Rocz Panstw Zakl Hig 2018;69(3):307-314

http://wydawnictwa.pzh.gov.pl/roczniki_pzh/

ORIGINAL ARTICLE

HYPOMETHYLATION OF THE *C-MYC* PROMOTER REGION INDUCED BY PHENOBARBITAL IN RAT LIVER

Katarzyna Urbanek-Olejnik[1], Monika Liszewska[1], Alicja Winczura[2], Agnieszka Hernik[1], Paweł Struciński[1], Jan K. Ludwicki[1]

[1]Department of Toxicology and Health Risk Assessment, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
[2]Department of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

ABSTRACT

Background. The changes in DNA methylation are considered as one of the early events in hepatocarcinogenesis.
Objective. We evaluated the ability of phenobarbital (PB) – the most widely used anticonvulsant worldwide and classical rodent liver carcinogen – to cause the promoter region of the *c-myc* protooncogene hypomethylation as well as changes of mRNA level of this gene. Moreover, the expression of Dnmt1 protein in rat treated with this compound was analyzed.
Material and Methods. Male *Wistar* rats received PB in daily oral doses of 92.8 mg kg^{-1} b.w. day^{-1} (at 24-h intervals; for one, three and fourteen days). Methylation of the *c-myc* promoter region was measured by PCR-based methylation-sensitive restriction enzyme analysis (MSRA). Levels of mRNA for *c-myc* and protein Dnmt1 were assayed using Real-Time PCR and Western Blot, respectively.
Results. The study showed that phenobarbital stimulated persistent changes in DNA methylation, i.e. loss of methylation in the promoter region of the *c-myc* gene and up-regulated its mRNA level. In addition, a significant increase in protein level of Dnmt1 in the *c-myc* over-expressing liver cells was observed.
Conclusion. The oppose relationship between Dnmt1 activity and methylation status of *c-myc* gene was demonstrated. The *c-myc* over-expression by demethylation might represent an important, early events in the mechanism of action (MOA) of phenobarbital.

Key words: *c-myc*, DNA methylation, Dnmt1, genes expression, liver, rats

STRESZCZENIE

Wprowadzenie. Zmiany metylacji DNA są rozważane jako jeden z wczesnych mechanizmów hepatokancerogenezy.
Cel pracy. Celem badań była ocena wpływu fenobarbiatlu (PB) - leku przeciwpadaczkowego, modelowego promotora raka wątroby - na poziom metylacji regionu promotorowego i ekspresji na poziomie mRNA protoonkogenu *c-myc*. Ponadto dokonano analizy poziomu ekspresji białka Dnmt1.
Materiał i metody. Samce szczurów szczepu *Wistar* otrzymywały PB w dawce 92,8 mg/kg m.c. x $dzień^{-1}$ jednorazowo, 3-krotnie i 14-krotnie. Ocenę poziomu zmian metylacji genu *c-myc* dokonano metodą MSRA (ang. Methylation-Sensitive Restriction Enzyme Analysis). Analizę względnego poziomu transkryptów genu *c-myc* i białka Dnmt1 przeprowadzano odpowiednio metodą PCR w czasie rzeczywistymi i techniką Western Blot.
Wyniki. W wyniku oddziaływania fenobarbitalu wykazano trwałe zmiany metylacji DNA - obniżenie metylacji w rejonie promotorowym genu *c-myc* i nadekspresję badanego genu na poziomie mRNA. Jednocześnie obserwowano statystycznie istotny wzrost poziomu białka Dnmt1.
Wnioski. Wykazano odwrotną zależność między aktywnością Dnmt1 a poziomem metylacji genu *c-myc*. Nadekspresja *c-myc* w wyniku demetylacji może stanowić istotne, wczesne zdarzenie w mechanizmie działania (ang. Mechanism of Action (MOA)) fenobarbitalu.

