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Epigenetic mechanisms in the regulation of the B7-H1 and IRF-1 expression in tumour cells

Epigenetické mechanismy v regulaci exprese molekul B7-H1 a IRF-1 v nádorových buňkách

Diploma Thesis

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Podpis

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Contents

Abstract

Interferon γ is an important T-cell helper type 1 (Th1) cytokine involves in antimicrobial immunity. It is a part of the inflammatory immune response in the site of infection. However, for its proper function, the regulation of immunity is necessary to avoid injury of the tissue caused by long-term inflammation. While interferon γ triggers expression of proinflammatory genes, it also regulates genes which inactivate immune response. The B7-H1 molecule belongs among these inhibitory regulators.

Furthermore, antitumour effect of interferon γ is well-known as well. After extensive experiments, interferon γ was tested as an immunotherapeutic drug against melanomas in clinical trials. However, the trials had to be terminated prematurely because of unsuccessful results. It started to be clear that interferon γ could have also a protumour effect. Interferon γ upregulates the expression of B7-H1 molecule which aids tumour in escape from immunity. The B7-H1 molecule possesses a binding site for interferon regulatory factor 1 (IRF-1) in its promoter region. This IRF-1 is induced by interferon γ – JAK/STAT signalling pathway.

In our previous research, we observed interferon γ induced DNA demethylation of promoters in genes that are involved in antigen presenting machinery. Additionally, DNA methylation of interferon regulatory factors was observed in different tumours. Owing to these facts, I wanted to clarify the possible role of DNA methylation of B7-H1 molecule via IRF-1 transcription factor after interferon γ treatment.

The elucidation of the B7-H1 regulation might contribute to better design of anticancer immunotherapy based on interferon γ.

Key words: B7-H1, IRF-1, interferon γ, DNA methylation

Abstrakt

Interferon γ patří mezi významné cytokiny T pomocných lymfocytů typu 1 (Th1), které se účastní protimikrobiální imunity, jejímž důsledkem je zánětlivá odpověď v místě infekce. Dlouhodobé účinky zánětu mohou vést k poškození tkáně v místě infekce, proto je důležité imunitní odpověď regulovat. Interferon γ toto zajišťuje spuštěním exprese genů, které tlumí zánětlivou odpověď. Mezi tyto regulátory se řadí molekula B7-H1.

Je velmi dobře známo, že interferon γ také vykazuje protinádorové účinky. Po mnoha dlouholetých výzkumech se začal testovat v klinických studiích jako možný lék při léčbě melanomů. Bohužel se od těchto testů muselo předčasně opustit kvůli nevyhovujícím výsledkům. Po těchto zkušenostech začalo být zřejmé, že interferon γ může také podporovat nádorové bujení. Interferon γ aktivuje zvýšenou expresi B7-H1, která je významným činitelem při úniku nádorových buněk imunitnímu systému. Propojení mezi molekulou B7-H1 a interferonem γ je transkripční faktor nazývaný interferonem regulovaný faktor 1 (IRF-1). IRF-1 má své vazebné místo v promotoru genu B7-H1 a jeho exprese je spouštěna JAK/STAT dráhou, kterou aktivuje interferon γ.

V našich předchozích pokusech jsme pozorovali schopnost interferonu γ zahájit demetylaci DNA v promotorech genů, které se účastní prezentace antigenu. Dále byla prokázána metylace DNA v promotorech genů faktorů IRF u nádorů žaludka. Díky těmto informacím jsem chtěla zjistit možnou úlohu demetylace DNA v regulaci molekuly B7-H1 po působení interferonu γ. Tato regulace by mohla být zprostředkována faktorem IRF-1.

Hlubší poznání řízení exprese B7-H1 by mohlo napomoci vylepšení protinádorové imunoterapie založené na účinku interferonu γ.

Klíčová slova: B7-H1, IRF-1, interferon γ, metylace DNA

List of abbreviations

5caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
Actb	Name of gene for β-actin
ADD domain	ATRX-DNMT3-DNMT3L domain
AID	Activation induce cytosine deaminase
Akt	Protein kinase B
Alu elements	Arthrobacter luteus element
APC	Antigen presenting cell
Apobec	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
B7-H1	B7 homolog 1
BAH1/2 domain	Bromo adjacent homology 1/2
Bcl-2	B-cell Lymphoma 2
BER	Base excision repair
BRCA1	Breast cancer 1
BSP	Bisulfite specific polymerase chain reaction
C57BL/6	C57 black 6, inbred strain of mouse
CD	Cluster of differentiation
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CIITA	Class II major histocompatibility complex transactivator
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXXC domain	Cystein-variable amino acid-variable amino acid-cystein domain
DC	Dencritic cells
DMSO	Dimethyl sulfoxid
dNTP	deoxy-Nucleotid Triphosphate
E2F	Helix-loop-helix transcription factor
E6	Early 6 genes of human papilloma virus
E7	Early 7 genes of human papilloma virus
FCS	Fetal calf serum
GAS	Gamma activated site
H2D^b	Name of first gene for MHC I
H2K^b	Name of second gene for MHC I
H3K27 me3	Trimethylation of lysine 27 of histone 3
H3K36me3	Trimethylation of lysine 36 of histone 3
H3K9ac	Acetylation of lysine 9 of hisone 3
H3K9me2, 3	Di-, trimethylation of lysine 9 of histone 3
H-MEMd medium	Cell culture medium for RVP3 cells
HPV-16	Human papillomaviruse, type 16

H-ras	Harvey rat sarcoma viral oncogene homolog, GTPase
ICAM1	Intercellular adhesion molecule 1
IDO	Indoleamin 2,3 dioxygenase
IFNGR1	Interferon gamma receptor 1
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl β-d-1-thiogalactopyranoside
IRF-1	Interferon regulatory factor 1
ISRE	Interferon stimulated response element
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
JAK 1, 2	Janus activated kinase 1, 2
LIF	Leukima inhibitory factor
LINE	Long interspersed element
LMP 2, 7	Low molecular mass polypeptide 2, 7
MDSC	Myeloid derived suppressor cell
MECL1	Multicatalitic endopeptidase complex subunit
MHC I, II	Major histocompability complex class I, class II
MLH1	Mutl homolog 1
MSH2	Muts homolog 2
MSP	Methylation specific polymerase chain reaction
MuLV	Murine leukemia virus
NaOAc	Sodium acetate
NK cell	Natural killer cells
NKG2D	Natural killer group 2 member d
NKT cells	Natural killer T-cells
NLS	Nuclear localization signal
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD-1	Programmed death receptor 1
PD-L1	Programmed death ligand 1
pGEM-T Easy Vector	Plasmid vector for the cloning PCR products (Promega)
PI3K	Phosphoinositide 3 kinase
PP2A	Protein phosphatase 2A
pVEJB	Plasmid constructed by inserting the *Bam*hi fragment of plasmid pEJ6.6 carrying the Ha-c-ras oncogene at the unique site of plasmid pVV2.
qPCR	Quantitative real-time PCR
RFTS domain	Replication focus targeting sequence domain
RPMI medium	Roswell park memorial institute medium
RVP3	Mice tumour cell line come from RVP3 sarcomas
SAM	S-adenosil methionin

SATR-1	Satellite sequence, located on chromosome 5
SH2	Src homology 2
SHP 1, 2	Src homology 2 containing tyrosine phosphatase 1, 2
SINE	Short interspersed elements
SMUG1	Singel-strand-selective monofinctional uracil-dna glycosilase 1
SOCS1	Suppressor of cytokine signalling 1
STAT 1	Signal transducer and activator transcription 1
TAP 1, 2	Transporter associated with antigen processing 1, 2
TBE	Tris-borate-EDTA
TC-1	Mice lung tumour cell line, parental cell line to TC-1/A9
TC-1/A9	Mice lung tumour cell line, daughter cell line of TC-1
TCP45	T-cell protein tyrosine phosphatase
TCR	T-cell receptor
TET	Ten-eleven translocation methylcytosine dioxygenase
TGD	Trigalactosyldiacylglycerol protein
TGFβ	Transforming growth factor β
Th1	T-helper cell type 1
TNFα	Tumour necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T-regulatory cell
VEGF	Vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, inert chromogenic sunstrate for β-galactosidase
ZAP70	Zeta-chain-associated protein kinase 70

Introduction

The idea of tumour growth suppression by immune system was established by Frank Macfarlane Burnet and Lewis Thomas in the late 1950´s. Their theory was called "Cancer Immunosurveillace" *(Dunn et al, 2004)*. After some decades of studies, it started to be clear while immune system is able to fight against tumours, it can also promote cancer development. Predominance of negative immune regulatory signals leads to inhibition of antitumour immunity. Influence of interleukine 10 (IL-10) and transforming growth factor β (TGFβ) cytokines, presence of T regulatory cells (Treg) and myeloid derived suppressor cells (MDSC) in cancer microenvironment, cell surface downregulation of MHC I and upregulation of B7-H1 co-inhibitory molecule, these all are the main effectors of tumour escape *(Mittal et al, 2014)*.

Cytokine interferon γ (IFNγ) is the crucial player of immune response. It is a well established inducer of proinflammatory immune response. It initiates expression of many genes which are involved in activation of antigen presenting cells, natural killer cells and in modulation of T-helper type 1 (Th1) cell immunity. This ability is used in antimicrobial as well as antitumour defence of organism. IFNγ was tested as an immunotherapeutic drug against melanoma in clinical trials because of its antiproliferative and proapoptotic capability. However, these trials had to be terminated prematurely as IFNγ proofed to have none or even negative effect in patients *(Zaidi and Merlino, 2011)*. B7-H1 upregulation after IFNγ stimulation belongs to one of these possible protumour signals.

B7-H1 plays role in inhibition of the T-cell mediated immune response by transferring the second signal via B7-H1 – PD-1 pathway. This leads to cessation of T-cell proliferation, IL-2 cytokine release and overall protein production. In general, B7-H1 molecule is expressed in immune privileged organs and in trophoblast of the placenta. Moreover, transformed cells are able to utilize B7-H1 inhibitory effect for their own benefit against T-cells immunity. In these days, the therapeutic antibody against B7-H1 molecule is being tested in clinical trials *(Seliger et al, 2008; Robert, Soria and Eggermont, 2013)*.

It is well known that IFNγ regulates B7-H1 molecule through interferon regulatory factor 1 (IRF-1). Our previous findings showed that IFNγ triggers promoter DNA demethylation of antigen presenting machinery genes (*TAPs, LMPs*). DNA demethylation of *TAP* and *LMP* genes lead to their transcription activity *(Annexe).* Similar observation was published by Thillainadesan and colleagues about TGFβ and its ability to cause DNA demethylation of target gene *(Thillainadesan et al, 2012)*. Further, DNA methylation proofed to be crucial in inhibition of *IRF* genes in gastric cancer *(Yamashita et al., 2010)*.

Based on our research experience and on literature, the aim of my diploma project was to determine the IFNγ induced cell surface upregulation of B7-H1 via DNA demethylation of *IRF-1* gene promoter. Elucidation of regulation of B7-H1 expression via IFNγ is important for realization undestanding how IFNγ immunity is negatively controlled and how it can be misused in cancer development. My project might contribute to the application of immunotherapy in clinical practise, mainly to the use of therapeutic antibodies against B7-H1.

Other goals were to gain my own work experience in laboratory. I learned how to search in genome browser databases and how to design primers. Finally, I learned methods which I used in the study of *IRF-1* gene regulation, namely quantitative real-time PCR, bisulfite conversion, methylation specific PCR, DNA cloning and bisulfite sequencing.

Literature review

1. The role of the B7-H1 co-inhibitory molecule in immune system and tumour invasion

1. 1. B7-H1 molecule characteristics

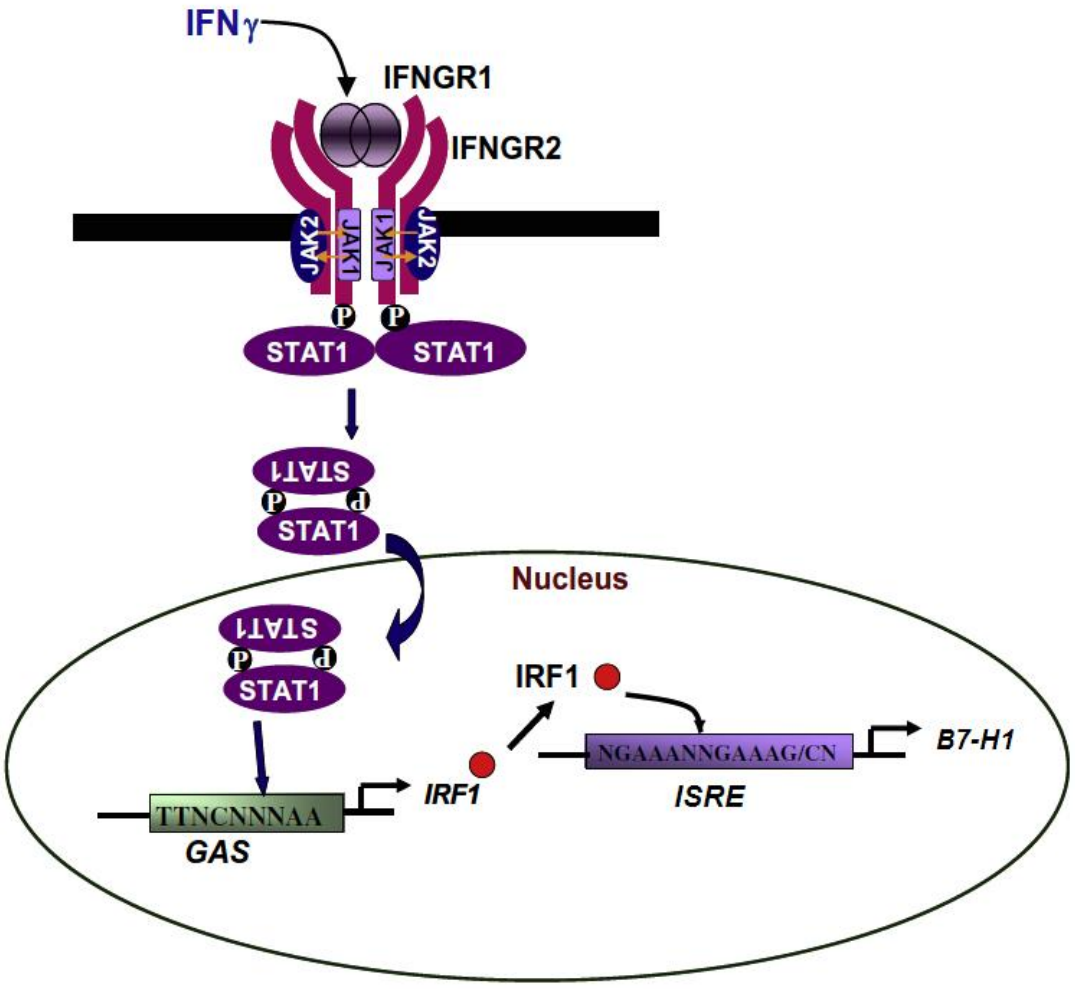
The connection of T-cell receptor (TCR) with major histocompatibility complex (MHC)-antigen peptide is the first event in the transformation of naive T-cell into an effector T-cell. A second key step is necessary for proper T-cell activation. Second signal is afforded by B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules linked with their CD28 receptor. However, an inverse signal is crucial for T-cell response regulation. For example, B7-1, B7-2 and B7-H1 provide this co-inhibitory signalling via binding to the inhbitory receptors CTLA-4 and PD-1 *(Seliger et al, 2008).*

B7-H1 molecule, also called CD274 or programmed death-ligand 1 (PD-L1), shares homology with B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules in variable (V) and constant (C) immunoglobulin domains. It is called B7 homolog 1 (B7-H1) because it has more homology in common with the B7-1 molecule *(Dong et al, 1999).*

The molecule is expressed on a variety of hematopoietic cells such as professional antigen presenting cells (APC), T-cells and natural killer cells (NK cells). Furthermore, it is expressed on cells of immune privileged organs such as corneal cells in the eye and trophoblast cells of the placenta. The expression of its mRNA was also detected in pancreatic islet cells, spleen, heart, lung *(Dong et al, 1999; Hori et al, 2006; Petroff et al, 2003; Fife et al, 2011).*

Finally, B7-H1 was described in most neoplasms such as lung cancer, ovarian cancer, hepatocellular carcinoma, anaplastic large cell lymphomas, myelodisplastic syndrom and glioma *(Dong et al, 2002; Yamamoto et al, 2009; Yao et al, 2009; Kondo et al, 2010; Shi et al, 2011).* The IFNγ cytokine is a regulator of the B7-H1 expression. IFNγ potentiates B7-H1 cell surface expression on noncancerous as well as on cancerous cells. The B7-H1 upregulation by IFNγ is dependent on interferon regulatory factor-1 which binds to a specific site in the B7-H1

gene promoter *(Fig.1.1.; Seliger et al, 2008; Lee et al, 2006; Lee et al, 2005).* The fact, that B7-H1 is expressed on tumour cells, indicates its possible benefit for tumour growth.



Adapted from: Saha, B., Jyothi Prasanna, S., Chandrasekar, B., Nandi, D. 2010. Cytokine

Fig.1.1: Induction of B7-H1 expression through IRF-1 transcription factor after interferon γ stimulation.

Ligation of IFNγ to its receptor causes phosphorylation of Janus tyrosine kinase 1 and 2 (JAK1, JAK2). Activated JAKs phosphorylate both interferon gamma receptors 1 (IFNGR1). Signal transducer and activator of transcription 1 (STAT1) binds to this phosphorylated IFNGR1 and is also phosphorylated. The STAT1s form a homodimer and translocate to the nucleus. STAT1 dimer recognizes specific sequences in promoter of *IRF-1* gene, called gamma interferon activated site (GAS). Further, IRF-1 transcription factor binds to interferon stimulated response elements (ISRE) in promoter of *B7-H1* gene, and induces its expression.

