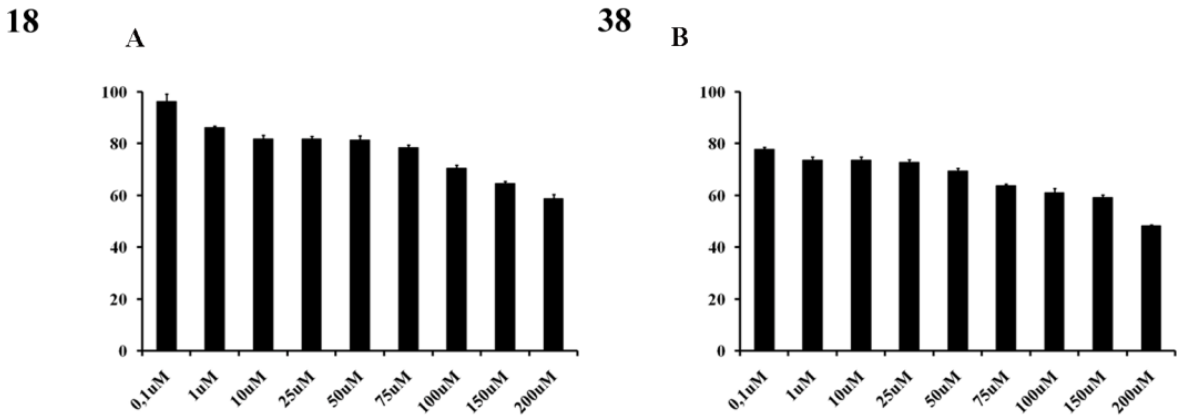

Figure 6. Cell toxicity assay after 7 days of treatment with increasing doses of SAM in GB. All cells, were plated at exactly the same density of viable cell per well in triplicates and were exposed to SAM at concentrations ranging from 0.1 to 200 µM in differentiating media for 7 days, the medium was refreshed with SAM every second day. Cell toxicity assay for 18 (A) and 38 (B).

D.I.4. The effect of SAM treatment on *Igf-1r* methylation profile

In order to assess if SAM induced cytotoxicity in NSCLC and/or GB cell lines interfered with *Igf-1r* promoter hypermethylation, DNA extracted from SAM treated cells was amplified with the unmethylated versus methylated primers and the MSP was compared with the untreated DNA. Surprisingly, SAM treatment did not modify the *Igf-1r* CpG island methylation profile, nor in the NSCLC (Figure 7), neither in GB cell lines (Figure 8). The same hemimethylated *Igf-1r* state was maintained irrespective of the concentration of SAM used.

