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Original research article

The effect of p73 isoforms on DNA methylation in human osteosarcoma cells

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Abbreviations:
CGI – CpG island
DBD – DNA-binding domain
FDR – false discovery rate
OD/TD – oligomerization/tetramerization domain
TAD – transactivation domain

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Abstract

The role of p73 protein, a member of the p53 family, in tumorigenesis is not fully understood. The TP73 *gene has two promoters and consequently can give rise to two main groups of isoforms: transactivating (TAp73) isoforms generated from P1 promoter, and ΔNp73 isoforms transcribed from P2 promoter which lack N-terminal region. The most common change in tumors is increased expression of ΔNp73, which acts as transdominant inhibitor of TAp73 and p53. DNA methylation is a form of epigenetic regulation, which refers to adding methyl groups to cytosine residues, thus regulating gene activity. Hypermethylation of CpG islands in promoter regions inhibits transcription of genes silencing their expression. Using inducible Tet-ON system, we examined the impact of increased expression of TAp73α and ΔNp73α isoforms on global DNA methylation in SaOS-2 human osteosarcoma cell line using Infinium HumanMethylation450 BeadChip. However, statistically significant change was not found upon induced expression of p73 isoforms. We concluded there was no significant impact of increased expression of TAp73α or ΔNp73α on SaOS-2 global methylation.*

INTRODUCTION

DNA methylation represents a pivotal epigenetic modification in eukaryotes important in health and disease. It comprises a covalent addition of a methyl group to the cytosine residues of CpG dinucleotides or CpHpG (H = A, T, C) creating s-methylcytosine at CpG sites *(1)*. DNA methylation is reversible and not inheritable, but important in the control of gene expression and alternative splicing regulation, regulation of embryonic development and a number of other biological processes *(1–3)*. CpG dinucleotides are randomly distributed throughout the genome and most of them are methylated, while some are clustered in the regions called CpG islands (CGIs) with over 200 bp and more than 50 % CG dinucleotides, generally non-methylated *(1)*. CGIs are commonly located in gene promoter regions, and their hypermethylation is frequently related to transcriptional repression. Therefore, DNA methylation of promoters and distal regulatory regions controls gene expression *(4)*.

The function of DNA methylation was originally focused on promoter-associated CGIs of genes which were silenced due to inhibited transcription. This epigenetic modification is a common mechanism of gene expression regulation associated with the tumorigenesis. The methylation of CGIs within certain gene promoters contributes to the silencing of key tumor suppressor genes such as *CDKN2A, RB1*, *CDH1* and *BRCA1*, while hypomethylation of others reactivates the expression of

genes with an oncogenic potential such as *HRAS* and *MYC (5)*. Therefore, DNA methylation is the primary driver of the stable modification of gene expression, a hallmark of cancer cells.

The p53 family of proteins encompasses p53, p63, and p73, which share high structural and functional resemblance. The discovery of p53 homologues, p63 and p73, undoubtedly has changed the conventions about this key tumor suppressor. All members of the p53 family have high structural similarity consisting of three main functional domains: the transactivation domain (TAD), DNA-binding domain (DBD), and oligomerization/tetramerization domain (OD/TD) (Figure 1A). However, despite many analogous roles, each member has its unique identity and functions. The family complexity has been enriched by the use of alternative promoters, alternative splicing, and diverse translation initiation sites *(6, 7)*. Consequently, numerous protein isoforms with diverse N- and C- termini are encoded.

The human *TP73* gene consists of 15 exons (designated as exons 1–14, plus one alternative exon 3') and can be transcribed into different mRNAs, subsequently producing different protein isoforms (Figure 1B). The transcription from promoter 1 (P1) located upstream of exon 1 gives rise to a set of transcriptionally active isoforms containing entire TAD, named TAp73. Transcription from the internal promoter P2 situated in the intron 3 produces N-terminally truncated isoforms that lack entire TAD, referred to as *Δ*Np73 isoforms *(8–10)*. The transcripts generated from P1 promoter can be alternatively spliced at the 5'-end producing other isoforms that do not have an entire TAD - *Δ*Ex2p73 (lacking exon 2), *Δ*Ex2/3p73 (lacking exons 2 and 3), and *Δ*N'p73 (containing alternative exon 3') *(10, 11)*. Interestingly, due to posttranslational modifications, the transcripts ΔNp73 and ΔN'p73 translate to the identical protein product *(9)*. Alternative splicing is even more frequent at the 3'-end, producing seven different transcripts. Therefore, combining transcription from two alternative promoters and splicing at 5'/3'-ends can hypothetically result in 35 different mRNAs, which can be translated into 28 diverse protein isoforms *(12)*. However, not all of them have been detected so far.