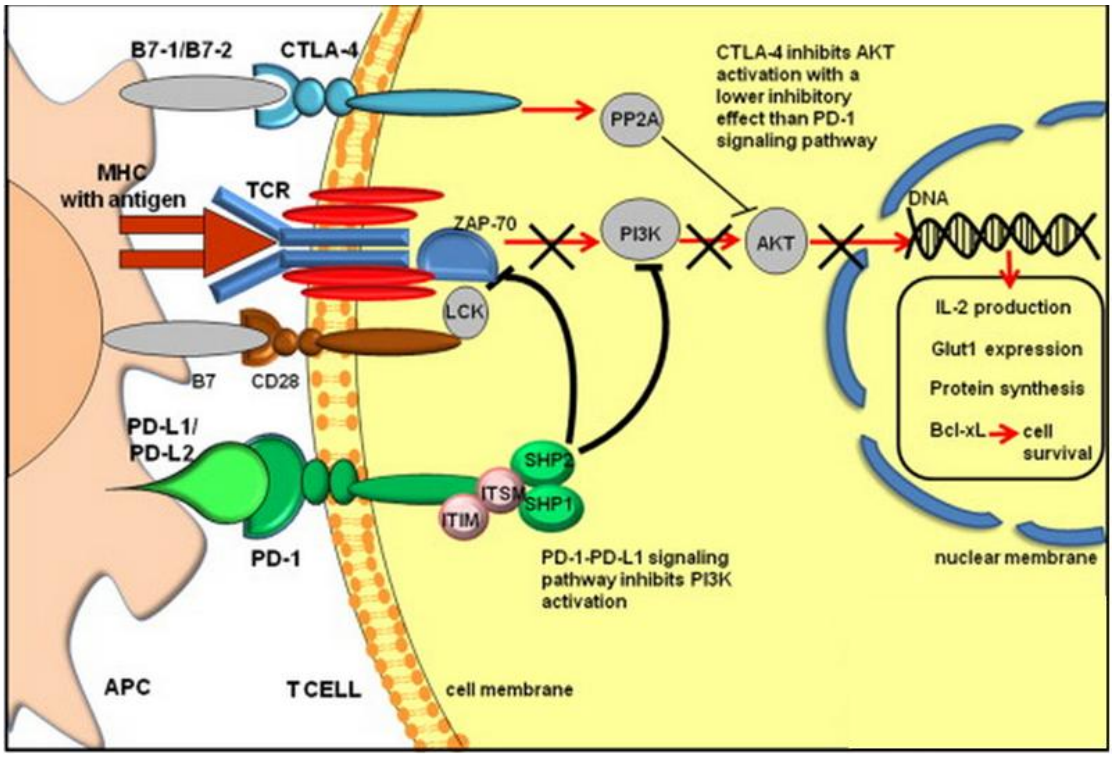
1. 2. Mechanism of T-cell inhibition by B7-H1 molecule

As it was mentioned above, B7-H1 is able to inhibit activation of T-cells and induce apoptosis of tumour specific T-cells. How is this T-cell inactivation generated?

It is well known that programmed death receptor (PD-1) is expressed on activated T- and B-cells and that this molecule can downregulate immune response. Freeman and his colleagues indentified that PD-1 counterpart is the B7-H1 and that their interaction reduces T-cell proliferation and cytokine production *(Freeman et al, 2000).* PD-1 receptor is a 55 kDa large transmembrane protein. It belongs to the immunoglobulin (Ig) receptor superfamily which contains the N-terminal IgV-like domain. PD-1 cytoplasmic domain includes immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). These motifs are present in inhibitory receptors. Their phosphorylation induces recruitment of proteins with inhibitory effect, for example Src homology region 2 domain-containing phosphatase-1 and -2 (SHP-1, SHP-2) *(Blank et al, 2005; Francisco et al, 2010; Lorenz, 2009).*

After further studies, it became clear that PD-1 can inhibit T-cell response through interference with phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) activity. This ability of PD-1 to block PI3K/Akt signalling pathway is provided by recruitment of the SHP-2 molecule to ITSM of PD-1 cytoplasmic tail *(Parry et al, 2005; Latchman et al, 2001).* For T-cell inactivation, PD-1 needs to colocalise with CD3 and CD28 receptors *(Chemnitz et al, 2004)*. This colocalization was validated by the observation that PD-1 is able to induce dephophorylation of Zeta-chain-associated protein kinase 70 (ZAP70 kinase) and thus inhibit CD3ζ chain-T-cell receptor (TCR) mediated signalling *(Sheppard et al, 2004).* SHP-2 association with ITSM and subsequent PD-1 mediated T-cell suppression was observed in PD-1-TCR microclusters. A component of TCR microclusters is also the CD28 receptor *(Yokosuka et al, 2012; Yokosuka et al, 2008).*

The PD-1 mediated negative regulation results in the reduction of protein synthesis, in IL-2 production by T-cells and in the decrease of T-cell proliferation and survival *(Fig.1.2.; Gianchecchi et al, 2013)*.



Adapted from: Gianchecchi, E., Delfino, D. V., and Fierabracci, A., 2013. Autoimmunity Reviews.

Fig.1.2: Inhibitory signalling pathway through interaction programmed death receptor (PD-1) and its ligand B7-H1 (PD-L1).

The second co-stimulatory signal induced by B7-CD28 interaction is important for full activation of T-cell. However, T-cell inhibition is mediated through B7-CTLA-4 or B7-H1 (PD-L1)-PD-1 binding. During the negative T-cell regulation mediated by B7-H1-PD-1 pathway, SHP-2 directly binds to phosphorylated ITSM motif in PD-1 receptor. Subsequently, SHP-2 inactivates ZAP-70 kinase, CD3ζ chain and PI3K kinase by direct dephosporylation. B7-CTLA-4 signalling pathway inhibits Akt kinase directly through activation of protein phosphatase 2 (PP2A). Inhibition of PI3K/Akt signalling pathway leads to reduction of protein synthesis, IL-2 cytokine production, proliferation and survival of T-cell.

1. 3. The significance of the B7-H1 molecule in cancer immunoediting

The role of immune system was thought to be limited only to the defence against microbic and parasitic infection. More than 50 years ago, Frank Macfarlane Burnet and Lewis Thomas proposed a theory that the protection from neoplastic disease by recognition and elimination of transformed cells belongs amongst function of immune system. This theory was called "Cancer Immunosurveillance". However, few following decades of experiments revealed that immune system is not only able to protect organism against tumour formation but it is also able to promote cancer development. Thus the theory of cancer immunosurveillance was modified to the hypothesis called "Cancer Immunoediting". Cancer immunoediting contain three phases – Elimination, Equilibrium and Escape *(Fig.1.3; Schreiber, Old and Smyth, 2011).*

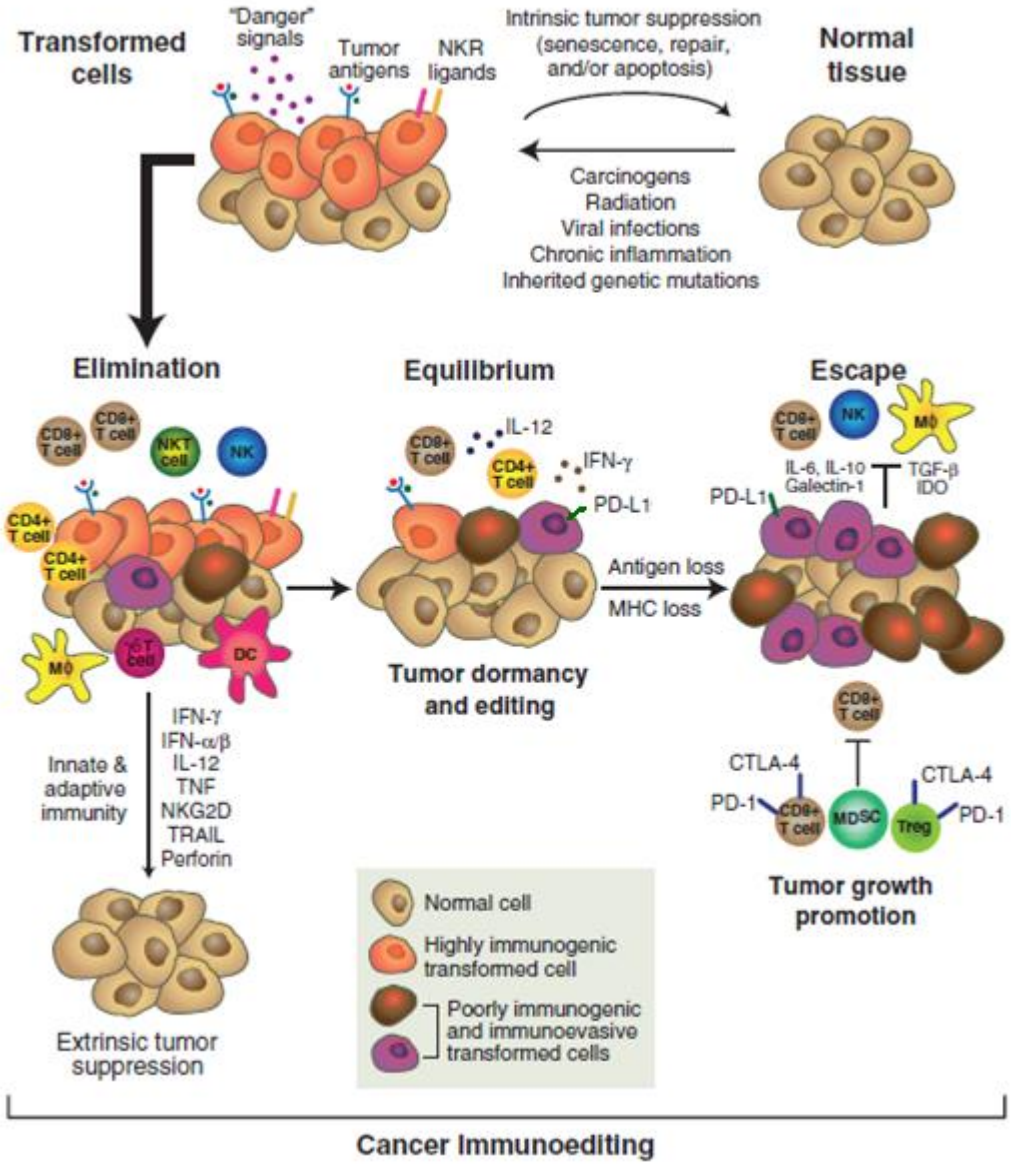
Immune system in the elimination phase is able to detect and eradicate transformed cancer cells before they become clinically noticeable. Immunogenicity is caused by $CD8^+$ T-cells and NK cells recognition of tumour antigens through MHC I glycoproteins and NKG2D ligands. Antigen presenting cells mainly DC present tumour antigens to $CD4^+$, $CD8^+$ and NKT cells. Moreover, the transformed cells express Fas and TRAIL receptors or NKG2D ligands which incite their apoptosis through the activation of T-cells and NK cells. Effector T-cells and activated NK cells secrete cytokines (IFNγ, TNFα), perforines and granzymes which inhibit cancer cell proliferation or induce tumour cell apoptosis *(Mittal et al, 2014).*

The following state – the equilibrium phase – is characteristic for functional dormancy of tumour cells held by active antitumour immune response. While some cancer cells are still destroyed by effector T-cells and NK cells, some transformed cells start to be unresponsive. This insensitivity is evolved by genetic and epigenetic changes which lead to the deficient antigen presentation and expression of immunosuppressive molecules, such as B7-H1, on tumour cells *(Mittal et al, 2014*).

Finally, in the escape phase of immunoediting, immuno-resistant cancer cells survive and the neoplasm becomes clinically detectable. These cells evade immune recognition by reduction of antigen presentation through MHC I downregulation, by acquisition of immunosuppression via B7-H1 cell surface expression, for instance. They increase their

survival by expression of antiapoptotic molecules, such as Bcl-2 and secret proangiogenic and immunosuppressive cytokines, for example VEGF, IL-6 or TGFβ. Furthermore, tumour microenvironment is infiltrated by immunoregulatory cells, Treg and MDSC, which inhibit effector T-cells by expression of immunoinhibitory molecules – iNOS, IDO, Arginase 1 – and cytokines – IL-10, TGFβ (*Mittal et al, 2014*).

In conclusion, B7-H1 is upregulated after IFNγ stimulation. It can inhibit T-cell immune activity via B7-H1 – PD-1 signalling pathway. Moreover, it is expressed in a vast variety of different cancers. Obviously, tumour cells use this molecule for inactivation of anticancer immunity (cancer immunoediting). These facts make the B7-H1 an interesting molecule for further deep investigation of its regulation. These days, an anti-B7-H1 antibody drugs are being examined as a new immunotherapeutic approach in clinical trials *(Robert, Soria and Eggermont, 2013)*. Further research will help develop better immunotherapy for eradication of neoplasm.



Adapted from: Schreiber, R. D., Old, L. J., and Smyth, M. J., 2011. Science.

Fig.1.3: Cancer immunoediting hypothesis.

The three phases of cancer immunoediting theory are Elimination, Equilibrium and Escape. During the elimination phase, transformed cells are detected and destroyed by effector T-cells ($CD8^+$, $CD4^+$ T-cells), NKT cells and activated NK cells. The antigen presenting cells (macrophages, DC) present tumour antigen to these effector cells. Production of different cytokines by T-cells and APCs leads to overexpression of MHC I and antigen presentation by tumour cells. TRAIL and perforines induce apoptosis of cancer cells.

In the equilibrium phase, tumour cells are held in functional dormancy by the effector T-cells ($CD8^+$, $CD4^+$ T-cells) and their products (IFNγ, IL-12). However, some transformed cells accumulate genetic and epigenetic changes which lead to a reduction of antigen presentation by downregulation of MHC I glycoproteins and expression of immunosuppressive molecule, such as B7-H1 (PD-L1). Cancer cells, in the escape phase, inhibit immune cell response by production of immunoregulatory cytokines IL-10, TGFβ or expression of coinhibitory molecule B7-H1 (PD-L1). Furthermore, tumour microenvironment is infiltrated by Treg and MDSC which also inactive effector T-cells. Taken together, under T-cell inhibitory conditions (presence of IL-10, TGFβ, B7-H1, Treg, MDSC, no MHC I-antigen presentation) tumour cells can escape from T-cell immune surveillance.

2. The role of interferon-regulatory factor-1 in immune system and tumour invasion

2. 1. Interferon-regulatory factor-1 characteristic

Interferon-regulatory factor-1 (IRF-1) was discovered at the end of 1980´s as a protein which bound to regulatory elements of cytokine interferon β gene (IFNβ) *(Yanai, Negishi and Taniguchi, 2012)*. Later, other groups described IRF-1 binding to the cytokine interferon α (IFNα) gene enhanceosome. The IRF-1 factor regulates transcription of type I interferon cytokines (IFNα, IFNβ). It also regulates transcription of IFNγ (type II interferon) induced target genes *(Paun and Pitha, 2007).* This factor belongs to interferon-regulatory factors family, which consist of nine members (IRF-1 – IRF-9). All IRF factors possess N-terminal DNA binding domain (DBD) which conforms to helix-turn-helix structure. DBD recognize DNA sequences called IFN-stimulated response element (ISRE). IRF-1 C-terminus contain IRF-associated domain 2 (AID2) mediates IRF interaction with transcriptional factor, cofactors and other members of the IRF family *(Yanai, Negishi and Taniguchi, 2012)*.

IRF-1 transcription factor is important for terminal differentiation of myeloid cells and partly plays a role in differentiation of CD8 and CD4 thymocytes. Further IRF-1 is also essential for the macrophages or T-cell activation via APC *(Simon, Desrois and Schmitt-Verhulst, 1997; Dror et al, 2007; Bauvois et al, 2009)*. It is also crucial for regulation of cell

cycle arrest because of transcription activation of cyclin-dependent kinase (CDK) inhibitor *p21* gene *(Kano et al, 1999; Yanai, Negishi and Taniguchi, 2012)*.

IRF-1 transcription factor upregulates lots of genes, for example gene for CDK ihnibitor p21, interleukine-17 receptor (IL-17R), leukemia inhibitory factor (LIF) or CD80 co-stimulatory molecule. IRF-1 regulates apoptosis, macrophages activation and second signal for full T-cell activation through above mentioned proteins. The B7-H1 co-inhibitory molecule, which is crucial T-cell immune response inhibitor, also belongs among target genes *(Lee et al, 2006)*. This suggests that IRF-1 is an important regulator of immune response after IFNγ stimulation.

2. 2. Interferon-regulatory factor-1 and its relation to tumour growth

IRF-1 transcription factor regulates apoptosis not only in nontransformed cells but also in cancer cells and its loss of function promotes tumour growth *(Kano et al, 1999; Kim et al, 2002; Liebermann and Hoffman, 2009)*. At first, its defect was described in patients with myelodysplastic syndrom (MDS) a preleukemic stage of human leukemias. MDS cells have frequent deletion within the long arm of chromosome 5 (5q) or loss of whole 5 chromosome. *IRF-1* gene was mapped in 5q31.1 chromosome region and the deletion of one or both *IRF-1* alleles in 5q31.1 locus was proven in MDS patients *(Willman et al, 1993)*. Later on, Maratheftis and colleagues demonstrated aberrant splicing of *IRF-1* mRNA and subsequent truncated expression of IRF-1 protein in MDS patients *(Maratheftis et al, 2006)*. Furthermore, higher expression of IRF-1 was proven to correlate with lower recurrence of hepatocellular carcinoma after surgical removal and better survival of these patients *(Yi et al, 2013)*.

Taken together, IRF-1 transcription factor is a tumour suppressor. However, it is also important for regulation of T-cell response after IFNγ proinflammatory stimulation. One of these pathways is mediated through transcription and translation of B7-H1 molecule which negatively regulates T-cell activity. Inhibition of T-cell immune response through IFNγ-IRF-1-B7-H1 pathway could be misused for cancer development *(Fig. 1.1; Lee et al, 2006)*.

3. The role of interferon γ in immune system and tumour invasion

3. 1. Interferon γ characteristics

IFNγ was discovered in 1965 as a protein which interfered with viral replication. It is the only element of type II interferon family because it is structurally unrelated and binds to a different receptor than members of type I interferon family (IFNα, IFNβ) *(Saha et al, 2010)*. Activated $CD4^+$ Th1, $CD8^+$ cytotoxic T-cells (CTL), NK cells, NKT cells and professional APC, such as dendritic cells (DC) and macrophages are the major sources of this cytokine *(Schroder et al, 2004)*.

IFNγ is crucial for antimicrobial immune response. Induction of this immunity is also caused by its ability to initiate expression of different genes which are involved in antigen presentation, immunomodulation of T-cells, NK cells and APC and have antiproliferative and apoptotic effect. Antigens are presented by MHC I and II glycoproteins. For efficient presentation, there are several steps in assembling of antigen peptides to MHC classes. Among proteins, which are involved in MHC I-antigen peptide presentation, are subunits of immunoproteasome LMP2, LMP7 and MECL-1, TAP transporters, α heavy chains and β_2-microglobulin of MHC I glycoprotein and Tapasin chaperon. The α and β subunits of MHC II glycoproteins, invariant chain chaperon (Ii), class II transactivator (CIITA) and more are important for presentation of antigens via MHC II. Inhibitors of CDK p21 and p27, retinoblastoma protein (Rb) and inhibitor of E2F transcription factor p202 are involved in the antiproliferative effect. Caspase 1 and Fas receptor-Fas ligand signalling induce apoptosis. Among immunomodulatory proteins belong CD80 and CD86 costimulatory molecules, IL-12 cytokine and chemoattractans. All of the genes cited above, and many more, are upregulated by IFNγ *(Schroder et al, 2004)*.