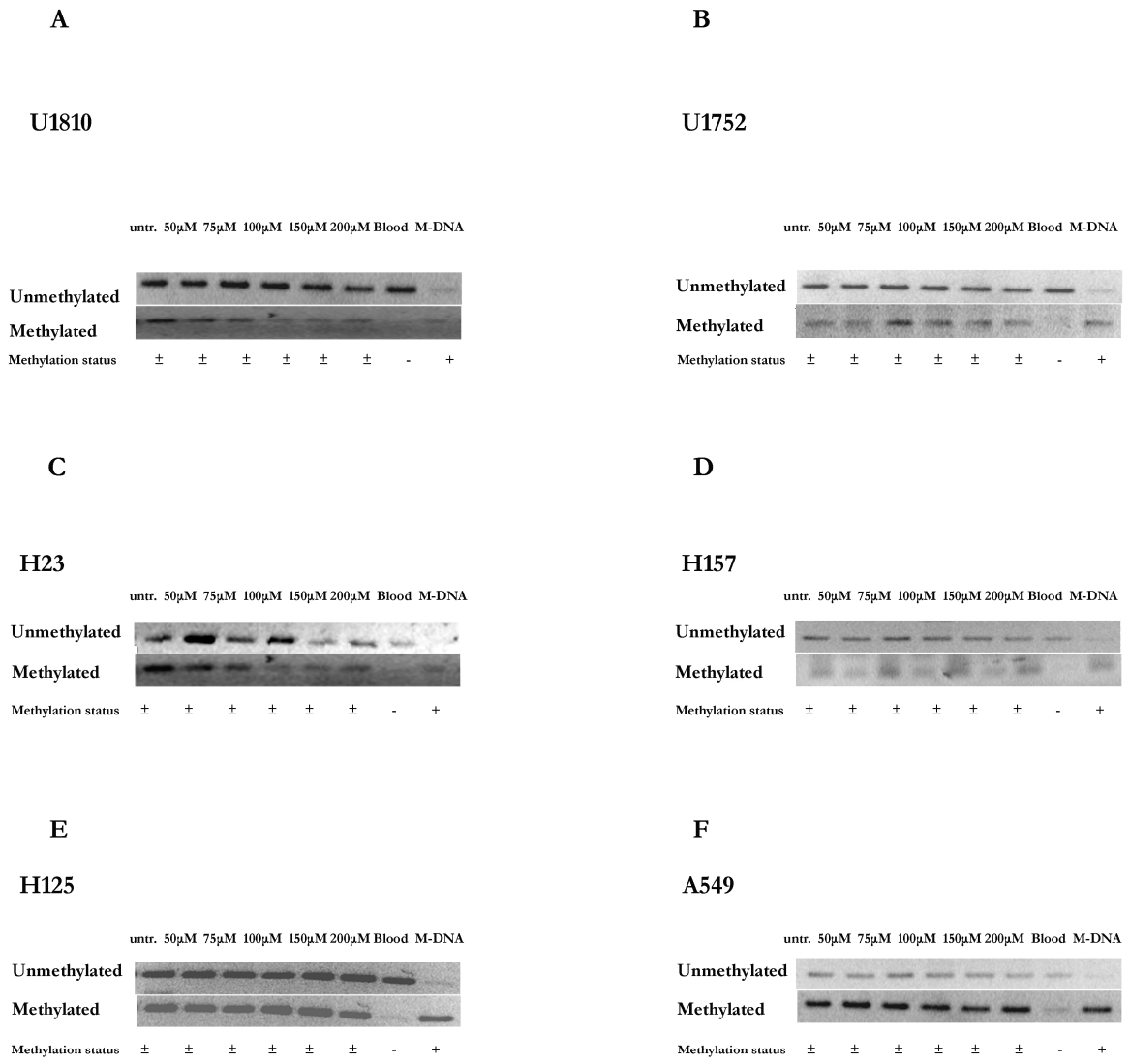

Figure 7. Methylation of *Igf-1r* promoter gene after SAM treatment in NSCLC. All cells lines were treated with increased doses of SAM for 7 days and the MSP was performed on the extracted DNA from the treated cells versus the untreated cells. The partial methylation status of *Igf-1r* was maintained after SAM treatment in all the NSCLC: U1810 (A), U1752 (B), H23 (C), H157 (D), H125 (E), A549 (F).

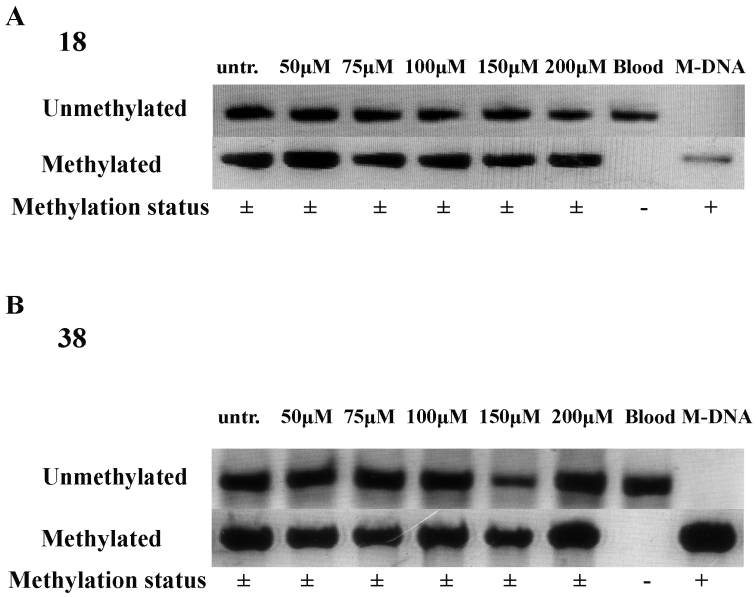

Figure 8. Methylation of *Igf-1r* promoter region after SAM treatment in GB. All cells lines were treated with increased doses of SAM for 7 days and the MSP was performed on the extracted DNA from the treated cells versus untreated cells. The partial methylation status of *Igf-1r* was maintained after SAM treatment in both GB cell lines: 18 (A) and 38 (B).

D.I.5. The effect of SAM treatment on IGF-1R expression pattern

As SAM did not influence the methylation state of the *Igf-1r* oncogene, it was expected that SAM had no profound effect either on IGF-1R protein expression levels. The protein was not significant affected by SAM treatment when compared to untreated cells in any of the NSCLC (Figure 9) or GB cell lines analyzed (Figure 10).