Słowa kluczowe: *c-myc, metylacja DNA, Dnmt1, ekspresja genów, wątroba, szczury*

INTRODUCTION

The introduction of short-term tests for the detection of genetic damage has partly solved the problem of an initial and early evaluation of carcinogenic potential of chemical substances. There is, however, a need to supplement them with tests aimed at identification of carcinogens which act through non-genotoxic, i.e. epigenetic mechanisms. According to current knowledge, epigenetic mechanisms play an important

Corresponding author: Katarzyna Urbanek-Olejnik, Department of Toxicology and Health Risk Assessment, National Institute of Public Health – National Institute of Hygiene, Chocimska 24, 00-791 Warsaw, Poland, tel. +48 22 54 21356, e-mail:kolejnik@pzh.gov.pl

role in carcinogenesis, including hepatocarcinogenesis [15, 40, 46, 49]. In spite of intensive research in this field, the selection of markers, which would serve as tests for the identification and evaluation of the interactions of non-genotoxic potential carcinogens, still remains an open issue.

Literature from the last years [22, 35, 37] indicates that using short-term changes in gene expression in target organ as effect of epigenetic alteration, may be one of the approaches to address the problem. It has been hypothesized that epigenetic alternations may be used as early biomarker in the evaluation of the carcinogenic potential of the chemicals [10, 22, 26, 27]. Furthermore, abnormal changes in DNA methylation and mRNA expression of *c-myc* are believed as an important stimulatory factors in the development of liver cancer [9, 30].

The *c-myc* protooncogene encodes a transcription factor which has a well described role in cell growth and proliferation [1, 5]. Moreover, direct role of *c-myc* in the control of DNA synthesis is suggested [6, 41]. The ability to induce DNA hypomethylation and/or *c-myc* protooncogene in mouse liver tumors has been reported for many non-genotoxic carcinogens, including peroxisome proliferators (PPs) and phenobarbital [2, 34, 38, 39]. However it should be pointed out that the most of long-term studies, which were already performed, did not detect early changes in the genome [16, 45].

Phenobarbital (PB) is classical rodent liver carcinogen whose carcinogenic potency may be linked with its ability to cause epigenetic effects. *In vitro* and *in vivo* tests demonstrated that the carcinogenic activity of PB is not a result of its genotoxic activity. IARC has classified PB to group 2B, as non-genotoxic agent, carcinogenic in animals and possibly carcinogenic in humans [17].

The purpose of this study was to determine the methylation status and expression of the *c-myc* protooncogene, which is one of the so-called immediate early genes associated with increased cell proliferation, in the liver of *Wistar* rats treated of PB up to 14 days. The ability of PB to affect Dnmt1 expression was also analyzed.

MATERIAL AND METHODS

Chemicals

Phenobarbital (95%) was obtained from "Galenus", Poland. Genomic DNA Purification Kit, the HpaII restriction enzyme and phage λ DNA were purchased from Fermentas, USA; FastStart Taq Polymerase was obtained from Roche, Germany. RNeasy® Mini Kit – Qiagen, Germany; RT-PCR – Advantage® RT-for-PCR Kit – Clontech, USA; Kapa™ SYBR® Fast qPCR Kit PCR – Kapa Biosystem, USA. Other chemicals were obtained from Sigma Chemical Company, USA, and were of the highest quality and purity.

Animals and outline of experiment

The studies were performed on male *Wistar* rats, *Crl:WI(Han)* weighing 200 ± 10 g. All procedures involving animals were performed according to national animal welfare regulations after authorization by the Local Ethics Committee for the conducting of research studies in live vertebrates (permissions No. 6A/01 and 23/2010).

All animals were housed in groups of five in cages at 22 – 24°C, relative air humidity 50 ± 10% and a 12-h light/dark cycle. Prior to the use, the rats were acclimated for 2 weeks before the start of the experiment. During the adaptation and experimental phases standard feed LSM and filtered tap water *ad libitum* was administered to the animals. The animals were observed daily and their body weight as well as food and water consumption were monitored.

PB was dissolved in olive oil (vehicle) immediately before use and was administered orally *via* gavage at doses of 92.8 mg kg^{-1} b.w. day^{-1} (dose corresponding to 1/10 of the LD_{50} value) for 1, 3 and 14 days (at 24-h intervals), respectively. The dose of PB was identical to this used in previous study [20].

Animals were anesthetized by inhalation isoflurane/O_2, then were euthanized by decapitation. Livers were removed, rinsed of surface blood, blotted to dryness, weighed, frozen in liquid nitrogen and stored at -80°C. Representative samples of liver tissue from the right lobe were taken for analysis.

Analysis of DNA methylation

After DNA isolation using the Genomic DNA Purification Kit (Fermentas), methylation status of selected fragment of *c-myc* gene was determined based on the restrictive analysis sensitive to methylation (MSRA), as described previously [21].