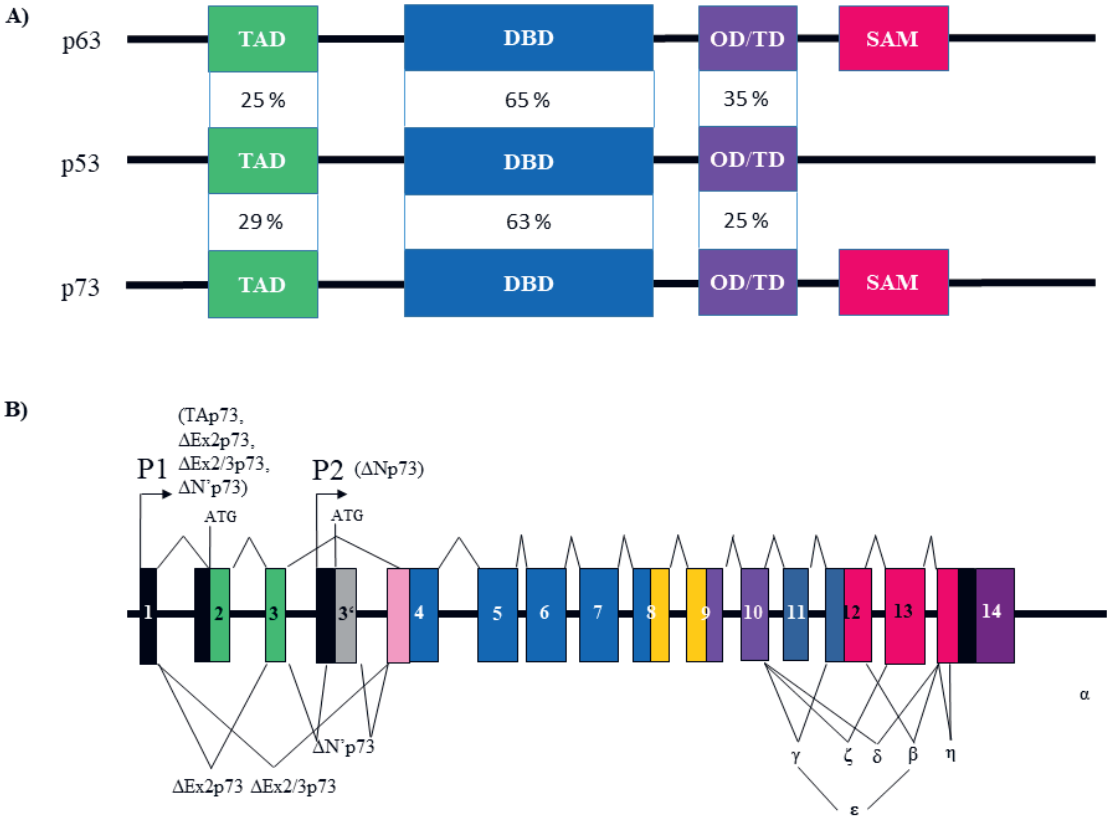

Figure 1. ***Homology between the p53 family members and the structure of the* TP73 *gene.*** *(**A**) Comparison of p53, p63 and p73 protein structure. All three proteins comprise three main structural domains: transactivation domain (TAD), DNA-binding domain (DBD) and oligomerization/tetramerization domain (OD/TD) and show high degree of homology. Percentage of homology of residues between particular domains is shown. In addition, p63 and p73 proteins have sterile α-motif (SAM) domain on the C-terminus. (**B**) The scheme of the human* TP73 *gene structure. The* TP73 *gene contains two promoters (P1 and P2) producing TAp73 and ΔNp73 isoforms. In addition, alternative splicing on 5'- and 3'-end produces ΔEx2/ΔEx2/3/ΔN'p73 and α/β/γ/δ/ε/ζ/η isoforms, respectively.*

While p53 is mutated in nearly all types of tumors, there is low frequency of the *TP73* gene mutations in cancer. Principally, p73 expression is often elevated in tumor tissues compared to the tissues of origin. Although both TAp73 and ΔNp73 isoforms are overexpressed in many tumors, dominant-negative ΔNp73 isoforms, rather than TAp73, are relevant components of tumor-associated p73 overexpression, functionally overriding an accompanying increase of TAp73 expression *(6, 13–15)*. ΔNp73 isoforms fail to induce cell cycle arrest and apoptosis and, moreover, abrogate the suppressive activity of p53 and TAp73 *(8)*.

In many cancers' types, significantly higher expression levels of ΔNp73 isoforms are found in p53 wild-type than in p53 mutant tumors supporting the concept that upregulation of ΔNp73 alleviates the selection pressure to mutate p53 *(8, 14)*. Also, this could explain the lack of p73 mutations in cancers. Therefore, ΔNp73 isoforms might act as pathophysiologically relevant oncogenes *(10)*. Correspondingly, ΔNp73 is an independent clinical prognostic marker for poor prognosis of cancer patients, as confirmed in several tumor types including colorectal cancer, neuroblastoma and ovarian carcinoma *(8, 12, 14, 16–18)*.

The methylation of 5' cytosine in CGIs of the P1 and exon 1 region of *TP73* might play a critical role in inactivating expression of TAp73 isoforms and has been reported in several cancers, including leukemias/lymphomas *(19)*. Interestingly, in hepatocellular carcinoma and gastrointestinal cancer positive correlation was found between p73 expression and DNA methylation in promoter regions of *TP73*, i.e. hypermethylation within the *TP73* promoter activates *TP73* gene expression. This is explained by close association among CTCF (a chromosomal networking protein), *TP53* and DNA methylation at the *TP73* promoter site which regulates *TP73* expression *(20)*.

Gomez et al. observed elevated expression of ΔNp73 in breast tumors in which *TP73* was found methylated close to P1 promoter *(21)*. In accordance with this study, Lai et al. reported that when promoter is hypermethylated, ΔNp73 is expressed at high levels while TAp73 expression remains low *(22)*. Considering that P1 contains more CGIs than P2, methylation affects more the transcription and expression of TAp73. Also, Daskalos and coworkers have found that P1 hypermethylation-associated reduction of TAp73 levels is relatively infrequent, while the P2 hypomethylation-associated higher expression of ΔNp73 mRNA is a frequent event in squamous cell carcinomas. In normal and leukemic lymphocytes ΔN 5' region (which includes P2 promoter) is completely methylated and as a consequence, the lack of ΔNp73 expression was observed in these cells. In contrast, this region was only partially methylated in the neuroblastoma cells expressing ΔNp73 *(23)*. Further reports detected the hypermethylation of the *TP73* in gastric carcinoma and cervical cancer *(24, 25)*. Interestingly, increased methylation of P2 promoter was found in cells resistant to cisplatin *(26)*. Therefore, the overexpression of p73 isoforms observed in several cancer types might influence the global DNA methylation status.