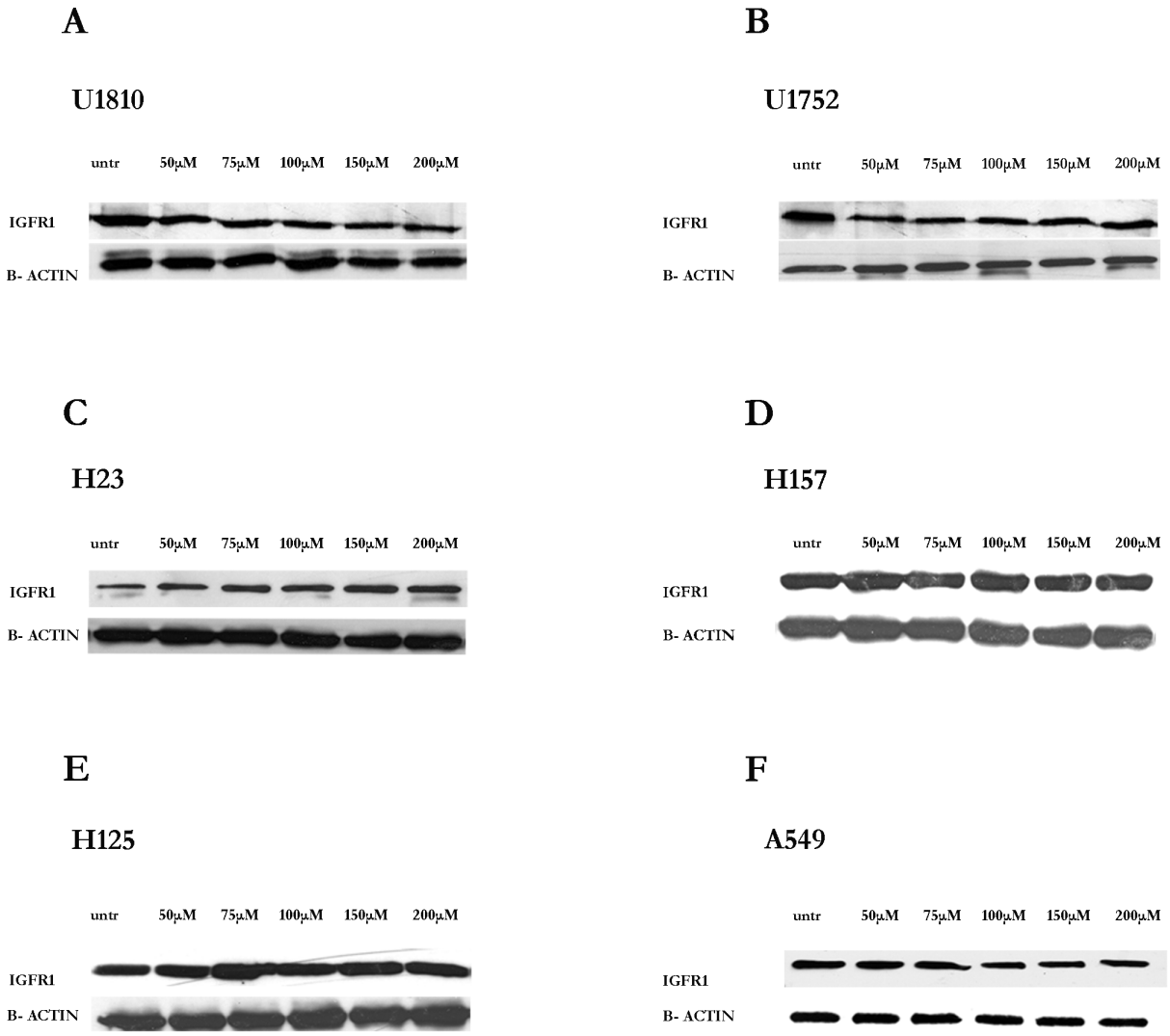

Figure 9. IGF-1R expression after SAM treatment in NSCLC. After 7 days of SAM treatment, cell lysates from untreated and treated cells were subjected to Western Blotting and IGF-1R expression levels were compared with the untreated cells in NSCLC: U1810 (A), U1752 (B), H23 (C), H157 (D), H125 (E), A549 (F). β-Actin was used as a loading control.

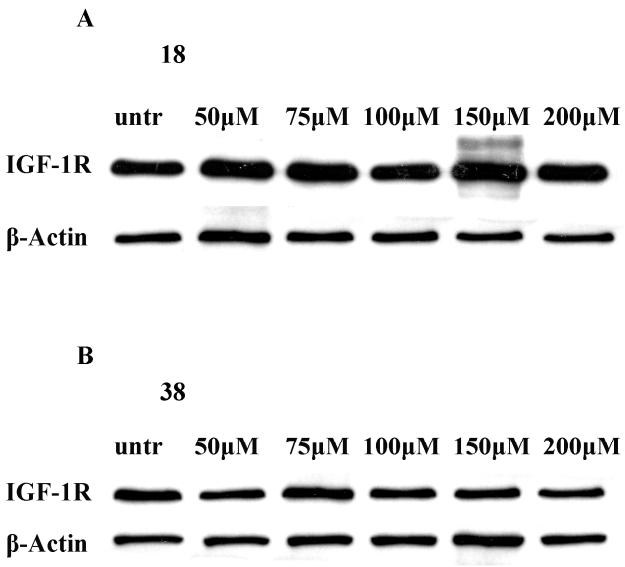

Figure 10. **IGF-1R expression after SAM treatment in GB cell lines.** After 7 days of SAM treatment, cell lysates from untreated and treated cells were subjected to Western Blotting and IGF-1R expression levels were detected in comparison with the untreated cells in GB cell lines: 18 (A) and 38 (B). β -Actin was used as a loading control.