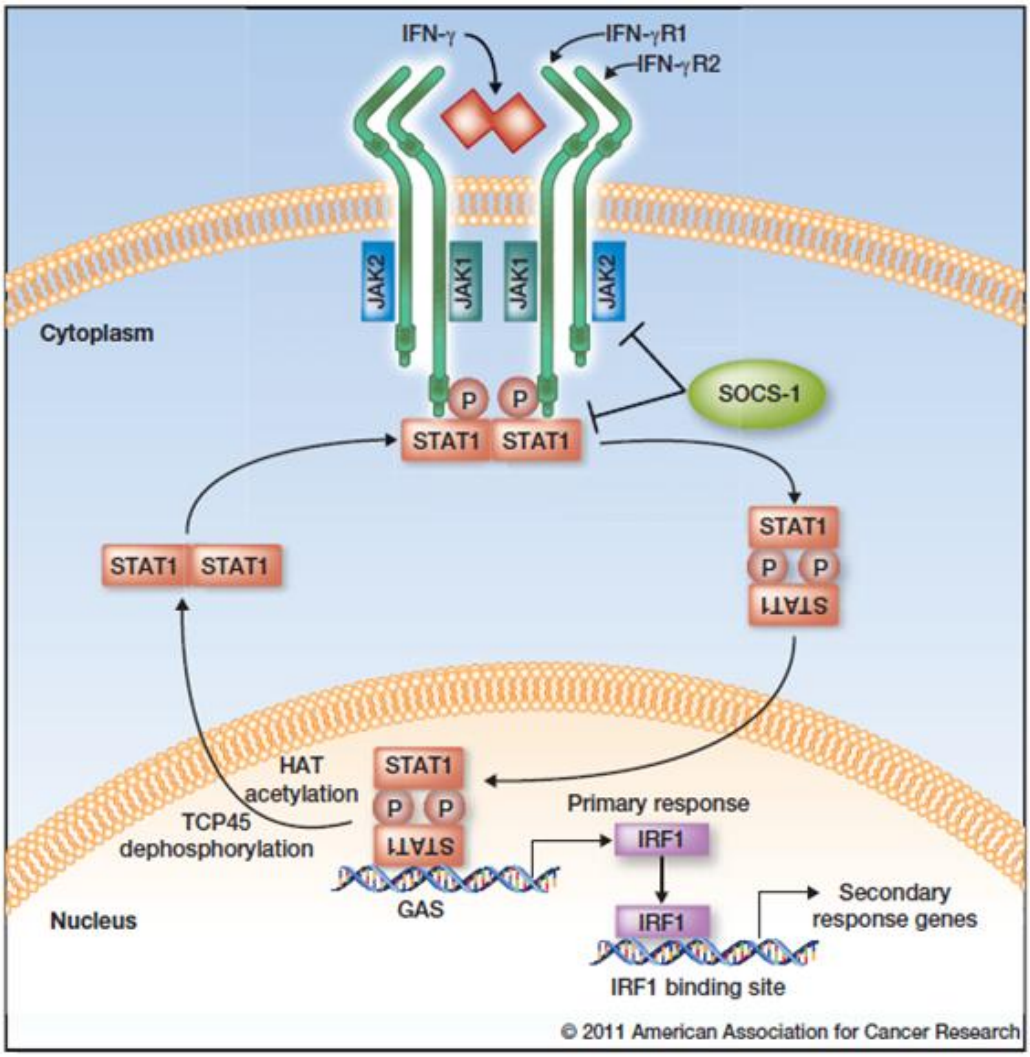
Because of activation of these genes, IFNγ is able to induce anti-viral and -bacterial immune response. IFNγ is a master regulator of many proinflammatory pathways which activates T-cell. This suggests an idea that IFNγ cytokine is an antitumour effector.

3. 2. Interferon γ signalling pathway

IFNγ signalling starts with attachment of IFNγ to its interferon gamma receptor (IFNGR) which is composed of two distinct subunits IFNGR1 and IFNGR2. Two cytoplasmic Janus activated tyrosine kinases (JAK), JAK1 and JAK2, constitutively associate with membrane-proximal region of IFNGR1 and 2 because receptor itself lacks catalytic activity. JAK1 cooperates with IFNGR1 and JAK2 with IFNGR2 *(Haan et al, 2006; Platanias, 2005)*. Upon the binding of IFNγ to its receptor, JAK2 kinases autophosporylate and allow the phosphorylation of JAK1 kinases. IFNGR1 subunits contain tyrosine residues which are important for signal transduction. Activated JAK1s phosphorylate these tyrosines and provide docking sites for Src homology 2 domains (SH2) of signal transduction and activator of transcription 1 (STAT1) *(Saha et al, 2010)*. Phosphorylation of two STAT1 proteins, on tyrosine residues, initiates formation of STAT1 dimers and the dissociation from their receptor. Homodimers are then translocated into the nucleus where they recognize special sequences in promoters of target genes called gamma activated sites (GAS). STAT1 dimers induce expression of primary response genes, such as IRF-1. The primary response genes then activate other genes called secondary response genes. Among the secondary activated genes belong, for example, TAP transporters, LMP immunoproteasomes, CIITA transcactivator, CD80 and 86 costimulatory molecules and more *(Zaidi and Merlino, 2011)*.

IFNγ signalling transduction needs to be regulated to avoid harmful consequences of overstimulation. We know that IFNγ also stimulates expression of its own inhibitory molecules called suppressor of cytokines signalling (SOCS). SOCS1 directly interact with JAK1 and JAK2 kinases and their catalytic activities *(Saha et al, 2010)*. Among others immediate inhibitors of IFNγ signalling pathway are the inhibitors of STAT1 homodimers. Intracellular lysin acetylation of STAT1 by CREB binding protein (CBP) histone acetylases induce binding of T-cell protein tyrosine phosphatase (TCP45). By this way, STAT1 is inhibited by dephosphorylation and translocated from the nucleus into the cytoplasm *(Fig.3.2; Krämer et al, 2009)*.

IFNγ induction of pro-inflammatory immunity is also regulated by the inhibition of T-cell immune response, for instance T-cell inhibition by B7-H1 co-inhibitory molecule.



Adapted from: Zaidi, M. R., and Merlino, G., 2011. Clinical Cancer Research

Fig.3.2: Signalling pathway of interferon γ.

Ligation of IFNγ into its receptor causes autophosphorylation of JAK2 which phosphorylates JAK1. Activated JAK1 phosphorylates tyrosine residues of both IFNGR1. This leads to generation of binding sites for STAT1 via its SH2 domain. Phosphorylated STAT1s form an active homodimer which then translocates to the nucleus. STAT1 dimer recognizes GAS sequences in promoters of target primary response genes, such as IRF-1. IRF-1 transcription factor induces expression of secondary response genes.

3. 3. The role of the interferon γ in tumour invasion

As it was mentioned in the chapter "*Importance of B7-H1 molecule in cancer immunoediting"*, IFNγ plays a considerable role in the elimination of transformed cells and in maintaining of tumour dormancy. This ability is caused by the induction of MHC I and II and CD80, CD86 co-stimulatory molecule upregulation. Thus APC can better present tumour antigens to T-cells. The effector T-cell can then destroy cancer cells by releasing granzymes and perforines. Activation of NK cells, important fighter against tumour cells, is also induced by IFNγ. Further on, IFNγ is able to cause expression of antiproliferative effectors, such as CDK inhibitors p21 and p27. Moreover, it stimulates expression of apoptotic executors, such as Fas ligand and receptor or caspase 1. Cytostatic and apoptotic IFNγ activity contributes to tumour growth suppression.

However, in some studies, it was observed that treatment of cancer cells by IFNγ can enhance tumour resistance to immune response. For instance a correlation of an aggressive phenotype of melanoma cells with high level of MHC II expression was described (*Hemon et al., 2011)*. Other research groups detected acceleration of tumour proliferation and resistance to NK cells after IFNγ treatment. Clinical trials also demonstrated unfavourable results for using IFNγ as a potential immunotherapeutic drug *(Zaidi and Merlino, 2011)*. How can IFNγ have these two opposite effects?

Indoleamine 2,3-dioxygenase (IDO) expression by cancer cells is dependent on IFNγ. IDO facilitates inhibition of T-cell proliferation by degradation of amino acid tryptophan *(Brody et al, 2009)*. Further, IDO activates Treg cells in tumour microenvironment *(Munn and Mellor, 2007).* IFNγ supports accumulation of immature myeloid cells, called MDSC, in tumour microenvironment. MDSC production of IL-10 and TGFβ cytokines lead to formation of Treg cells as well *(Ostrand-Rosenberg and Sinha, 2009)*. Treg cells and MDSC are well-known negative regulators of effector T-cell activity. Among other T-cell inhibitors which are induced by IFNγ stimulation include B7-H1 co-inhibitory molecule *(Seliger et al, 2008)*.

It is obvious that long-term inflammation caused by IFNγ elicits antiinflammatory signals which could lead to inhibition of IFNγ effect. This regulation helps to prevent tissue injuries caused by inflammation. However, it is also used by transformed cells to spread out in organism.

4. DNA methylation

DNA methylation belongs to epigenetic modifications associated with gene silencing in mammals. The reason why this alteration is related to the transcriptional gene regulation is that the cytosine bases are modified by DNA methyltransferases in promoter regions called CpG islands.

4. 1. CpG islands

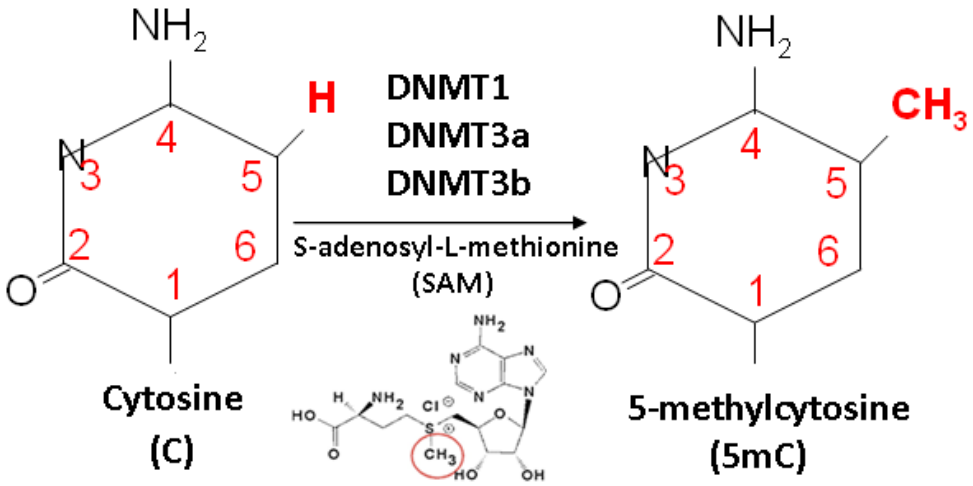
CpG islands are dinucleotides of cytosine (C) and guanine (G) connected through phosphodiester bond (p). Even though they comprise less than 1 % of total genomic DNA, they are very frequent (60-70 %) in promoters. They remain unmethylated in somatic cells *(Cross and Bird, 1995; Illingworth and Bird, 2009)* and their size ranges from 0.5 to 5 kb *(Singal and Ginder, 1999)*. According to the calculations of authors Antequera and Bird, first half of CpG islands is associated with housekeeping genes and the second half with tissue-restricted genes *(Antequera and Bird, 1993; Illingworth and Bird, 2009)*. Chromatin of promoters with CpG islands is often highly acetylated, lacks H1 histone and has a nucleosome-free region. This is the sign of open chromatin structure which enables the interaction of transcription factors to their promoter´s binding sites *(Cross and Bird, 1995)*.

4. 2. DNA methyltransferases

DNA methylation represents addition of methyl group from S-adenosyl-L-methionin (SAM), which acts as a methyl donor, to the 5th carbon of cytosine ring in the CpG islands *(Fig.4.2.1)*. This reaction is catalyzed by DNA (cytosine 5-)methyltransferases, or briefly DNA methyltransferases (DNMTs) *(Singal and Ginder, 1999; Robertson, 2002)*. In mammals, there are two families of enzymes involved in DNA methylation; DNMT1 and DNMT3 family *(Cheng and Blumenthal, 2008)*. Their structures are composed of two parts, N-terminal region and C-terminal region containing catalytic domain. The N-terminal regions are not similar between DNMT1 and DNMT3 enzymes. These DNMTs contain several different domains which bind to specific factors or domains that directly regulate their activity.

In contrast to the N-terminal region, C-terminal regions are homologous in DNMTs. They contain conservative motifs with catalytic function. A single exception is DNMT3L. It does not have some specific domain and motifs in the N-terminal and C-terminal part but it binds DNMT3a and b enzymes and modulates their activity (*Klose and Bird, 2006; Ryazanova et al, 2012)*.

According to the differences in the presence or absence of domains in N-terminal part, DNMTs also vary in their time of action. DNMT1 associates with replication foci during the S phase of cell cycle, because it can recognize the unmethylated CG sites of newly synthesized DNA strands, thanks to its CXXC domain *(Ryazanova et al, 2012)*. This suggests that DNMT1 is required for the maintenance of DNA methylation pattern after replication. This is why DNMT1 is also called “maintenance” methyltransferase *(Robertson, 2002)*. On the contrary, family of DNMT3 enzymes is involved in *de novo* methylation because of high expression in embryonic stem cells and low expression in differentiated cells. The DNMT3s are caled “*de novo*” methyltransferases *(Bird, 2002)*.



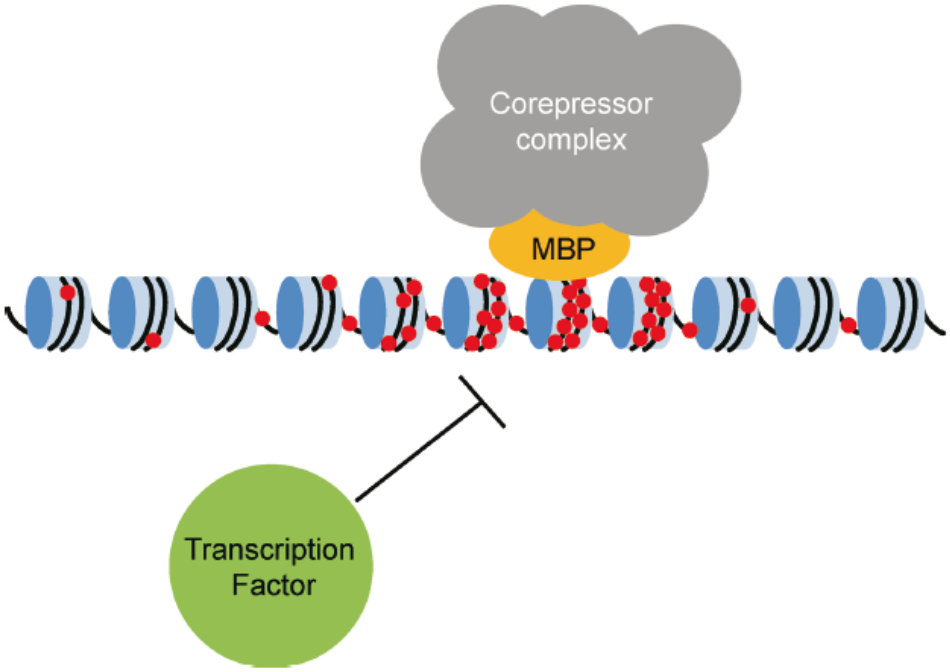
Adapted from: Leung C.-M., Tsai, K.-W-, Pan, H.-W., 2013. Available from: http://www.intechopen.com/books/gastric-carcinoma-new-insights-into-current-management/dna-methylation-in-aggressive-gastric-carcinoma

Fig.4.2.1: Methylation of cytosine.

DNA methyltransferases (DNMT1, 3a, 3b) use S-adenosyl-L-methionin as source of methyl group during cytosine methylation. DNMTs bind methyl group to the 5th carbon of cytosine ring.

4. 3. Possible mechanisms of transcriptional silencing by DNA methylation

As I have mentioned above, DNA methylation is linked with gene silencing through inhibition of gene transcription. Two possible mechanisms of this repression have been described. First is, that, methylation of cytosine themselves prevents binding of transcription factors into their DNA recognition sites. The second machinery is the recruitment of methyl-CpG binding proteins (MBPs) with co-repressor complexes into methylated sites *(Fig.4.3; Klose and Bird, 2006; Fukushige and Horii, 2013)*. Histone deacetylases (HDACs) and histone methyltransferases (HMTs) are involved in these complexe. They modify the surrounding chromatin by deacetylation and methylation of histones *(Hendrich et al, 2001; Fukushige and Horii 2013)*. This implies the connection between DNA methylation status and chromatin remodeling processes in silencing of genes.



Adopted from: Fukushige, S. and Horii A., 2013. The Tohoku Journal of experimental medicine

Fig.4. 3: Two possible mechanisms of transcription repression by DNA methylation.

The presence of methylated cytosines (red dots) directly inhibits binding of transcription factors to their recognition sites.

DNA methylated locuses are distinguished by methyl-CpG binding proteins (MBPs) that are linked with co-repressor complexes. These complexes contain histone deacetylases (HDACs) and methyltransferases (HMTs). This shows the interaction of DNA methylation with chromatin remodeling.

