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PREUČEVANJE IZRAŽANJA GENA *BCL7A* V RAKAVI CELIČNI LINIJI NB4

STUDY OF *BCL7A* GENE EXPRESSION IN NB4 CANCER CELL LINE

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Magistrsko nalogo sem pripravila v raziskovalnem centru GENYO (Centro Pfizer - Universidad de Granada - Junta de Andalucía de Genómica e Investigación Oncológica) v Granadi.

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CONTENTS

TABLE OF FIGURES

CONTENT OF TABLES

TABLE OF EQUATIONS

POVZETEK

Rak velja za enega od vodilnih vzrokov smrti v razvitih državah in predstavlja resen zdravstveno-socialni problem. Po zadnjih podatkih naj bi število obolelih v naslednjih letih močno naraslo. Kljub nenehno aktivni raziskovalni dejavnosti na tem področju številna vprašanja v zvezi z nastankom raka še vedno ostajajo nepojasnjena. Gre za večstopenjski proces, ki se razlikuje med različnimi vrstami raka in v katerega so vpleteni tako genetski kot tudi epigenetski dejavniki. Še posebej kritične so genetske ali epigenetske spremembe v protoonkogenih in tumor-zaviralnih genih, saj lahko sprožijo spremenjeno izražanje teh genov in posledično preobrazbo normalnih celic v rakave celice. Ugotovljeno je bilo, da so spremembe v izražanju genov eden od vodilnih vzrokov za začetek procesa kancerogeneze. Eden od kompleksov s pomembno vlogo pri uravnavanju izražanja genov je kromatin-remodelacijski kompleks SWI/SNF, ki je bistvenega pomena za pravilno delovanje bioloških procesov. Predhodne raziskave so pokazale, da je kompleks SWI/SNF najpogosteje mutiran kromatin-remodelacijski kompleks pri raku, kar je vzbudilo veliko raziskovalne pozornosti na področju onkologije. Kromatin-remodelacijski kompleks SWI/SNF s pomočjo energije ATP spreminja strukturo nukleosoma. S tem prepisovalnim mehanizmom omogoča ali preprečuje dostop do verige DNA, kar ga tesno povezuje s procesi, kot so prepisovanje DNA, podvojevanje DNA in popravljalni procesi DNA.

Kompleks SWI/SNF so odkrili in prvotno preučevali v glivah. Pred kratkim so odkrili različico kompleksa tudi pri sesalcih, pri katerih so odkrili nove stabilne podenote, ki jih pri glivah niso našli. Ena izmed novo odkritih podenot, imenovana *BCL7A*, je bila predmet naše raziskave. Točna biološka vloga podenote *BCL7A* še ni znana, vendar dosedanje študije nakazujejo na tumor-supresorsko vlogo, saj je pri raku, še posebej pa pri hematoloških malignih boleznih, stopnja izražanja *BCL7A* močno znižana ali popolnoma zavrta. Številne študije so dokazale, da je utišanje *BCL7A* posledica genetske mutacije ali nenormalnih epigenetskih procesov, najpogosteje hipermetilacije promotorja. Za preučevanje *BCL7A* smo uporabili celično linijo NB4, vrsto akutne promielocitne levkemije M3. Skupina, v kateri sem sodelovala pri raziskovalni dejavnosti, je predhodno ugotovila, da je izražanje gena *BCL7A* v tej celični liniji močno znižano. Želeli smo preučiti vzrok tega utišanja, ki bi lahko bil genetski (mutacija) ali epigenetski.

Predhodno so z metodo po Sangerju analizirali vseh 6 eksonov *BCL7A* v NB4, vendar niso odkrili mutacij, ki bi lahko vplivale na izražanje gena. Tako smo se osredotočili na epigenetske spremembe, pri katerih za razliko od genetskih ne pride do sprememb v nukleotidnem zaporedju. Te spremembe obsegajo predvsem kemijske spremembe v DNA in histonih ter vplivajo na spremenjeno izražanje genov. Dodatno je bilo ugotovljeno, da tvorba *PML-RARα* v preučevani celični liniji vpliva na spremembo metilacije DNA, ki je pri raku pogost pojav. Eksperimentalno delo smo nadaljevali ob predpostavki, da gre za pojav prekomerne metilacije (domnevno) tumor supresorskega gena, kar bi povzročilo njegovo utišanje in zavrlo zmožnost preprečevanja raka.

Preučevanja metilacije DNA smo se lotili z metilacijsko specifičnim PCR, ki je zelo hitra in enostavna kvalitativna metoda. Osredotočili smo se na promotorsko regijo in dokazali prisotnost hipermetilacije *BCL7A* promotorja v celični liniji NB4. Prekomerni metilacijski proces smo želeli oceniti natančneje, kvantitativno, zato smo nadaljevali z delovno precej intenzivnejšo in dolgotrajnejšo metodo bisulfitnega sekvenciranja z uporabo TA klonirnega sistema. Rezultati so pokazali, da je več kot 50 % CpG v preučevanem promotorskem zaporedju metiliranih.

Ker so epigenetske spremembe za razliko od genetskih reverzibilne, smo želeli na celični liniji NB4 izvesti eksperiment z demetilacijskim sredstvom decitabinom in opazovati možno obnovo izražanja *BCL7A*. Decitabin je pirimidinski analog, ki zavira delovanje DNA metiltransferaz in izniči metilacijski utiševalni učinek. S tem eksperimentom smo dosegli ponovno izražanje *BCL7A* na stopnji mRNA pri določeni koncentraciji in času izpostavitve. Z izvedbo Western blot smo dokazali obnovljeno izražanje tudi na stopnji proteina.

Glede na naše rezultate lahko predpostavimo, da je izražanje *BCL7A* zaradi promotorske hipermetilacije neznačilno utišano v celični liniji NB4. Zato bi bila celična linija NB4 primeren (*BCL7A* pomanjkljiv) celični model za preučevanje biološke vloge BCL7A v fenotipskih študijah po izzvani obnovitvi izražanja.

Ključne besede:
kromatin-remodelacijski kompleks SWI/SNF, *BCL7A*, razvoj raka, hipermetilacija promotorske regije, hematološke maligne bolezni.

ABSTRACT

Cancer continues to rank among one of the leading causes of death. The key element in cancer cell transformation is the change in gene expression patterns. One of the complexes that has an important role in gene expression regulation and has been studied by our research group is SWI/SNF chromatin-remodelling complex. It regulates the expression of genes by affecting the availability of DNA during the transcription process.

More specifically, we studied *BCL7A* subunit, which is a recently discovered subunit of this complex. There is significant evidence that the expression of this subunit is down-regulated in cancer, especially in haematological malignancies. This down-regulation may contribute to tumour development, therefore it is suggested that *BCL7A* has a tumour suppressor activity, since many tumour suppressor genes are silenced in cancer.

We used NB4 cell line as cell model, which was derived from acute promyelocytic leukaemia patients. The expression of a *BCL7A* is down-regulated in this cell line, and the aim was to study the cause of this down-regulation. Firstly, we assumed that the consequence for the absence of expression could be a genetic mutation. Sanger sequencing of all 6 exons of *BCL7A* gene in NB4 cell line was performed without detecting any mutation of greater importance. We continued with studying epigenetic aberrations, more precisely aberrant methylation process, which is increasingly recognized as an important factor in the pathogenesis of cancer as many tumour suppressor genes can be inactivated through this epigenetic mechanism. Methylation specific polymerase chain reaction method showed the presence of hypermethylation in the promoter region. Furthermore, we proceeded with bisulfite sequencing analysis with a TA cloning system to obtain qualitative results, whereby we detected that more than 50% of the promoter region is methylated in this cell line.

Additionally, we tried to restore *BCL7A* expression through treatment with demethylating agent decitabine. At a lower concentration and longer exposure time of a chemical we detected *BCL7A* expression restoration not only at mRNA level but also at protein level.

Based on this we were able to determine that promoter hypermethylation of BCL7A gene occurs in NB4 cell line and down-regulates its expression.

Keywords: Chromatin remodelling complex SWI/SNF, *BCL7A* subunit, cancer development, promoter hypermethylation, haematological malignancies.

LIST OF ABBREVIATIONS

AML – acute myeloid leukaemia or acute myelogenous leukaemia

APL – acute promyelocytic leukaemia

ATP – adenosine triphosphate

BAF – BRG1 associated factors

BCL7A gene – B-cell chronic lymphocytic leukaemia/lymphoma 7A in Homo sapiens gene

BiSeq primers – primers designed for amplification of previously bisulfite converted sequence in bisulfite analysis

bp – base pair(s)

BRG1 – brahma related gene or *SMARCA4* gene

CNT – control (sample)

DAC – decitabine or 5-aza-2'-deoxycytidine

DCO – 3,3-dicloro-2-phosphonomethyl-acrylic acid

ddTTP – dideoxythymidine triphosphate

DSMZ - Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

EtBr – ethidium bromide

Fw – forward (primer)

gDNA – genomic DNA

gDNA Meth – commercial genomic completely methylated DNA

gDNA Unmeth – commercial genomic completely unmethylated DNA

HDAC(s) – histone deacetylases(s)

IARC – International Agency for Research on Cancer, part of the World Health Organization

IPTG – isopropyl-β-D-1-tiogalaktopiranozid

LB – Luria-Bertani broth

M – methylated version (MSP)

M3 – or APL, a subcategory of AML by the French-American-British classification of AML

MRT – malignant rhabdoid tumour

MSP – methylation specific polymerase chain reaction

mSWI/SNF – mammalian SWI/SFN complex

Na_2EDTA – ethylenediaminetetraacetic acid, disodium salt, dihydrate

NP-40 – tergitol-type NP-40 or nonylphenoxypolyethoxylethanol

OV_4 – orthovanadate (sodium orthovanadate Na_3OV_4)

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline with Tween 20

PCR – polymerase chain reaction

PML – promyelocytic leukaemia gene

PMSF – phenylmethylsulfonyl fluoride

RA – retinoic acid

RARα – retinoic acid receptor α gene

RT – room temperature

Rv – reverse (primer)

SD – standard deviation

SDS – sodium dodecyl sulphate

SOC medium – super optimal broth, a nutrient rich bacterial growth medium

SWI/SFN complex – switching/sucrose non-fermentable

SNF5 – subunit of SWI/SNF chromatin remodelling complex essential for hepatocyte differentiation (sucrose non-fermentable 5)

TAE – tris-acetate pH8 and EDTA

TEMED – tetramethylethylenediamine

Tm – melting temperature

TRI (reagent) – trizol reagent

U – unmethylated version (MSP)

WB – western blotting

INTRODUCTION

Cancer is one of the leading causes of death in developed countries worldwide and is therefore a serious social health problem. According to the latest report of IARC, there were an estimated 14.1 million cancer cases around the world in 2012, of these 7.4 million cases were in men and 6.7 million in women. Unfortunately, this number is expected to increase to 24 million by 2035 (1).

There is a large number of research studies dedicated to solving some of the many questions raised by this complex disease. Carcinogenesis manifests as a multistep process where an accumulation of genetic and epigenetic alterations in a cell occurs and transforms a normal cell into a malignant cell that has the ability to proliferate uncontrollably and may even invade other tissues. These genetic and epigenetic alterations are especially critical if located in (proto)-oncogenes and/or in tumour suppressor genes, and as a result changes in gene expression regulation occur (2). Gene expression regulation allows cells to express certain proteins when needed. Therefore, it is one of the most important and complex processes of biology. Changes in gene expression patterns are key in cancer cell transformation, through an increase in the expression of genes that promote carcinogenesis (oncogenes) that are normally silenced and/or through a decrease in the expression of genes that prevent it (tumour suppressor genes) and that must be active to block the process of transformation to a malignant cell (3). During last years, next-generation sequencing performed on tumour genomes has highlighted the importance of epigenetic factors in tumour development. Among these epigenetic agents, one of the most frequently altered in tumours was the chromatin remodelling complex SWI/SNF (4).

1.1. The SWI/SNF complex and its importance in cancer

DNA is tightly packed up and compacted around histone octamer protein forming nucleosome, the basic structural unit of chromatin. In eukaryotic cells there is a number of chromatin remodelling complexes, but they are mainly abundant in mammalian cells (5). The importance of these multiprotein complexes lies in their ability to displace nucleosome position to expose or hide a promoter or regulatory region. There are two different types of chromatin remodelling complexes: those that covalently modify nucleosomes and those that consume ATP to mobilize nucleosomes and modulate chromatin compaction. SWI/SNF complex uses the energy released upon the hydrolysis of ATP to destabilize interactions between DNA and histones, and modifies the accessibility of information contained in DNA to the cellular machinery in order to activate

or block gene expression (4, 5). Due to this, these complexes are closely related with processes such as transcription, replication and DNA repair (5).

Genes encoding SWI/SNF subunits were initially identified in screens of yeast genes that regulate mating type switching and sucrose metabolism (SNF – sucrose non-fermentable) (5, 6). Recently, an equivalent homolog of the yeast complex has been discovered in mammals, consisting of additional subunits. Mammalian SWI/SNF complex (mSWI/SNF), also termed as the BAF complex, is a polymorphic assembly made of at least 13 subunits encoded by 26 genes, which generates an extensive diversity of complexes with a specialized function in specific tissues (7).

There is increasing evidence demonstrating the importance of the activity of mSWI/SNF complex in cancer development. The first evidence of its function as a tumour suppressor was obtained after studying one of the subunits of this complex, SNF5. This unit was frequently mutated in malignant rhabdoid tumours (MRTs), both germline and spontaneous tumours. Research conducted in vivo corroborated its role as a tumour suppressor, since mice SNF5 negative developed tumours that were histologically similar to human tumours (8, 9). Moreover, the catalytic subunit of mSWI/SNF complex BRG1 was found to be mutated in human primary tumours (10, 11, 12) and other subunits of the mSWI/SNF complex, BAF250 (ARID1A) and BAF180 (PBRM1), were found more frequently mutated in clear cell of ovarian cancer and renal carcinoma (13, 14, 15).

In 2013, a meta-analysis of the overall mutational rates of every subunit was made in 44 different sequencing studies. The obtained results lead us to the conclusion that mutations in the mSWI/SNF complex appear to be present in nearly all tumour types with an overall mutation rate of approximately 20% in every sample analysed (**Figure 1**). Additionally, mutations affecting more than one subunit are found to be more frequent in many cancer types. The altered subunit varies between different tumour types and their tumour function is still not precisely known, but it is indicated that a specific subunit may have protective cancer role in a specific tissue (7).