D.II. Methylation profile of p16 and Rb1 in NSCLC and GB cell lines

D.II.1 p16/Rb1 methylation in NSCLC and GB cell line

In the second part of this study, the methylation profile of *p16* and *Rb1* tumor suppressor genes was addressed in the same cell lines as in the first study.

After bisulphit treatment, PCR with specific primers that specifically recognize the unmethylated versus methylated *p16*/*Rb1* fragments were used.

Amplification with the methylated primers for *p16* was obtained in two NSCLC: H23 and U1810 (Figure 11A) and in one of the primary GB cell lines, in 18 cells (Figure 11B). Amplification with the unmethylated primers for *p16* was obtained in U1752 and H157 NSCLC cells (Figure 11A) and respectively in the 38 GB cell line (Figure 11B). A549 cell line has been previously characterized with the *p16* gene depleted [16]. For *Rb1* tumor suppressor gene, amplification with the methylated primers was obtained in none of the cell lines studied. Only the unmethylated allele was amplified when unmethylated primers have been used (Figure 11A,B).

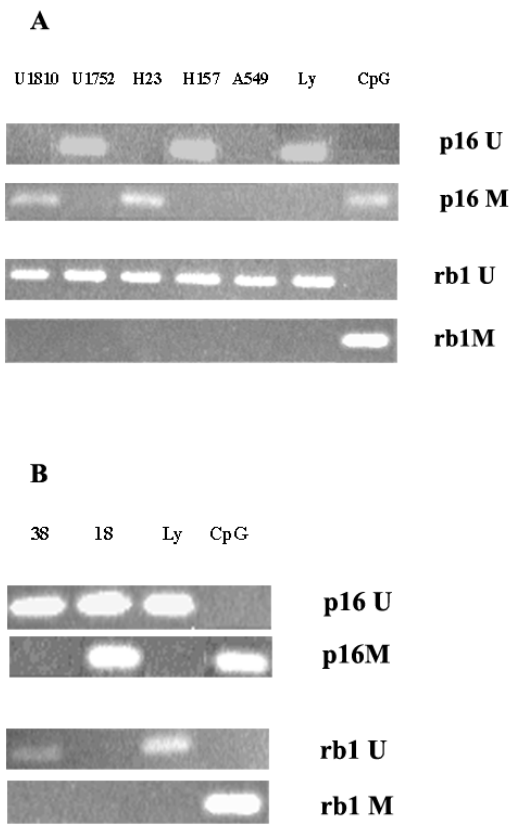

Figure 11. Methylation profile of *p16* and *Rb1* in NSCLC and GB cell lines. The presence of the visible PCR product in methylated lanes indicates the methylated allele of *p16*/*Rb1*, and the unmethylated lanes indicates the unmethylated allele of *p16/Rb1* in (A) NSCLC: U1810, U1752, H23, H157 and A549 and in (B) GB cell lines: 38 and 18; Ly (positive control for unmethylated band); CpG, Universal Methylated Human Genomic DNA, (positive control for the methylated band);

D.II.2 P16 and RB1 protein expression in NSCLC and GB cell lines

Next, P16 and RB1 protein expression levels were assayed by Western Blot.

P16 was endogenously detected in the same cell lines where amplification with the unmethylated primers has been obtained, respectively in U1752, H157 NSCLC cells (Figure 12A) and in 38 GB cell lines (Figure 12B). As expected, P16 protein expression was absent in U1810 and H23 NSCLC cells (Figure 12A), and also in 18 GB cells (Figure 12B), the same cell lines where the bisuphit modified DNA was amplified only with the methylated primers. RB1 was endogenously expressed in all the cell lines analysis (Figure 12A,B).

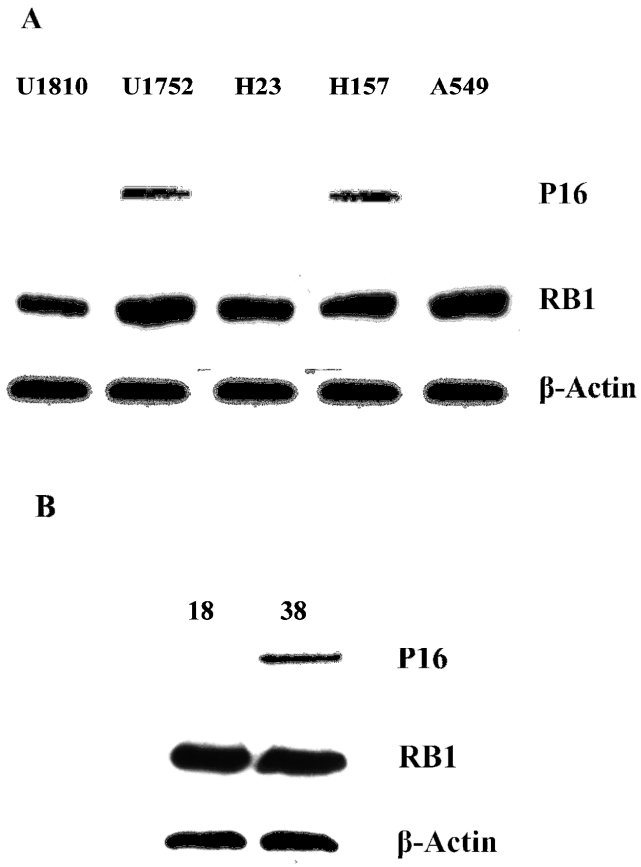

Figure 12. P16 and RB1 protein determination by Western Blot. (A) Protein expression levels in U1752, U1810, H23, H157, H125 and A549 NSCLC and (B) in 18 and 38 GB cell lines; β-Actin was used as a loading control.