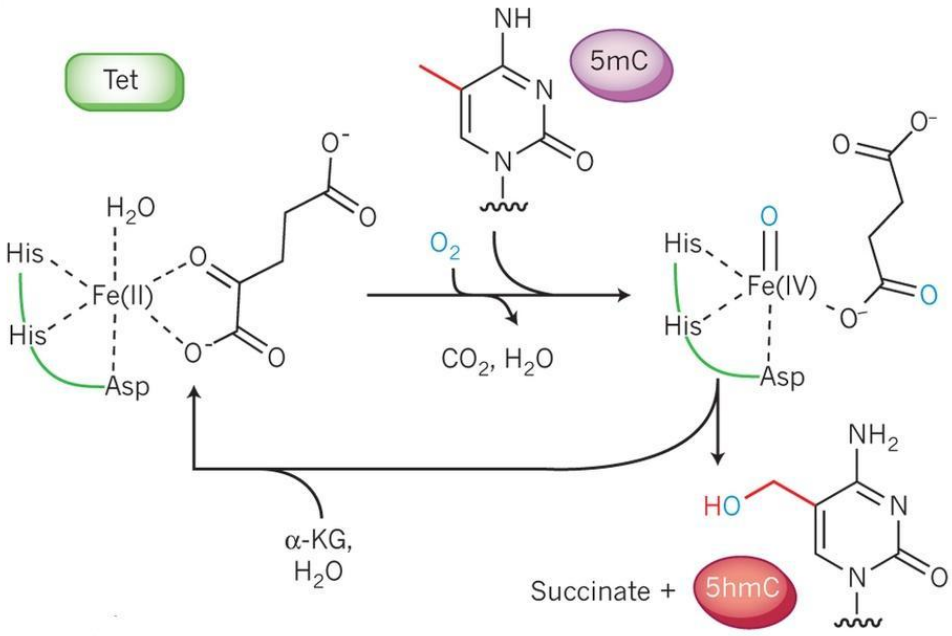
4. 4. Passive and active demethylation of cytosine in CpG dinucleotides

Epigenetic modifications are crucial during embryonic development. The epigenetic reprogramming in precursors of mammalian germ cells, called primordial germ cells (PGMs) belongs among these changes. This reprogramming includes DNA demethylation on a genome wide level *(Hajkova et al, 2002).*

It has been suggested that two mechanisms of DNA demethylation occur. The first is called passive demethylation because this modification emerges after DNA replication when DNA methyltrasferases are inhibited *(Kagiwada et al, 2013; Ohno et al, 2013)*. Active demethylation, the second possible mechanism of methyl group removal from cytosine, occurs during cell cycle and is enzymatically catalyzed. In 1999 Bhattacharya and his colleagues published that MBD2 enzyme, which can bind methylated DNA, also has a demethylase activity *(Bhattacharya et al, 1999).* However, it was claimed that the breakage of stable carbon-carbon bond between methyl group and 5th C of cytosine is thermodynamically unfavorable. Further experiments illustrated that the fertilized oocytes, from mice lacking the MBD2 enzyme, were endowed with active demethylation. Moreover, MBD2 is a transcriptional repressor thus it is not able to demethylate DNA *(Ng et al, 1999; Santos et al, 2002)*. Therefore, the question of active DNA demethylation has been re-introduced.

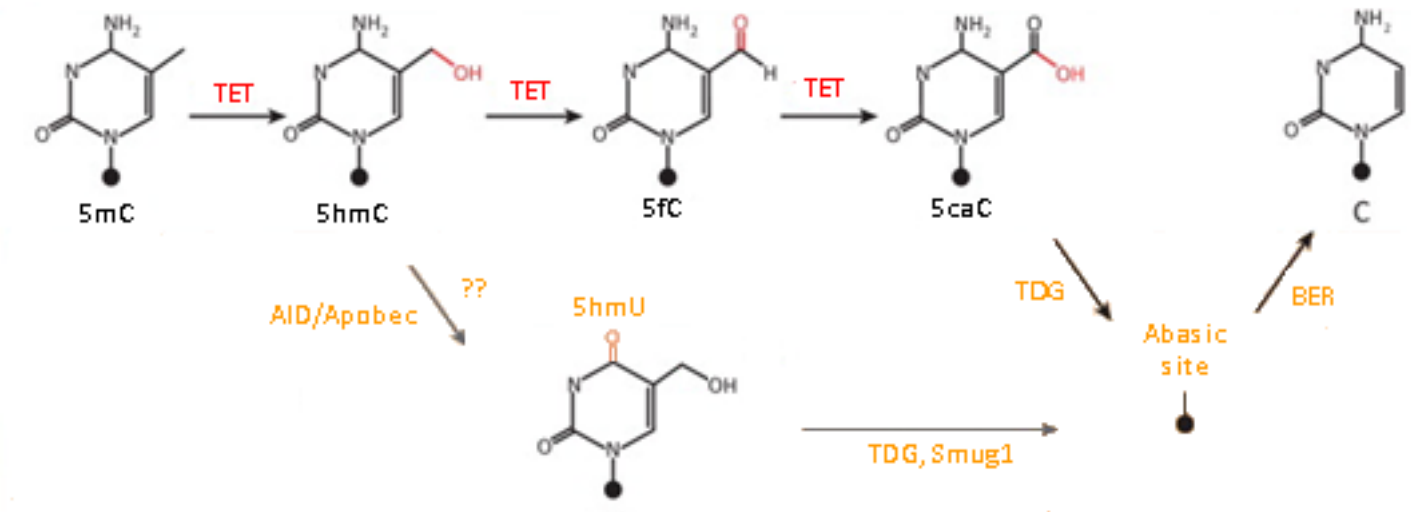
Enzymes which could participate on active demethylation in mammals were discovered based on a study of DNA demethylation in the parasite *Trypanosoma brucei (Kohli and Zhang, 2013)*. This group includes ten-eleven translocation (TET) enzyme family with three members, TET1, 2 and 3. These proteins are able to oxidize the 5mC to 5hydroxymethyl cytosine (5hmC) with the use of α-ketoglutarate and Fe(II) as cofactors *(Fig.4.4.1) (Piccolo and Fisher, 2013).* After this modification of 5mC several possible pathways occur lead to its excision. First is deamination of 5hmC by AID/Apobec enzymes which results in generation of 5 hydroxymethyl uracil (5hmU). 5hmU is subsequently removed by glycosylases, such as Smug1 or TDG. The abasic site is restored by adding of unmodified cytosine through BER pathway. However, some dubieties exist about this AID/Apobec-BER pathway because 5hmC is a poor substrate for AID enzyme *(Piccolo and Fisher, 2013).* Other possible pathways were

suggested. TETs are able to oxidize 5hmC to 5 formylcytosine (5fC) and then to 5 carboxylcytosine (5caC). These modified bases are potential targets for TGD glycosylase and can be cut out by them to create abasic site. Addition of unmethylated cytosine can be re-established by BER pathway *(Fig.4.4.2; Piccolo and Fisher, 2013; Kohli and Zhang, 2013)*.



Adapted from: Kohli, R. M., and Zhang, Y. 2013. Nature

Fig.4.4.1: 5 methylcytosine (5mC) is oxidized to 5 hydroxymethylcytosine (5hmC) by TET enzymes.
Active site Fe(II) of TET enzyme coordinates the oxidation of α-ketoglutarate by molecular oxygen. This results in high-valent Fe(IV) oxo-intermediate. Fe(IV) oxo-intermediate transfers oxygen to 5mC thereby yield 5hmC and succinate. Fe(IV) oxo-intermediate regenerates to Fe(II) species



Adapted from: Piccolo, F. M. and Fisher, A. G.2013. Trends in Cell Biology

Fig.4.4.2: Active demethylation of 5 methylcytosine (5mC) through several possible pathways.

5mC is oxidized to 5 hydroxymethylcytosine (5hmC) by TET enzymes. 5hmC can probably be deaminated by AID/Apobec complex to 5 hydroxymethyluracil (5hmU). 5hmU can be recognized by TDG or Smug1 glycosylases which can cut it out from the DNA strand and create the abasic site. This single strand DNA damage can be restored by base excision repair (BER) pathway. More probable pathway of active demethylation is oxidation of 5hmC to 5 formylcytosine (5fC) following another oxidation to 5 carboxylcytosine (5caC). 5caC is then excised by TGD and the newly-emerged abasic site is again repaired by BER pathway.

4. 5. Role of DNA methylation in tumour invasion

DNA methylation in promoters of genes is related to their transcriptional inhibition. It is likely that transformed cells can use this epigenetic modification for alternation of genes expression status and thus evade to anti-tumour immunity. DNA hypomethylation and hypermethylation of promoters are observed in cancers too *(Fukushige and Horii, 2013)*.

Hypermethylation of satellite sequences and repetitive transposable elements, such as LINE, SINE and Alu elements, is important for maintaining of genomic integrity and stability in non-transformed cells. However, DNA demethylation of SATR-1 satellite region was detected in 63 % of breast tumour cell lines and in 86 % of primary breast tumours *(Costa et al, 2006)*. Hypomethylation of LINE and Alu elements was also observed in patients with epithelial ovarian cancer and glioma *(Chen et al, 2013; Akers et al, 2014)*. These observations indicate that activated transposable elements can integrate into random sites in the genome, leading to mutagenesis *(Kulis and Esteller, 2010)*. Decreased genomic methylation can also activate expression of latent virus genes in human genome which could direct to tumour generation *(Badal et al, 2003)*.

On the other hand, promoter hypermethylation silencing of genes with undeniable anticancer effect was also described in literature. Among the genes which are inhibited belong DNA repair genes, such as *MLH1*, *MSH2* and *BRCA1 (Kulis and Esteller, 2010; Gomes et al, 2014)*, pro-apoptotic genes, for instance *DAPK1 (Christoph et al, 2006)* and well-known gene for tumour suppressor Rb protein *(Kulis and Esteller, 2010)*.

DNA hypermethylation in promoter region of tumour suppressor genes is a hallmark of many cancers and could be used as an early diagnostic marker in clinical practise. However, DNA methylation changes are linked with histone modification. Hence, it should be remembered that some genes could be silenced only by histone alternation *(Hellebrekers et al, 2006).*

Materials and Methods

5. Cell lines

I have used mouse tumour cell lines TC-1, TC-1/A9 and RVP3. TC-1 cell line was generated from primary lung epithelial cells of C57BL/6 mice. These cells were immortalized by HPV-16 E6 and E7 oncogenes and subsequently transformed with plasmid pVEJB expressing activated human *H-ras* gene. TC-1 cells express MHC I glycoproteins and have decreased B7-H1 protein expression on their cell surface *(Lin et al, 1996)*. Increase in MHC I and B7-H1 expressions occur after influence of IFNγ.

TC-1/A9 cells are derived from TC-1 cell line. Smahel and his colleagues immunized mice twice with plasmids containing HPV-16 E7 gene and subsequently challenged these mice with TC-1 tumour cells. Then, they excised developed tumours from immunized mice and derived cell lines with reduced MHC I protein expression *(Smahel et al, 2003)*. Therefore, TC-1/A9 cell line is characterized by deficient MHC I and B7-H1 protein expression. Recovery of their expression comes after IFNγ treatment.

RVP3 cell line comes from RVP3 sarcomas. These tumours originate from C57BL/6 mice injected with *Rous-sarcoma virus-Prague (Bubenik et al, 1967).* RVP3 cells have also deficient expression of MHC I and B7-H1 proteins on their surface. These expressions are not reconstituted after IFNγ influencing.

I have used TC-1 as a positive control cell line and RVP3 as a negative control cell line.

6. Cell culture

TC-1 and TC-1/A9 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 % fetal calf serum (FCS, PAN Biotech), 0.5 μg/ml amphotericine (Sigma-Aldrich) and 40 μg/ml gentamycin (Sigma-Aldrich). RVP3 cells were cultured in H-MEMd medium supplemented with 10 % FCS (PAN Biotech), 0.5 μg/ml amphotericine (Sigma-Aldrich) and 40 μg/ml gentamycin (Sigma-Aldrich). Cell cultures were cultivated in

plastic tissue culture flasks with 25 cm^2 or 75 cm^2 growth area (TPP Zellkultur und Labortechnologie made in Switzerland) in Sanyo CO_2 Incubator at 37°C, 5% CO_2 (MCO-18AC: 6.0 cu.ft. CytoGROW GLP Series CO2 Incubator). Cell handling was done in Telstar Bio-II-A laminar flow box. Cells were centrifuged for 10 min at 170 x g in Hettich Zentrifugen Universal 32. Calculation of cells was made by automated cell counter Countess (Invitrogen).

First of all, cells were defrosted from liquid nitrogen storage. Ampoule with cells was put into a container with warm water to defrost cells quickly. The volume of ampoule was dropped into flask with 5 ml of medium. Then, it was centrifuged for 10 min at 170 x g to get rid of cytotoxic dimethyl sulfoxid (DMSO). Supernatant was strained and cells were resuspended in 10 ml of medium with subsequent calculating. $5x10^6$ cells were cultivated in tissue culture flasks with 75 cm^2 growth area to regenerate. They were passaged every third day. During passaging, they were washed twice by 1x PBS and detached by 0.5% trypsin in PBS. Effect of trypsin was stopped by addition of medium containing serum. Then, cells were centrifuged for 10 min at 170 x g. Further, cell pellet was resuspended in fresh medium, calculated on automated cell counter and transferred into fresh warmed medium (10 ml) at desired density $1x10^6$ cells/25 cm^2 per flask.

On the next day, cells were treated with murine IFNγ (50 U/ml) for 48 hours. After that, cells were harvested, separated into three parts for RNA/DNA isolation and flow cytometry analysis.

7. Flow cytometry

For investigation of MHC I glycoprotein and B7-H1 protein expression on tumour cell surfaces, flow cytometry analysis was used. After centrifugation and removal of medium, cells were washed in flow cytometry RIA buffer (50 ml 10x PBS, 450 ml deionized water, 5 ml FCS and 5 ml azid) once and finally resuspended. 1.5 µl of rat anti-mouse CD16/CD32 antibody (0.5 mg/ml, clone: 2.4G2, BD Biosciences Pharmingen) was added to cells for inhibition of nonspecific antibody binding into Fc receptors. Samples were divided into 3 groups (izotype control, MHC I and B7-H1). Staining was done in tissue culture test plate with

96 wells (TPP 92697) in concentration $0.15x10^6$ cells per well. Further, it was added 0.9 µl of mouse IgG2a κ izotype control antibody (0.2 mg/ml, BD Biosciences Pharmingen) as a negative control, 0.9 µl of mouse anti-mouse H-$2D^b$ antibody (0.2 mg/ml, clone: KH95, BD Biosciences Pharmingen) and 0.9 µl of mouse anti-mouse H-$2K^b$ antibody (0.2 mg/ml, clone: AF6-88,5, BD Biosciences Pharmingen) for analysis of MHC I glycoproteins and 0.9 µl rat anti-mouse CD274 antibody (0.2 mg/ml, clone: MIH5, BD Biosciences Pharmingen) for B7-H1 protein analysis. Next, plates were incubated on ice in dark for 30 min. After incubation, wells were washed twice using 200 µl of flow cytometry buffer and centrifuged for 5 min at 170 x g (Hettich Universal/K2S). Finally, samples were analyzed (in 96 well plates) on BD LSRII flow cytometer (BD Biosciences). Results were evaluated in FlowJo software version 7.6.5 (Tree Star Inc.).

8. RNA isolation

RNA was isolated from tumour mouse cell lines mentioned above owing to use it for mRNA expression analysis of MHC I glycoprotein, B7-H1 and IRF-1 proteins in tumour cells with or without IFNγ treatment by quantitative real-time polymerase chain reaction (qPCR). RNeasy Mini Kit (Qiagen) was used for RNA isolation according to the protocol attached in the kit. 600 µl of lysis buffer RLT was added into the pellet of harvested cells and mixed by pipetting. Lysate was passed through a blunt 20-gauge needle (0.9 mm diameter) 5 times to ensure well spreading of a buffer in the pellet. Then, 600 µl of 70% ethanol was added and the mixture was vortexed. 700 µl of samples was transferred into RNeasy spin columns placed in a 2 ml collection tubes. Columns were centrifuged for 30 sec at 8000 x g in Centrifuge 5415R (Eppendorf). This step was repeated until the depletion of the lysate. The flow-through was discarded after each centrifugation. Next, 700 µl of wash buffer RW1 was added and the mixture was centrifuged for 30 sec at 8000 x g. This step was repeated with 500 µl of RPE wash buffer. Another 500 µl of RPE buffer was added and the mixture was centrifuged for 2 min at 8000 x g. RNeasy spin columns were placed into the new 2 ml collection tubes and centrifuged for 1 min at full speed (16000 x g) to get rid of RPE buffer

residues. Finally, RNeasy spin columns were placed into new 1.5ml microcentrifuge tubes. 50 µl of eluate RNeasy-free water was added into each sample and centrifuged for 1 min at 8000 x g. RNAs were eluated into 1.5 ml microcentrifuge tubes. Concentration of RNA was measured using Nanodrop ND-1000 Spectophotometer (NanoDrop Technologies, Inc). RNA samples were stored in freezer at -70°C.

9. Reverse transcription

To detect the level of mRNA of selected genes using quantitative real-time polymerase chain reaction (qPCR) method, RNA had to be transcribed into complementary DNA (cDNA). 1000 ng of RNA were used in each reaction. The reaction mixture and thermal program are stated in the following tables *(Tab.9.1, 9.2.)*. Total volume of each reaction was 20 µl. The reverse transcription took place in GeneAmp PCR system 2700 thermocycler (Applied Biosystems).

Tab.9.1: Reaction mixture of reverse transcription

Composition of reaction mixture	Volume
$MgCl_2$ Solution (25 mM, Applied Biosystems)	4 µl
10x PCR Buffer II (contains no $MgCl_2$, Applied Byosystems)	2 µl
PCR dNTP mix (10 mM, Top Bio)	2 µl
RNase Inhibitor (20 U/µl, Applied Biosystems)	1 µl
Random Hexamers (50 µM, Applied Biosystems)	1 µl
MuLV Reverse Transcriptase (50 U/µl, Applied Biosystems)	1 µl
Isolated RNA	1000 ng
Distilled water DNase/RNase free (Gibco by Life Technologies)	Volume adjusted to total volume of 20 µl

Tab.9.2: Thermal program of reverse transcription

Temperature	Time
42°C	45 min
99°C	5 min
10°C	∞

10. Quantitative real-time polymerase chain reaction (qPCR)

The mRNA expression level of *H2D*^{b} and *H2K*^{b} (for mouse MHC I glycoprotein), *CD274* (for mouse B7-H1 protein) and *IRF-1* (for mouse IRF-1 transcription factor) genes was assessed using qPCR. *Actb* (for mouse β-actin protein) was used as a reference gene. The reaction mixture and thermal program used are stated below *(Tab.10.1; 10.2)*. The qPCR reactions were run in plastic 384 well plates in light cycler LC480 (Roche). Total volume of one reaction was 10 µl. Sequences of primers for mouse *Actb*, *H2D*^{b}, *H2K*^{b}, *CD274* and *IRF-1* genes are listed in the table below *(Tab.10.3)*. Student T-test was used for statistical analysis.