Here we examined the impact of TAp73α and ΔNp73α overexpression on global DNA methylation. The aim of our research was to understand how the two most abundant p73 isoforms - TAp73 and ΔNp73 - affect the global DNA methylation pattern. Although we expected that our research will lead to new insights into the impact of increased expression of TAp73 or ΔNp73 on the overall methylation of the genome, and contribute to the elucidation of the functions and mutual relationships of the p73 protein isoforms, there was no statistically significant impact of increased expression of TAp73α or ΔNp73α on the methylation of SaOS-2 promoter regions.

MATERIALS AND METHODS

SaOS-2 Tet-ON cells

SaOS-2 Tet-ON cell line, a human p53-null osteosarcoma cell line that expresses the rtTA transactivator in the presence of an inducer (ATCC® Number: HTB-85TM, designation: SaOS-2), was grown in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (Thermo Fisher Scientific, USA), 100 units/mL penicillin (Sigma Aldrich, USA), 100 µg/mL streptomycin (Sigma Aldrich, USA), 2 mM L-glutamine (Sigma Aldrich, USA), and 10 mM HEPES (Sigma Aldrich, USA) in a humidified atmosphere of 5 % CO_2 at 37 °C.

We used SaOS-2 TAp73 Tet-ON and SaOS-2 ΔNp73 Tet-ON cell lines expressing TAp73α or ΔNp73α, respectively, controlled by the tetracycline promoter. To induce the expression of relevant gene, we exposed the cells to tetracycline-derivative doxycycline in the final concentration of 2 µg/mL for 24 h.

Protein isolation and western blot

Proteins were extracted in PBS supplemented with protease inhibitors (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail; Roche Diagnostics, Switzerland). Pellets were sonicated (1 mm probe, 2 × 15 sec) and protein concentration was determined by the Pierce BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA). After isolation, 30 µg of total proteins were separated on 8 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Merck Millipore, USA). The membrane was incubated with primary mouse anti-p73 ER-15 antibody which detects the α forms of p73 (Oncogene Research Products, USA; 1:100) or anti-vimentin antibody used as loading control (Lab Vision, USA; 1:1000), and secondary HRP-conjugated anti-mouse (Cell Signaling,

USA; 1:5000). Proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, USA) on Alliance 4.7 imaging system (UVItec, UK).

Extraction of genomic DNA

Genomic DNA was extracted from cells by adding 500 µL of lysis buffer (50 mM Tris-HCl, pH 8.5; 1 mM EDTA; 0.5 % Tween 20) and proteinase K (Sigma Aldrich, USA) to a final concentration of 100 µg/mL followed by phenol/chloroform method. The DNA was dissolved in TE buffer.

Quantitative analysis of DNA methylation using Infinium HumanMethylation450 BeadChip

The Infinium HumanMethylation450 BeadChip (Illumina Inc., USA) provides comprehensive analysis of CpG sites, allowing the examination of over 485,000 methylation sites per sample, with a resolution of one nucleotide. It covers 99 % of gene regions from the RefSeq collection, with an average of 17 CpG sites per gene region, distributed across promoters, 5' UTRs, first exons, the genes themselves, and 3' UTRs. It also covers 96 % of CpG islands and associated regions. The Illumina's Infinium measures DNA methylation using quantitative genotyping of bisulfite-converted genomic DNA.

500 ng of genomic DNA samples were bisulfite-converted using the Zymo EZ-96 DNA Methylation Kit (Zymo Research Corporation, USA). The incubation conditions recommended for Infinium analysis were used (16 cycles at 95 °C for 30 s, and at 50 °C for 60 min). The DNA was processed for analysis using the Illumina standard kit (Illumina Inc., USA). After bisulfite conversion, the DNA was denatured, isothermally amplified, fragmented, and hybridized to the Infinium HumanMethylation450 BeadChip. The chips were then washed, dried, and scanned on the Illumina iSCAN (Illumina Inc., USA). The obtained values were normalized with an internal control, and the DNA was quantified using the Illumina's GenomeStudio Software module v1.8 (Illumina Inc., USA).

Statistical analysis

Initial data processing and normalization were performed using the standard software support of Illumina's GenomeStudio Software. Normalized ratios of methylated signals and the sum of methylated and unmethylated signals (β-values) were used for further analysis using the R computing platform and the Bioconductor package *(27, 28)*.

Using R computing platform, a t-test was performed for each pair of samples being compared to identify differentially methylated loci for each CpG site (~480,000), as well as for more complex structures such as CGIs (~28,000). The adjustment of p-values for multiple comparisons was done by converting the p-values from the t-test into q-values, using a FDR of 0.1 as the threshold for statistically significant differences.

RESULTS AND DISCUSSION

TAp73α and ΔNp73α proteins are expressed upon induction in SaOS-2 Tet-ON cells

SaOS-2 Tet-ON, inducible gene expression stable human p53-null osteosarcoma cell line, expresses TAp73α or ΔNp73α upon exposure to tetracycline-derivative doxycycline (Dox). The expression of genes of interest was examined upon induction with Dox using Western blot analysis with the specific monoclonal antibody. We confirmed the expression of TAp73α or ΔNp73α upon induction (Figure 2).