D.II.3 The effect of 5Aza-dC on cell viability

It is well established that treatment with 5Aza-dC leads to cancer cell death by interfering with DNA demethylation. To explore the importance of 5Aza-dC induced demethylation in NSCLC and GB cells survival, cells in culture were exposed to increased doses (ranging from 0.5-5μM) of 5Aza-dC and cell viability was analyzed by MTT assay.

The growth inhibition profiles of treated versus untreated cell lines is depicted in Figure 13. 5Aza-dC treatment resulted in dose-dependent decrease in cell viability in all NSCLC cell lines (U1810, H23, U1752, H157, A549) (Figure 13A). In the case of GB cell lines, 18 GB cells were more sensitive to 5Aza-dC, when compared to 38 GB cells (Figure 13B).

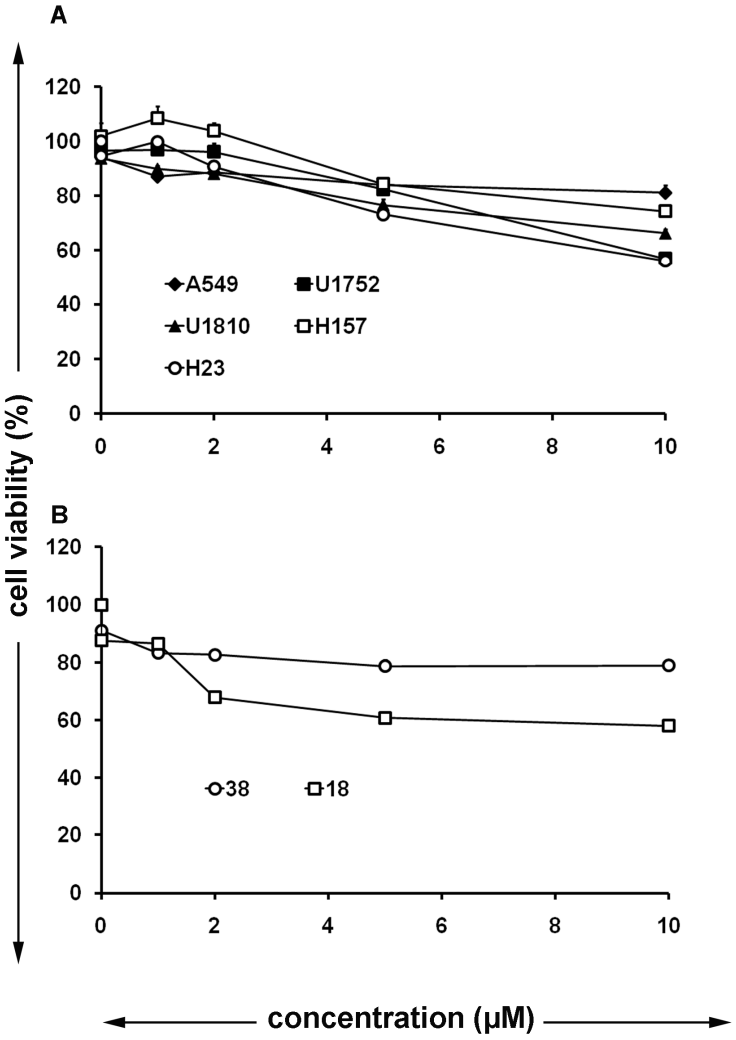

Figure 13. The effect of 5-Aza-dC on cell growth inhibition. All cells, were plated at exactly the same density of viable cell per plate in triplicates and were exposed to 5-Aza-dC at concentrations ranging from 0.5 to 10μM in differentiating media for 5 days. Cell toxicity assay for U1752, U1810, H23, H157, H125 and A549 NSCLC (A) and for 18 and 38 GB cell lines (B). Cell viability for each experiment was normalized against the corresponding non-treated control.

D.II.4 The effect of 5Aza-dC treatment on *p16* gene/protein re-expression

To investigate if 5Aza-dC suppressed cell growth in NSCLC and GB cells interfered with *p16* demethylation, 5Aza-dC treated DNA was subject to MSP and compared to untreated DNA. 5Aza-dC treatment restored *p16* gene expression in a concentration dependent manner. In U1810 and H23 NSCLC cells, at 5μM 5Aza-dC, *p16* was amplified with the umethylated primers (Figure 14B,D), while in 18 GB cells, 2μM 5Aza-dC was sufficient for the *p16* re-expression (Figure 14F).