Tab.10.1: Reaction mixture of quantitative real-time PCR

Composition of reaction mixture	Volume
LightCycler 480 SYBR Green I Master mix (Roche)	5 µl
Forward primer (0.1 mM, Generi Biotech)	0.15 µl
Reverse primer (0.1 mM, Generi Biotech)	0.15 µl
Distilled water DNase/RNase free (Gibco by Life Technologies)	2.7 µl
cDNA	2 µl

Tab.10.2: Thermal program of qPCR

Program	Temperature	Time	Cycles
Denaturation	95°C	2 min	1
Denaturation	95°C	25 sec	45
Annealing	60°C	45 sec	
Elongation	72°C	1 min	
Incubation	80°C	5 sec	1

Tab.10.3: Primer sequences for *Actb*, *H2D^b*, *H2K^b*, *CD274* and *IRF-1* genes

Gene	Forward primer sequence	Reverse primer sequence
Actb	5´CCAGAGCAAGAGAGGTATCC 3´	5´GAGTCCATCACAATGCCTGT 3´
H-2D^b	5´CGCGACGCTGCTGCGCACAG 3´	5´TACAATCTCGGAGAGACATT 3´
H-2K^b	5´CGCGACGCTGCTGCGCACAG 3´	5´TACAATCTGGGAGAGACAGA 3´
CD274	5´GGAAGATGAGCAAGTGATTCAG 3´	5´CAATGAGGAACAACAGGATGG 3´
IRF-1	5´GCCCGGACACTTTCTCTGATG 3´	5´AGACTGCTGCTGACGACACACG 3´

11. DNA isolation

From all mouse tumour cell lines mentioned above, DNA was also isolated in order to use it in a subsequent DNA methylation analysis of *IRF-1* promoter region. DNA was isolated by DNeasy Blood and Tissue Kit (Qiagen) according to the protocol attached in the kit. Cells were harvested and cell pellets were washed with 200 µl of 1x PBS in sterile condition in laminar flow box (Telstar Bio-II-A). After washing, 20 µl of proteinase K was added for protein degradation and samples were transferred into 1.5 ml microcentrifuge tubes. Then, in unsterile condition, 200 µl of lysis buffer AL was supplemented, vortexed and incubated at 56°C for 10 min in thermal block digital bath BTD Grant (P-Lab). After incubation, 200 µl of 96% ethanol was added and mixture was vortexed. Next, samples were transferred into DNeasy Mini spin columns placed in 2ml collection tubes and centrifuged for 1 min at 8000 x g in Centrifuge 5415R (Eppendorf). Flow-throughs were poured out and new collection tubes were placed. After each centrifugation, flow-throughs and collection tubes were discarded and new collection tubes were replaced. Next, 500 µl of wash buffer AW1 was added into each sample and centrifuged for 1 min at 8000 x g. Subsequently, 500 µl of wash buffer AW2 was added and samples were centrifuged again for 3 min at maximum speed. Finally, spin columns with samples were placed into 1.5ml microcentrifuge tubes; 200 µl of elution buffer AE was added and centrifuged for 1 min at 8000 x g. Samples were eluted into AE buffer and their concentrations were measured on Nanodrop ND-1000 Spectophotometer (NanoDrop Technologies, Inc). DNA samples were stored at -70°C.

12. Bisulfite conversion

To find out, if *IRF-1* gene promoter was methylated in TC-1/A9 tumour cells, methylation specific PCR (MSP) was used and subsequently bisulfite sequencing. Previously, it was necessary to modify DNA by sodium bisulfite which converts unmethylated cytosines to uracils, while methylated cytosines remain unchanged *(Fig.12.1; Fukushige and Horii, 2013)*. This process is called bisulfite conversion.

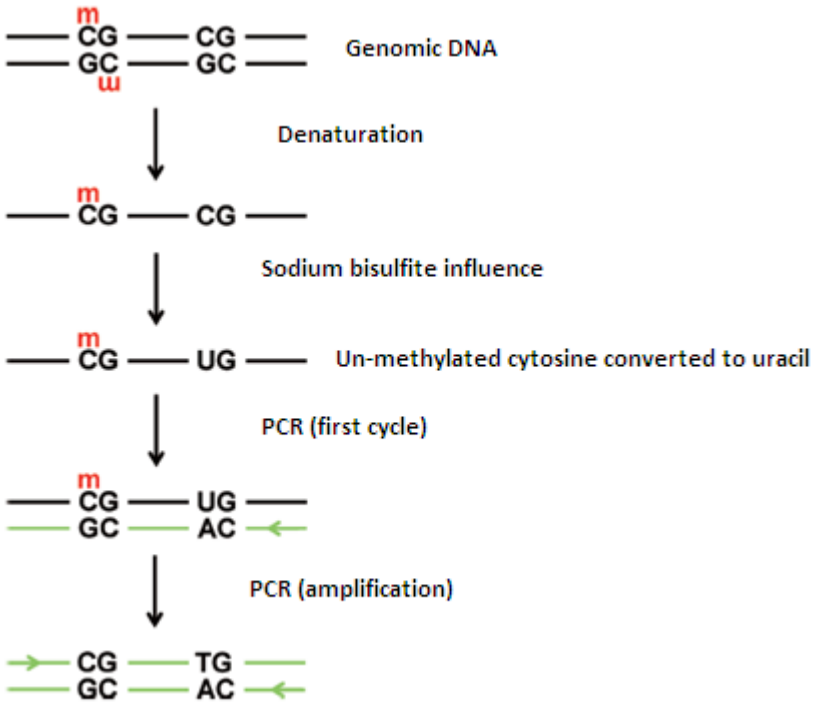
Conversion was done using EpiTect Bisulfite Kit (Qiagen) according to the attached protocol. 2000 ng of DNA was used per reaction. Bisulfite mix was dissolved by adding 800 µl RNase-free water. Reaction mixture and thermal program used are stated below *(Tab.12.1; 12.2)*. Total volume of reaction was 140 µl. The bisulfite conversion took place in GeneAmp PCR system 2700 thermocycler (Applied Biosystems). Converted DNA samples were stored at -20°C.

Tab.12.1: Reaction mixture of bisulfite conversion

Composition of reaction mixture	Volume
Bisulfite mix	85 µl
DNA Protect buffer	35 µl
DNA	2000 ng (max 20 µl)
RNase-free water	Volume adjusted to total volume of 140 µl

Tab.12.2: Thermal program of bisulfite conversion

Program	Temperature	Time
Denaturation	95°C	5 min
Incubation	60°C	25 min
Denaturation	95°C	5 min
Incubation	60°C	85 min
Denaturation	95°C	5 min
Incubation	60°C	175 min
Hold	20°C	∞



Adapted from: Fukushige, S. and Horii A., 2013. The Tohoku Journal of experimental medicine

Fig.12.1: Bisulfite conversion

Firstly, DNA is denaturated. Then, influence of sodium bisulfate convert unmethylated cytosine to uracil and methylated cytosine remain unchanged. During PCR reaction uracil is changed to thymine.

13. Methylation Specific Polymerase Chain Reaction (MSP)

At first, the methylation status of three specific CpG sites in *IRF-1* gene promoter was analyzed by MSP. Two sets of primers were used for methylated DNA and unmethylated DNA. One set of primers contains CpG sites and it is desgined for recognition of methylated DNA. Second set does not include CpG sites, so it is specific for unmethylated DNA. Primers were designed by MethPrimer software *(Li and Dahiya, 2002; available from: http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)*. Primer sequences are listed in the table below *(Tab.13.3)*. 100 ng of bisulfite DNA was used per reaction. Reaction mix was prepared on ice and run in thermocycler GeneAmp PCR system 2700 (Applied Biosystems). Total volume was 25 µl. PCR product was 150 bp. Reaction mixtures and thermal program used are stated below *(Tab.13.1; 13.2)*. CpG sites are marked as a red letters in primer sequences. Resulting samples were analysed gel by electrophoresis.

Tab.13.1: Reaction mixture of MSP

Composition of reaction mixture	Volume
$MgCl_2$ Solution (25 mM, Applied Biosystems)	3 µl
10x Taq Buffer Complete (Top Bio)	5 µl
PCR dNTP mix (10 mM, Top Bio)	1 µl
Forward primers (0.1 mM, Generi Biotech)	0.25 µl
Reverse primers (0.1 mM, Generi Biotech)	0.25 µl
Combi Taq polymerase (1U/µl, Top Bio)	2.5 µl
Bisulfite converted DNA	100 ng
Distilled water DNase/RNase free (Gibco by Life Technologies)	Volume adjusted to total volume of 25 µl

Tab.13.2: Thermal program of MSP

Program	Temperature	Time	Cycles
Denaturation	95°C	2 min	1
Denaturation	95°C	2 min	35
Annealing	55°C	2 min	
Elongation	73°C	1 min 30 sec	
Incubation	73°C	10 sec	1
Hold	4°C	∞	∞

Tab.13.3: Methylated and unmethylated primer sequences for *IRF-1* gene

Gene	***IRF-1***
Methylated Forward Primer	5´ GGTGGTTAGAGGGATTTTAGTATTTCG 3´
Methylated Reverse Primer	5´ CACCTTTACTACAAAAACGATTCG 3´
Unmethylated Forward Primer	5´ GGTTAGAGGGATTTTAGTATTTTGG 3´
Unmethylated Reverse Primer	5´ ACCTTTACTACAAAAACAATTCACA 3´

14. Electrophoresis

Samples from MSP were analzyed on 1.5% agarose gel [0.5 g of agarose powder (Sigma) was dissolved in 50 ml of 0.5x Tris-Borate-EDTA buffer (TBE, Sigma)]. A microwave oven was used to heat a mixture and accelerate dissolving. Finally mixture was cooled down. 5 µl of GelRed Nucleic Acid (10.000 x in water, Biotium) was added into mixture to stain DNA. Then it was poured out into a prepared form and comb was placed inside. Gel solidified for 20 min and was transferred into the bath with 0.5x TBE buffer. Samples (5 µl) and DNA Step ladder 50bp (5 µl, Sigma) were mixed with 6x Gel loading buffer (1 µl, Sigma) and applied into gel. 100 V for 20 min were used for electrophoresis. The gel was analyzed using UV transluminator 2000 (Biorad) and GelCapture software.

15. Bisulfite sequencing analysis

Lastly, methylation status of IRF-1 gene promoter was analysed by bisulfite sequencing in order to confirm MSP results. Converted DNA was amplified by bisulfite specific polymerase chain reaction (see below), cleaned and used for DNA cloning (see below). Finally, vectors with cloned DNAs were isolated and sequenced. Analysis of bisulfite sequencing products was done in the service laboratory (Institute of Microbiology ASCR in Prague, Czech Republic). Data were evaluated with BiQ Analyser software.

15. 1. Bisulfite specific polymerase chain reaction (BSP)

BSP analyzes much more CpG sites then MSP. In the contrary to MSP, only one set of primers was used. These primers do not contain CpG sites, so they recognize converted DNA irrespective of methylated/unmethylated DNA. Primers were designed by Methprimer software *(Li and Dahiya, 2002; available from: http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)*. Primer sequences are listed in the table below *(Tab.15.1.3).* For BSP, 500 ng of converted DNA was used in each reaction. The BSP took

place in GeneAmp PCR system 2700 thermocycler (Applied Biosystems). Total volume was 20 µl. PCR product was 348 bp. Reaction mixtures and thermal program used are stated below *(Tab.15.1.1; 15.1.2)*.

Tab.15.1.1: Reaction mixture of BSP

Composition of reaction mixture	Volume
$MgCl_2$ Solution (25 mM, Applied Biosystems)	2.4 µl
10x Taq Buffer Complete (Top Bio)	4 µl
PCR dNTP mix (10 mM, Top Bio)	0.8 µl
Forward primer (0.1 mM, Generi Biotech)	0.2 µl
Reverse primer (0.1 mM, Generi Biotech)	0.2 µl
Combi Taq polymerase (1U/µl, Top Bio)	2 µl
Bisulfite converted DNA	500 ng
Distilled water DNase/RNase free (Gibco by Life Technologies)	Volume adjusted to total volume of 20 µl

Tab.15.1.5: Thermal program of BSP

Program	Temperature	Time	Cycles
Denaturation	95°C	3 min	1
Denaturation	95°C	35 sec	16
Annealing	58°C	2 min	
Elongation	72°C	1 min 40 sec	
Denaturation	95°C	30 sec	25
Annealing	54°C	2 min	
Elongation	72°C	1 min 50 sec	
Incubation	72°C	10 min	1
Hold	4°C	∞	∞

Tab.15.1.3: Primer sequences for BSP of *IRF-1* gene

Gene	Forward primer sequence	Reverse primer sequence
IRF-1	5´ AGGTGGTTAGAGGGATTTTAGTATTT 3´	5´ ACCACTCTAAACTACCAAAAAACC 3´

15. 2. Purification of BSP products

Subsequently, it was necessary to purify BSP products. The specificity of the BSP and lenght of desired products were checked on gel as described above (see page 49, chapter "Electrophoresis"). Then, *IRF-1* products (348 bp) were cut out from the gel and purified by QIAEX II Gel Extraction Kit (Qiagen) according to the attached protocol. Slices of gel were weighed and the solubilization buffer QX1 was added (3 volumes of the gel´s weight). Next, 30 µl of vortexed QIAEX II buffer was added and incubated for 10 min at 50°C to solubilize agarose and bind DNA. Every 2 min of incubation, samples were mixed by vortexing to keep QIAEX II in suspension. After 10 min, samples were centrifuged for 30 sec at maximum speed (16000 x g). Supernatants were removed and pellets were resuspended with 500 µl of QX1 buffer by vortexing to remove agarose residues. Then, samples were centrifuged for 30 sec at maximum speed once again. Supernatants were removed by pipetting and pellets were washed twice with 500 µl of wash buffer PE to dispose of salt residues. Samples were centrifuged for 30 sec at maximum speed and supernatants were removed by pipetting. Next, pellets were dried for 30 min at 40°C in thermal block digital bath BTD Grant (P-Lab). 20 µl of RNase-free water was added into each sample to eluate DNA and centrifuged for 30 sec at maximum speed. Finally, supernatants, containing purified DNA, were put into clean 1.5 ml microcentrifuge tubes. Concentrations of purified DNAs were measured on Nanodrop ND-1000 Spectophotometer (NanoDrop Technologies, Inc). Samples were stored in -20°C.

15.3. DNA cloning

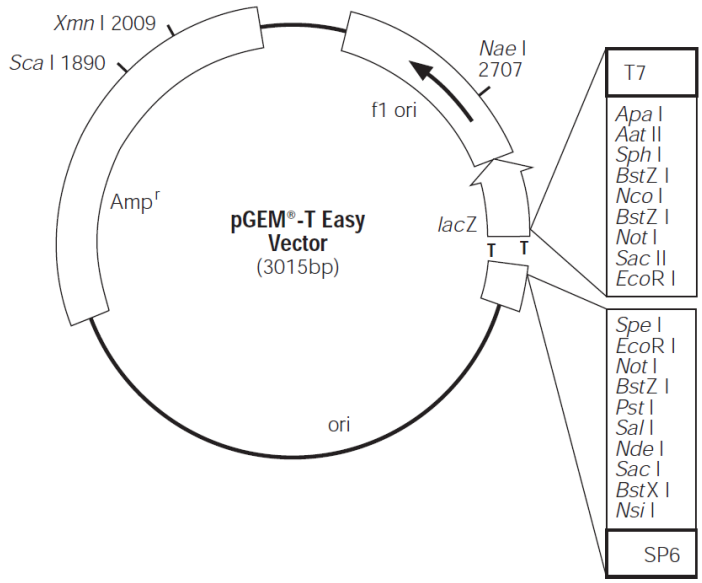
DNA products originated from BSP were cloned. DNA cloning comprised ligation of BSP products into plasmid vector pGEM-T Easy Vector; transformation of ligation products into JM109 bacterial competent cells; isolation of plasmid DNAs; bisulfite sequencing and finally, precipitation of sequenced DNA.

15. 3. 1 Ligation

Purified DNA from BSP was ligated into pGEM-T Easy Vector (Promega). Ligation was prepared under sterile condition in laminar flow box Hera Safe Heraeus (Thermo Scientific). Reaction mixes were prepared according to protocol from pGEM-T Easy Vector System kit (Promega) on ice. Reactions run through night at 12°C in thermocycler ProFlex PCR System (Applied Biosystems). Total volume was 10 µl. As a positive control for insertion, the Control Insert DNA attached in the kit was used. The composition of reaction mixture is described below *(Tab.15.3.1)*. PCR products were inserted into multiple cloning regions which are located inside α-peptide coding region of β-galactosidase enzyme in *lac operon (Fig.15.3.1).*

Tab.15.3.1.: Reaction mixture of ligation

Composition of reaction mixture	Volume	Volume
2x Rapid Ligation Buffer (Promega)	5 µl	5 µl
pGEM-T Easy Vector (50 ng/µl, Promega)	1 µl	1 µl
Purified BSP product (purified DNA)	3 µl	-
Control Insert DNA (4 ng/µl, Promega)	-	3 µl
T4 DNA Ligase (3U/µl, Promega)	1 µl	1 µl



Adapted from: pGEM-T and pGEM-T Easy Vector Systems, Technical Manual No.042. Promega

Fig.15.3.1: pGEM-T Easy Vector map

pGEM-T Easy Vector contain *lac operon* with *lacZ* start codon, replication origin of filamentous phage f1 and ampiciline resistance. Inside *lac operon*, there are insertion site flanked with thymidine and multiple cloning region with multiple restriction sites. T7 and SP6 RNA polymerase promoters are placed in the terminations of multiple cloning regions.

15. 3. 2 Transformation of ligation products

After ligation, vectors with inserts were transformed into JM109 High Efficiency Competent Cells (Promega) according to the protocol in pGEM-T Easy Vector System kit (Promega). Transformation was performed by heat shock process. As a positive control for viability of the cells, the Competent Cells Control DNA attached in the kit was used. 50 µl of competent cells were mixed with 10 µl of ligation products and put on ice for 30 min. Then, samples were transferred into the thermal block digital bath BTD Grant (P-Lab) for 2 min at 42°C. After that, samples were directly returned on ice. Next, solutions were transferred into plastic tubes with 500 µl of LB media in laminar flow box Hera Safe Heraeus (Thermo Scientific) and shaking in Shaking Incubator NB-205 (N-Biotek) for 1 hour at 37°C for regeneration. Finally, cells were seeded into plates with LB medium and ampicillin (prepared

in IMG) and with supplemented 20 µl of chromogenic substrate X-gal (50 ng/ml) and 4 µl of isopropyl β-D-thiogalactopyranosidase (IPTG). Plates were stored in incubator NB-201 (N-Biotek) at 37°C through night.

The next day, white colonies were collected into plastic tubes with 1 ml of LB medium and 100 µl of ampicillin. Inhibition of active β-galactosidase enzyme by PCR product insertion was subsequently identified by color screening of bacterial colonies. Blue colonies did not contain PCR insert because they had an active β-galactosidase enzyme which was able to degrade X-gal into subsequent blue color product. However, white colonies contained inactive β-galactosidase enzyme because of PCR product insertion. So, such colonies were unable to degrade X-gal substrate on a blue product and remained white. 10 white colonies were taken from each sample (control sample and IFNγ treated sample). Tubes were shaking in Shaking Incubator NB-205 (N-Biotek) for 24 hours at 37°C.