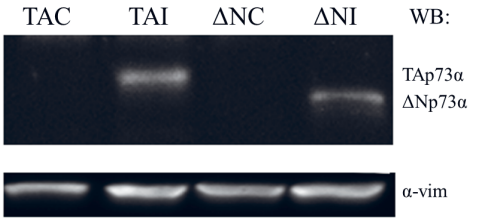

Figure 2. ***Western blot analysis of TAp73α and ΔNp73α expression after induction in SaOS-2 cells.*** *The induction of the Tet-ON system based on SaOS-2 cells was established to selectively express human TAp73α or ΔNp73α isoforms. For induction doxycycline was added at a concentration of 2 µg/mL and the cells were collected 24 h after. For Western blot analysis, total protein (30 µg) was loaded on an 8 % polyacrylamide gel and transferred onto a nitrocellulose membrane. Nonspecific binding was reduced by blocking with 5 % non-fat dry milk in TBS-Tween 0.01 %. The expression of p73 isoforms was revealed using the anti-p73 antibody ER-15 (1:100), and anti-α-vimentin (1:1000) was used as a loading control.*

Analysis of global DNA methylation in SaOS-2 Tet-ON cells depending on the expression of individual p73 isoforms

We used Illumina's Infinium HumanMethylation450 BeadChip to analyze global DNA methylation of 480 000 CpG sites. The results have shown correlation between each partner (control and induced cell lines). Percentages of methylation were derived by Illumina's GenomeStudio Software. Analysis of the obtained results (grouped and standardized using GenomeStudio Software) from the methylation analysis of individual samples, revealed predominantly significant differences in DNA methylation between two cell lines before the acti-

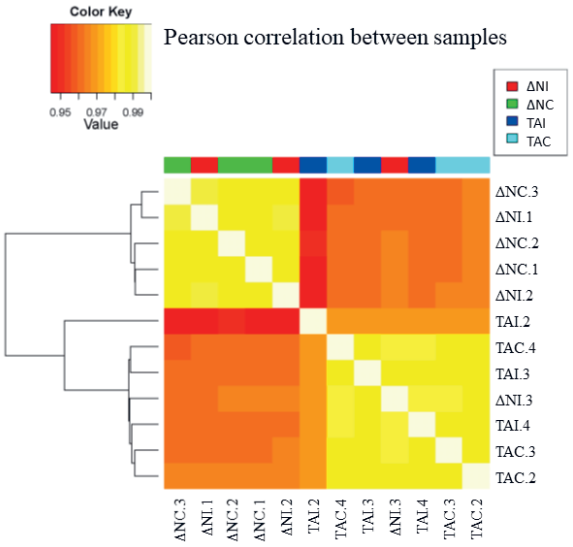

Figure 3. ***Correlation between all sample pairs.*** *The expression of TAp73α or ΔNp73α was induced in SaOS Tet-ON by adding of doxycycline in the medium in three independent experiments. TAp73I and ΔNp73I represent the samples of cell lines induced by doxycycline, while TAp73C and ΔNp73C are their controls (cells without induction). DNA methylation was determined using Infinium HumanMethylation450 BeadChip and average ratio calculated for each cell line (doxycycline induced TAI and ΔNI, and the controls ΔNC and TAC) using GenomeStudio Software. High mutual correlation between all sample pairs (Pearson correlation > 95 %) indicates the technical validity of the procedure and the quality of the data.*

vation of specific p73 isoform (Figure 3). There was no statistically significant difference in methylation of the genome between induced cells and their controls.

Since we found a statistically significant difference in the methylation of the 12,091 CGIs in the samples before the induction of the isoforms, but no such difference was found when comparing the same samples after induction of the individual isoforms, it is likely that a change in genome methylation did occur in the samples after activation. However, this change was present in both samples in very small numbers with opposite signs, so after statistical processing and calculating the average value of the three measurements, it was not statistically significant.

Further, the data were standardized by determining the β-value for each individual locus *(29)*. The β-value represents the standardized ratio of methylated versus the sum of methylated and unmethylated signals, or the ratio of methylated cytosines to the total sum of methylated and unmethylated cytosines at a particular locus. The histogram comparing the average β-values of individual samples shows the expected distribution of methylated and unmethylated cytosines (Figure 4). In all samples, an almost identical distribution of the frequencies of methylated versus unmethylated cytosines was observed - a significantly higher frequency of loci with low β-values (up to 0.2). The frequency of loci in the β-value range of 0.2-0.7 was low, with a higher frequency of loci with higher