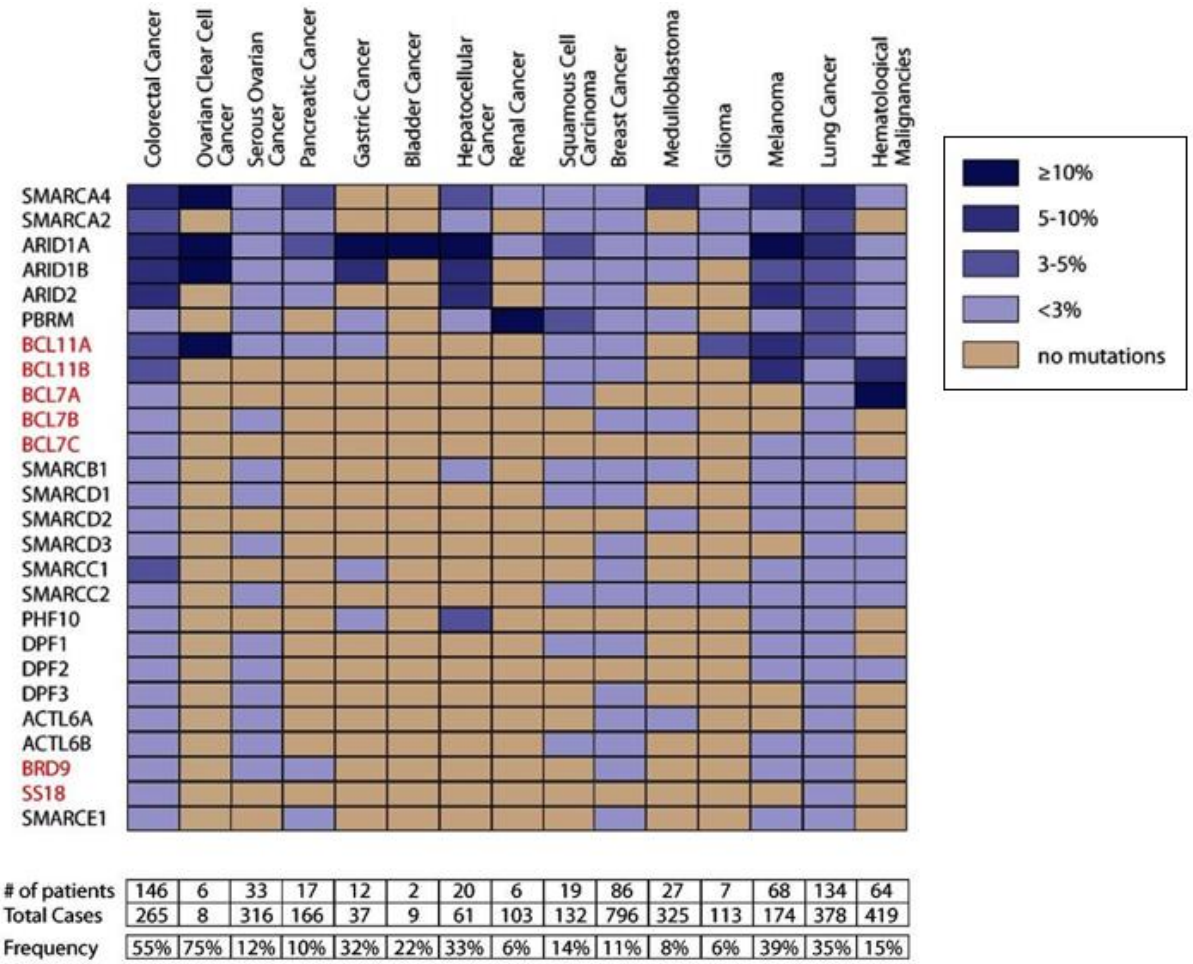

Figure 1: Mutation frequency in mSWI/SNF complex in different cancer types (7).

Data from different studies on the same tissue/cancer type has been summarized to acquire the individual frequency for each subunit. At the bottom part the overall frequency of mutation occurrence in mSWI/SNF complex has been calculated separately for each cancer type. New stable subunits discovered only in mammalian SWI/SNF complex are highlighted in red (7).

1.2. BCL7A – a novel subunit of mammalian SWI/SNF complex

SWI/SNF complex was first discovered in yeast, where it has been studied extensively. However, new subunits, not found in yeast, have been identified recently as stable subunits in mammalian SWI/SNF complexes (7). Among these newly discovered subunits in mammalian SWI/SNF complex *BCL7A* was found. Currently, the biological role of *BCL7A* is still unknown, but it has been found mutated in a large number of cancer types, especially hematopoietic

malignancies, and it has been postulated as a tumour suppressor gene within this latter type of cancers.

Mutation rate of this subunit was reviewed in previous deep-sequencing studies and it was found that *BCL7A* presents a high mutation rate in haematological malignancies: 19.7% of non-Hodgkin lymphomas (16) and 21.7% of multiple myelomas (17). Another report showed that *BCL7A* overexpression has been associated with the germinal centre (GC) phenotype in diffuse large B cell lymphoma in comparison with an activated B-cell like (ACB) phenotype (18). Moreover, since *BCL7A* is cytogenetically located in chromosome 12 (12q24.13), it is directly involved in three-way translocation t(8;14;12)(q24.1;q32.3;q24.1) in Burkitt lymphoma (19). All these studies indicate that genetic alterations in *BCL7A* gene may play an important role in B cell malignancies.

Leaving aside B-cell malignancies specifically and considering not only the mutational status of *BCL7A*, some reports found *BCL7A* expression inactivated in other haematological malignancies by a different mechanism, e.g. epigenetic mechanisms, such as promoter hypermethylation. *BCL7A* was found to be hypermethylated in 48% of biopsies from patients with cutaneous T-cell lymphoma (CTCL) (20), where the loss of *BCL7A* expression has been associated with poor prognosis (21). Also, *BCL7A* gene locus was found to be recurrently deleted in patients with cutaneous T-cell lymphoma (22).

Further investigation is needed in relation to the *BCL7A* gene in myeloid cell lines. The *BCL7A* role in lymphoid, as well as in myeloid cell lines, has been the investigated subject matter of the ongoing project of the research group in which I participated.

1.3. NB4 cell line – acute promyelocytic leukaemia (*PML-RARα*)

NB4 cell line is derived from a patient with acute promyelocytic leukaemia (APL), which is an M3 subcategory of acute myelogenous leukaemia (AML), where malignant promyelocytes, immature myelocytes are being abnormally accumulated in bone marrow. NB4 cell line is genetically characterised by translocation t(15;17)(q24;q21) resulting in *PML-RARα* fusion protein between the retinoic acid receptor α gene and the promyelocytic leukaemia gene. In the absence of RA, *PML-RARα* is shown to have a transcriptional repressive function that is associated with the blockage of hematopoietic differentiation. If RA is present, *PML-RARα* activates transcription and stimulates differentiation (23). Moreover, it was found that in the

absence of RA, *PML-RARα* induces the production of more condensed chromatin structure. Modifications of chromatin structure at target promoters are suggested to be an important mechanism of leukaemogenesis. It was shown that *PML-RARα* causes abnormal recruitment of HDACs (23) which have an ability to regulate gene methylation, and reciprocally DNA methylation recruits histone deacetylases (HDACs) to promoter regions (24). Both phenomena lead to the formation of a more closed chromatin structure and transcriptional repression. Due to this, it is claimed that *PML-RARα* has a general impact on both transcriptome and methylome (25).

Aberrant DNA methylation is one of the typical features in AML cancer types, causing the silencing of tumour suppressor genes (23, 26). It would be interesting to study whether this occurs in the case of *BCL7A* – suspected tumour suppressor, since recent results of the research group in which I participated showed almost absent *BCL7A* gene expression in NB4 cell line.

1.4. Epigenetics in cancer

Epigenetic mechanisms are crucial to maintain tissue-specific gene expression patterns. They have a pivotal role in normal cell growth, cell differentiation, genome stability, and are overall essential for normal gene expression regulation and organism development (27).

Failure of the proper maintenance of epigenetic mechanisms results in inappropriate activation or inhibition of signalling pathways, which leads to altered gene function and consequently to malignant cellular transformation (28).

Cancer, which used to be seen mainly as a genetic disease, is now more and more recognized as a disruption of epigenetic mechanisms along with genetic alterations. Epigenetic alterations can complement or even precede genetic abnormalities and may be fundamental initiating events in some forms of cancer. The field of cancer epigenetics is a rapidly evolving field where it has been postulated that with cancer, the reprogramming of every epigenetic machinery component may occur (28). Scientists mostly draw attention to mechanisms such as DNA methylation, histone modification and RNA-associated silencing (27, 28).

1.4.1. Aberrant methylation – Promoter hypermethylation

The methylation of DNA is one of the most important epigenetic processes. It occurs more frequently within CpG-rich regions of DNA, where a methyl group is covalently added to

cytosines by enzymes DNA methyltransferases (29). The addition of a methyl group condenses chromatin structure and consequently affects transcription process. Due to this, DNA methylation is involved in transcriptional silencing of genes, without a change in their coding sequence (27, 30).

Critical is aberrant methylation, such as hypermethylation of normally unmethylated promoter region or exon 1 in tumour suppressor genes. Tumour suppressor genes prevent carcinogenesis and once they are silenced, the promotion of carcinogenesis may occur. It is suggested that aberrant DNA methylation may be among the earliest changes that occur during carcinogenesis (30). Scientists believe that it would be interesting to study promoter hypermethylation in cancer cells from a clinical point of view as its detection can be exploited in the diagnosis and prognosis of cancer patients (28, 29).

1.4.2. Methods to detect and study promoter hypermethylation

There are several methods to study the methylation process (**Figure 2**). In our experimental work we used methylation specific PCR to obtain qualitative results and bisulfite sequencing with cloning system to additionally obtain quantitative results (circled in **Figure 2**).

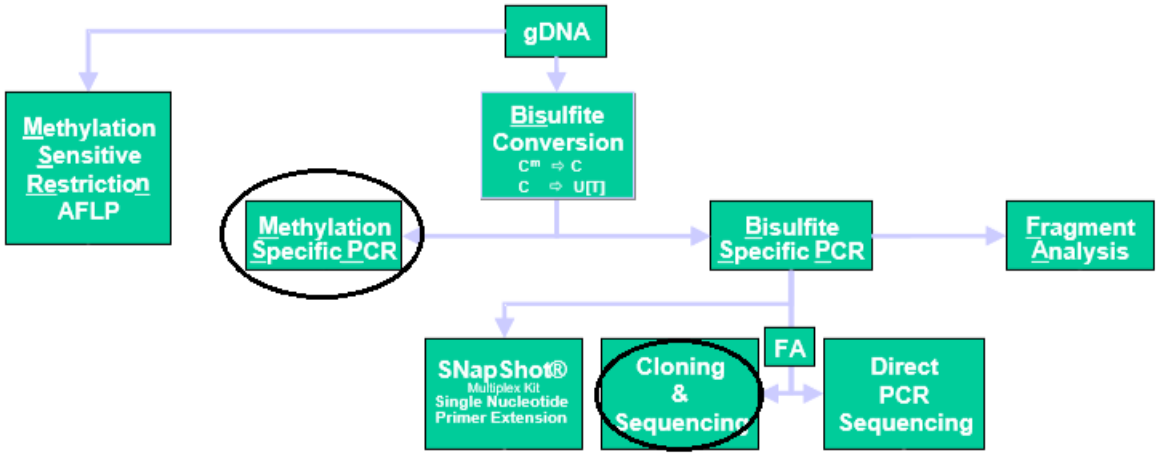

Figure 2: Workflow options for methylation detection and study. FA; fragment analysis (31).

1.4.2.1. DNA bisulfite conversion

For methylation studies we first need to do a DNA bisulfite conversion, where all unmethylated cytosines are converted into uracils, while methylated (5-methylcytosine) cytosines are left unconverted (32).

We need to denature DNA at 95°C in order to separate the paired strands of the genomic DNA. This is followed by a three-step conversion (**Figure 3**) with sodium bisulfite, generating a uracil. Methylated cytosines are not susceptible to the conversion step. In order to reveal the methylation status, the procedure can be followed by PCR or sequencing methods. For a successful bisulfite conversion the purity of gDNA is of greater importance. Moreover, putting an excessive amount of gDNA per reaction or gDNA not being completely denaturated prior to the bisulfite treatment may result in incomplete bisulfite conversion (32, 33).

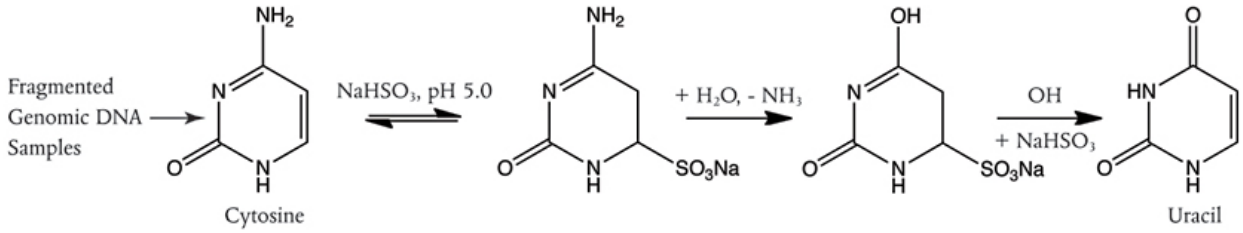

Figure 3: Bisulfite conversion reaction (33).

1.4.2.2. Methylation specific polymerase chain reactions (MSP)

MSP is a very undemanding method to evaluate the methylation status of CpG islands in a specific region. Qualitative results can be obtained easily and quickly, a very small quantity of DNA is needed, the method is very sensitive (it detects already 0.1% methylated alleles of a given CpG island locus) and specific for methylation of virtually any block of CpG sites in a CpG island. As mentioned before, a pre-step is required, where we modify DNA with sodium bisulfite. Secondly, we perform PCR amplification with primers specific for methylated versus unmethylated DNA. Next, we carry out the gel separation, ethidium bromide staining and direct visualization under UV illumination.

It is important that we design two different types of primers (M pair and U pair) in order to distinguish methylated DNA from unmethylated DNA in bisulfite-modified DNA, taking advantage of the sequence differences resulting from bisulfite modification (34).

When designing MSP primers, we should take into consideration that primers need to contain at least one CpG site within their sequence. Preferably, this CpG site is to be located in the very 3'-end of the sequence in order to discriminate maximally methylated DNA against unmethylated DNA. Primers for methylated DNA (M pair) and for unmethylated DNA (U pair) should contain the same CpG sites and should preferably have a similar annealing temperature. However, they may differ in length and start position (35).

1.4.2.3. Bisulfite sequencing analysis with cloning

Bisulfite sequencing is considered to be the "gold standard" for methylation analysis and it gives a more accurate assessment of the methylation status of a sample. Although it is a difficult and labour-intensive procedure it is used for both routine practice and discovery analysis (31).