15. 3. 3 Isolation of plasmid DNA

Plasmid DNA from bacterial colonies was isolated for following sequencing using QIAprep Spin Miniprep Kit (Qiagen) according to the attached protocol. Colonies were harvested, centrifuged for 3 min at 6800 x g and the pellets were resuspended in 250 µl with resuspension buffer P1. Mixtures were transferred into 1.5 ml microcentrifuge tubes. Next, 250 µl of lysis buffer P2 was added and mixed by inverting the tubes 5 times. Further, 350 µl of neutralization buffer N3 was added and again mixed by inverting the tubes 5 times. Tubes were centrifuged for 10 min at maximum speed (16400 x g) in Centrifuge 5424, Eppendorf. Subsequently, supernatants were transferred into QIAprep spin columns by pipetting and again centrifuged for 1 min at 16400 x g. In all the following steps, flow-throughs were poured out. Samples were washed with 500 µl of binding buffer PB and centrifuged for 1 min at 16400 x g. Next, samples were washed using 750 µl of washing buffer PE and centrifuged for 1 min at 16400 x g. Columns with samples were transfered into new collection tubes and centrifuged for 1 min at 16400 x g to remove buffer residues. Finally, columns were placed into clean 1.5 ml microcentrifuge tubes. 50 µl of elution buffer EB was added to eluate

plasmid DNA. Samples were centrifuged for 1 min at 16400 x g and their DNA concentration was measured on Nanodrop ND-1000 Spectophotometer (NanoDrop Technologies, Inc).

15. 4. Bisulfite sequencing

Plasmid DNA was sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the attached protocol. 500 ng of DNA was used per reaction. Total volume was 20 µl. Reaction mixture and thermal program used are stated below *(Tab.15.4.1; 15.4.2)*. The bisulfite sequencing took place in GeneAmp PCR system 2700 thermocycler (Applied Biosystems).

Tab.15.4.1: Reaction mixture of bisulfite sequencing

Composition of reaction mixture	Volume
BigDye Terminator v 3.1 Cycle Sequencing RR-100 reaction mix (Applied Biosystems)	4 µl
5x BigDye Terminator v 3.1 Cycle Sequencing Buffer (Applied Biosystems)	2 µl
M13 (-21) Control Forward Primer (Applied Biosystems)	1 µl
Plasmid DNA	500 ng
Distilled water DNase/RNase free (Gibco by Life Technologies)	Volume adjusted to total volume of 20 µl

Tab.15.4.2: Thermal program of bisulfite sequencing

Program	Temperature	Time	Cycles
Denaturation	96°C	10 sec	25
Annealing	50°C	5 sec	
Elongation	60°C	4 min	
Hold	10°C	∞	1

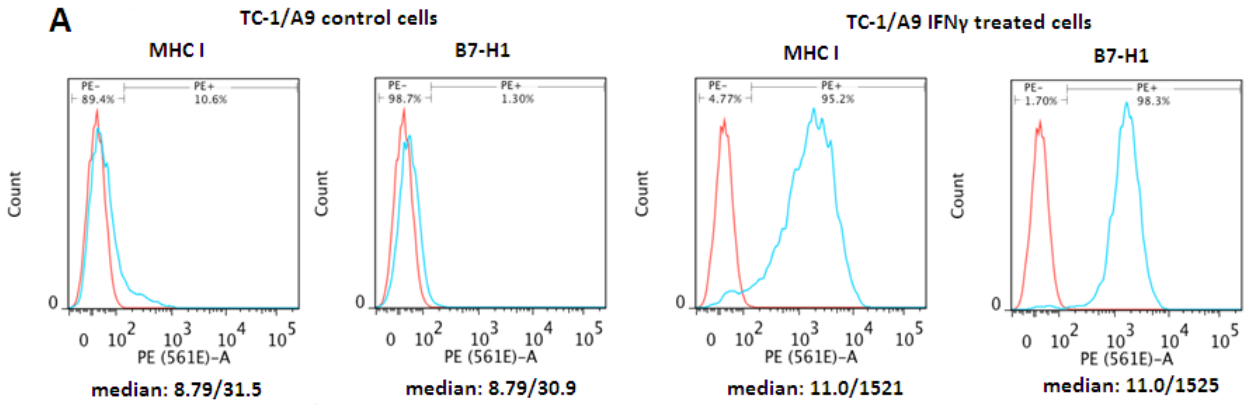
15. 5. Precipitation of bisulfite sequencing products

After the step, the products were purified. They were transferred into 1.5 ml microcentrifuge tubes and 3 µl of sodium acetate (NaOAc, 3 M) with 1 µl of glycogen (20 mg/ml) and 50 µl of 96% ethanol were added. Solutions were mixed by vortexing and incubated for 15 min at room temperature. Further, samples were centrifuged for 30 min at 16000 x g in cooled centrifuge (4°C, Centrifuge 5415R, Eppendorf). Supernatants were discarded by pipetting. Next, pellets were washed twice by addition of 250 µl of 70% ethanol and centrifuged for 15 min at 16000 x g. Finally, pellets were dried in thermal block digital bath BTD Grant (P-Lab) for 30 min at 40°C degree. Dried samples were hand over to the service center for DNA sequencing at Institute of Microbiology, ASCR (Prague, Czech Republic) for analysis of sequencing reactions. Raw data were further processed with BiQ Analyser software.

Results

16. Cell surface expression of MHC class I glycoproteins and B7-H1 proteins on TC-1/A9 tumour cells increases after the IFNγ treatment

MHC I glycoproteins and B7-H1 co-inhibitory proteins are surface molecules. It is known that their expression increases after IFNγ influence. To investigate, if TC-1/A9, TC-1 and RVP3 cells also respond to this treatment, they were influenced with murine IFNγ (50 U/ml) for 48 hours. After two days, cells were harvested and analyzed by flow cytometry to determine changes in MHC I and B7-H1 protein expression on the cell surface. The expression of both proteins increased in TC-1/A9 and TC-1 cells *(Fig.16 A, B)*. RVP3 cells showed no response to IFNγ *(Fig.16 C)*.



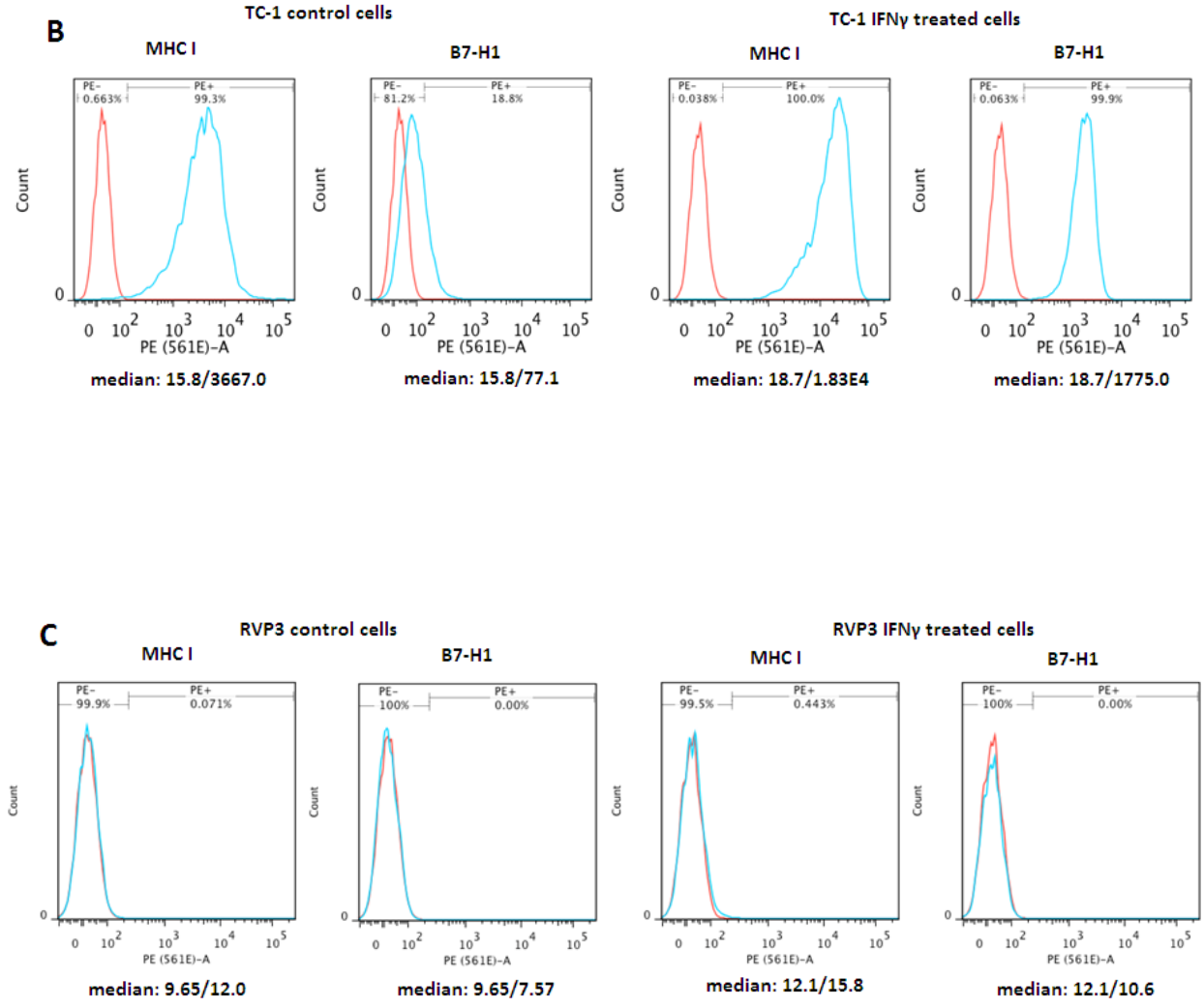


Fig.16: Expression of MHC I and B7-H1 molecules in response to IFNγ treatment.

Mouse tumour cell lines TC-1/A9, TC-1 and RVP3 cells were treated with IFNγ for 48 hours. A: TC-1/A9 cells are MHC I and B7-H1 negative. However, cell surface expression of these proteins increases after IFNγ treatment. B: TC-1 cells are positive for MHC I glycoprotein and weak positive for B7-H1 protein. Expression of these proteins also increased after IFNγ treatment. These cells were used as a positive control. C: On contrary to previous mentioned cell lines, RVP3 cells are negative for MHC I and B7-H1 proteins and have no response to IFNγ treatment. RVP3 cell line was used as a negative control. Red lines correspond to izotype control; blue lines to MHC I or B7-H1 protein expression. First number, under the chart, means median value of izotype control; second number means median value of MHC I or B7-H1 protein expression. Experiment was repeated twice.

17. Basal mRNA expression of H2D^b and H2K^b, B7-H1 and IRF-1 genes in parental TC-1 and daughter TC-1/A9 mice tumour cell lines

First of all, the basal mRNA level of *H2D^b, H2K^b* and *B7-H1* genes was determined with qPCR. The purpose of this measurement was to confirm that the above mentioned genes are also downregulated on transcriptional level, not only on protein level (flow cytometry data), in TC-1/A9 cells compare to parental TC-1 cell line. *B7-H1* gene has a binding site in the promoter region for IRF-1 transcription factor and this factor is also induced by IFNγ. Owing to these facts, *IRF-1* gene mRNA level was detected too. The basal mRNA expression of genes was not measured in RVP3 cells because they do not respond to IFNγ stimulation *(Fig. 16 C)*. The transcription of *H2D^b* and *H2K^b* was significantly downregulated in TC-1/A9 cells than in parental TC-1 cells. The transcription of *B7-H1* and *IRF-1* gene was decreased in TC-1/A9 cells compare to TC-1 cells *(Fig.17 A)*.

A

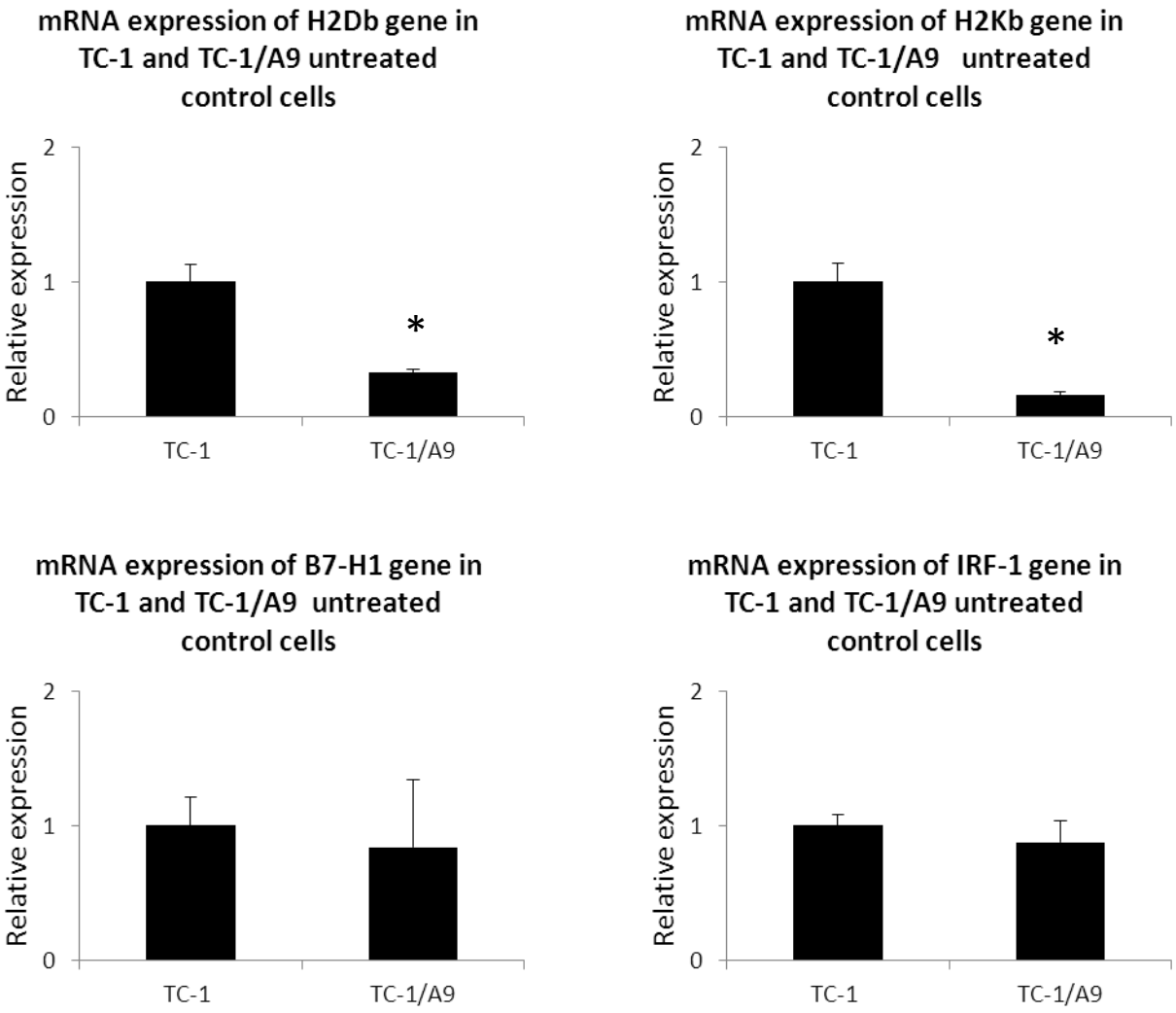


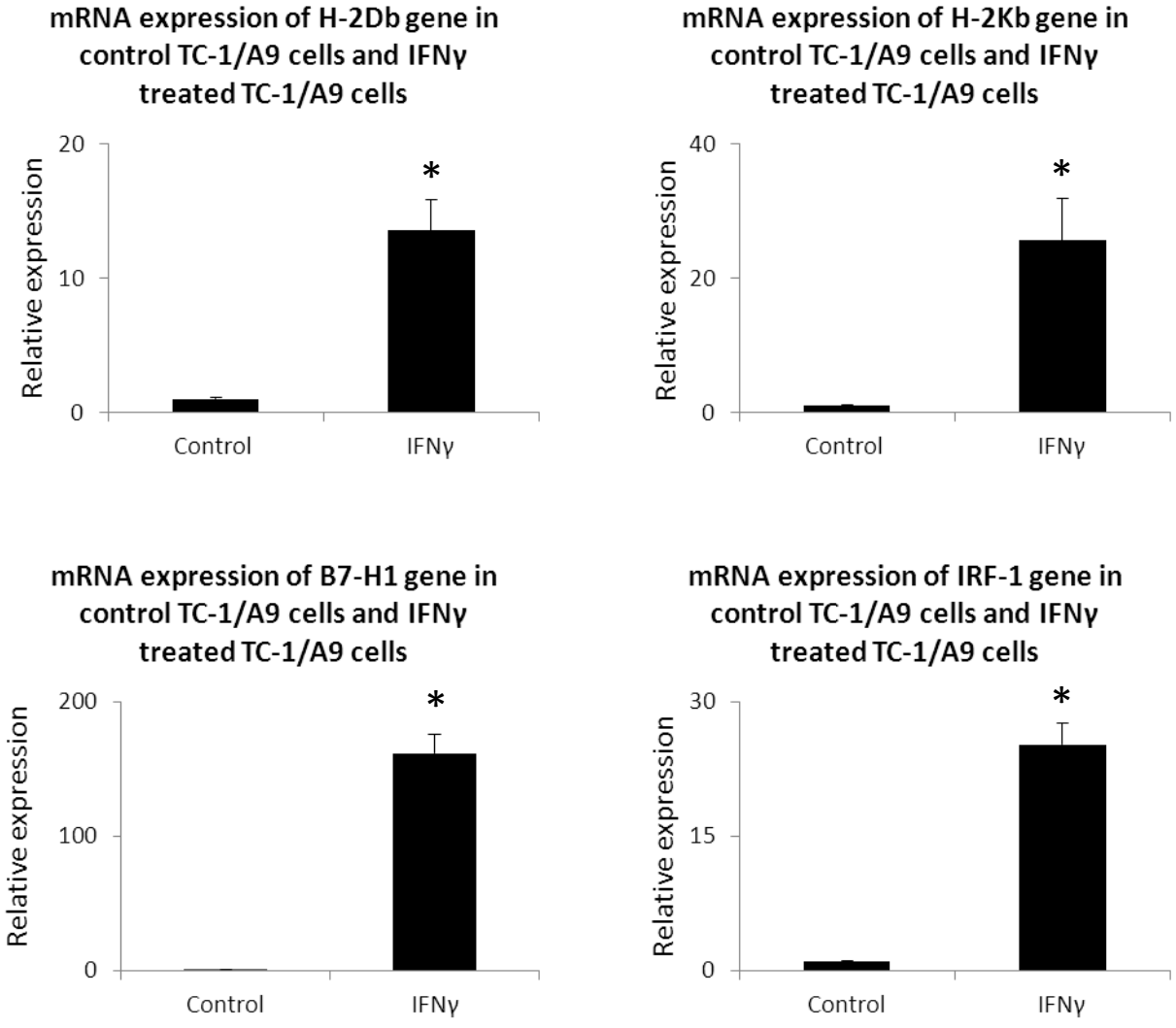
Fig.17: Basal mRNA expression of *H2D^b, H2K^b, B7-H1 and IRF-1* genes in parental TC-1 and its daughter TC-1/A9 cell lines.