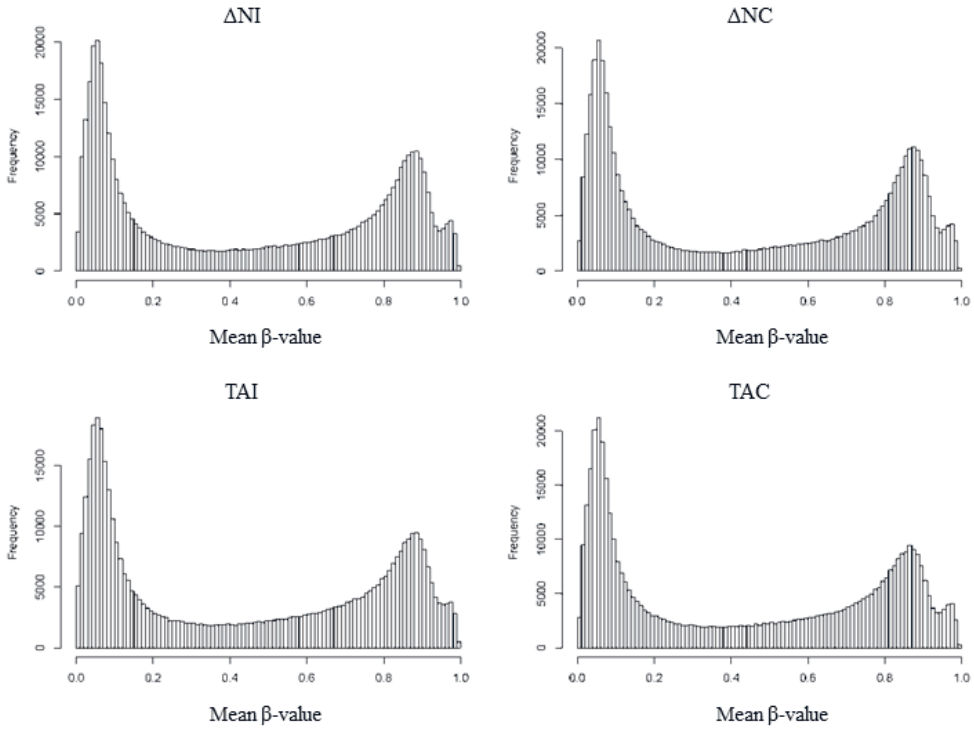

Figure 4. ***Histogram of the frequency of methylation β-values for individual samples.*** *ΔNI represents the mean β-values of samples with the induced ΔNp73α isoform, while ΔNC average represents the mean β-values of their controls (the same cells without induction). Likewise, TAI represents the mean β-values of samples with the expressed TAp73α isoform, and TAC represents the mean β-values of their controls. The β-values are determined and graphs are made using R computing platform.*

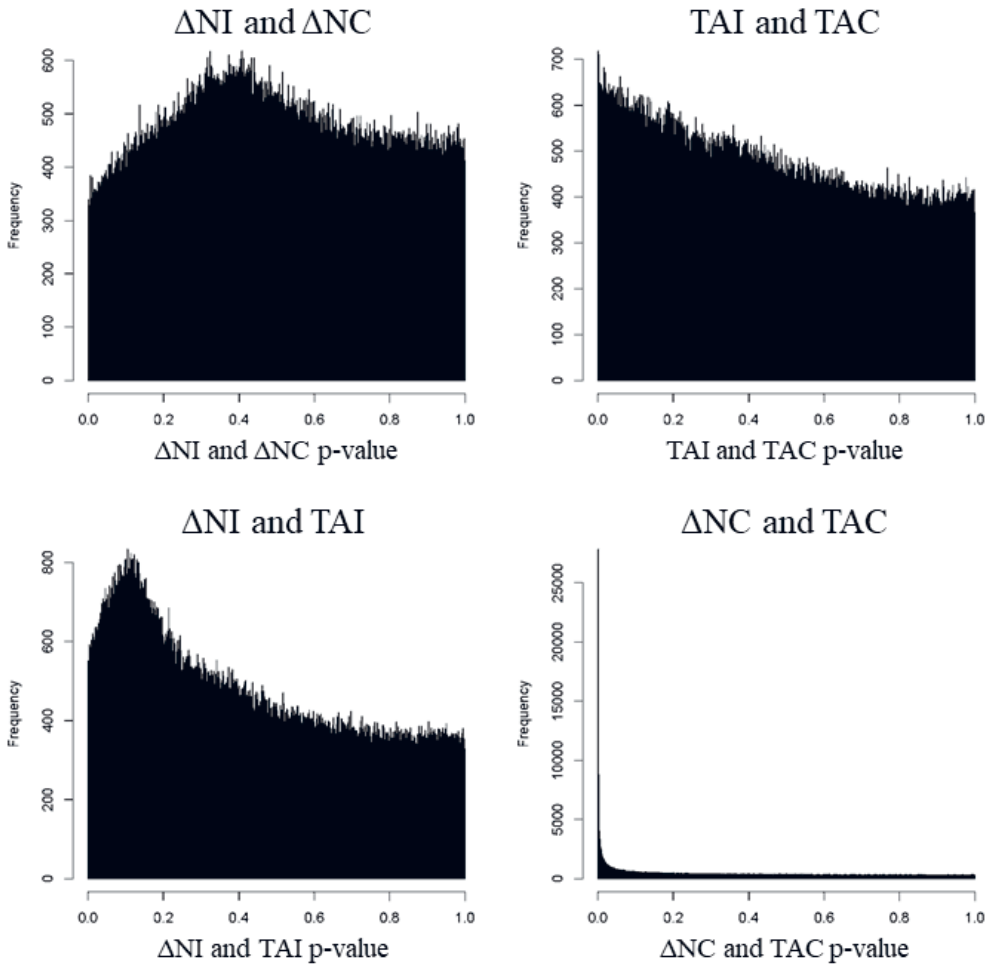

Figure 5. ***Comparison of methylation between samples.*** *A pairwise comparison of the methylation of individual loci for each cell sample (ΔNI samples with the expressed ΔNp73α isoform, ΔNC their control; TAI samples with the expressed TAp73α isoform, and TAC their control). The p-values are calculated and graphs made using R computing platform.*

β-values (above 0.7), although the latter was still about half the frequency of loci with lower β-values (for β-value of 0.1, the frequency was around 20,000, while for 0.9, it was around 10,000).