P16 protein expression levels were restored at the same concentrations: at 5μM of 5Aza-dC for the U1810 and H23 NSCLC cells (Figure 14A,C) and already at 2μM 5Aza-dC for 18 GB cells the 16KD band corresponding to the P16 protein was detected (Figure 14E).

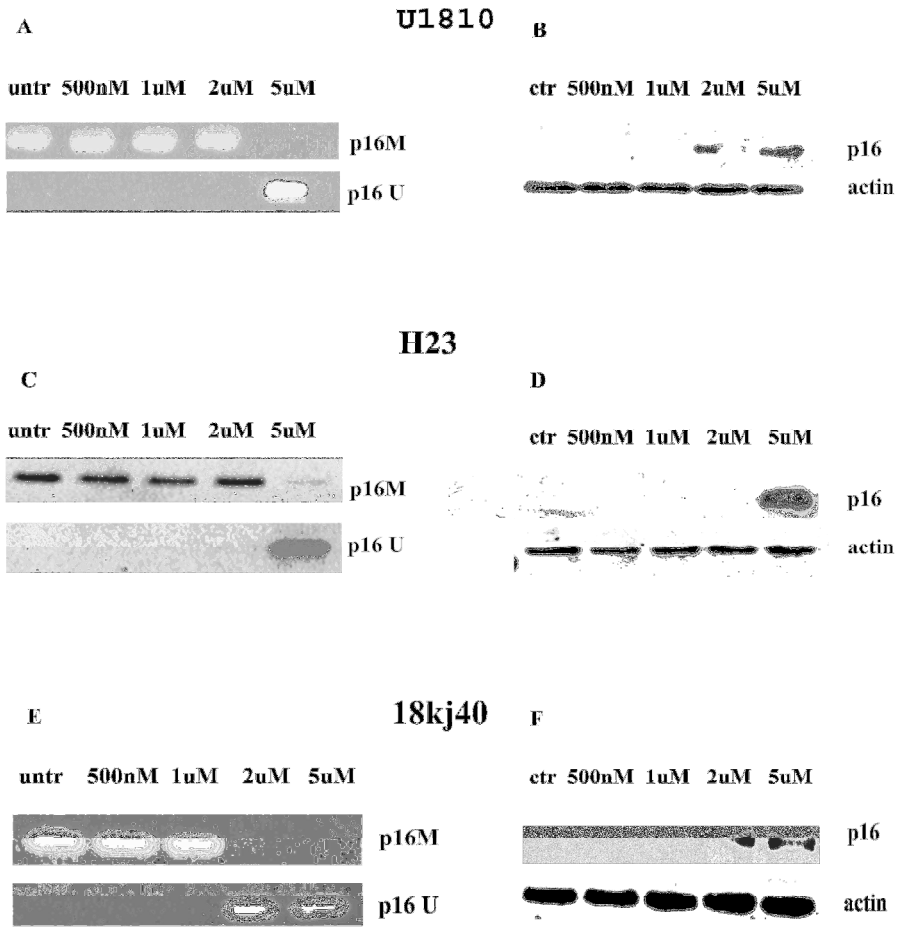

Figure 14. The effect of 5-Aza-dC treatment on p16 re-expression. All cells lines were treated with increased doses ranging from 0.5-5μM of 5Aza-dC for 5 days and MSP was performed on treated versus untreated cells. (A, C) In U1810 and H23, MSP showed amplification with the unmethylated primers and (B, D) re-expression of the protein at 5μM 5Aza-dC treatment. In 18 GB cell line *p16* amplification was obtained with the unmethylated primers (E) and the protein re-expression at 2μM 5Aza-dC dose (F).

E. Discussions

Three different mechanisms including DNA methylation, histone modification and miRNA inducing silencing are associated with the initiation and maintainance of epigenetic mechanisms.

Global hypomethylation is a hallmark of most cancer genomes and contributes to malignancy by either affecting directly the overexpression of oncogenes and activation of latent retrotransposons or by inducing chromosome instability.

In lung cancer, several studies suggested gene promoter methylation analysis as a powerful tool for early detection, diagnosis and prognosis [17].

Moreover, recent studies have showed that CpG island methylation pattern varies from cell type to cell type [18]. For this reason, three major NSCLC subtypes have been selected in this study: large cell lung cancer (U1810), squamous cell carcinoma (U1752, H125 and H157) and adenocarcinoma (H23, A549) and 2 primary GB cell lines (18 and 38).

In GB, *Igf-1r* was identified as a potential genetic marker [19] and in lung cancer the oncogene was associated with poor prognosis. In contrast to the well studied mechanisms of IGF-1R expression [2], the mechanisms underlying *Igf-1r* oncogene overexpression in cancer are less well characterized. Unlike other tyrosine kinase receptors, neither amplifications nor mutations of the *Igf-1r* were described in cancer, excepting the *Igf-1r* locus amplification at band 15q2 in a small number of melanoma and breast cancers [1].