As previously mentioned, initially DNA bisulfite conversion must be performed where all non-methylated cytosines are being deaminated to form uracil. Secondly, PCR amplification is normally performed in order to obtain a sufficient amount of bisulfite converted DNA, which generates amplicons, whereby uracil is being replaced by thymine. First, the primers for this PCR amplification need to be designed by a software programme where the user defines his criteria. When designing the primers for the amplification of bisulfite converted DNA, we should take into account that primer annealing sites need to be without CpGs, yet flanking the CpG rich region in order for PCR product to contain a large number of CpG motifs. Additionally, primers should be relatively long to achieve a desirable high Tm. We should avoid longer poly(T) sequence in annealing sites as it results in non-specific primer annealing and poor amplification due to polymerase slippage. The PCR product can then be sequenced directly or first cloned by a cloning system and then sequenced (30). For our experiment we decided to perform bisulfite sequencing method with cloning (TA cloning system) of the amplified products since it provides higher analytical sensitiveness (31, 36).

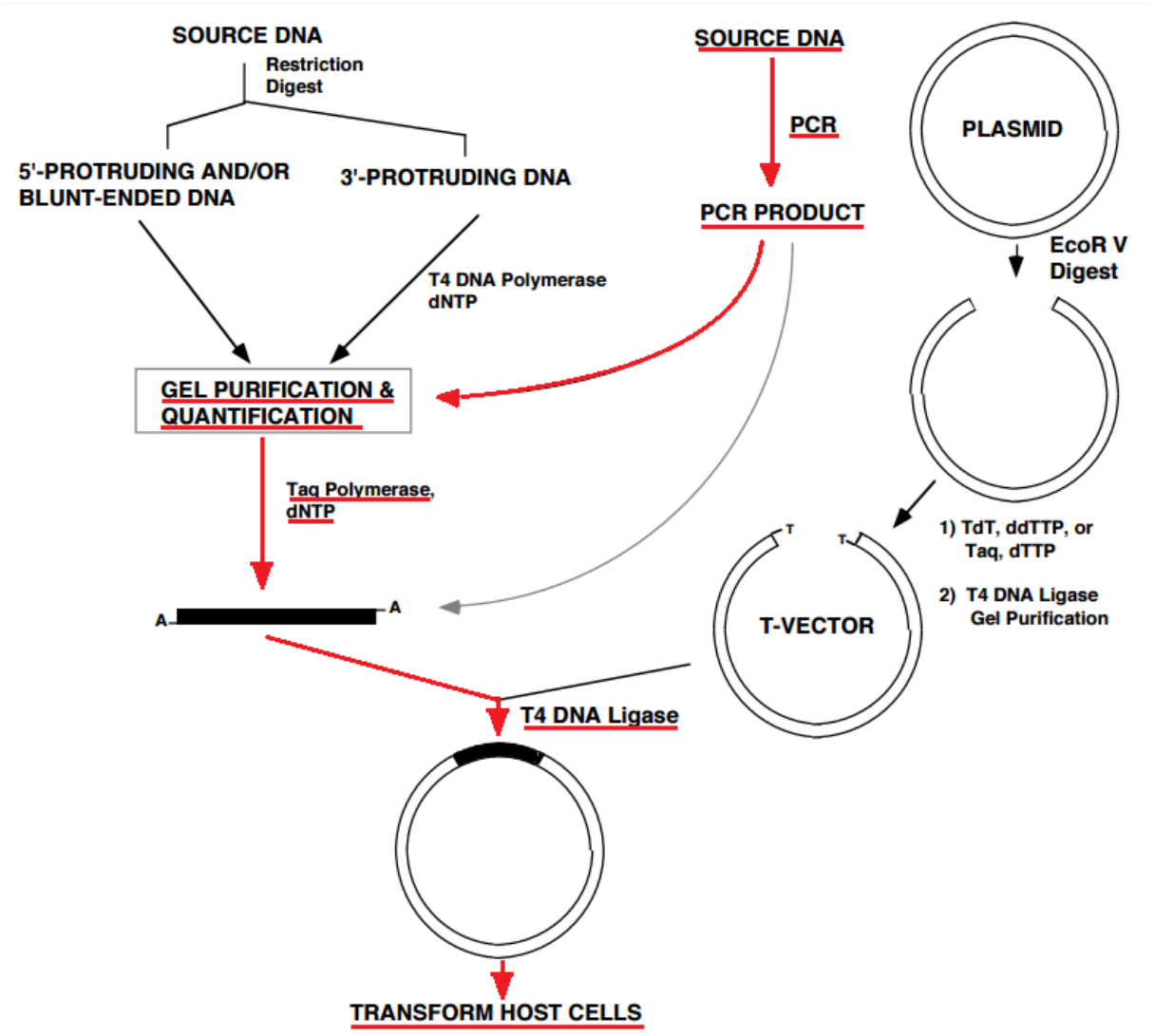

Figure 4: Schematic diagram of cloning procedures using T-vectors (36).

The procedure we used in our experiment (underlined in red colour, **Figure 4**) is one of the easiest, labour- and time-saving as well as the most efficient technique for the cloning of PCR products, where simply an A-tail fragment of interest is inserted into T-tailed vector.

In advance, a single 3'-adenine overhang needs to be added to each end of the insert (A-tailing procedure) in order to fit into the T-vector. This is created by performing a PCR using Taq DNA Polymerase that lacks 3' to 5' proofreading activity and usually adds this A-overhang to the insert. Preferably the insert should have a guanine at the 5' end since this increases the probability of adenine to be tagged to the 3' end. T-vector is previously cut with a blunt-end restriction enzyme (EcoRV), linearized and tagged with an overhanging thymine using terminal

transferase and ddTTP by the manufacturer. DNA fragment is then directly ligated into the vector, forming a cyclic molecule that can be autonomously replicated in host cells. After transformation the positive colonies can be identified by restriction mapping of the mini-prepared plasmids. The insert is released by digestion with restriction enzyme from multiple cloning sites (EcoRI) and the insert size is confirmed by agarose gel electrophoresis. Finally, the sequencing is performed to determine the identity and the orientation of the clone, and the methylation status of the sequence of interest can be assessed (36).

1.4.3. Epigenetic therapy with demethylating agents

Epigenetic alterations are intrinsically reversible. It was discovered that a possible restoration of normal state can be achieved by epigenetic treatment (28). This scientific discovery initiated sudden development of epigenetic drugs and therapies. A large number of drugs have been found effective in restoring aberrant epigenetic state to normal. Many of them are being tested in clinical trials, while several of them have been used in clinics so far. Due to the identification of cancer-associated changes in DNA methylation as attractive targets for therapeutic intervention, drugs based on the inhibition of DNA methylation (demethylating drugs) are being developed (37).

Decitabine or 5-aza-2'-deoxycytidine is a clinically approved DNA-methyltransferase inhibitor, effective in myeloid malignancies treatment. Decitabine incorporates into DNA as a deoxycytidine analogue, irreversibly covalently binds with DNA-methyltransferases at methylation target sites and decreases cytosine methylation process. Subsequently, the expression of silenced genes is reactivated. Based on previous studies, it was shown that lower doses are more effective than higher doses due to decitabine's cytotoxic effect (38).

2. OBJECTIVES OF THE WORKING THESIS

As mentioned before, the biological role of *BCL7A* is still unknown. The main and final objective of the project in which I participated is to establish a cell line model deficient in *BCL7A* expression. Afterwards, this model could be used for phenotypic studies after *BCL7A* expression restoration in order to elucidate the role of *BCL7A* in haematological malignancies.

The research group in which I carried out individual research work for my master thesis has already evaluated *BCL7A* expression levels by qPCR and Western Blot from a large battery of haematological cell lines. The results showed that in the case of NB4 cell line, *BCL7A* gene expression is almost absent. Given that this gene is assumed to be linked with a tumour suppressor role, it would be interesting to study the cause of this down-regulation and to define whether the *BCL7A* expression downregulation is caused by any genetic variation (mutations) or epigenetic aberration.

The research group has already obtained the *BCL7A* sequencing results for NB4 cell line and they have not detected any important genetic aberration in any of the six exons of *BCL7A*. Therefore, we mostly focus on epigenetic aberration, more precisely on promoter hypermethylation. The aberrance of DNA methylation is one of the typical features observed in cancers such as acute myeloid leukaemia. However, the mechanistic link between the aberrance of DNA methylation and leukaemogenesis is not well understood, therefore it continues to be investigated. Aberrant promoter methylation contributes to gene silencing of tumour suppressor genes in cancer. We want to investigate if the cause for *BCL7A* downregulation in NB4 cancer cell line occurs by promoter hypermethylation. In order to study the methylation status of promoter region, we plan to perform MSP to obtain qualitative results, and indirect bisulfite sequencing method (with TA cloning system) to obtain quantitative results of the methylation status.

Moreover, epigenetic aberrations are potentially reversible and can be restored to normal state. If we confirm the presence of hypermethylation in *BCL7A* promoter region in NB4 cell line, we plan to continue our experiment by treating NB4 with demethylating agent decitabine or 5-aza-2'deoxycytidine in order to observe the possible restoration of *BCL7A* expression.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory equipment

During our work we used the following laboratory equipment (**Table I**).

Table I: Laboratory equipment.

LABORATORY EQUIPMENT	MANUFACTURER, MODEL
Bacteria incubator	BINDER, C170 CO2 Incubator
Biomolecular imager of gels and blots	GE Healthcare Life Sciences, ImageQuant™ LAS 4000
Centrifuge	Corning® LSE™ High Speed Centrifuge
Centrifuge, Refrigerated	Thermo Scientific™ Sorvall Legend Micro 17R Centrifuge
Cube Dry Bath Incubator	Cleaver scientific, TCDB-01 The Cube Dry Bath Incubator (one block unit)
Detection system (Protein quantification, Bradford)	GloMax®-Multi Detection System, Promega
Electrophoresis Power Supply	VWR International, Model 302 (300V, 90W, 500mA)
Freezer to store samples at –80°C	Thermo Scientific Forma 900 Series Ultra Low Temperature Freezer Model 995
Gel Imaging System	Avegene Life Sciences, LIAS Slite140
Genetic Analyzer (Sanger sequencing)	Applied Biosystems, 3130 (4-capillary) Genetic Analyzer
Incubated Tabletop Orbital Shaker	Thermo Scientific, MaxQ HP 420
Microcentrifuge	Eppendorf™ MiniSpin™
Microwave	Daewoo, KOR – 63A5
PCR machine, thermal cycler	Thermo Scientific™ Arktik™
PCR machine, thermal cycler (with the ability of temperature gradient)	BioER LifeECO™ Thermal Cycler
PCR machine, thermal cycler	Applied Biosystems, Veriti 96-Well Fast Thermal Cycler
Pipettes	Eppendorf Research Plus, - Single-Channel, Adjustable (0.1–2.5 µL, 0.5-10 µL, 10–100 µL, 100–1000 µL)
Power Supply (WB)	BioRad, PowerPac™ Basic
Real-Time PCR machine	Applied Biosystems, 7500 Real-Time PCR Systems

Spectrophotometers	Thermo Scientific™ NanoDrop™ 2000 and 2000c
Thermomixer	Eppendorf, Thermomixer comfort
Vacuum system (Kitasato)	Merck Millipore

3.1.2. Reagents and biological material

Chemical compounds, commercial kits and biological material used during experimental work are listed below (**Table II, III** and **IV**).

Table II: Chemical compounds.

CHEMICAL COMPOUNDS	**MANUFACTURER**
APS (Ammonium persulfate)	Panreac AppliChem
10% SDS (Sodium dodecyl sulphate)	VWR International
40% Acrylamide/bisacrylamide	Panreac AppliChem
Agarose A9539	Sigma – Aldrich
Ampicillin	Biowest
Anti-BCL7A primary antibody	Sigma – Aldrich
Anti-mouse secondary antibody	GE Healthcare
Anti-rabbit secondary antibody	GE Healthcare
Anti-tubulin primary antibody	Sigma – Aldrich
Bovine albumin serum	USBiological
Bradford reagents	Amresco
Chloroform	Sigma Aldrich
Coumaric	Sigma – Aldrich
dATP	Sigma – Aldrich
DNA Gel Loading Dye (6X)	Thermo Scientific™
Ethanol	VWR International
EtBr	Alfa Aesar
Foetal bovine serum	Gibco®
gDNA Meth – commercial completely methylated genomic DNA	Applied Biosystems
gDNA Unmeth – commercial completely unmethylated genomic DNA	Applied Biosystems
Hydrochloric acid 37%	VWR International
IPTG (Isopropyl-β-D-1-tiogalaktopiranozid)	Sigma – Aldrich
Isopropanol	VWR International
Luminol	Sigma – Aldrich
$MgCl_2$	Sigma – Aldrich
PBS	Biowest
Penicillin	Biowest
RNase-free water	Thermo Scientific™
RPMI 1640 medium	Biowest

Skimmed milk powder	Nestle
SOC medium	New England Biolabs (UK)
Streptomycin	Biowest
SYBR Green Supermix Universal iTaq	Bio-Rad Laboratories
Taq Polymerase	Sigma – Aldrich
TEMED	Alfa Aesar
TRi® Reagent	Gibco
Tris-(hydroxymethyl-)-aminomethane	VWR International
Tween 20	AMRESCO
X-Gal	Sigma – Aldrich

Table III: Commercial kits.

COMMERCIAL KITS	**MANUFACTURER**
DNA-spin™ Plasmid DNA Purification Kit	iNtRON Biotechnology
EZ DNA Methylation-Gold™ Kit (Catalogue Nos. D5005 & 5006)	Zymo Research
GenElute ™ Gel Extraction Kit	Sigma-Aldrich
GenElute ™ PCR Clean-Up Kit	Sigma-Aldrich
Kapa HiFi Hot start Uracil + Ready mix PCR kit	Kapa Biosystems
pGEM®-T Easy Vector system I. (A1360): pGEM®-T Easy Vector (50 ng/µl) Control Insert DNA (4 ng/µl) T4 DNA Ligase (3 Weiss units/µl) 2X Rapid Ligation Buffer	Promega
RevertAid RT Reverse Transcription Kit	ThermoFisher Scientific

Table IV: Biological material.

BIOLOGICAL MATERIAL	**MANUFACTURER / ORIGIN**
NB4 cell line	DSMZ Repository, Germany
Agar plate LB broth + ampicillin 100 µg/mL	Homemade, Genyo, Granada
DH5α chemically competent E. coli.	Homemade, Genyo, Granada

3.2. Methods

3.2.1. Extraction of genomic DNA

The acute promyelocytic leukaemia cell line NB4 was provided by DSMZ Repository (dsmz.de). The extraction of genomic DNA was performed by a homemade protocol that obtains high quality DNA. First, we prepared an extraction buffer (proteinase K buffer) from 20 mM

Tris-HCl pH 7.5 (we need to adjust pH to 7.4), 4 mM Na_2EDTA and 100 mM NaCl. We added 1 mL of extraction buffer to every cell pellet sample and homogenized it by repetitive pipetting. Next, we added 10% of SDS to the pellet and re-suspended by 15-second vortexing. Proteinase K solution (20 mg/mL) was added immediately, following an overnight incubation at 55°C. Next morning we cooled down the samples to room temperature (RT), added 5.3 M NaCl solution and vortexed shortly. After the centrifuging step at 13000 rpm for 5 minutes at 4°C, cold isopropanol (stored at –20°C) was added. We mixed the specimen gently by inverting the tubes at least 5–6 times, and left them at RT 5 minutes to precipitate DNA. Next, we removed DNA directly by a wide bore tip and transferred it to a microfuge tube. We centrifuged it at maximum velocity for 15–20 minutes and discarded the supernatant. Then the washing of sample with 1 mL of 70% ethanol followed. We centrifuged it again at maximum velocity for 15–20 minutes, left DNA at 37°C to dry and finally re-suspended DNA in dH_2O.