After determining the methylation status for each individual cell sample (ΔNI, ΔNC, TAI, and TAC), we compared the samples with each other in order to identify differentially methylated loci for each CpG site individually (approximately 480,000 in total), as well as more complex structures such as CGIs (around 28,000), and CGIs associated with promoter regions (about 18,000). A t-test was performed for each pair of samples. The histograms of p-values for the four comparisons (ΔNI vs. ΔNC, ΔNI vs. TAI, TAI vs. TAC, and ΔNC vs. TAC) show that the most significant difference in methylation was observed between the control cell lines, i.e., the cell lines in which p73 isoforms were not induced by doxycycline (ΔNC and TAC) (Figure 5).

The β-values of individual samples were compared to each other (ΔNI vs. ΔNC, TAI vs. TAC, ΔNI vs. TAI, and ΔNC vs. TAC). By comparing a total of 39,170 loci for each sample, we found a statistically significant difference in genome methylation only in the comparison of cell samples before the induction of p73 isoforms (ΔNC vs. TAC). Thus, with a false discovery rate (FDR) < 0.1, a difference in methylation between these two samples was found at a total of 12,091 loci, and with an FDR < 0.05, at 8,715 loci. We found that genome methylation did not change statistically significantly at any locus after induction of TAp73α or ΔNp73α expression.

We confirmed this result by comparing the distribution of average β-value differences in the individual comparisons and the corresponding p-values between the 100 statistically most significant differences in each comparison (Figure 6). The differences in the comparison of ΔNC and TAC are statistically more significant than those in the other three comparisons. Similar results can be observed when comparing the methylation levels of CGIs (Figure 6, panel 4 – second column). In this case, the average β-value was calculated for each CpG sequence within each sample, and then different sample types were compared.

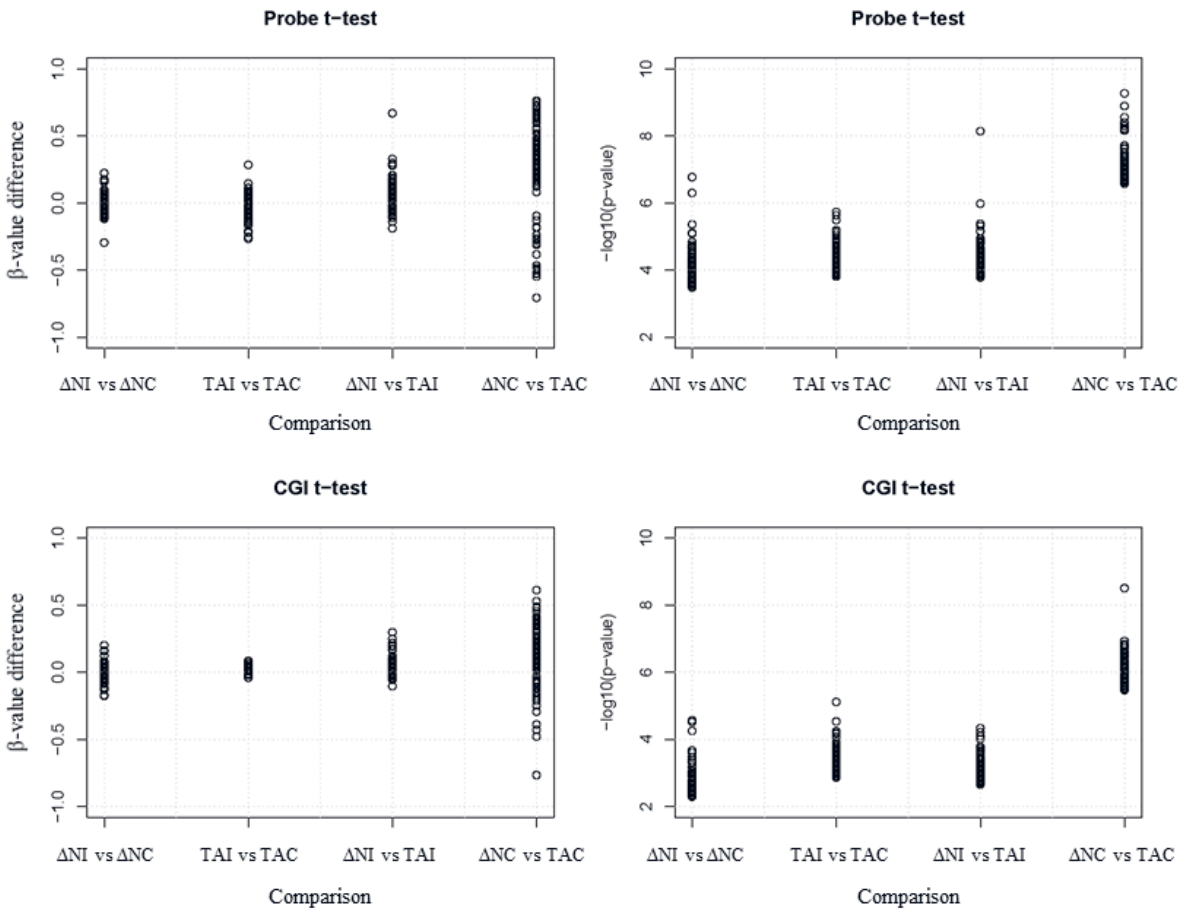

Figure 6. ***Comparison of methylation levels of CpG sequences (CGI).*** *First, the β-value was calculated for each CpG sequence in each sample, and then individual samples were compared. The statistics and graphs are made using R computing platform.*

By comparing β-values (the ratio of methylated cytosines to the total sum of methylated and unmethylated cytosines at a given locus) in induced cells with their controls, SaOS-2 Tet-ON cells before induction of the corresponding isoform, although a difference was observed, we did not find statistically significant differences in methylation status. This indicates that the induction of the ΔNp73α or TAp73α isoforms does not significantly affect the methylation profile of the cells.