A: The mRNA expression level of *H2D^b, H2K^b, B7-H1* and *IRF-1* genes was decreased in TC-1/A9 cells compare to TC-1 cells. *H2D^b* and *H2K^b* genes were significantly decreased in TC-1/A9 cells. *Actb* was used as a reference control. Relative expression was calculated using C_T values. The levels of relative gene expression were presented as fold changes compared to the levels found in control samples. Student T-test was used for statistical analysis. Differences between IFNγ treated and control samples with $P< 0.05$ were considered to be statistically significant and marked by star (*). All samples were run in biological triplicates. Experiment was repeated twice.

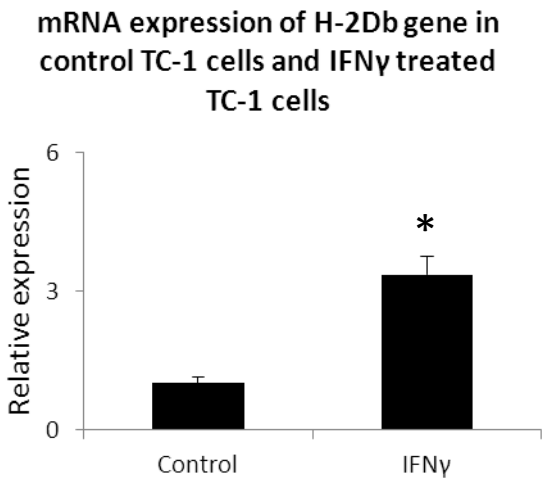
18. The mRNA expressions of H2D^b and H2K^b, B7-H1 and IRF-1 genes increase after IFNγ treatment in TC-1/A9 and TC-1 tumour cells but not in RVP3 tumour cells

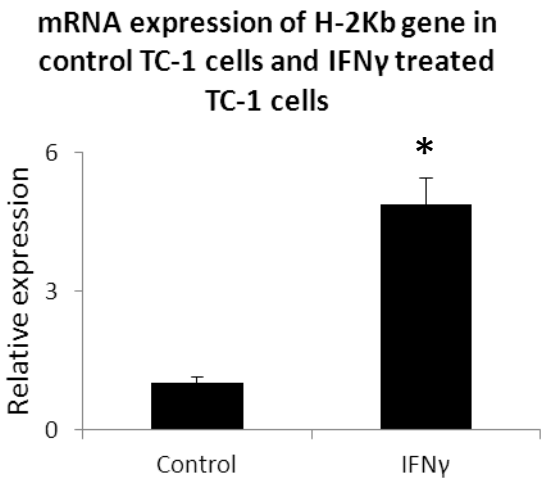
Next, I wanted to establish, if the augmented protein expression after IFNγ stimulation is regulated on the level of transcription. The mRNA level of *H2D^b*, *H2K^b*, *B7-H1* and *IRF-1* genes has significantly risen in IFNγ treated TC-1/A9 and TC-1 cells compared to control cells *(Fig. 18 A, B)*. In RVP3 cells, mRNA expression also slightly increased after IFNγ treatment, however the change was not statistically significant *(Fig. 18 C)*. It is apparent that gene expression is restored by IFNγ stimulation in TC-1/A9 as well as TC-1 cells.

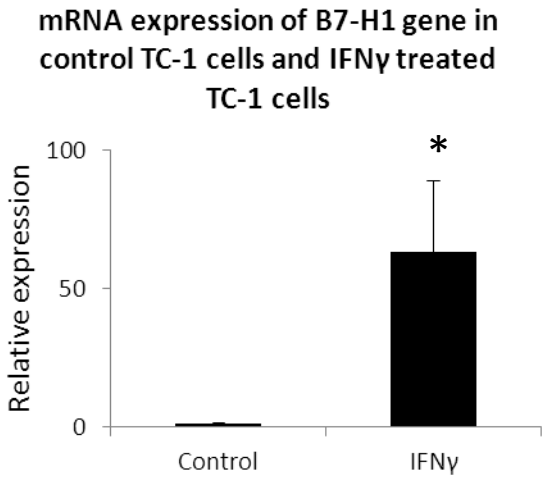
A

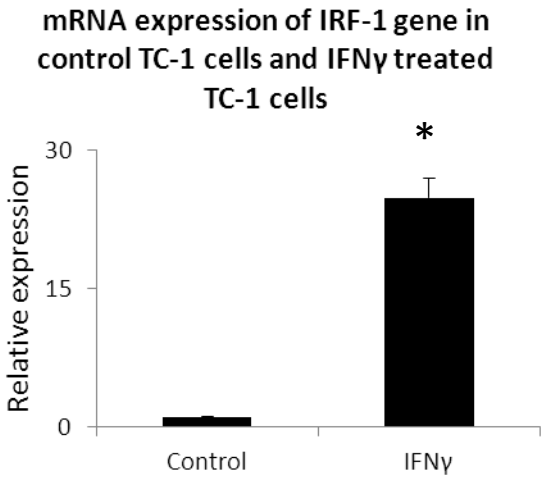


B


mRNA expression of H-2Db gene in control TC-1 cells and IFNγ treated TC-1 cells
Relative expression
6
3
0
*
Control
IFNγ


mRNA expression of H-2Kb gene in control TC-1 cells and IFNγ treated TC-1 cells
Relative expression
6
3
0
*
Control
IFNγ


mRNA expression of B7-H1 gene in control TC-1 cells and IFNγ treated TC-1 cells
Relative expression
100
50
0
*
Control
IFNγ


mRNA expression of IRF-1 gene in control TC-1 cells and IFNγ treated TC-1 cells
Relative expression
30
15
0
*
Control
IFNγ

C

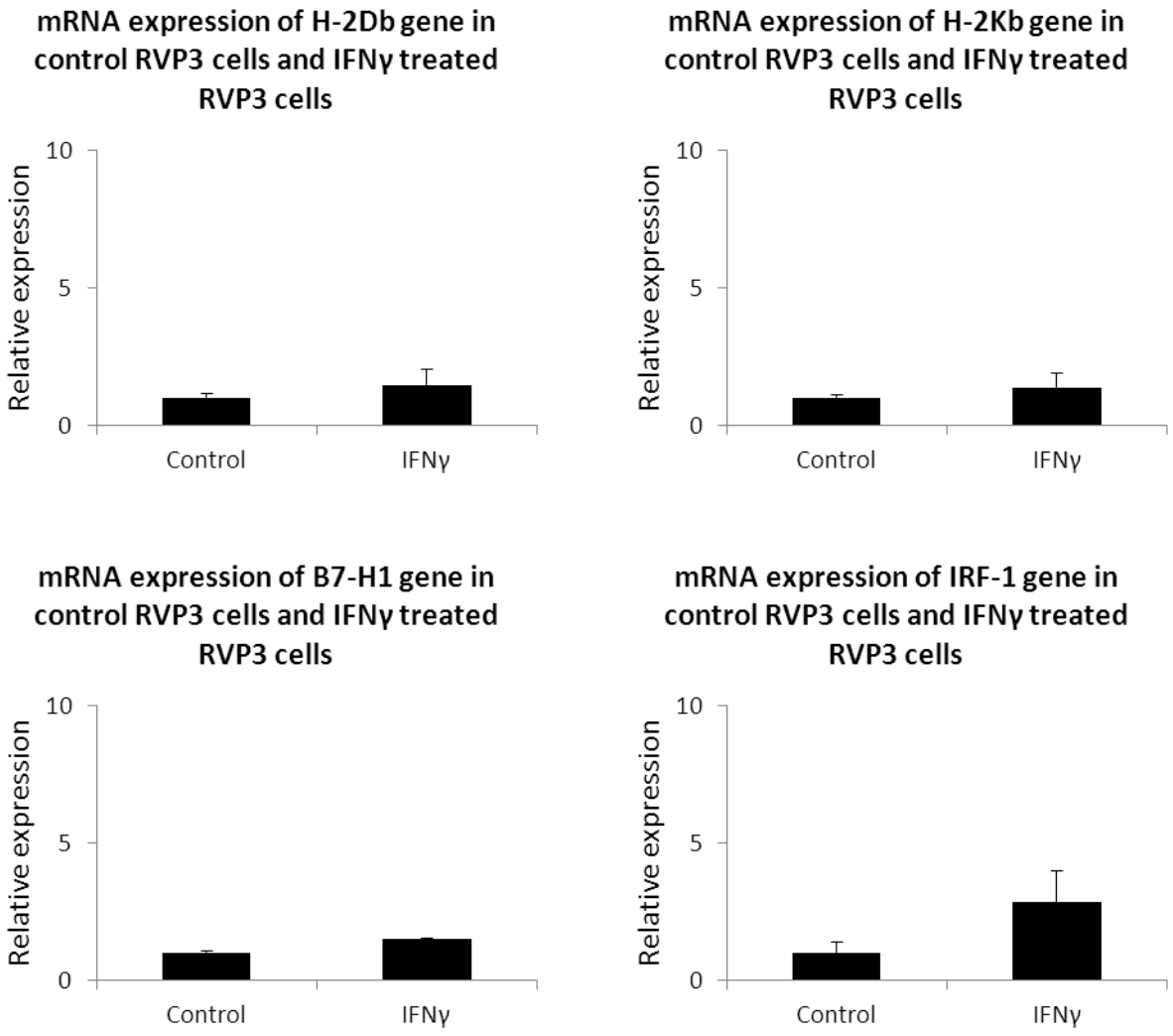


Fig. 18: The Relative mRNA expression level of *H-2D*^{b} and *H-2K*^{b}, *B7-H1* and *IRF-1* genes after 48h of IFNγ treatment in TC-1/A9, TC-1 and RVP3 tumour cell lines.

Mouse tumour cell lines TC-1/A9, TC-1 and RVP3 were treated by IFNγ cytokine for 48 hours. The mRNA expression of selected genes was analyzed by qPCR. Expression levels of *H-2D*^{b} and *H-2K*^{b} genes were used for determination of MHC I protein. A: In TC-1/A9 cells, mRNA level of all analyzed genes increased after the treatment. B: In TC-1 cells, the mRNA expression level of selected genes also increased after the treatment. C: In contrary, in RVP3 cells, mRNA expression of declared genes slightly increased but it was not statistically significant. *Actb* was used as a reference control. Relative

expression was calculated using C_T values. The levels of relative gene expression were presented as fold changes compared to the levels found in control samples. Student T-test was used for statistical analysis. Differences between IFNγ treated and control samples with $P< 0.05$ were considered to be statistically significant and marked by star (*). All samples were run in biological triplicates. *Control* means control cells; *IFNγ* means IFNγ treated cells. Experiment was repeated twice.

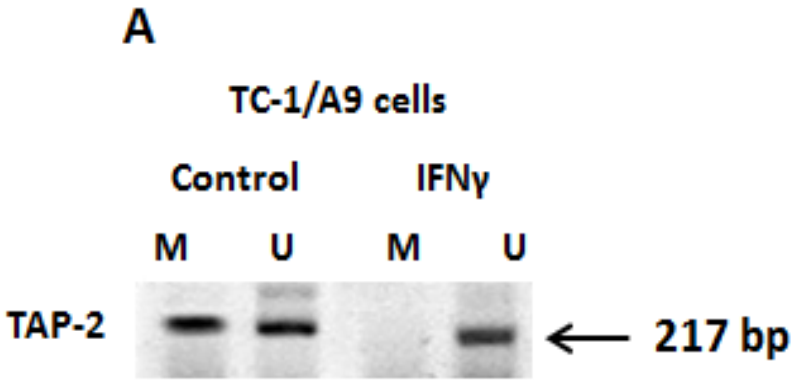
19. DNA demethylation of the IRF-1 promoter region was observed after IFNγ treatment using MSP

Flow cytometry and qPCR data showed upregulation of MHC I, B7-H1 and IRF-1 expression after IFNγ treatment. Our previous research showed DNA demethylation of *TAP-2* gene (and other antigen presenting machinery genes) after IFNγ treatment *(Fig. 19.1 A; Annexe)*. *TAP-2* promoter demethylation was accompained by upregulation of *TAP-2* mRNA level *(Annexe)*. TAP-2 belongs to antigen presenting machinery group of proteins which help with proper MHC I antigen presentation. We demonstrated that IFNγ induced DNA demethylation of *TAP*-2 (and other antigen presenting machinery genes) leads to upregulation of MHC I on cell surface of TC-1/A9 cells *(Annexe)*.

According to the acquired data from flow cytometry and qPCR analysis, that IFNγ upregulates expression of *B7-H1* and *IRF-1* gene, I wanted to find out if IFNγ is able to cause DNA demethylation of their promoter region as well as in *TAP-2* promoter. First of all, I wanted to determine CpG islands in *B7-H1* promoter. However, I have found that there are no CpG sites in this mouse gene. On the other hand, CpG islands are presented in *IRF-1* gene. Thus, I have focused on methylation analysis of *IRF-1* gene promoter area.

Three CpG sites were analyzed by MSP. TC-1/A9 cells are deficient to B7-H1 cell surface expression. Thus *IRF-1* gene promoter is silenced by DNA methylation in control TC-1/A9 cells. On the contrary, IFNγ caused DNA demethylation of *IRF-1* promoter region (activation of *IRF-1* gene) and this was associated with increased B7-H1 cell surface expression in TC-1/A9 cells *(Fig.19.2 A; 16 A)*.

In contrary to TC-1/A9 cells, *IRF-1* promoter showed no changes in methylation status after IFNγ treatment in TC-1 and RVP3 cell *(Fig.19.2 B, C)*. TC-1 cells are not deficient to B7-H1 molecule expression on cells surface. Thus, they have active *IRF-1* gene (nonmethylated promoter) in control cells as well as in IFNγ treated cells. On the other side, RVP3 cells are deficient to B7-H1 cell surface expression and they do not respond to IFNγ stimulation. Thus, it is obvious that this type of deficiency is not caused by DNA methylation but in different manner.



Adapted from: Vlkova, V., Stepanek, I., Hruskova, V., Senigl, F., Mayerova, M., Sramek, M., Simova, J., Bieblova, J., Indrova, M., Hejhal, T., Derian, N., Klatzmann, D., Six, A., Reinis, M. Unpublished data. Manuscript attached in Annexe.

Fig.19.1: Methylation and demethylation status of *TAP-2* gene promoter in TC-1/A9.

TC-1/A9 cell line was treated with IFNγ for 48 hours and analyzed by MSP. The expected size of MSP products were 217 bp. A: Methylation and unmethylation of *TAP-2* promoter was detected in control cells. After IFNγ stimulation, demethylation of *TAP-2* promoter was observed only in IFNγ treated cells.

M means methylation of CpG sites in *TAP-2* promoter; *U* means unmethylation of CpG sites in *TAP-2* promoter; *control* means untreated cells; *IFNγ* means IFNγ treated cells. Experiment was repeated three times.

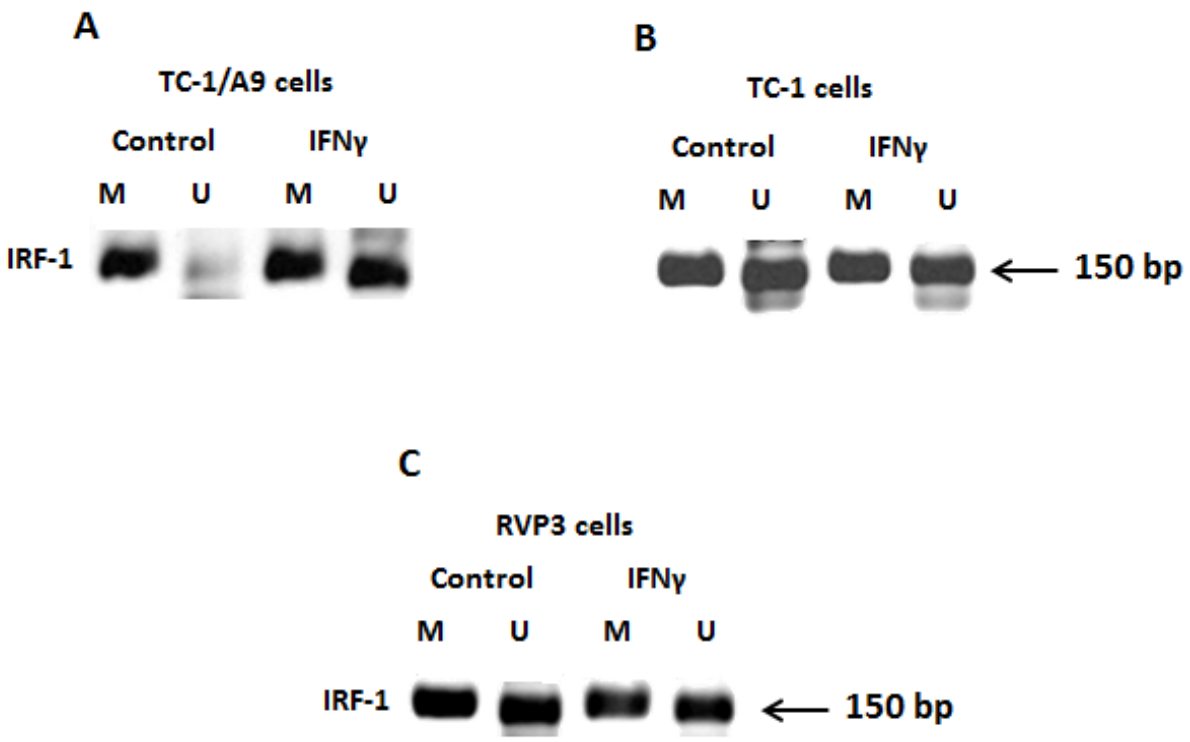


Fig.19.2: Methylation and demethylation status of *IRF-1* gene promoter in TC-1/A9, TC-1 and RVP3 cell lines.