3.2.2. Quantification of genomic DNA

DNA quantification was performed by NanoDrop™ 2000 Spectrophotometer. We vortexed every sample before we measured its concentration. First, a blank measurement was made with dH_2O. The concentration of NB4 sample was 635 ng/L.

3.2.3. DNA bisulfite conversion

Firstly, we needed to bisulfite converse our DNA samples. We used commercial kit EZ DNA Methylation-GoldTM Kit, by Zymo Research (Catalogue Nos. D5005 & 5006). We followed the procedure of the protocol by the manufacturer available at:
http://www.zymoresearch.com/downloads/dl/file/id/57/d5005i.pdf (39).
The input of DNA amount should be 500 pg – 2 µg DNA per treatment (39). The concentration of DNA in our NB4 sample was 635 ng/L. We added 1.58 µL of NB4 sample (1000 ng), and then we added dH_2O up to 20 µL (30). The final concentration was 50 ng/µL.
Additionally, we bisulfite converted commercial genomic DNA that was completely methylated (gDNA Meth) and the completely unmethylated gDNA (gDNA Unmeth) to use them as methylated and unmethylated standards.

3.2.4. Methylation specific PCR (MSP)

We performed MSP with bisulfite converted NB4 sample, commercial genomic DNA that is completely methylated (gDNA Meth), commercial genomic DNA that is completely unmethylated (gDNA Unmeth) and a blank one (without our DNA template).

When performing MSP, or in general to amplify the bisulfite-treated DNA, it is recommended to use a "hot start" polymerase. The bisulfite-treated DNA is normally AT-rich, since the majority of cytosines are converted into uracils. Due to this low GC composition, non-specific PCR products are to be generated more easily during the amplification process. By using the "hot start" polymerase we prevent DNA extension at lower temperatures, and more specific amplicons with little or no by-product are formatted (31).

We used commercial Polymerase; Kapa HiFi HotStart Uracil+ Ready mix PCR kit (Kapa Biosystems) KAPA HiFi HotStart Uracil+ ReadyMix that already contains hot-start DNA Polymerase, reaction buffer, dNTPs and $MgCl_2$ (at a final concentration of 2.5 mM). KAPA HiFi HotStart Uracil+ DNA Polymerase has a 5' → 3'polymerase and a 3' → 5' exonuclease (proofreading) activity.

The primers we used are schematically depicted in **Figure 5.** The theoretical size of product is 104 bp and it harbours 12 CpGs.

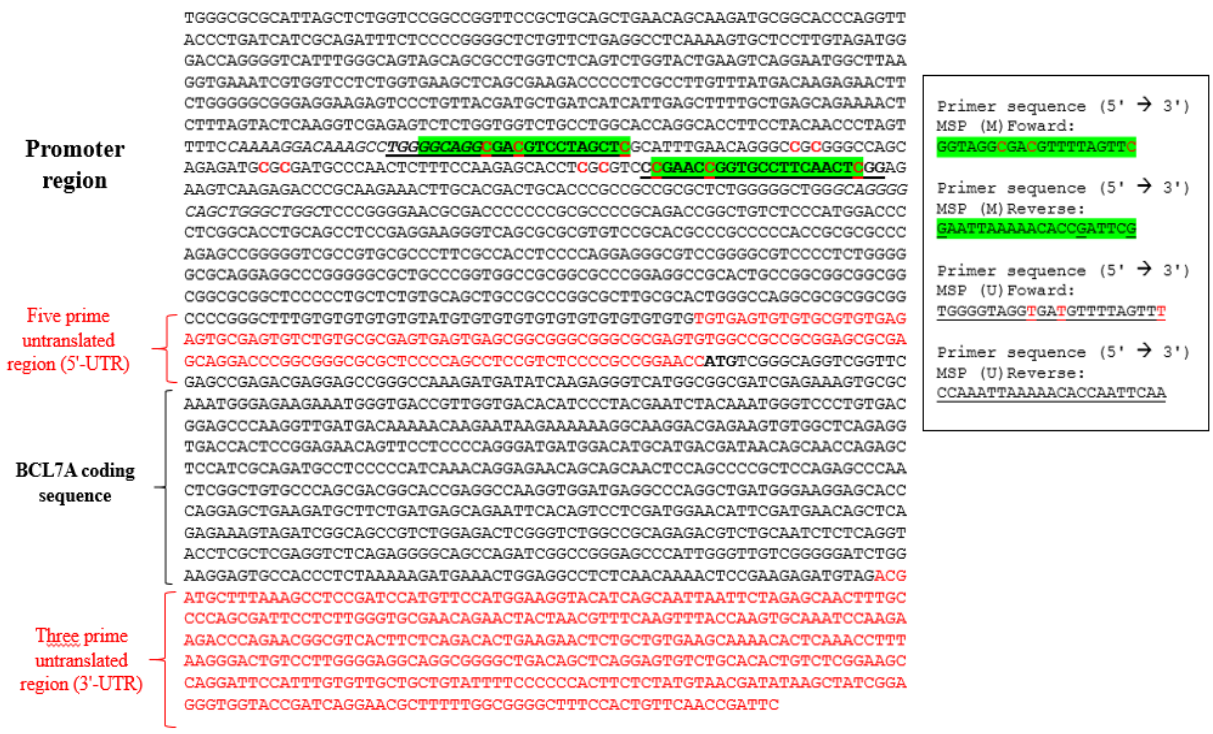

Figure 5: Schematic representation of *BCL7A* gene sequence used in MSP experiment. MSP primer positions are coloured in green. Source of the sequence: UCSC Genome Browser (genome.ucsc.edu).

Table V: Primer information used in MSP experiment (20).

Oligonucleo-tide name	**Sequence**	**Length**	**Tm**	**Manufacturer**
MSP (M) Fw	5'- GGTAGGCGACGTTTTAGTTC -3'	20 bp	68°C	Invitrogene, Life Technologies
MSP (M) Rv	5'- GAATTAAAAACACCGATTCG -3'	20 bp	62°C	Invitrogene, Life Technologies
MSP (U) Fw	5'- TGGGGTAGGTGATGTTTTAGTTT -3'	23 bp	68°C	Invitrogene, Life Technologies
MSP (U) Rv	5'- CCAAATTAAAAACACCAATTCAA -3'	23 bp	63°C	Invitrogene, Life Technologies

The PCR reaction was carried out in a final volume of 25 µL (**Table VI**) under specified conditions (**Figure 6**). We prepared two master mixes: for methylated (M) and unmethylated (U) version. All compounds were vortexed before added.

Table VI: The reaction mix for MSP experiment.

Methylated (M) version		Unmethylated (U) version	
dH_2O	up to 25 µL (10.00 µL)	dH_2O	up to 25 µL (10.00 µL)
KAPA HiFi HotStart Uracil+ Ready Mix	12.50 µL	KAPA HiFi HotStart Uracil+ Ready Mix	12.50 µL
Primer Fw (10 µM)	0.75 µL	Primer Fw (10 µM)	0.75 µL
Primer Rv (10 µM)	0.75 µL	Primer Rv (10 µM)	0.75 µL
DNA sample	1.00 µL	DNA sample	1.00 µL
	25.00 µL		**25.00 µL**

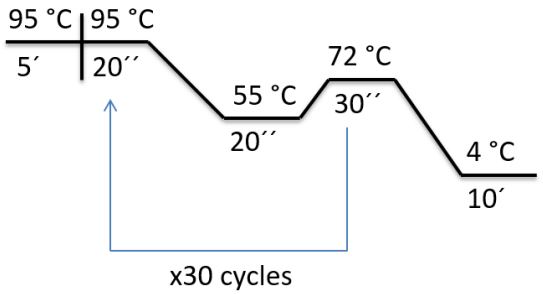

Figure 6: Schematic representation of PCR parameters.

After the PCR, the DNA Gel Loading Dye (6X) was added to the sample in ratio dye: sample = 1:5. Everything was loaded to an agarose gel (2% w/v), and ran for 1 hour at 130 V (constant voltage) using 1X TAE buffer, exposed to ethidium bromide staining and directly visualised under UV illumination (Avegene Life Sciences, LIAS Slite140).

3.2.5. Bisulfite sequencing analysis with TA cloning system

We decided to perform a bisulfite sequencing analysis with TA cloning system in order to get quantitative, more detailed results about methylation pattern. Bisulfite converted NB4 DNA sample needs to be amplified previously to get a sufficient amount of the sample for the following procedure.

3.2.5.1. PCR amplification of the sequence of interest

We specifically designed and ordered primers (**Table VII**) to anneal to bisulfite converted DNA sequence of interest that is depicted in **Figure 7**.

Table VII: Primer information used in bisulfite sequence analysis.

Name: BiSeq Primer Fw4 (Sigma-Aldrich)	Length (bp)	CpGs in product	Product size (bp)	Tm (°C)
5'- TAGAAAATTTTTTAGTATTTAAGGT -3'	25	61	579	53.0
Name: BiSeq Primer Rv3 (Sigma-Aldrich)	Length (bp)	CpGs in product	Product size (bp)	Tm (°C)
5'- TACACAAAACAAAAAAAACC -3'	20	61	579	54.3

Figure 7: Schematic representation of *BCL7A* gene sequence used in bisulfite sequencing analysis. BiSeq primer positions are highlighted in grey. Source of the sequence: UCSC Genome Browser (genome.ucsc.edu).

PCR reactions were carried out using Kapa HiFi Hot start Uracil+ ReadyMix that already contains KAPA HiFi HotStart Uracil+ DNA Polymerase, reaction buffer, dNTPs and MgCl2 (2.5 mM). As a template we used 50 ng of bisulfite converted DNA from NB4 sample. We performed 6 reactions (sextuplicates) and a blank one. The following table shows PCR reaction mix components. The PCR reaction was carried out in a final volume of 25 µL. All compounds were vortexed before added.

Table VIII: PCR reaction mix for amplification step for bisulfite sequencing analysis.

PCR mix for Bisulfite sequencing method	
dH_2O	up to 25 µL (10.00 µL)
KAPA HiFi HotStart Uracil+ Ready Mix	12.50 µL
BiSeq Primer Fw4 (10 µM)	0.75 µL
BiSeq Primer Rv3 (10 µM)	0.75 µL
DNA sample	1.00 µL (50 ng)
	25.00 µL

The PCR amplification of our sequence of interest was quite difficult and it took us a great amount of time and a number of repetitions before we finally found the right conditions for a successful reaction (see results below, **Figure 12**).

After the amplification we added the DNA Gel Loading Dye (6X) to the samples in ratio dye: sample = 1:5. Everything was loaded to agarose gel (1.5% agarose from Sigma, A 9539), put to separation for 1 hour at 130 V (constant voltage), exposed to EtBr staining and directly visualised under UV illumination (Avegene Life Sciences, LIAS Slite140). The result corresponded to the expected length of PCR product. We cut the bands at size 579 bp and united sextuplicates 3 by 3 in order to concentrate our samples.

3.2.5.2. Gel purification step

For the purification of both DNA samples from agarose gel we used GenElute Gel Extraction Kit (Sigma-Aldrich). This kit is designed for rapid purification of DNA amplicons of 100 bp – 10 kb in length. The gel first needs to be solubilized and DNA fragments are to be extracted and purified by a combination of silica-based membrane technology and simplicity of spin or vacuum centrifuge columns. DNA recovery is normally around 50–55%, but in some cases it may be as high as 80% (40). When performing gel purification, we followed the A section "Spin procedure for Agarose Gels" of the protocol by the manufacturer available online at: http://pws288.byu.edu/Portals/89/Simga%20Gel%20Elution%20Kit.pdf. The only modification we introduced during the procedure was in the last elution step. We added 25 µL instead of 50 µL of pre-heated Elution Solution to the membrane to increase the concentration of eluted DNA, and incubated it for 5 minutes instead of 1 minute at RT.

3.2.5.3. A-tailing procedure

This is an additional step we needed to take in our case. For PCR amplification of bisulfite converted DNA we used KAPA HiFi HotStart Uracil+ DNA Polymerase which has a proofreading activity and therefore does not generate fragments with 3'A-tail. When using a polymerase that generates blunt-ended fragments we first need to do an A-tailing step in order to ligate the DNA fragment later into the T-vector. Due to this, only one insert will be ligated into the vector in contrast with blunt-ended cloning, where multiple insertions can occur (41).

During the procedure we followed the protocol written by the manufacturer. To optimize cloning efficiency, normally we need to adjust the amount of DNA we use in the A-tailing reaction. After the amplification step we measured the concentration of DNA, which was 23 ng/µL. When molar concentration is low, larger volumes of the PCR fragment are needed (41). **Table IX** demonstrates the reaction mix volumes of added compounds.

Table IX: Reaction mix for A-tailing procedure.

Reaction mix for A-tailing procedure	**Volumes**
Taq DNA Polymerase 10X Reaction Buffer	2.5 µL
MgCl2	2.0 µL
dATP (10 mM)	0.5 µL (final concentration of 0.2 mM)
Taq Polymerase Sigma	1.0 µL (5 units)
PCR product	19.0 µL (437 ng of product)
	25.0 µL

We prepared the reaction mix with adapted volumes and made incubation at 70°C for 35 minutes.

3.2.5.4. PCR clean-Up step

After the A-tailing procedure it is recommended to perform an additional rapid purification of our double-stranded PCR product from excess components of the PCR reaction by using GenElute PCR Clean-Up Kit. We decided to do it only for one sample to see the difference. We followed the procedure of the protocol by the manufacturer available online at: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/na1020bul.pdf.

3.2.5.5. Ligation Step

DNA fragment was then ligated into the vector to form a cyclic molecule which can be autonomously replicated in host bacteria cells. We used pGEM®-T Easy Vector from Promega (**Figure 8**) that has already been linearized and tagged with single thymine overhangs on both 3' ends by the manufacturer.

pGEM®-T Easy Vector sequence reference points:	
T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

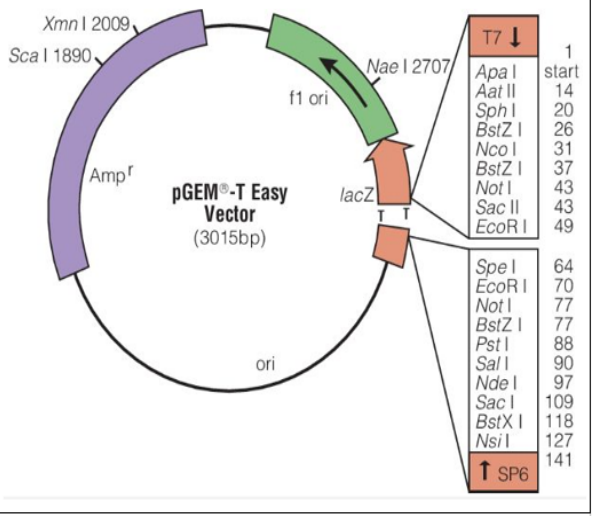

Figure 8: pGEM®-T Easy Vector Map and Sequence Reference Points (41).