RNA extraction and Real-time PCR for c-myc

Total RNA was extracted from frozen livers using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA synthesis was done in a 20 µl reaction mix starting with 1 µg of total RNA using the reverse transcription system of Clontech (Advantage RT-for-PCR kit) following the manufacturer's recommendations. Real-time PCR was performed on an MiniOpticon system (Bio-Rad) using Kapa Sybr®Fast qPCR Kit (Kapa Biosystems, USA). *c-myc* (GeneBank No NM_12603) cDNA was amplified using primers: 5'-CTGCTGTCCTCCGAGTCCTC-3'(F), 5'-GGGGGTTGCCTCTTTTCCAC-3'(R). *b-actin* (GeneBank No V01217) was used as the endogenous control and amplified using the following primers: 5'-GTGGGTATGGGTCAGAAGG-3'(F)

and 5'-CAATGCCGTGTTCAATGGGG-3'(R). Cycling conditions of Real-Time PCR were defined: 95°C for 2 min, followed by 40 cycles of denaturing at 95°C for 30 s, 55°C for 20 s, 72°C for 30 s, 80°C for 5 s. This number of cycles had been previously estimated to be the optimal one for detecting the signal in the linear range. PCR products were verified by melting curves and were also run on a 6% polyacrylamide gel to confirm the appropriate size. All real-time PCRs were done in triplicates. Amplification and threshold calculations were generated using the CFX Manager Software v1.6 (Bio-Rad). Relative gene expression was calculated using Pfaffl model, which provides for correction of data as regards differences in the efficacy of amplification of individual transcripts [32]. In the model the gene mRNA level in test samples is estimated relative to the level of the same gene in control samples and normalized to the mRNA level of a selected reference gene (*β-actin*).

Western blot analysis

Proteins from the animal's liver were isolated in T-Per Mammalian Tissue Reagent (ThermoFisher Scientific, USA) containing protease inhibitor (Complete Protease Inhibitor Cocktail, from Roche, Switzerland). Equal amounts of the protein (25 mg) were mixed with Laemmli buffer and boiled 5 min. Protein samples were then separated on 10% acrylamide gel in the presence of sodium dodecyl sulphate (SDS) and β-mercaptoethanol (SDS-PAGE) and transferred onto Immobilon-P membranes (Milipore, USA) using semi-dry electroblotting. The membranes were blocked in TBST (137 mM NaCl; 20 mM Tris-Cl; 0,1% Tween-20; pH 7,6) containing 5% non-fat milk and subsequently incubated overnight at 4°C with monoclonal mouse b-actin and polyclonal goat Dnmt1 antibodies (Santa Cruz), diluted respectively 1:5000 and 1:2000 in 5% milk in TBST. The membranes were washed four times for 10 min in TBST and then incubated with horseradish peroxidase (HRP) conjugate goat anti-mouse IgM antibodies and donkey anti-goat IgM antibodies (1:5000 dilution, Santa Cruz, USA) for 1 hour. The membranes were again washed four times for 10 min in TBST and then analyzed *via* an enhanced chemiluminescence method (Immobilon™ Western Chemiluminescent HRP Substrate, Millipore) and ultra-sensitive photographic X-ray Kodak film according to the manufacturer's instructions. The films were scanned and used for densitometry analysis by Image Quant v5.2. Dnmt1 proteins expression level was normalized relative to β-actin expression level.

Statistical analysis

The *c-myc* promoter methylation frequency was calculated with Fisher's test ($p<0.05$). REST-384 software tool was used to estimate the relative mRNA levels of particular genes. The software allows to compare mRNA levels in two groups (the control group and the test group) and to assess the statistical significance of demonstrated differences in mRNA levels using randomization tests (*Pair Wise Fixed Reallocation Randomization Test*) [31]. Proteins level was expressed as the mean ± SEM for five animals. The two-tailed Student's *t* test was employed to calculate the statistical significance between control and treated groups ($p<0.05$).

RESULTS

DNA methylation status of c-myc gene

The methylation-sensitive restriction endonuclease HpaII was used to assess the methylation status in the promoter regions of *c-myc* gene. A scheme of the *c-myc* gene promoter region is illustrated in Figure 1.