This can be partially explained by the fact that we used cancer cells for analysis, whose methylation status is already specific. Therefore, the additional expression of the ΔNp73α and TAp73α isoforms does not have a significant impact on the methylation of the genome.

Genome methylation status in relation to gene expression levels in response to TAp73α and ΔNp73α expression

We compared our results of genome methylation changes upon the induction of *TP73* gene isoforms of interest with the gene expression results obtained by the transcriptome analysis of p53-null cells overexpressing TAp73α or ΔNp73α *(30, 31)*.

TAp73α can modulate the expression and methylation of various cellular genes in the absence of p53

First, we compared our data with the results of changes in the transcriptional response of genes after induction of TAp73α expression in the same model that we used, SaOS-2 Tet-ON cells *(30)*. In the mentioned study by Koeppel and coworkers, global binding sites for TAp73α and TAp73β isoforms were examined within the genome of MDA-MB231, HCT116, HEK293, and SaOS-2 cell lines. After globally detecting their binding sites, the transcriptional response of the genome to isoform binding was analyzed using RNA sequencing (RNA-seq). Koeppel and coworkers have shown that both TAp73 isoforms bind to common, as well as isoform-specific, target sites and have different effects on target genes.

We have selected 20 genes that showed the most significant changes in expression upon induction of TAp73α in SaOS-2 Tet-ON cells calculated as logarithmic values and compared them with the differences in the β-values of methylation of the same genes between cells with induced TAp73α isoform (TAI) and control cells (TAC) and shown in Table 1.

Table 1. ***Comparison of changes in methylation of target genes after TAp73α induction with changes in expression of the same genes after TAp73α activation.*** *The differences in β-values of methylation for individual genes in our experiment after TAp73α activation (TAI-TAC) are shown, along with the logarithmic values of changes in their expression from the study by Koeppel et al. (30). Positive values indicate increased expression and negative values indicate reduced expression. The analysis was done using R computing platform.*

GENE SYMBOL	GENE NAME	TAI-TAC β-value	$\log_2$
BACE2	beta-site APP-cleaving enzyme 2	-0.023	-8
SFN	stratifin	0.002	7.79
CYYR1	cysteine/tyrosine-rich 1	-0.007	-7.73
DPYSL4	dihydropyrimidinase-like 4	-0.005	-7.48
EMB	embigin	-0.007	-7.25
MIER2	mesoderm induction early response 1, family member 2	-0.020	-6.98
SLC8A3	solute carrier family 8 (sodium/calcium exchanger), member 3	-0.010	-6.87
NRXN1	neurexin 1	0.020	-6.82
NPTX2	neuronal pentraxin II	-0.009	-6.75
RARRES2	retinoic acid receptor responder (tazarotene induced) 2	0.007	6.52
TP73	tumor protein p73	-0.005	6.47
ZNF682	zinc finger protein 682	-0.058	-6.3
IL17RA	interleukin 17 receptor A	0.038	-6.21
C3orf54	chromosome 3 open reading frame 54	-0.005	6.13
PLD5	phospholipase D family, member 5	-0.047	-5.91
EMID2	EMI domain containing 2	0.001	-5.7
NIPAL4	NIPA-like domain containing 4	0.010	5.64
THEM4	thioesterase superfamily member 4	-0.003	-5.58
ICAM1	intercellular adhesion molecule 1	-0.011	5.55
PARP11	poly (ADP-ribose) polymerase family, member 11	0.021	-5.55

Figure 7 shows the relationship between gene expression changes from the study by Koeppel et al. and the changes in the methylation of the same genes that we investigated in our study. As mentioned, in our work, we did not find a statistically significant change in genome methylation after TAp73α activation. When comparing the changes in expression of 20 genes with the changes in methylation of these same genes in our experiment, we found a pattern in six genes that could explain the change in gene expression with changes in methylation. For three genes showing increased expression after TAp73α activation, we observed reduced methylation in our study. Similarly, for three genes showing decreased expression, we found increased methylation in our experiment. When comparing data for the remaining 16 genes, we did not find any changes in methylation in our study that could be linked to the changes in expression found in the study by Koeppel et al. *(30)*.

When comparing our results with those of gene expression changes after TAp73α activation, we were unable to demonstrate an association between altered CpG island methylation in the promoter regions of genes and changes in their expression. This also suggests that the changes in the expression response after TAp73 activation might be mediated by other epigenetic mechanisms.

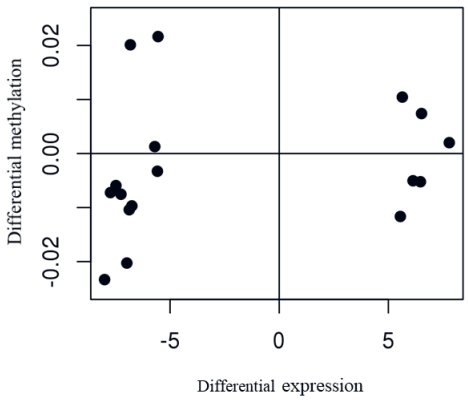

Figure 7. ***Comparison of differences in gene expression with differences in CGI methylation after induction of the TAp73α isoform in SaOS-2 Tet-ON cells.*** *The differential methylation expressed as β-value is correlated to the logarithmic values of changes in gene expression as presented in Table 1.*

ΔNp73α can modulate the expression and methylation of various cellular genes in the absence of p53

Next, we compared the genome methylation results of SaOS-2 Tet-ON cells after the induction of the ΔNp73α expression with the gene expression results obtained by the microarray analysis *(31)*. In the study by Kartasheva and coworkers, more than 30 genes were found to be altered upon overexpression of ΔNp73α in H1299 cell line (p53-null). We identified eighteen of these genes in our model and subsequently determined their methylation status. Table 2 shows comparison of the differences in DNA methylation of 18 genes in ΔNp73α expressing cells (ΔNI) and the control group (ΔNC), expressed as β-values, with the logarithmic values of changes in their expression level in response to ΔNp73α from the study by Kartasheva et al.