First, we needed to optimize the Insert : Vector molar ratio. The concentration of PCR product was estimated with a spectrophotometer NanoDrop™ 2000. The concentration of the first sample after PCR clean-up was 8 ng/µL and the concentration of the second sample without the clean-up step was 12 ng/µL.

Equation *1*: Calculation of appropriate amount of insert for ligation reaction in ng. The size of pGEM®-T and pGEM®-T Easy Vectors is approximately 3 kb and they are supplied at a concentration of 50 ng/µl. The size of the DNA insert is 579 bp (41).

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio}$$

Table X: Calculation of appropriate amount of DNA insert (in ng).

Insert : Vector molar ratio	Calculation of appropriate amount of insert
3:1	$\frac{50\text{ng vector} \times 0.58\text{kb insert}}{3.0\text{kb vector}} \times \frac{3}{1} = 28.8\text{ ng insert}$
5:1	$\frac{50\text{ng vector} \times 0.58\text{kb insert}}{3.0\text{kb vector}} \times \frac{5}{1} = 48\text{ ng insert}$

Previously, ligations with many different Insert : vector molar ratios were performed in order to find the optimal one. This experiment was performed with 3:1 and 5:1 ratio, since these two seemed the most promising to give best results, with both samples – the previously additionally purified (8 ng/µL) and the one without PCR clean-up (12 ng/µL). We also performed a reaction using positive control to determine transformation efficiency.

We calculated the volumes we used for the ligation reaction mix. X is the amount of the PCR product that needs to be calculated, depending on the used insert : vector molar ratio. T4 DNA Ligase was added as the last one.

Table XI: Volumes used for ligation reaction mix.

Reaction Component	Standard Reaction	Positive Control	3:1 Insert : Vector molar ratio Sample 1 (c=8 ng/µL)	5:1 Insert : Vector molar ratio Sample 1 (c=8 ng/µL)	3:1 Insert : Vector molar ratio Sample 2 (c=12 ng/µL)	5:1 Insert : Vector molar ratio Sample 2 (c=12 ng/µL)
2X Rapid Ligation Buffer	5 µL	5 µL	5.6 µL	8 µL	5.4 µL	6 µL
pGEM®-T Easy Vector (50ng)	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
PCR product	X µL	/	3.6 µL (28.8 ng)	6 µL (48 ng)	2.4 µL (28.8 ng)	4 µL (48 ng)
Control DNA Insert	/	2 µL	/	/	/	/
T4 DNA Ligase (3 Weiss units/µl)	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
Nuclease-free water	up to 10 µL	1 µL	/	/	1 µL	/
Final volume	**10 µL**	**10 µL**	**11.2 µL**	**16 µL**	**10.8 µL**	**12 µL**

The reaction mix was put to overnight incubation at 4°C. The ligation results in the formation of a cyclic molecule (insert + vector) that can be further replicated in transformed host cells (36).

3.2.5.6. Transformation using DH5α chemically competent E. coli

Transformation was carried out by a "home protocol". First, the agar plates for each ligation reaction were prepared. We dried them for 30 min at 37°C. Secondly, we mixed 100 µL of 0.1 M IPTG with 50 µL of x-GAL (20mg/mL), put on the agar plate next to the flame and left the mixture to incubate at 37°C for approximately 1 hour. In the meantime, we defrosted homemade chemically competent E. coli DH5α, stored at –80°C. We used 3 µL of previously mentioned ligation mix to which we added 50 µL of DH5α. The tubes were gently flicked to mix, and placed on ice for 30 minutes. The cells were then heat-shocked for approximately 45 seconds in a Cube Dry Bath Incubator at exactly 42°C, and put back to ice for 2 minutes. Following this incubation time we added 250 µL of SOC media without antibiotics and put tubes under stirring (250–300 rpm) for 60 minutes at 37°C. We used SOC medium, since it is richer in nutrients (2% tryptone, 0.5% yeast extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2 and 10 mM MgSO4). After the incubation we put the bacteria mixture (ligation mix/SOC medium) next to flame to the previously prepared (IPTG/X-Gal) agar plate. The plates were exposed to overnight incubation at 37°C (optimal 16 hours).

3.2.5.7. Picking the colonies

Next day we identified and picked suitable colonies with sterile pipette tip and put them into polypropylene tubes with previously added 3 mL of LB medium with antibiotics to inoculate. We put colonies to overnight incubation with stirring for 12–16 hours at 230 rpm and 37°C.

3.2.5.8. Mini prep

Next day we continued with the "mini prep" procedure (DNA-spin™ Plasmid DNA Purification Kit, iNtRON Biotechnology) following the commercial protocol available at http://www.bulldog-bio.com/iNtRON/DNA-spin_user_manual.pdf. The volume of pre-heat elution added was 30 µL instead of 50 µL.

3.2.5.9. Digestion with EcoRI restriction enzyme

The insert was then released by digestion with EcoRI from the multiple cloning site (**Figure 9**). We followed indications of Promega commercial house to perform digestion. First, we

quantified the mini-prepared samples and made the calculations needed to prepare the reaction mix for every sample.

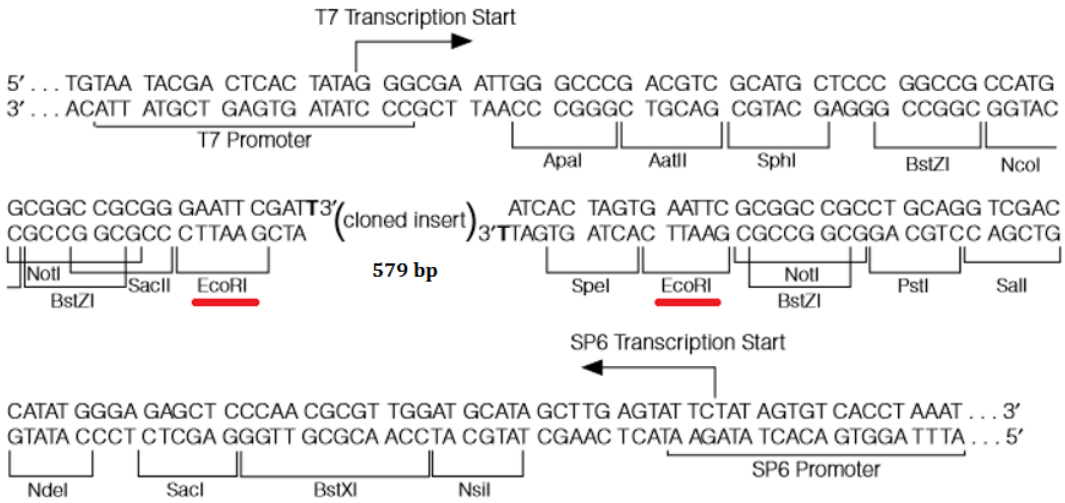

Figure 9: Promoter and multiple cloning sequence of pGEM®-T Vector (41). The top strand corresponds to the RNA synthesized by T7 RNA polymerase and the bottom strand shown corresponds to the RNA synthesized by SP6 RNA polymerase (41).

We quantified the concentration of the plasmid DNA, made the calculations for the sample amount needed (X is volume in µL for 1 µg of DNA to be added) and prepared the reaction mix for the digestion step (**Table XII**).

Table XII: Reaction mix for digestion step.

Digestion Reaction Mix	
dH_2O	17.5µL – X
Plasmid (1µg)	X µL
Buffer EcoRI	2.0 µL
EcoRI	0.5 µL
	Σ = 20 µL

Digestion took place for 1.5 hours at 37 °C, although the incubation time could be reduced. It is then recommended to inactivate the enzyme following manufacturer's instructions. To separate the DNA bands of different sizes we loaded the samples to 1% agarose gel (Sigma), put them to separation for 1 hour at 130 V (constant voltage), exposed them to EtBr staining and visualised them directly under UV illumination (Avegene Life Sciences, LIAS Slite140).

The bands of interest were detected and the samples that showed the band that was corresponding to the size of the sequence of interest (579 bp) were sent to the genomic unit of the research centre (GENYO – The Genomics and Genotyping Unit) in order to be sequenced. We sent 5µL of the sample selected together with T7 forward and additionally M13 reverse primer (**Table XIII**) of concentration 3 ng/µL.

Table XIII: T7 Fw Primer and M13 Rv Primer information.

Oligonucleotide name	Sequence	Tm	Manufacturer
T7 Fw Primer	5'- TAATACGACTCACTATAGGG - 3'	47°C	Sigma-Aldrich
M13 Rv Primer	5'- CAGGAAACAGCTATGAC - 3'	47°C	Sigma-Aldrich

3.2.6. Cell culture, Decitabine (DAC) Treatment

The NB4 cell line was cultured in RPMI 1640 supplemented with 0.5 mM L-glutamine, 10% foetal bovine serum and antibiotics (1% streptomycin/penicillin). It was incubated at 37°C at 5% CO_2 and 90% humidity.

For the experiment we cultured 1.2×10^6 cells into every T-25 flasks to a final volume of 6 mL, using 9 flasks for different conditions (**Figure 10**). We decided to treat NB4 cell line with 2 µM and 4 µM DAC concentration for 72 hours and 96 hours in duplicates and we needed one flask for control (untreated NB4). In total we needed 10.8×10^6 cells.

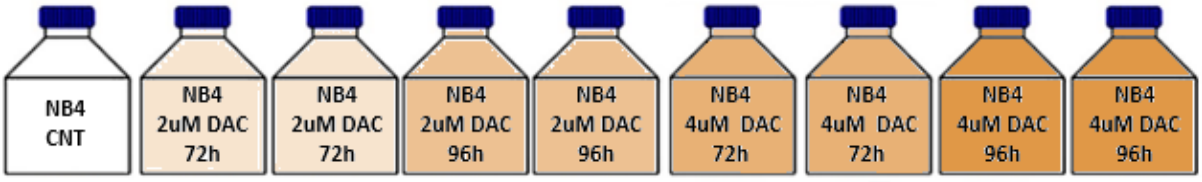

Figure 10: Schematic representation of DAC treatment experiment conditions.

We added 60 µL of 1 mM DAC to 30 mL of RPMI medium to prepare the 2 µM concentration of DAC and 120 µL to 30 mL of RPMI medium to prepare the 4 µM concentration. The control stayed untreated. The cultures were left to grow. We were preparing and changing the media with suitable DAC concentration daily. The treatment affected the viability of cells and caused many cells to die, therefore we merged the duplicates to singles. The pellets were collected at 72-hour and 96-hour time point for further RNA and protein extraction. The control was

collected at the end (96 h). To perform the following extraction of intact total RNA we added 500 µL of TRI® Reagent (Gibco), while collecting the pellets. The samples were then stored at –80°C or used immediately.

3.2.7. Extraction of intact total RNA

If stored at –80°C, we first defrosted the samples, vortexed them and incubated at RT for 5 minutes. Next, we added 100 µL of chloroform, vigorously vortexed for about 15 seconds, and incubated at room temperature for 2–3 minutes. The samples were then centrifuged at 12,000 g for 15 minutes at 4°C, leaving us with the supernatant to which we added 250 µl of 100% isopropanol, vortexed again and left overnight at 4°C. Next day the samples were centrifuged at 12,000 g for 10 minutes at 4°C and the supernatant was discarded. The pellet was washed with 500 µL of 75% ethanol and vortexed, centrifuged again at 7500 g for 5 minutes at 4°C. After discarding the supernatant, the pellet was left turned around to dry for approximately one hour, being subsequently re-suspended in 20 µL of RNase-free water. After heating to about 55–60°C for 10–15 minutes, we measured the concentration of extracted RNA with NanoDrop ™ 2000 Spectrophotometer.

3.2.8. cDNA retrotranscription

For reverse transcription of extracted total RNA and to obtain cDNA we used RevertAid RT Reverse Transcription Kit (ThermoFisher Scientific), following manufacturer's instructions available at https://tools.thermofisher.com/content/sfs/manuals/MAN0012955_RevertAid_RT_UG.pdf. The cDNA was stored at a final volume of 20 mL at –20°C for less than one week.

3.2.9. Real-Time Quantitative PCR

We quantified previously extracted cDNA and made dilutions to prepare samples at 10ng/µL. Quantitative PCR was performed using SYBR Green Supermix Universal iTaq (Bio-Rad Laboratories) with the following reaction mixture in triplicates (**Table XIV**).

Table XIV: qPCR reaction mixture.

BCL7A		*GAPDH*	
cDNA (10 ng/µL)	4 µL	cDNA (10 ng/µL)	4 µL
SYBR Green Supermix	5 µL	SYBR Green Supermix	5 µL
qPCR *BCL7A* Fw Primer	0.5 µL	qPCR *GAPDH* Fw Primer	0.5 µL
qPCR *BCL7A* Rv Primer	0.5 µL	qPCR *GAPDH* Rv Primer	0.5 µL

Table XV: qPCR primer information.

Oligonucleotide Name	Sequence	Tm	Manufacturer
qPCR *BCL7A* Fw Primer	5'- CAAGAGGGTCATGGCGGCGA - 3'	54°C	Sigma-Aldrich
qPCR *BCL7A* Rv Primer	5'- GCTCCGTCACAGGGACCCAT - 3'	54°C	Sigma-Aldrich
qPCR *GAPDH* Fw Primer	5'- GAAGGTGAAGGTCGGAGTC - 3'	50°C	Sigma-Aldrich
qPCR GAPDH Rv Primer	5'- GAAGATGGTGATGGGATTTC - 3'	50°C	Sigma-Aldrich

Cycle parameters were as follows: denaturing for 15 seconds at 95°C, and annealing and extension for 60 seconds at 60°C for 40 cycles.

The data were analysed using 7500 software version 2.0.4 (Applied Biosystems). The *BCL7A* results were normalized to housekeeping gene *GAPDH* (reference control gene, stably expressed also after treatment), and analysed using $\Delta\Delta C_t$ method.

The experiment was repeated with the condition that showed the most desired outcome. Further, we used this condition in the next experiment to additionally verify the protein level.

3.2.10. Protein extraction

Protein extraction was performed by adding 30 mL of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DCO, 0.1% SDS, 50 mM Tris HCl pH 7.5, 0.2 mM PMSF, 0.7 mM OV_4 and a cocktail of inhibitors 1x Pierce ™ from Thermo Scientific) to the cell pellets, re-suspending and leaving it on ice for 10–15 minutes. After centrifugation at 17,000 g (13300 rpm) for 10 minutes at 4°C, the supernatant from the protein extraction was collected for further analysis. The protein concentration of the extracts was determined by the Bradford protein assay (Amresco).