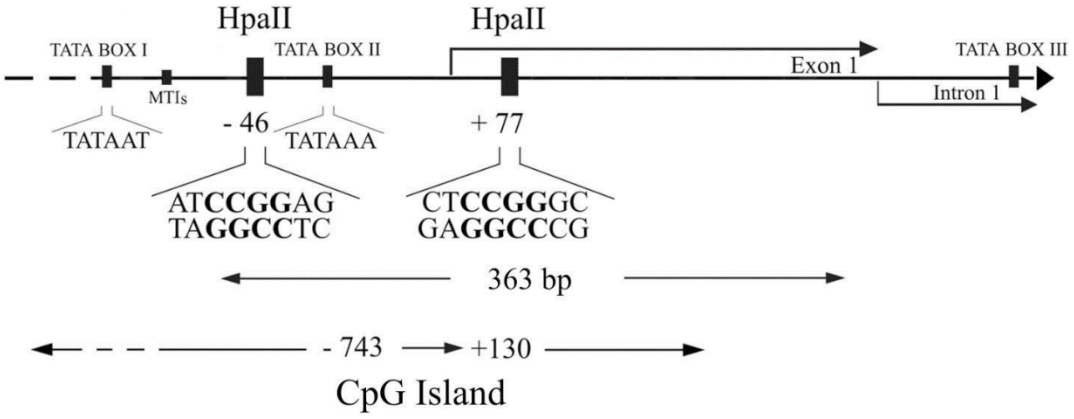

Figure 1. Scheme of the *c-myc* promoter region

The *c-myc* promoter region contains CpG Island at position -743 bp-+130 bp, which was defined according to Gardiner-Garden and Frommer [11]. Within this region are located two CpG sites recognized by the methylation-dependent enzyme HpaII at position –46 bp and +77 bp. Two of the three independent non-overlapping promoters (TATA BOX I and II) occur in a region rich in CpG dinucleotides.

The ability of PB to induce *c-myc* hypomethylation in male rat liver is presented in Table 1 and Figure 2.

The *c-myc* promoter region was unmethylated at a frequency of 50% (in 8 out of 16 samples analyzed) after single dose of PB (Table 1). Three doses of the PB, administered every 24h, induced a further increase demethylation of *c-myc*. The unmethylated cytosines (in 10 out of 16 DNA samples) were indicated in HpaII-digested DNA from the liver of male rats exposed to PB at dose of 92.8 mg kg^{-1} b.w., which exceeded control by 63% - a frequency 13% higher than this obtained after a single dose of compound ($p<0.05$). After 14 days of exposure, the *c-myc* promoter region was also unmethylated at a frequency of 63% (in 10 out of 16 samples) ($p<0.05$). Hypomethylation of *c-myc* gene did not occur in the control rats.

Table 1. Summary of methylation-sensitive digestion with HpaII of DNA isolated from the liver of rats treatment with PB for 1, 3 or 14 days[1]

Group of rats	1 day	3 days	14 days
	(M/U)	(M/U)	(M/U)
PB	8/8*	6/10*	6/10*
Control	8/0	8/0	8/0

[1]DNA material from the livers of four PB-treated rats was digested with HpaII in two independent repeats. Each restricted DNA sample was used for two independent PCR runs. Total the 16 DNA samples were analyzed. The results for each DNA sample from the control rats are presented for one PCR. M - DNA samples in which investigated CpG sites were methylated; U – DNA samples in which investigated CpG sites were unmethylated; (*) Significance calculated using Fisher's test.