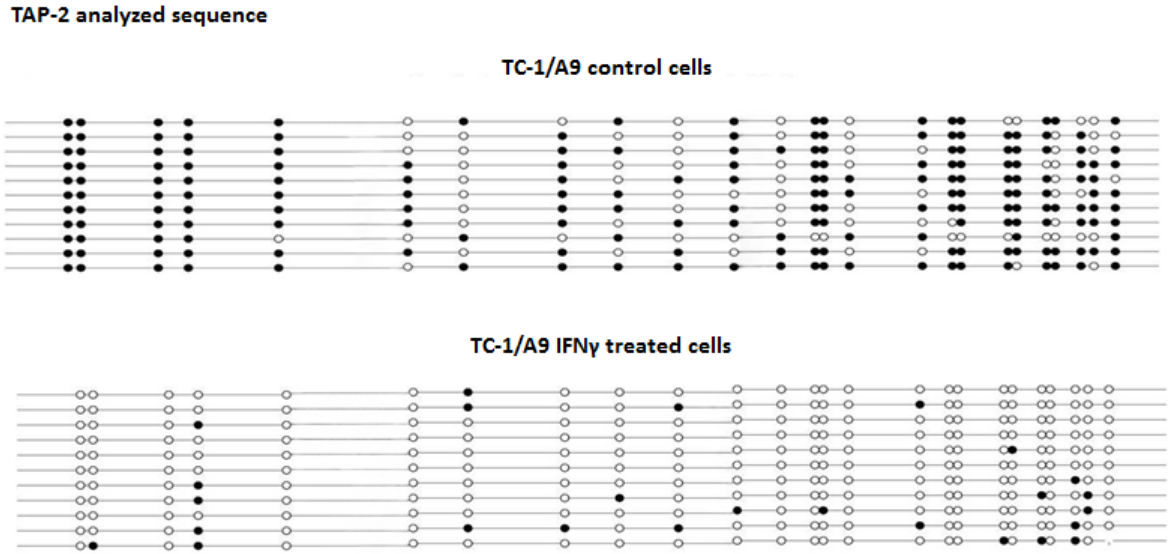
All cell lines were treated with IFNγ for 48 hours and analyzed by MSP. The expected size of MSP products were 150 bp. Three CpG site were analyzed by MSP. A: Methylation of *IRF-1* promoter was detected only in TC-1/A9 control cells. Unmethylated band was detected as well, however it was too weak. After IFNγ stimulation, demethylation of *IRF-1* promoter was increased in TC-1/A9 cells. B: Regardless of IFNγ treatment, methylation and unmethylation *IRF-1* promoter region was detected in TC-1 cells. C: Methylation and unmethylation of IRF-1 promoter region was also detected in control and IFNγ treated RVP3 cells.

M means methylation of CpG sites in *IRF-1* promoter; *U* means unmethylation of CpG sites in *IRF-1* promoter; *control* means untreated cells; *IFNγ* means IFNγ treated cells. Experiment was repeated twice.

20. DNA demethylation of the IRF-1 promoter after IFNγ stimulation was not confirmed by bisulfite sequencing

Data from MSP of *TAP-2* gene were confirmed by bisulfite sequencing which showed that *TAP-2* promoter was demethylated after IFNγ treatment *(Fig.20.1)*. I wanted to confirm MSP results of *IRF-1* promoter region as it was determined in *TAP-2* promoter. I chose bisulfite sequencing as well. I analyzed *IRF-1* promoter region from TC-1/A9 cell lines because of limited financial options.

CpG sites, which were detected by MSP, were also presented in the region analyzed by bisulfite sequencing. A total of 44 CpG sites were analyzed by bisulfite sequencing. I used 10 clones with DNA insert from control and IFNγ treated cells. But only 7 clones with control DNA insert and 6 clones with IFNγ treated insert were suitable for analysis with BiQ Analyser. In control cells, it was detected that some CpG sites were methylated. After IFNγ stimulation, these sites were demethylated *(Fig.20.2)*. This indicates that IFNγ can cause some demethylation of CpG islands. However, majority of CpG sites in IRF-1 promoter were un-methylated in control cells. Thus, it is difficult to proclaim that IFNγ causes demethylation of IRF-1 gene promoter in general.



Adapted from: Vlkova, V., Stepanek, I., Hruskova, V., Senigl, F., Mayerova, M., Sramek, M., Simova, J., Bieblova, J., Indrova, M., Hejhal, T., Derian, N., Klatzmann, D., Six, A., Reinis, M. Unpublished data. Manuscript attached in Annexe

Fig.20.1: Bisulfite sequencing of *TAP-2* promoter region in TC-1/A9 cells before and after 48 hours of IFNγ treatment.

TC-1/A9 cells were treated by IFNγ for 48 hours. Black and white spots represent individual CpG sites in *TAP-2* promoter. A: In TC-1/A9 control cells, CpG sites were mostly methylated (black spots). After IFNγ treatment, most of methylated CpG sites in promoter from control cells were demethylated (white spots).

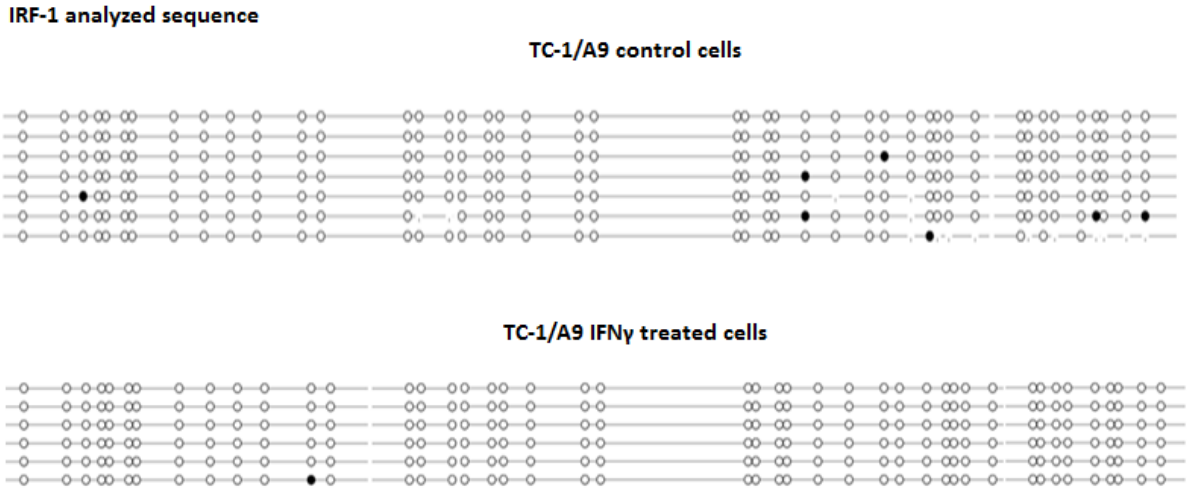


Fig.20.2: Bisulfite sequencing of *IRF-1* promoter region in TC-1/A9 cells before and after 48 hours of IFNγ treatment

TC-1/A9 cells were treated by IFNγ for 48 hours. Black and white spots represent individual CpG sites in *IRF-1* promoter. In TC-1/A9 control cells, CpG sites were mostly unmethylated (white spots) and few sites were methylated (black spots). After IFNγ treatment, every methylated CpG sites in promoter from control cells were demethylated. Experiment was repeated twice.

Discussion

IFNγ ´s biological function is associated with host cell defense against viral and bacterial contagion. The ability of IFNγ to fight against infection is owing to the upregulation of MHC I and II glycoproteins on the cell surface of antigen presenting cells and other genes which are important to the inflammatory response. Expression of MHC molecules helps prime and present bacterial and viral antigens by professional antigen presenting cells and modulates T-cell immune response. Th1 immunomodulation via IFNγ suggested an idea for IFNγ application in cancer treatment *(Zaidi and Merlino, 2011).* However, IFNγ also increases expression of molecules which regulate T-cell response. The B7-H1 (CD274, PD-L1) belongs among these molecules. This co-inhibitory molecule is most expressed in hematopoietic malignancies and also in solid tumours *(Seliger et al, 2008).* B7-H1 is a ligand for PD-1 receptor on T cells. This binding elicits inhibitory signals when engaged with T-cell receptor (TCR). The outcome of the inhibitory signalling is a decrease in protein synthesis, proliferation, cell survival and IL-2 production by T cells *(Francisco, Sage and Sharpe, 2010)*. Induction of $CD8^+$ T-cell apoptosis by tumour cells through B7-H1 – PD-1 signalling pathway was also documented in several studies *(Dong et al, 2002; Shi et al, 2011)*. B7-H1 molecule inhibition by specific antibodies improves the effect of irradiation and immunotherapeutic vaccination to cancer growth. In addition, a decline in the number of Treg and MDSC infiltration in tumour microenvironment was observed. Both cell types are well-known protectors of neoplasm against T-cell antitumour immunity *(Deng et al, 2014; Duraiswamy, Freeman and Coukos, 2013).* Furthermore, therapeutic antibody against B7-H1 was also effective in clinical studies of human cancer immunotherapy *(Robert, Soria and Eggermont, 2013)*

Because of eminent importance of B7-H1 in tumour growth advantage, it would be interesting to determine how B7-H1 could be regulated by IFNγ. According to literature and my experience, B7-H1 molecule expression, as well as the expression of MHC I glycoprotein, increases after IFNγ treatment in mice tumour cells on mRNA and protein level *(Fig. 16, 18 A, B).* IFNγ can cause DNA demethylation of *TAP-2* promoter and other genes which are coupled

with antigen presenting machinery and which are also upregulated after IFNγ treatment *(Fig.19.1 A; 20.1; Annexe)*. Further, cytokine TGFβ can also cause DNA demehylation of $p15^{ink4b}$ promoter region (*Thillainadesan et al, 2012)*. According to these facts, I wanted to shed more light on the possible DNA demethylation changes of *B7-H1* promoter. Consistent with the UCSC Genome Browser databases, mouse *B7-H1* promoter region does not possess any CpG site. Nevertheless, B7-H1 co-inhibitory molecule is regulated by IRF-1 transcription factor which has its binding site in the *B7-H1* promoter region *(Lee et al, 2006).* IRF-1 transcription factor is one of the first activated genes after IFNγ stimulation through JAK/STAT signalling pathway *(Fig.3.2; Zaidi and Merlino, 2011).* Further, IRFs were observed silenced by DNA methylation of their promoters in gastric cancers *(Yamashita et al, 2010)*. So, these informations suggested possible *IRF-1* DNA methylation silencing in our TC-1/A9 tumour cell line. IRF-1 upregulation after IFNγ treatment in mouse tumour cells was determined on mRNA level and correlated with upregulation of *B7-H1* mRNA level *(Fig.18 A, B)*.

CpG islands of IRF-1 transcription factor were determined in the UCSC Genome Browser. *IRF-1* promoter contains 93 CpG sites which comprise 761 bp. At first, MSP was used for investigation of three specific CpG sites DNA methylation in *IRF-1* gene promoter. These CpG sites were methylated in control untreated TC-1/A9 cells *(Fig.19.2 A)*. TC-1/A9 cells are deficient to MHC I and B7-H1 molecule cell surface expression. *TAP-2* gene silencing causes MHC I expression deficiency in these cells *(Annexe)*. It seems that B7-H1 expression deficiency could be caused by the similar way as MHC I cell surface downregulation. Deficient B7-H1 cell surface expression could be caused by silencing of IRF-1 transcription factor in TC-1/A9 cells. DNA methylation of *IRF-1* gene promoter indicated its inactivity by MSP methods. This silencing also correlates with lower mRNA expression level of *IRF-1* gene in control TC-1/A9 cells *(Fig.17 A)*. After IFNγ treatment, DNA demethylation of *IRF-1* promoter was increased in TC-1/A9 cells *(Fig.19.2 A)*. This means that IFNγ induces DNA demethylation of *IRF-1* promoter region. This demethylation was sufficient to enable *IRF-1* and *B7-H1* transcription and translation *(Fig.18 A)*. DNA methylation and unmethylation of promoter region of *IRF-1* gene was observed in both treated and untreated TC-1 and RVP3 cells *(19.2 B,*

C). TC-1 cells constitutively express MHC I and in a low level B7-H1 molecules. These molecules are up-regulated after IFNγ influence *(Fig. 18 B)*. This means that *H2D*^{b}, *H2K*^{b} and *B7-H1* genes are always active in untreated and treated cells. On the contrary, RVP3 cells are deficient in MHC I and B7-H1 cell surface expression so I assumed that they would have the same methylation status as TC-1/A9 cells. However, it is not known by which means the MHC I and B7-H1 deficiency is maintained. According to flow cytometry data *(Fig.16 C)*, RVP3 do not respond to IFNγ treatment which was also confirmed by the mRNA level of *H2D*^{b}, *H2K*^{b}, *B7-H1* and *IRF-1 (Fig.18 C)*. This suggests that MHC I and B7-H1 deficiency in RVP3 cells could be caused by the defect in IFNγ receptor or signalling pathway.

As I was analysed only three CpG sites in *IRF-1* gene promoter with MSP method, it was necessary to confirm the results. I used bisulfite sequencing analysis. CpG sites analyzed by MSP were included in the sequence analyzed by bisulfite sequencing. Unexpectately, only few CpG sites in IRF-1 promoter in control untreated TC-1/A9 cells were found methylated. However, the vast majority of CpG islands were unmethylated *(Fig.20.2)*. After IFNγ treatment, methylated CpG sites were further demethylated. Nevertheless, the whole promoter was demethylated as well *(Fig.20.2)*.

The discrepancy between MSP and bisulfite sequencing results can be caused by the quantity of CpG sites which were analysed. Primers for MSP were designed with three CpG islands inside them. As a consequence, the PCR reaction amplified only three specific CpG sites. They were detected on agarose gel as bands. On the other hand, primers for bisulfite sequencing are designed without CpG sites and thus all CpG sites located within the sequence bordered by primers were analyzed. In my case, 44 CpG islands by bisulfite sequencing were detected. Further, whole CpG islands size of *IRF-1* gene promoter is 761 bp. I analyzed only 348 bp large sequences because of technic reason. Product for BSP should have size only about 300 bp. When size of product is bigger, the BSP does not run well. It is possibility that I analyzed region which is not important for epigenetic regulation of *IRF-1* gene promoter. The analysis of second part of *IRF-1* promoter would confirm or disprove the possibility of *IRF-1* promoter regulation by DNA methylation. Another reason for MSP/bisulfite sequencing differences could be in bisulfite conversion. Conversion could run

inaccurately and MSP results were thus false positive *(Sasaki et al, 2003)*. In conclusion, this shows that the results from the bisulfite sequencing are more relevant than the results from the MSP analysis.

For absolute statement that *IRF-1* gene promoter is not inhibited by epigenetic modifications, it would be necessary to further investigate the histone modification changes in promoter by chromatin immunoprecipitation analysis (ChIP). Di- and trimethylation of lysine 9 on histone H3 (H3K9me2 or H3K9me3) as well as trimethylation of lysine 27 and 36 on histone H3 (H3K27me3; H3K36me3) and loss of acetylation of lysine 9 on histone H3 (H3K9ac) and trimethylation of lysine 4 on histone H3 (H3K4me3) is always linked with inhibition of gene transcription *(Rose and Klose, 2014).* Interactions between DNA methyltransferases and histone methyltrasferases or between DNA methyltransferases and epigeneticaly modified the N-terminus of histones are present in silenced genes *(Lehnertz et al, 2003; Dhayalan et al, 2010).* Likewise, it was observed that histone methylation precedes DNA methylation *(Feldman et al, 2006).* Finally, Hellebekers and colleagues determined that histone modifications and not promoter DNA methylation are responsible for silencing of intercellular adhesion molecule-1 (ICAM-1) in tumour cells *(Hellebrekers et al, 2006)*. These data suggest that *IRF-1* gene promoter might be silenced by the presence of di- or trimethylated lysine 9 on H3 histone and trimethylated lysin 27 and 36 on H3 histone but not by DNA methylation.

In conclusion, these results indicate that B7-H1 upregulation after IFNγ treatment is not going through DNA demethylation of *IRF-1* gene promoter, although the first MSP data suggested this possibility. It would be interesting to examine histone modifications in *IRF-1* promoter, epigenetic changes of its enhancer or other members of JAK/STAT pathway which could lead to the inhibition of B7-H1 expression in TC-1/A9 cells. For instance, DNA methylation of STAT-1 and its consequent mRNA reduced level was determined in human squamous cell carcinomas and ovarian cancers *(Xi et al, 2006; Chang et al, 2012).* As well as, association of STAT-1 with lysine 4 mono- and trimethylation of histone H3 indicates possible epigenetic regulation *(Robertson et al, 2008)*. This could signify that STAT-1 potential

inhibition by epigenetic silencing might also reflect the deficient cell surface expression of B7-H1 on TC-1/A9 tumour cells.

Conclusion

Interferon γ plays important role in the induction of immunity. It affects antimicrobial and anticancer immune response. On the other hand, it sets off expression of immune inhibitory genes such as B7-H1. The B7-H1 molecule is associated with effect in tumour escape from immune surveillance. This link suggests the IFNγ supports tumour growth.

Our previous research proposed that IFNγ can trigger DNA demethylation in promoters of genes which are associated with antigen presenting machinery and thus IFNγ activates their transcription *(Annexe)*. Further studies also demonstrated other cytokines which can induce DNA demethylation of target genes. Based on these facts, I wanted to show the IFNγ epigenetic effect on other genes.

At first, I chose B7-H1 coinhibitory molecule because of its relation with tumour escape and its deficient cell surface expression on mouse tumour cell line TC-1/A9. Its mRNA expression was also lower in TC-1/A9 cells then in parental TC-1 cell line. The B7-H1 upregulation was restored by IFNγ which was shown on flow cytometry results and on mRNA expression level. For determination of DNA methylation of B7-H1 gene, it was necessary to define CpG sites in promoter region. However, I found no CpG islands in mouse B7-H1 promoter according to the UCSC Genome Browser databases. Nevertheless, B7-H1 molecule is regulated via IRF-1 transcription factor because this factor has binding site in B7-H1 promoter region. Moreover, *IRF-1* gene expression is triggered by IFNγ. According to the UCSC Genome Browser databases, *IRF-1* promoter possesses CpG islands. Further, it evinced lower mRNA expression level in TC-1/A9 cells compare to parental TC-1 cells and it was upregulated after IFNγ treatment too. Finally, I studied DNA methylation changes in IRF-1 promoter as a response to IFNγ stimulation.

First of all, the DNA methylation/demethylation of IRF-1 promoter region was analyzed with MSP method. Results were very optimistic because, in control TC-1/A9 cells, the strong methylated band was detected and only weak unmethylated band was presented. After IFNγ treatment of TC-1/A9 cells, strong unmethylated band was shown. These results proposed that IFNγ is able to demethylate promoter of IRF-1 gene.

However, only three CpG sites in IRF-1 promoter were analyzed by the MSP method. For confirmation of the MSP results, I used the bisulfite sequencing which detected 44 CpG islands. In control TC-1/A9 cells, few methylated CpG sites were detected but the majority was unmethylated. After IFNγ treatment, previously methylated CpG sites became demethylated. But the majority stayed unmethylated. Taken together, I did not confirm that the activity of IRF-1 transcription factor is regulated by alterations in DNA methylation induced by IFNγ.

Nevertheless, it would be worth analyzing the histone modification changes in IRF-1 promoter because they also modify transcription of genes epigenetically. Furthermore, one study showed that only histone modifications caused gene transcription silencing and no DNA methylation was observed. The investigation of gene regulation by IFNγ is important for deeper cognition of regulatory mechanisms and in future it could be used for better design of anticancer immunotherapy based on IFNγ effect.

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Annexe