The *Igf-1r* gene expression levels are determined to a large extent at the transcriptional level. The possibility that an altered methylation status of the *Igf-1r* oncogene is responsible for the high expression levels in cancer was investigated in the first part of this study. 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), the *c-fms* oncogene, the *erbB2/neu* and other oncogenes such as *c-myc*, *hox11*, *h-ras* and *c-ros*.

Analysis of the *Igf-1r* promoter region with the MethPrimers software identified two CpG islands rich in CG dinucleotides. Amplification using specific primers for the methylated versus unmethylated *Igf-1r* alleles revealed a partial methylation status of the *Igf-1r* promoter region in all three subgroups of NSCLC and in both GB cell lines studied. Partial methylation of the gene promoter has been defined as the presence of amplified products with both, methylated and unmethylated primer pairs. However, the functional significance of this methylation status of a gene is not well understood. Studies on *hTert* CpG methylation also identified a partial methylation status of this gene *in vitro* and all expressed the telomerase, suggesting that the hemimethylated *hTert* is not inhibitory of telomerase gene expression.

A partial methylation profile of the mouse adenine phosphoribosyltransferase (*Aprt*) promoter region has been shown to be maintained both in vivo and *in vitro* [20].

The correlation between the methylation profile of a gene and the corresponding encoded protein is very well established. Surprisingly, all NSCLC and GB cell lines expressed IGF-1R at the cell surface irrespective of the hemimethylated profile of *Igf-1r* that characterized all cells.

The premise which led to the continuation of the study was that the stable maintenance of a partial methylation profile may have a functional significance, since the partial methylation profile of *Igf-1r* was absent from healthy blood and also from the universal methylated DNA.

In order to identify the possible function of this specific methylation pattern, the DNA methyltransferase reaction was stimulated by *in vitro* treatment with SAM, the principal methyl donor group in all biochemical reactions.

Exogenous administration of SAM has been associated with DNA hypermethylation inducing cytotoxicity in cancer cells [21] and more recently, SAM has been identified to be an attractive agent for treatment of liver cancer [22].

The possibility that SAM induces *Igf-1r* hypermethylation *in vitro* and increase cell death by IGF-1R downregulation was further addressed in this study. All histological variants of NSCLC cell lines were sensitive to SAM treatment but differences were evident in antiproliferative responses between the different cell lines. However, the level of SAM-induced cytotoxicity was not related to the NSCLC cell subtype.

In GB cell lines, 38 cells were more sensitive to SAM when compared to 18 cells.

*S*ince SAM induced cell death in different proportions in all cell lines analyzed, it was next investigated whether SAM induced *Igf-1r* CpG island hypermethylation could mediate the drug cytotoxicity in the cells. By analyzing the methylation profile of *Igf-1r* oncogene and the levels of expression of the corresponding protein after treatment with gradually increasing concentrations of SAM, surprisingly, changes in the *Igf-1r* CpG island methylation could be observed in none cell lines studied.

These results indicate that partial *Igf-1r* methylation can exist in *Igf-1r* -positive cells and is not inhibitory of *Igf-1r* gene expression.

Therefore, *Igf-1r* CpG island methylation is unlikely to play a substantial role in the regulation of *Igf-1r in vitro*. SAM induced cell death may be due to the induction of other hypomethylated genes in these specific cell lines, since the citotoxic response can not be attributted to *Igf-1r* changes in the methylation profile.

In the second part of the study the methylation profile of *p16*/*Rb1* in the same cell lines has been analyzed.

The amplified *p16* promoter products contained both methylated in U18102 and H23 NSCLC and 18 GB cell line and unmethylated PCRs in U1752 and H157 NSCLC cells and 38 GB cell line respectively.

p16/*Rb1* methylation status in U1810 and H23 NSCLC cell lines, or in 18 and 38 GB cell lines has never been investigated. Surprisingly, we observe no methylation of the *Rb1* tumor suppressor gene in any of the tumor cell lines analyzed, neither in the cell lines with methylated *p16* nor in the cell lines expressing endogenous P16, although there is evidence that *Rb1* gene is often inactivated in lung cancer [23].

H23, U1810 NSCLC and 18 GB cell lines with methylated *p16* were characterized by the absence of the protein, while the U1752 and H157 and 38 GB cell lines expressed endogenously P16 and the MSP was obtained only with the unmethylated primers.

5Aza-dC is a demethylating agent which is well known for its ability to induce the expression of genes silenced by *de novo* methylation.
In the cell lines where *p16* was initially methylated and no protein was detected, 5Aza-dC treatment resulted in P16 re-expression. In U1810 and H23, P16 protein was reactivated at the concentration of 5µM 5Aza-dC and in the GB cell line 18 at 5µM 5Aza-dC. These results could explain why the brief treatment of human tumor cell lines with 5Aza-dC led to an increase in the suppression of tumor cell growth.
However, only 2 NSCLC cell lines had a dormant *p16* gene, suggesting that *p16* methylation may be a common event during the establishment of cell lines *in vitro*.
P16 upregulation in these cells may also explain how 5Aza-dC suppresses cell growth; however the absence of P16 up-regulation in the other cell lines suggests that other growth factors in different pathways may be potential targets for *de novo* methylation.