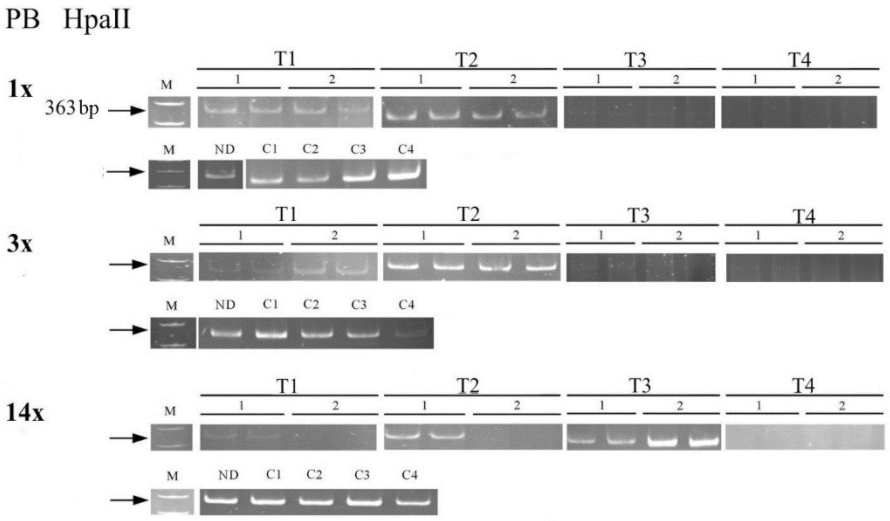

Figure 2**.** Effect of PB on the methylation status of the *c-myc* gene in the liver of *Wistar* rats

Methylation status of the *c-myc* investigated CpG sites in rat liver determined by methylation sensitive restriction enzyme analysis (MSRA). The rats were administered PB for 1, 3 or 14 days in doses 92,8 mg kg^{-1} b.w. day^{-1}. The absence of a visible PCR product (363 bp) indicates the presence of unmethylated investigated CpG sites. T1-T4 – DNA isolated from rat livers administered with PB; C1-C4, DNA isolated from the livers of control rats. Number 1 and 2 – represents two of DNA digestion with HpaII. ND (not digested) – positive control of PCR. The arrows in the left margin indicate the size of the bands (363 bp) for *c-myc.*

Representative results of methylation sensitive restriction enzyme analysis (MSRA) showing effect of PB on the methylation status of the *c-myc* promotor region in the rat liver are illustrated in Figure 2.

In the analyzes of 4 independent samples of DNA isolated from the livers of rats exposed to a single dose of PB, HpaII endonuclease digestion yielded no PCR product of the size of 363 bp in samples T3 and T4. This shows that no cytosine methylation occurs in the examined CpG sequences. Further reduction of cytosine methylation levels at the examined positions –46 bp and +77 bp was observed after 3 and 14 days of exposure to PB. No PCR product was obtained in samples T3 and T4 as well as in one run of the restriction analysis conducted on sample T1 after 3 doses of PB. No DNA methylation was observed following prolonged exposure (14 days) to PB in samples T1, T2 (one run) and T4, as evidenced by the lack of 363 bp product. In four control samples (C1–C4), restriction analysis afforded the appropriate PCR product.

mRNA expression of c-myc gene

DNA methylation has an essential role in the transcriptional regulation of gene expression and has been recognized as an important factor in the activation of protooncogenes such as *c-myc* [9]. Therefore, we assessed the effect of exposure to PB on the level of the *c-myc* mRNAs in rat liver. Changes of *c-myc* mRNA levels induced by studied compound are presented in Table 2.

Table 2. Effect of PB on the mRNA expression of the *c-myc* gene in the liver of *Wistar* rats[2]

c-myc	Expression ratio		
	1	3	14
PB	1,719*	1,934*	1,666*

[2]Expression levels of *c-myc* were analyzed by real-time RT-PCR. The data were normalized to the housekeeping gene *β-actin* and expressed as the relative to control value. (*) Significance calculated using randomisation test.

As shown in the Table 2, PB caused a sustained increase the transcript levels of *c-myc* as compared with the control group. The expression of mRNA of the *c-myc* gene was increased by 70 and 90% after a single dose and 3 doses of the compound, respectively.

This tendency was also observed after 14 days of exposure to PB.

DISCUSSION

Literature from the last years indicates that investigations of the response of the cellular genome to the action of chemical agents give the possibility of both in-depth understanding of the mechanisms of their action as well as form the basis for earlier evaluation of their carcinogenic potential [35, 46].

The results of studies assessing the effect of non-genotoxic carcinogens (NGCs) on the methylation levels of key genes involved in regulation of the cellular cycle and apoptosis may be particularly important due to the role of DNA methylation in regulation of gene expression [28, 29, 30] and the NGCs' capability to interfere with processes of cell proliferation and apoptosis at the early stages of the tumorigenic process [23, 33].