3.2.11. Western Blotting

After the extraction of protein samples, 80μg of total protein was loaded onto a 10% polyacrylamide gel, electrophoresis was carried out under denaturing conditions for 20 minutes at 60 V and for 30–40 minutes at 110 V, followed by a wet transfer to PVDF membrane for 90 minutes at 110 V. The membrane was blocked with 3% skimmed milk powder in PBS and 0.1% Tween 20 (depending on the recommendations of the trading house of the antibodies used), and incubated with the primary antibody solution – anti-BCL7A 1:375 diluted in milk (Sigma - Aldrich) or α-tubulin 1:400 (Sigma - Aldrich) overnight at 4°C. After incubation three 15-minute washes of the membrane with PBS-T were performed. The membrane was subsequently incubated with the appropriate secondary antibody – anti-rabbit 1:2000 or anti-mouse 1:1000 (GE Healthcare) for 90 minutes at RT and washed again with PBS-T three times. The secondary antibody was conjugated to horseradish peroxidase, therefore detection was performed by chemiluminescence technique using a solution of 3 mL luminol (Sigma Aldrich), 30 μL of coumaric (Sigma Aldrich) and 0.9 μL of H_2O_2. ™ Image Quant 4000 (GE Healthcare) luminescence detector was used.

4. RESULTS

4.1. Methylation specific PCR (MSP)

Methylation specific polymerase chain reaction (MSP) was performed in order to obtain qualitative results of the presence of hypermethylation in the promoter, CpG island-region, predicted by bioinformatic tools. The results showed the presence of methylation pattern.

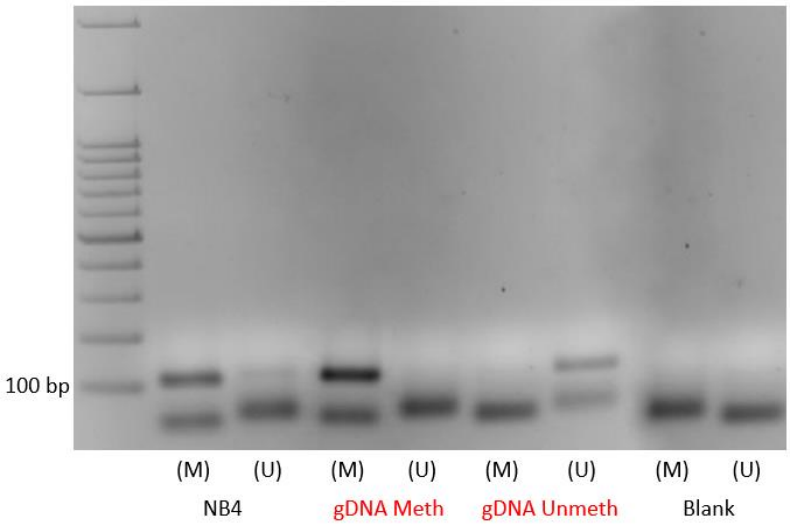

Figure 11: MSP experiment results.

NB4 cell line showed a methylated pattern. The specificity of primers amplification for methylated (M) or unmethylated (U) version was validated by using commercial genomic DNA that is completely methylated (gDNA Meth) or unmethylated (gDNA Unmeth) and a blank version where the DNA template was absent in order to see if any interference is present, which could cause erroneous analytical results.

4.2. Bisulfite sequencing analysis with TA cloning system

4.2.1. The PCR amplification of bisulfite converted DNA

The PCR amplification of bisulfite converted DNA sequence of interest turned out to be quite challenging. We performed a higher number of experiment repetitions with different DNA amount and magnesium concentration, using different DNA polymerases and several different

oligonucleotide pairs, with many modifications in PCR scheme (annealing temperature, number of cycles, annealing times, extension times …) before being successful (**Figure 12**).

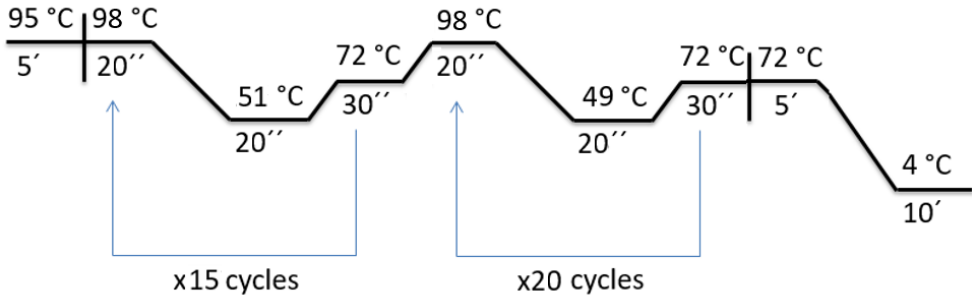

Figure 12: Schematic representation of effectual PCR reaction parameters for amplification of bisulfite converted DNA of interest.

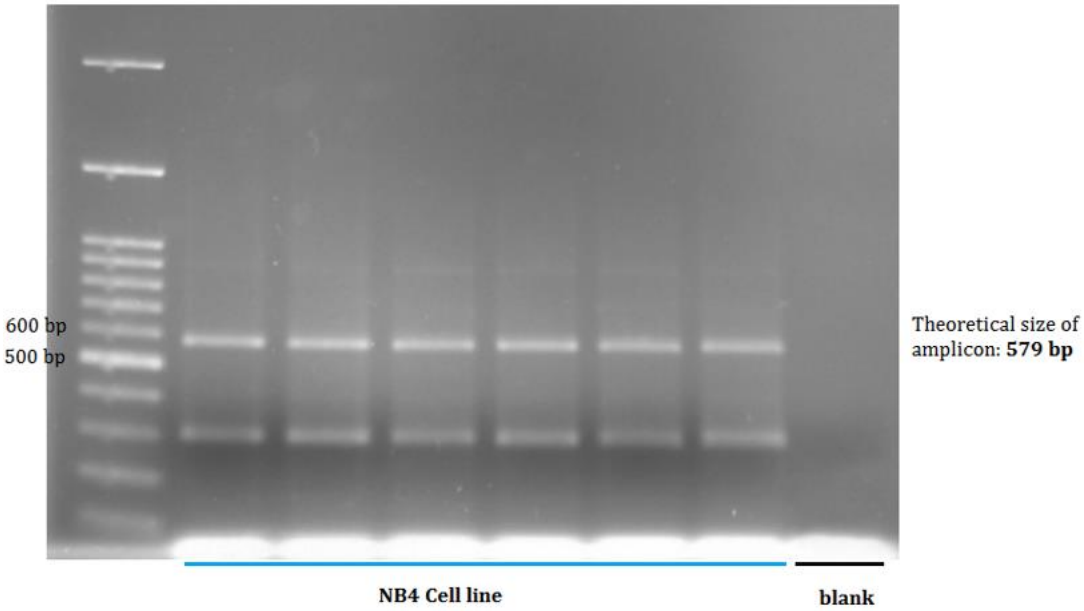

Figure 13: PCR results of amplification of bisulfite converted DNA sequence of interest.

The theoretical size of our PCR product is 579 bp. The size of the obtained PCR bands corresponds to the theoretical size of the amplicon.

4.2.2. Transformation using DH5α

After the transformation step we identified and picked several colonies (**Table XVI**).

Table XVI: Number of colonies grown and picked.

	3:1 Insert : Vector molar ratio Sample 1 (c=8 ng/µL)	**5:1 Insert : Vector molar ratio Sample 1 (c=8 ng/µL)**	**3:1 Insert : Vector molar ratio Sample 2 (c=12 ng/µL)**	**5:1 Insert : Vector molar ratio Sample 2 (c=12 ng/µL)**	**Positive Control**
Number of white colonies identified	12	32	10	16	Full plate
Number of white colonies picked	6	10	6	6	/

We identified and picked 6 white colonies of 3:1 ratio sample 1 (8 ng/µL), 10 colonies of 5:1 ratio sample 1 (8 ng/µL) and 6 white colonies, each of 3:1 and 5:1 ratio sample 2 (12 ng/µL).

4.2.3. Digestion

We measured the plasmid DNA concentration for our 16 samples. Further, we calculated the required volume to add 1000 ng of DNA and dH_2O up to 17.5 µL (**Table XVII**) for the digestion reaction mix. Sample 4 was excluded from the experiment due to a lower plasmid concentration.

Table XVII: Plasmid DNA concentration measurement.

	Sample number	Plasmid conc. [ng/µL]	Plasmid volume [µL] for 1 µg (X)	V water [µL] (17.5 µL- X)
3:1 Insert : Vector molar ratio Sample 1 (c=8 ng/µL)	1	126	7.9	9.6
	2	74	13.5	4.0
	3	71	14.1	3.4
	4	41	17.5	0.0
	5	**172**	**5.8**	**11.7**
	6	121	8.3	9.2
5:1 Insert : Vector molar ratio Sample 1 (c=8 ng/µL)	7	206	4.9	12.6
	8	184	5.4	12.1
	9	69	14.5	3.0
	10	97	10.3	7.2

	11	152	6.6	10.9
	12	179	5.6	11.9
	13	98	10.2	7.3
	14	185	5.4	12.1
	15	448	2.2	15.3
	16	151	6.6	10.9
3:1 Insert : Vector molar ratio Sample 2 (c=12 ng/μL)	17	138	7.2	10.3
	18	132	7.6	9.9
	19	121	8.3	9.2
	20	186	5.4	12.1
	21	128	7.8	9.7
	22	81	12.3	5.2
5:1 Insert : Vector molar ratio Sample 2 (c=12 ng/μL)	23	127	7.9	9.6
	24	113	8.8	8.7
	25	105	9.5	8.0
	26	142	7.0	10.5
	27	102	9.8	7.7
	28	91	11.0	6.5

After EcoRI digestion we detected released inserts that corresponded to the size of our sequence of interest (**Figure 14** and **Figure 15**).

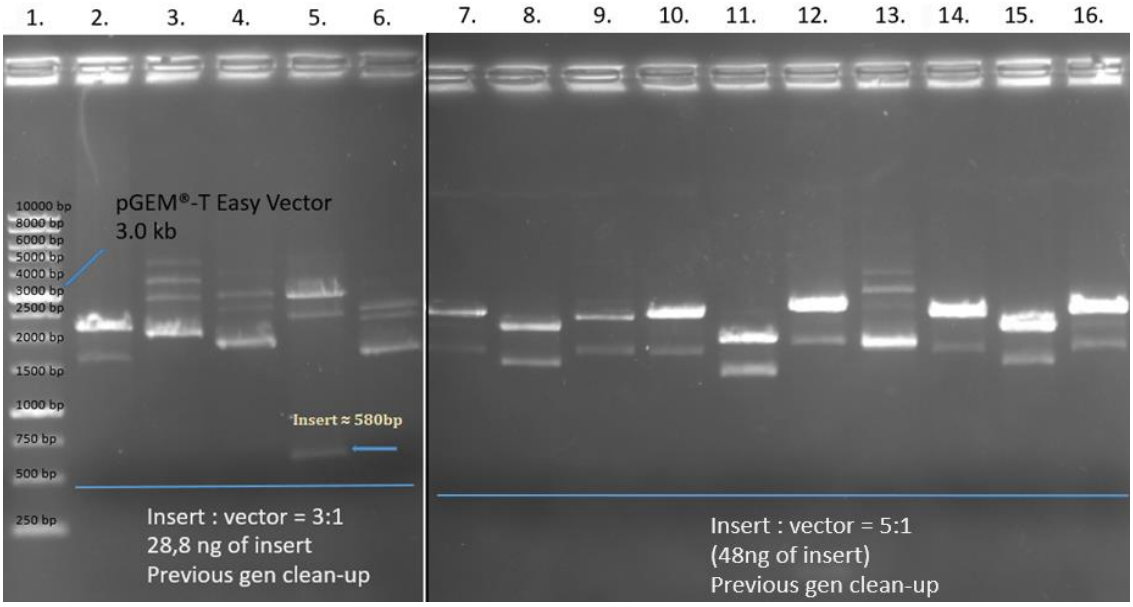

Figure 14: Agarose gel electrophoresis result after digestion with EcoRI (Sample 1 with previous purification step).

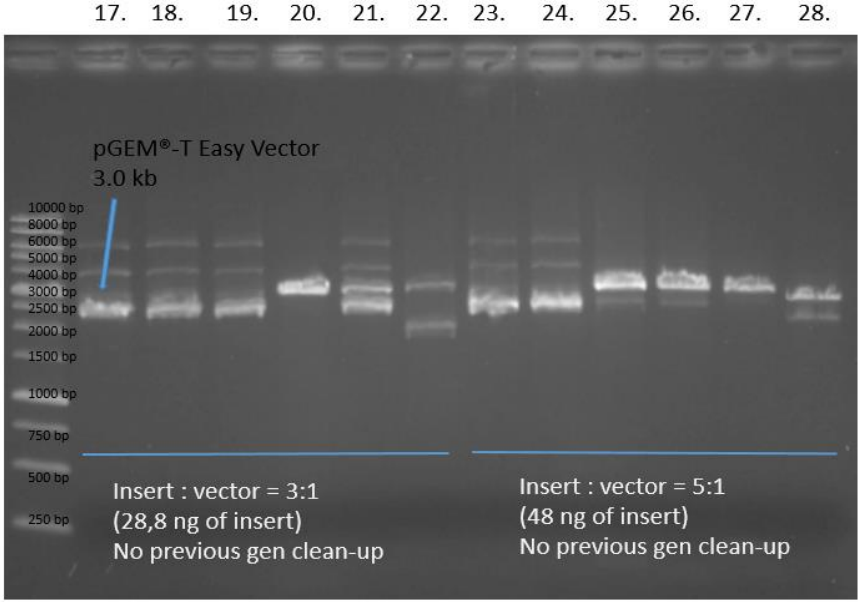

Figure 15: Agarose gel electrophoresis result after digestion with EcoRI (Sample 2 without previous purification step).

As it can be seen, only the band of sample number 5 corresponded to the size of our sequence of interest and was sent to the genomic unit to Sanger sequencing for further assessment (**Figure 16** and **Figure 18**).