The induction of other genes in combination with p16, may also explain the observed growth suppression in the U1752, H157 NSCLC cells and 38 GB cell. Whether the gradual decrease in *p16* expression during subsequent cell passages arose from the *de novo* methylation of this gene or the selection of cells unaffected by 5Aza-dC needs to be determined. Other genes besides growth regulatory genes may also be activated by 5Aza-dC, resulting in growth suppression.

Alternative explanations for these phenomena could be the cytotoxic activity of the drug or the induction of other cellular physiological changes, which normally slow cell growth.

In contrast to NSCLC cells, 18 primary GB cell line with the initial profile of methylated *p16* showed a substantial decrease in cell viability after 5Aza-dC treatment, if compared with the 38 cell line expressing endogenously P16.

F. Conclusions

In summary, the major findings of this study are:

- 2 CpG islands have been identified in the promoter region of *Igf-1r* oncogene
- *Igf-1r* promoter is partially methylated in NSCLC and GB cell lines
- SAM inducing cell death does affect neither the *Igf-1r* promoter methylation status, nor the IGF-1R protein expression.
- DNA methylation is not the responsible mechanism for the overexpression of *Igf-1r* in NSCLC and GB cells.
- Other mechanism than DNA methylation is responsible for the high expression of *Igf-1r* in NSCLC and GB cancer cell lines.
- *p16* tumor suppressor gene is hypermethylated in U1810 and H23 NSCLC and in 18 GB cell line.
- 5Aza-dC treatment affected the cell viability and rescued P16 expression in the cell lines with initially methylated *p16*.
- *Rb1* tumor suppressor gene is methylated in none of the cell lines studied.

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

Reactivation of growth-regulatory genes silenced by *de novo* methylation suggests clinical applications in which genes inactivated by hypermethylation in human tumors can be induced by 5Aza-dC during anticancer therapy.

In addition, the methylation profile of oncogenes and/or tumor suppressor genes, along with other molecular markers, may be used for individual prognosis.

H. Selected Bibliography

1. Almeida, A., et al., *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.
2. Dricu, A., 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.
3. Cosaceanu, D., 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.
4. Kiaris, H., et al., *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.
5. Xiao, X.Y., et al., *Chromosomal alteration in Chinese sporadic colorectal carcinomas detected by comparative genomic hybridization.* Diagn Mol Pathol, 2007. **16**(2): p. 96-103.
6. Figueroa, J.A., et al., *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.
7. Xie, Y., et al., *Expression of insulin-like growth factor-1 receptor in synovial sarcoma: association with an aggressive phenotype.* Cancer Res, 1999. **59**(15): p. 3588-91.
8. Chakravarti, A., J.S. Loeffler, and N.J. Dyson, *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.
9. Serrano, M., G.J. Hannon, and D. Beach, *A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4.* Nature, 1993. **366**(6456): p. 704-7.
10. Herman, J.G., et al., *Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers.* Cancer Res, 1995. **55**(20): p. 4525-30.
11. Merlo, A., et al., *5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers.* Nat Med, 1995. **1**(7): p. 686-92.
12. Brodin, O., L. Lennartsson, and S. Nilsson, *Single-dose and fractionated irradiation of four human lung cancer cell lines in vitro.* Acta Oncol, 1991. **30**(8): p. 967-74.
13. Hagerstrand, D., et al., *Characterization of an imatinib-sensitive subset of high-grade human glioma cultures.* Oncogene, 2006. **25**(35): p. 4913-22.
14. Herman, J.G., et al., *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.
15. 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.
16. Bai, M., Y. Jin, and X. Zhang, *[Effect of exogenous p16 gene on biological behaviors of human lung cancer].* Zhonghua Jie He He Hu Xi Za Zhi, 2001. **24**(8): p. 465-8.

17. Tomizawa, Y., et al., *Clinicopathological significance of epigenetic inactivation of RASSF1A at 3p21.3 in stage I lung adenocarcinoma.* Clin Cancer Res, 2002. **8**(7): p. 2362-8.
18. Costello, J.F., et al., *Aberrant CpG-island methylation has non-random and tumour-type-specific patterns.* Nat Genet, 2000. **24**(2): p. 132-8.
19. Hui, A.B., et al., *Detection of multiple gene amplifications in glioblastoma multiforme using array-based comparative genomic hybridization.* Lab Invest, 2001. **81**(5): p. 717-23.
20. Turker, M.S., et al., *A partial methylation profile for a CpG site is stably maintained in mammalian tissues and cultured cell lines.* J Biol Chem, 1989. **264**(20): p. 11632-6.
21. Shukeir, N., et al., *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.
22. 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.
23. Kelley, M.J., et al., *Differential inactivation of CDKN2 and Rb protein in non-small-cell and small-cell lung cancer cell lines.* J Natl Cancer Inst, 1995. **87**(10): p. 756-61.