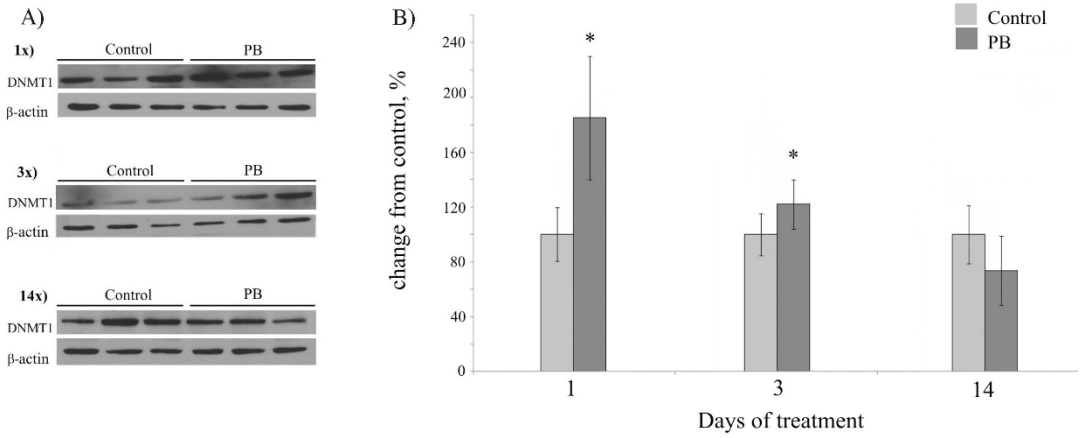

Figure 3. Western blot analysis of Dnmt1 protein in the liver of control rats and rats treated with PB

The signal intensity from the chemiluminescent detection was analyzed by ImageQuant software. β-actin was used as a loading control. A) The representing images of Dnmt1 and β-actin protein expression. B) Quantitative analysis of Dnmt1 protein level. Control values at each time point were considered as 100%. (*) Significantly different from the control at the same time point, $p<0,05$; These results are representative of three independent experiments.

Expression of Dnmt1 protein

The DNA methylation process is catalyzed by three independently coded DNA methyltransferases (DNMTs): Dnmt1, Dnmt3a and Dnmt3b. The main enzyme responsible for reproduction of methylation patterns upon DNA replication in somatic mammalian cells is Dnmt1. Disruption in its activity and/or expression may lead to alteration in DNA methylation [9, 19, 28, 42].

The Western blot analysis of Dnmt1 levels revealed statistically significant differences in the levels of the tested protein in the livers of rats exposed to the examined compound (Figure 3).

Over-expression of Dnmt1 was observed in protein extracts isolated from the livers of rats exposed to the single dose of PB. Dnmt1 expression level was 60% higher than that in the control group. Upon continued exposure of animals to PB, statistically significant increase in Dnmt1 levels (by 22%) was observed only after 3 days of exposure to studied compound.

The results reported in this work indicate that phenobarbital (PB) stimulated hypomethylation of the tested sequences of the *c-myc* promoter region, which is one of the pathways for regulating gene expression at a transcription level. Indeed, we found that expression of *c-myc* was up-regulated after short (1 and 3 doses at 24-h intervals) and prolonged exposure (14 doses at 24-h intervals) of the animals to PB. The relationship of the methylation status of promoters and gene transcription level was also reported with respect to other non-genotoxic liver carcinogens. Hypomethylation of *c-myc* gene has been found in the liver of rodent exposed to peroxisome proliferators (PPs), such as a model PP, Wy-14,643 [12] and dibuthyl phthalate [21]. The prolonged (7-8 weeks) treatment with dibromoacetic acid (DBA) induced hypomethylation of promoter regions of the *c-myc* and *Igf2* (insulin-like growth factor 2) protooncogenes in mouse liver. DBA also increased the mRNA

expression of the two genes that was associated with their hypomethylation in mouse liver [43]. In other study, it has been shown [2], that phenobarbital causes hypomethylation of Ha-*ras* (encodes a protein that is involved primarily in regulating cell division) and increased expression of the protooncogene in 50% of B6C3F1 mice. Moreover, Lempiäinen et al. [24] and Luisier et al. [25] found *Cyp2b10*, the cytochrome P450 member, to be concomitantly hypomethylated and transcriptionally activated in the liver of phenobarbital-treated mice.