4.2.4. Sanger sequencing

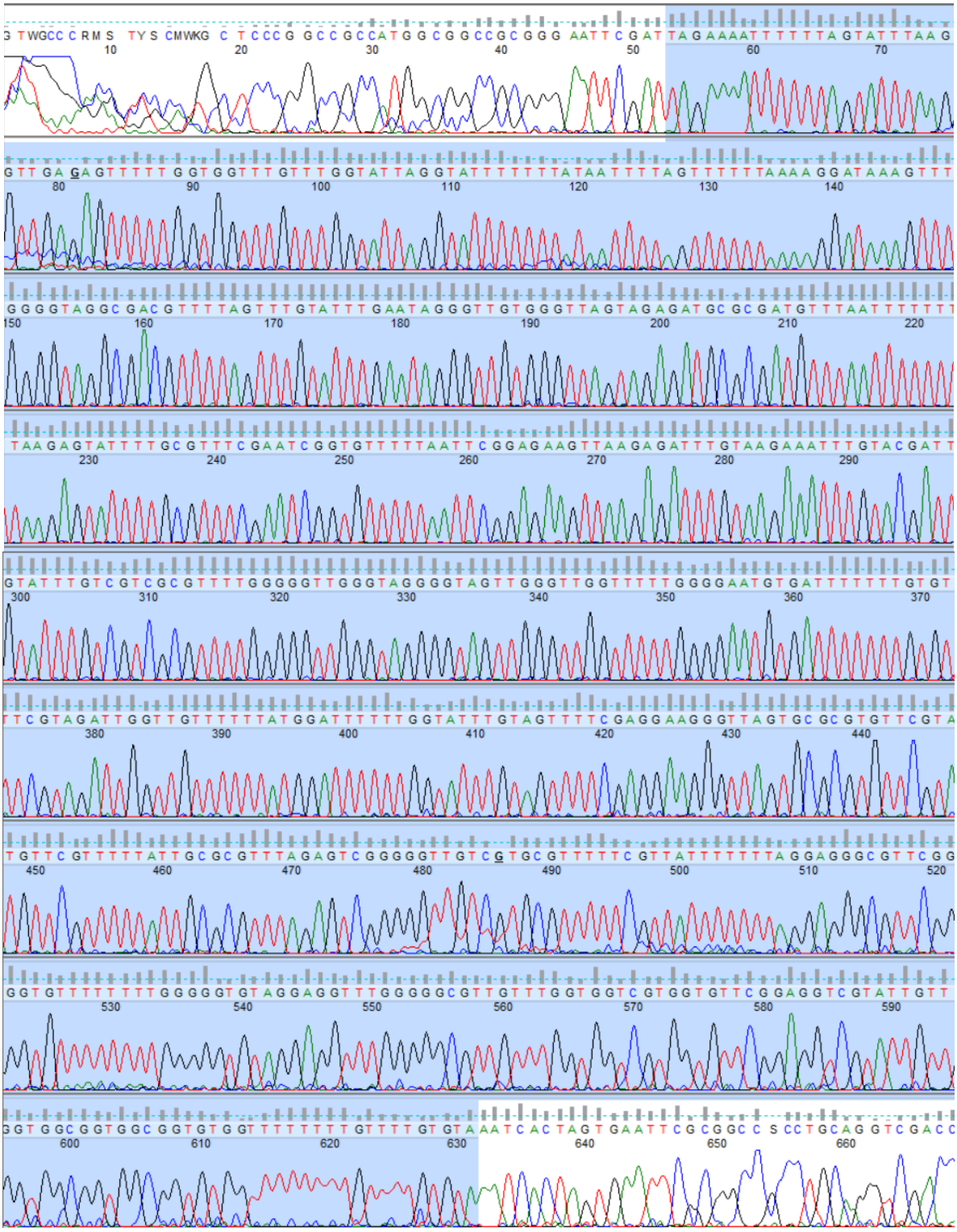

Figure 16: Result of sequencing reaction for sample 5 with T7 Fw primer produced a useable sequence. The sequence of inserted DNA fragment (579 bp) is highlighted in blue.

In the further assessment of the sample we counted the CpG in our DNA fragment. 32 CpG island showed to be methylated in analysed sequence out of 61 in theoretical sequence, representing 52.46% of the methylation rate (**Figure 17**).

Sample: 5. (T7 Fw Primer)

TAGAAAATTTTTTAGTATTTAAGGTTGAGAGTTTTTGGTGGTTTGTTTGGTATTAGGTATTTTTTT
ATAATTTTAGTTTTTTAAAAGGATAAAGTTTGGGGTAGGCGACGTTTTAGTTTGTATTTGAATAG
GGTTGTGGGTTAGTAGAGATGCGCGATGTTTAATTTTTTTTAAGAGTATTTTGCGTTTCGAATCG
GTGTTTTTAATTCGGAGAAGTTAAGAGATTTGTAAGAAATTTGTACGATTGTATTTGTCGTCGCG
TTTTGGGGGTTGGGTAGGGGTAGTTGGGTTGGTTTTTGGGGAATGTGATTTTTTTGTGTTTCGTAG
ATTGGTTGTTTTTTATGGATTTTTTGGTATTTGTAGTTTTCGAGGAAGGGTTAGTGCGCGTGTTCG
TATGTTCGTTTTTATTGCGCGTTTAGAGTCGGGGGTTGTCGTGCGTTTTTCGTTATTTTTTTAGGA
GGGCGTTCGGGGTGTTTTTTTTGGGGGGTGTAGGAGGTTTGGGGGCGTTGTTTGGTGGTCGTGGTG
TTCGGAGGTCGTATTGTTGGTGGCGGTGGCGGTGTGGTTTTTTTTGTTTTGTGTA

32x CpG / 61 = 52,46%

Figure 17: Schematic representation of CpG islands in sample 5 sequence (T7 Fw Primer).

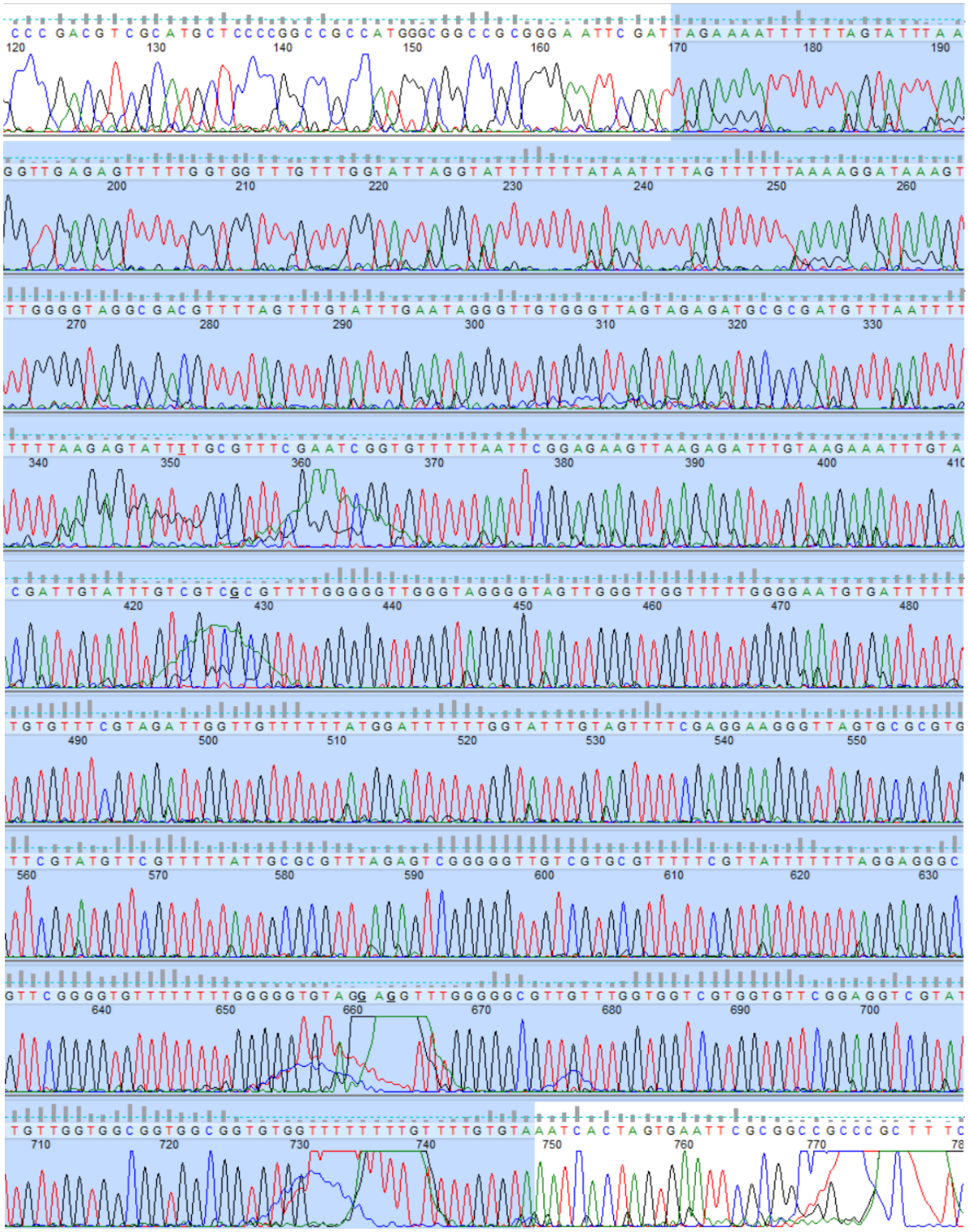

Figure 18: Result of sequencing reaction for sample 5 with M13 Rv. The sequence obtained with reverse primer has a lower quality with more background interference as the sequence with forward primer. The sequence of inserted DNA fragment (579 bp) is highlighted in blue.

The results obtained with reverse primer fully corresponded to previously presented results obtained with forward primer. Out of 61 CpG islands in theoretical sequence 32 showed to be methylated in analysed sequence, representing 52.46% of the methylation rate (**Figure 19**).

Sample: 5. M13 Rv Primer (ALREADY REVERSE COMPLEMENT)

TAGAAAATTTTTTAGTATTTAAGGTTGAGAGTTTTTGGTGGTTTGTTTGGTATTAGGTATTTTTTT
ATAATTTTAGTTTTTTAAAAGGATAAAGTTTGGGGTAGGCGACGTTTTAGTTTGTATTTGAATAG
GGTTGTGGGTTAGTAGAGATGCGCGATGTTTAATTTTTTTTAAGAGTATTTTGCGTTTCGAATCG
GTGTTTTTAATTCGGAGAAGTTAAGAGATTTGTAAGAAATTTGTACGATTGTATTTGTCGTCGCG
TTTTGGGGGTTGGGTAGGGGTAGTTGGGTTGGTTTTTGGGGAATGTGATTTTTTTGTGTTTCGTAG
ATTGGTTGTTTTTTATGGATTTTTTGGTATTTGTAGTTTTCGAGGAAGGGTTAGTGCGCGTGTTCG
TATGTTCGTTTTTATTGCGCGTTTAGAGTCGGGGGTTGTCGTGCGTTTTTCGTTATTTTTTTAGGA
GGGCGTTCGGGGTGTTTTTTTTGGGGGGTGTAGGAGGTTTGGGGGCGTTGTTTGGTGGTCGTGGTG
TTCGGAGGTCGTATTGTTGGTGGCGGTGGCGGTGTGGTTTTTTTTGTTTTGTGTA

32x CpG / 61 = 52,46%

Figure 19: Schematic representation of the CpG islands in sample 5 sequence (M13 Rv Primer). The inserted sequence amplified by forward and reversed primer corresponds to the theoretical sequence.

4.3.Real-Time Quantitative PCR analysis

With real-time quantitative PCR experiment we wanted to analyse the relative changes in *BCL7A* gene expression (on mRNA level) based on the PCR signal of treated samples compared to untreated control using ΔΔCt method.

We used Daudi cell line, which expresses *BCL7A* gene, to create a standard curve. The data were analysed with programme 7500 software version 2.0.4 (Applied Biosystems), and the graphics of standard curve showed that the efficiency of PCR reaction for *GAPDH* and *BCL7A* was between 90–110% (**Figure 20**). Due to that, the standard curve calculation is not needed and the analysis using $\Delta\Delta C_t$ method (**Equation *2*** and **Table XVIII**) is sufficient (42).

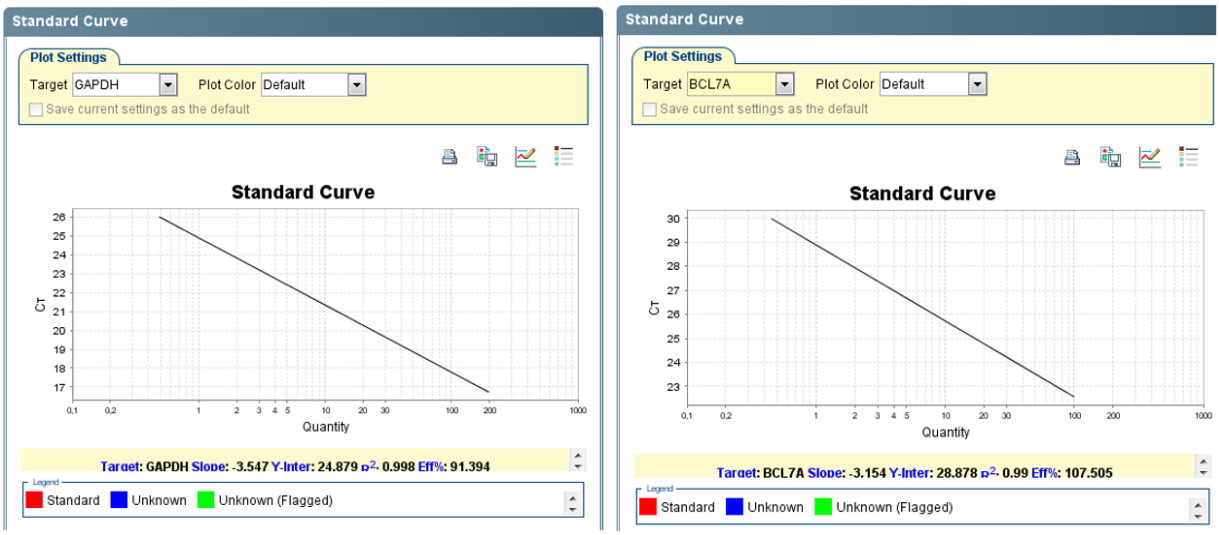

Figure 20: Quantitative PCR standard curve graphics given by 7500 software version 2.0.4 (Applied Biosystems) for GAPDH and BCL7A measurements. The efficiency for GAPDH is shown to be 91.394% and for BCL7A 107.505%.

Equation *2*: Relative expression (fold change) formula.

$$Fold\ change = 2^{-\Delta\Delta Ct}$$

Value Ct or threshold cycle is the number of cycles needed for sample fluorescence signal to exceed the background noise.

Table XIX: ΔΔCt method results from DAC experiment.