Figure 8 illustrates the relationship between changes in gene expression from the study by Kartasheva et al. and the changes in the methylation of the same genes that we detected in our study. The data show that for genes exhibiting upregulated expression of ΔNp73α, there was a reduction in methylation, although it was not statistically significant. For genes with reduced expression after ΔNp73α activation, we did not find such a clear association. In our study, we were unable to demonstrate the expected increase in methylation levels for this group of genes.

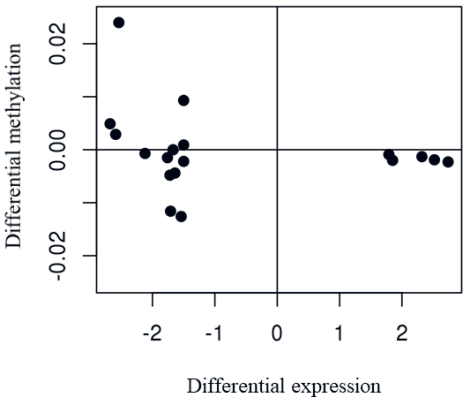

Figure 8. ***Comparison of differences in gene expression with differences in methylation of CpG islands after overexpression of the ΔNp73α isoform.*** *The differential methylation expressed as β-value is correlated to the logarithmic values of changes in gene expression as presented in Table 2.*

Based on our results, altered methylation of genes is not the dominant mechanism by which the TAp73α and

Table 2. ***Comparison of changes in methylation of target genes after ΔNp73α induction with changes in expression of the same genes after ΔNp73α overexpression.*** *The differences in β-values of methylation for individual genes in our experiment are shown, along with the logarithmic values of changes in their expression from the study by Kartasheva et al. (29). Positive values indicate increased expression and negative values indicate reduced expression.*

GENE SYMBOL	GENE NAME	ΔNI-ΔNC β-value	$\log_2$
CCNA2	cyclin A2	-0.0116	2.74
CDC6	cell division cycle 6 homolog (S. cerevisiae)	-0.002	-2.68
EGR1	early growth response 1	-0.0023	-2.59
ODC1	ornithine decarboxylase 1	0.0049	-2.54
UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	0.0029	2.52
FOSL1	FOS-like antigen 1	0.0240	2.32
CSRP2	cysteine and glycine-rich protein 2	-0.0019	-2.12
PRAME	preferentially expressed antigen in melanoma	-0.0013	1.85
AKAP1	A kinase (PRKA) anchor protein 1	-0.0007	1.79
GCH1	GTP cyclohydrolase 1	-0.0009	-1.76
MPHOSPH10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	-0.0015	-1.72
PTPRCAP	protein tyrosine phosphatase, receptor type, C-associated protein	-0.0048	-1.71
F2R	coagulation factor II (thrombin) receptor	8.62E+08	-1.67
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-0.0044	-1.64
TCEA1	transcription elongation factor A (SII), 1	-0.0126	-1.54
GOLPH3	Golgi phosphoprotein 3 (coat-protein)	-0.0022	-1.5
SIAH2	seven in absentia homolog 2 (Drosophila)	0.0009	-1.5
CARS	cysteinyl-tRNA synthetase	0.0093	-1.5

Δ*N*p73α isoforms regulate gene expression. Possibly the changes in gene expression are caused by epigenetic mechanisms not related to methylation, such as histone modifications or regulation with miRNAs. Additionally, since we used different cell lines, it is possible that cell-specific proteins influence the genes' expression or signaling pathways that prevent significant changes in methylation in the specific cell line.

However, when we compared the individual genes from Kartasheva et al.'s study, for which we observed methylation changes in our cells, we found that for all five genes with increased expression after Δ*N*p73α activation (*EGR1*, *CSRP2*, *PRAME*, *CDC6*, *GCH1*), we observed decreased methylation after Δ*N*p73α induction compared to cells in which the isoform was not activated *(31)*. Although these differences were not statistically significant, they suggest that decreased methylation may be a mechanism by which Δ*N*p73α influences the increased expression of these target genes. Of the thirteen genes with reduced expression, we found increased methylation in six of them, while in the remaining seven, the methylation was decreased after Δ*N*p73α activation.

In conclusion, we did not find a clear statistically significant association between changes in methylation and gene expression after activating the Δ*N*p73α and TA-p73α isoforms. This may indicate that methylation is not the primary epigenetic mechanism regulating gene expression in this context. It is possible that other epigenetic mechanisms play a more important role in this process.

CONCLUSIONS

Infinium HumanMethylation450 BeadChip was confirmed for rapid detection on large numbers of methylated CpG`s (approx. 480 000 CpG sites). Methylation status for 39170 CGIs in SaOS-2 gene promoter regions was showed. Methylation changes in promoter region followed by activation of p73 isoforms were noticed. However, there was no statistically significant impact of increased expression of TAp73α or Δ*N*p73α on SaOS-2 promoter regions.

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