Furthermore, we have demonstrated that PB induces a transient increase of Dnmt1 expression. According to current knowledge, Dnmt1 is considered the main enzyme responsible for the maintenance of the normal methylation pattern in repeated replication cycles that precede cell division [2]. Dnmt1 plays an essential role in the control of cellular cycle. Synthesis of Dnmt1 is induced of the moment when cells enter S-phase of the cellular cycle [18, 19]. This process is associated with the requirement of methylation of the newly synthesized DNA strand in hemimethylated sequences. This indicates that Dnmt1 activity/ expression inhibition can cause loss of methylation during DNA synthesis. However, we found the methylation status of *c-myc* gene was not associated with decreased of Dnmt1 expression. It is suggested that Dnmt1 protein level was not a critical determinant of promoter demethylation of *c-myc* gene. On the other hand, a well-established link between expression of Dnmt1 and DNA replication, as mentioned previously, was also confirmed by results from this work and our previous studies [20, 47] conducted under identical experimental conditions (the same dose level and dosing regimen). Treatment of rats with PB for 1 and 3 days produced an increase in nuclear Dnmts activity and DNA synthesis (previous study) [20] and Dnmt1 protein level (this study). Taking into account Dnmt1 activity/expression and DNA synthesis, our results are consistent with a study by Ge et al. [13] who not only reported increased cell proliferation and Dnmts activity, but also DNA hypomethylation of the *c-myc* gene after single administration of Wy-14,643 to mice. However, a long-term (22 weeks) exposure to Wy-14,643 [39, 38] had no effect on *c-myc* methylation, although up-regulation of *c-myc* proteins was detected. It is worth noting, that Wy-14,643-induced global hypomethylation of DNA was associated with a decrease in Dnmt1 protein levels and increased cell proliferation. Thus, coexistence of hypomethylation of *c-myc* gene and transient over-expression of Dnmt1, reported in this paper, indicates that there must be a more complex mechanisms responsible for these findings.

One of the hypotheses regarding the carcinogenic potential of PB [8, 44] takes into account potential induction of oxidative stress as a result of PB-stimulated metabolism, particularly of CYP2B induction, leading to generation of reactive oxygen species (ROS) and DNA damage. Moreover, the integrated transcriptomic and metabolomic analyses of livers and plasma of the *Fisher F344* rats exposed to PB for up to 14 days [48], also suggest that oxidative stress is involved in the development of early lesions that are caused by this compound. On the other hand, there is a growing conviction that oxidative stress has significant consequences for epigenetic processes, including DNA methylation [7, 29, 50].

It is widely known that oxidative damage can lead to formation of a variety of modified bases in DNA [4] as well as can be involved in the 5-hydroxymethylcytosine (5hmC) formation in a demethylation pathway [14]. Considering above assumption we cannot exclude conversion of promoter 5mC to cytosine (without Dnmt1) through a 5hmC intermediate for PB-induced demethylation of *c-myc* gene.

CONCLUSION

In conclusion, the results of our study showed early changes in the genome of the liver of *Wistar* rats exposed to a tumor-promoter such as phenobarbital. Decreased methylation in the promoter region of the *c-myc* gene and increased level of its mRNA in the presence of a transient increase in Dnmt1 expression were found. Given the ability of *c-myc* gene to collaborate in the regulation of key cancer-related processes, i.e. cell proliferation and apoptosis, its deregulated expression (overexpression) by epigenetic events, can play crucial role for PB toxicity, including its carcinogenic activity.

Further study will be needed to verify these results by use more sensitive method to quantify methylation levels. Moreover, the additional experiments are required to establish the details of the mechanisms that are responsible for PB-stimulated demethylation of *c-myc* gene as well as to unravel a link between change of methylation status *c-myc* and induction of oxidative stress by PB.

Acknowledgements

This study was supported by the Polish Grant no. 2 P05D 028 26 and NN404 123438. The authors would like to thank Professor Barbara Tudek from the Institute of Biochemistry and Biophysics Polish Academy of Science in Warsaw for providing access to the laboratory equipment.