Sample name	GAPDH C_t average	BCL7A C_t average	ΔC_t	$\Delta\Delta C_t$	$2^{-(\Delta\Delta Ct)}$	Fold change SD	n (sample size)
CNT	17.277	26.050	8.773	0.000	1.000	0.296	3
2 µM 72 h	17.039	25.480	8.441	–0.332	1.259	0.505	3
2 µM 96 h	24.579	29.550	4.971	–3.802	13.951	6.322	2
4 µM 72 h	18.465	26.278	7.813	–0.960	1.945	0.439	2
4 µM 96 h	19.627	27.361	7.734	–1.040	2.056	1.208	3

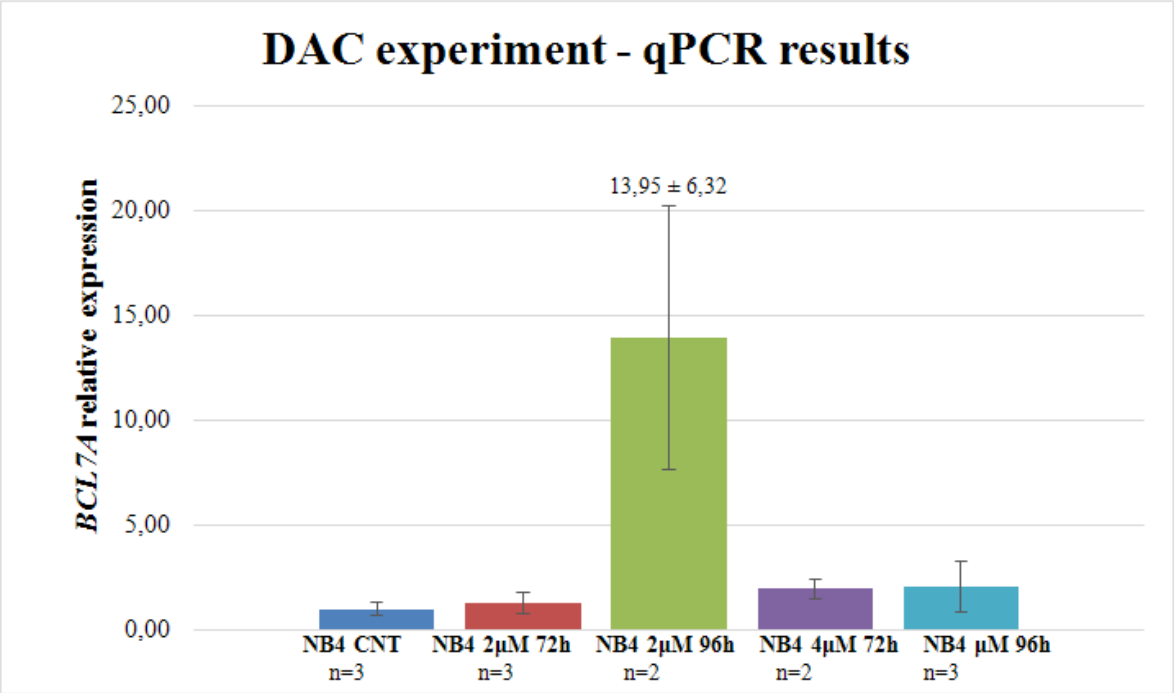

Figure 21: Quantitative PCR results of DAC treatment experiment, where error bars present standard deviation.

The results showed an increase of *BCL7A* expression at mRNA level after being exposed to decitabine treatment. The data have been normalized to a control CNT.

The most evident increase of *BCL7A* mRNA level was shown after 96-hour treatment at 2 µM of decitabine (**Figure 21**), therefore we repeated the DAC experiment with this condition and control (**Table XIX** and **Figure 22**) to verify the expression restoration at mRNA level and also to collect cells in order to additionally perform a western blot analysis.

Table XX: ΔΔCt method results from DAC experiment repetition (96 hours, 2 µM).

Sample name	GAPDH Ct average	BCL7A Ct average	ΔCt	ΔΔ Ct	$2^{-(\Delta\Delta Ct)}$	Fold change SD	n (sample size)
CNT	20.701	28.993	8.293	0.000	1.000	0.109	3
2 µM 96 h repetition	22.828	28.208	5.380	–2.913	7.532	1.372	3

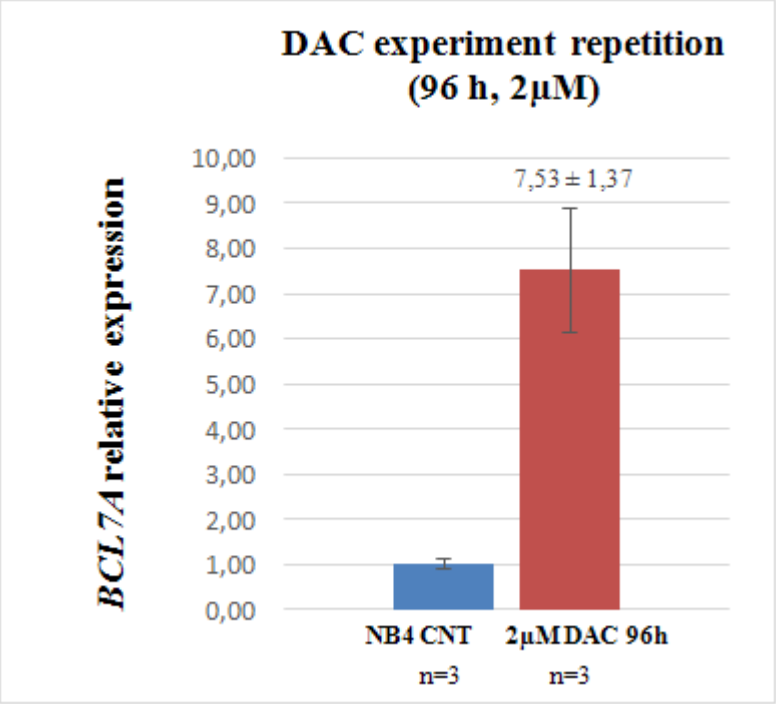

Figure 22: Quantitative PCR results of DAC experiment repetition (96 hours, 2 µM DAC).

The results of repeated experiment with 96-hour 2 µM-decitabine treatment showed the increase of *BCL7A* expression at mRNA level (relative expression 7.53 ± 1.37). The data have been normalized to a control CNT.

4.4. Western blotting

We performed WB using NB4 sample from the repeated experiment with 96-hour 2 µM-DAC treatment to verify whether *BCL7A* expression is increased at protein level.

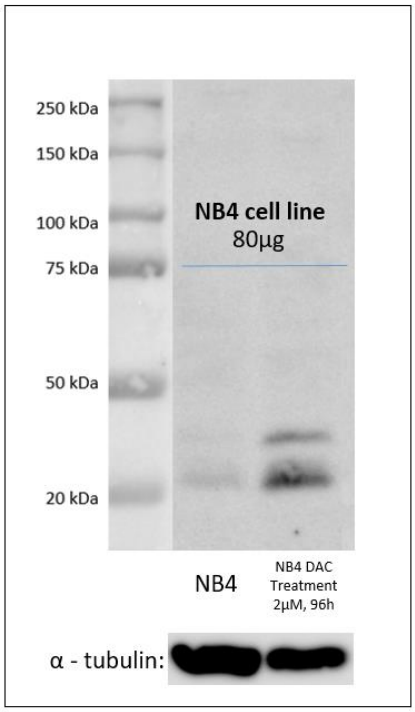

Figure 23: WB results for NB4 cell line treated with DAC 2 µM, 96 h. For loading control we used α-tubulin.

As it can be seen, the protein expression in the treated sample is visibly increased compared to the untreated control. With this experiment we ascertained that the restoration of protein expression of longer and shorter isoform occurs after DAC treatment under certain conditions – concentration and exposure time.

5. DISCUSSION

The main and final objective of the project in which I participated is to establish a cell line model deficient in *BCL7A* expression to study its biological role. In the case of myeloid leukaemias there is not much information available on *BCL7A*, therefore, it would be a challenge to elucidate the role of *BCL7A* in this kind of haematological malignancies. In first screenings, NB4 appeared to be a great candidate for cell line model to study the role of *BCL7A* in myeloid leukaemia.

We compared the results obtained in MSP for NB4 cell line with other myeloid cell lines in order to determine whether the downregulation that occurs in NB4 cell line is aberrant or rather a common phenomenon present in myeloid cell lines. Considering our results, only two cell lines out of five show *BCL7A* promoter hypermethylation. Other investigated myeloid cell lines showed unmethylated pattern and higher BCL7A expression as it can be seen by western blot. Moreover, NB4 and HL60 are both acute promyelocytic leukaemias, but they showed completely contrary results (**Figure 24**). Due to this, we assumed that the methylation in NB4 could be departing from the normal state and we decided to carry out further investigation.

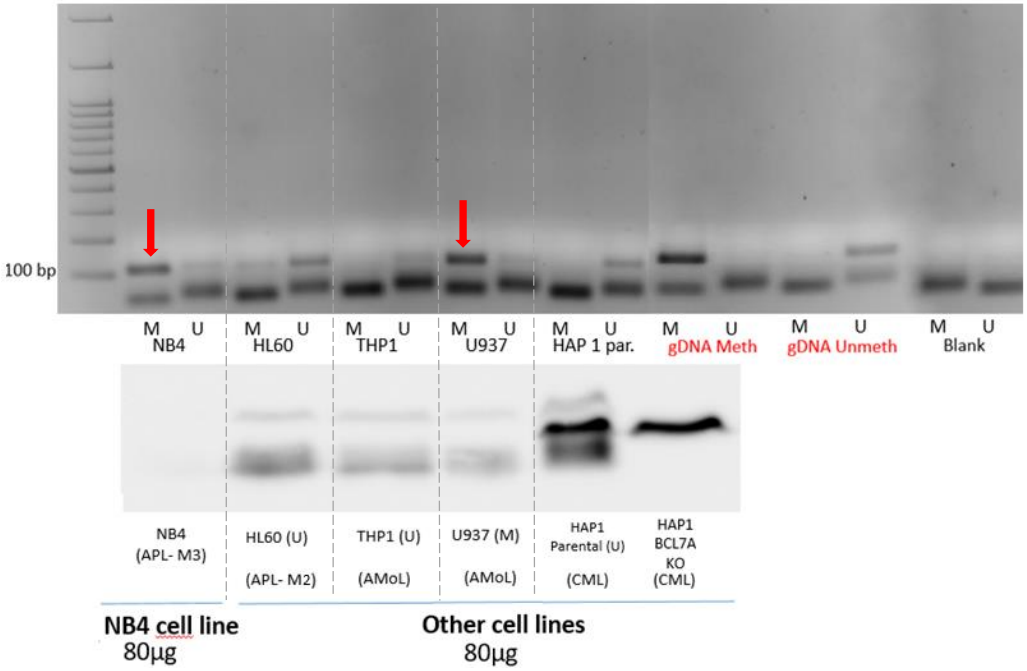

Figure 24: NB4 results in comparison with data from previously performed experiments with other myeloid cell lines.

Acute promyelocytic leukaemia M3 is genetically characterised by a translocation t(15;17)(q24;q21) that renders a mutant *PML-RARα* with a transcriptional repressive function. We propose that the translocation causes transcriptional repression/downregulation of *BCL7A* in NB4 cell line by inducing abnormal histone de-acetylation and DNA methylation.

In the presence of RA, *PML-RARα* has proven to be a less potent transcriptional repressor. As a future experiment we would suggest to perform RA treatment in NB4 to observe the direct restoration of *BCL7A* expression, to elucidate if *BCL7A* is among the target promoters of *PML-RARα* and possibly to discuss if *BCL7A* is linked with differentiation/maturation process in myeloid cells.

To evaluate qualitative *BCL7A* methylation state with bisulfite sequencing analysis in NB4 cell line, we obtained only one positive colony and corresponding sequence after several repetitions of the experiment. The procedure of bisulfite sequence analysis is very long and labour-intense. Due to the lack of time at the end of practical training, we were not able to perform more repetitions of the experiment to obtain a higher number of positive clones. For the final qualitative evaluation of *BCL7A* methylation state with bisulfite sequencing analysis in NB4 cell line, a higher number of sequences must be analysed for sufficient sample size and valuable statistical strength.

During our experiment we observed a very low number of white colonies, indicating an insufficient number of clones containing the PCR product of interest. This low number of white colonies could be due to the unsuccessful ligation step or lower quality and quantity of the inserted DNA.

When performing the transformation step with the control insert, we obtained a very high number of white colonies (full plate), demonstrating that apparently the ligation step was successful. The only difference between the transformation step of our samples and the positive control was the inserted DNA. The purity and concentration of inserted DNA are important factors for successful ligation reaction.

Considering our results, the only positive colony we obtained was when we used a PCR insert that was previously cleaned up after the A-tailing procedure, although being of a lower

concentration. Therefore, the purification step of the PCR fragment is much recommended to remove contaminants in the PCR product and to improve ligation efficiency. We could improve the purification by following kit manufacturer's recommendations (Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281)) since it is possible that the one we used in our experiment (GenElute ™ PCR Clean-Up Kit, Sigma-Aldrich) contained compounds/substances that contaminated our PCR product and promoted inhibition in the ligation step.

Secondly, to obtain a more concentrated and pure DNA insert it would be recommended to avoid the A-tailing step. In our lab we performed the amplification of bisulfite converted DNA using several different polymerases, but the amplification was successful only with KAPA HiFi HotStart Uracil+ DNA Polymerase which has a proofreading activity and does not generate fragments with 3'A-tail. This requires the A-tailing procedure to follow in the next step. It would be recommended to skip this A-tailing part, since we think it may reduce the concentration of DNA insert and affect the ligation process in TA cloning procedure. In order to skip A-tailing, we would need to perform the amplification of bisulfite converted DNA with an effectual polymerase without proofreading activity, leaving the fragments with 3'A-overhangs.

With DAC treatment we confirmed that *BCL7A* promoter is hypermethylated and the use of this therapeutic agent allowed us to promote *BCL7A* expression restoration in NB4 cancer cell line at both mRNA and protein level. This experiment additionally confirmed that *BCL7A* expression is strongly downregulated due to aberrant methylation process, and can be restored using demethylating agent DAC at a lower concentration and longer exposure time. We observed a high number of death cells during the treatment in cell culture due to a dual effect of decitabine on cancer cells. At a lower concentration, DAC reactivates silenced genes and triggers differentiation, however at a higher concentration; DAC induces apoptosis of NB4 cells and has a cytotoxic effect (43).

6. CONCLUSION

- We confirmed DNA methylation in *BCL7A* promoter region in NB4 cell line by MSP. Based on our results, we claim that *BCL7A* expression is strongly downregulated in NB4 cell line due to promoter hypermethylation.
- To evaluate qualitative methylation state in *BCL7A* with bisulfite sequencing analysis in NB4 cell line, a higher number of sequences must be analysed for sufficient sample size and valuable statistical strength.
- *BCL7A* expression restoration at mRNA and protein level in NB4 cancer cell line was achieved by decitabine treatment.
- All in all, our data demonstrate that *BCL7A* expression has been aberrantly down-regulated in NB4 cell line by promoter hypermethylation. Hence, NB4 would be a suitable cell line model deficient in *BCL7A* expression to study its biological role after *BCL7A* expression restoration.

7. LITERATURE

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