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. *Amente S., Lania L., Majello B.*: Epigenetic reprogramming of Myc target genes. Am J Cancer Res 2011; 1(3): 413-18.
2. *Bachman A.N., Phillips J.M., Goodman J.L.*: Phenobarbital induces progressive patterns of GC-rich and gene-specific altered DNA methylation in the liver of tumor-prome B6C3F1 mice. Toxicol Sci 2006; 91(2): 393-405. doi: 10.1093/toxsci/kfj155.
3. *Cedar H., Bergman Y.*: Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 2009; 10(5): 295-304. doi:10.1038/nrg2540.
4. *Dahl C., Grønbæk K., Guldberg P.*: Advances in DNA methylation: 5-hydroxymethylcytosine revisited. Clin Chim Acta 2011; 412(11-12): 831-6. doi:10.1016/j.cca.2011.02.013.
5. *Dang C.V.*: MYC, metabolism, cell growth, and tumorigenesis. Cold Spring Harb Perspect Med. 2013; 3(8). pii: a014217. doi: 10.1101/cshperspect.a014217.
6. *Dominguez-Sola D., Gautier J.*: MYC and the control of DNA replication Cold Spring Harbor Perspectives in Medicine 2014; 4(6). pii: a014423. doi:10.1101/cshperspect.a014423.
7. *Donkena K.V., Young Ch.Y.F., Tindall D.J.*: Oxidative stress and DNA methylation in prostate cancer. Obstet Gynecol Int 2010; 2010: 302051. doi:10.1155/2010/302051.
8. *Dostalek M., Brooks J.D., Hardy K.D., Milne G.L., Moore M.M., Sharma S., Morrow J.D., Guengerich F.P.*: In vivo oxidative damage in rats is associated with barbiturate response but not other cytochrome P450 inducers. Mol Pharmacol 2007; 72(6): 1419-24. doi: 10.1124/mol.107.040238.
9. *Du Y.P., Peng J.S., Sun A., Tang Z.H., Ling W.H., Zhu H.L.*: Assessment of the effect of betaine on p16 and c-myc DNA methylation and mRNA expression in a chemical induced rat liver cancer model. BMC Cancer 2009; 9: 261. doi:10.1186/1471-2407-9-261.
10. *Fielden M.R., Adai A., Dunn R.T., Olaharski A., Searfoss G., Sina J., Aubrecht J., Boitier E., Nioi P., Auerbach S., Jacobson-Kram D., Raghavan N., Yang Y., Kincaid A., Sherlock J., Chen S.J., Car B.; Predictive Safety Testing Consortium, Carcinogenicity Working Group.*: Development and evaluation of a genomic signature for the prediction and mechanistic assessment of nongenotoxic hepatocarcinogens in the rat. Toxicol Sci 2011; 124(1): 54-74. doi: 10.1093/toxsci/kfr202.
11. *Gardiner-Garden M., Frommer M.J.*: CpG islands in vertebrate genomes. J Mol Biol 1987; 196(2): 261-82.
12. *Ge R., Tao L., Kramer M. P., Cunningham M.L., Pereira M.A.*: Effect of peroxisome proliferators on the methylation and protein level of the c-myc protooncogene in B6C3F1 mice liver. J Biochem Mol Toxicol 2002; 16(1): 41-47.
13. *Ge R., Wang W., Kramer P.M., Yang S., Tao L., Pereira M.A.*: Wy-14,643-induced hypomethylation of the c-myc gene in mouse liver. Toxicol Sci 2001; 62(1): 28-35.
14. *Globisch D., Münzel M., Müller M., Michalakis S., Wagner M., Koch S., Brückl T., Biel M., Carell T.*: Tissue Distribution of 5-Hydroxymethylcytosine and Search for Active Demethylation Intermediates. PLoS ONE 2010; 5: e15367. doi:10.1371/journal.pone.0015367.
15. *Herceg Z.*: Epigenetic Mechanisms as an Interface Between the Environment and Genome. Adv Exp Med Biol 2016; 903: 3-15. doi:10.1007/978-1-4899-7678-9_1.
16. *Hernández L.G, van Steeg H., Luijten M., van Benthem J.*: Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. Mutat Res 2009; 682(2-3): 94-109. doi:10.1016/j.mrrev.2009.07.002.
17. *IARC*. Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Thyrotropic Agents. Lyon: International Agency for Research on Cancer. 2001; 79: 161-288.
18. *Jeltsch A., R.Z. Jurkowska.*: New concepts in DNA methylation. Trends Biochem Sci 2014; 39(7): 310-8. doi:10.1016/j.tibs.2014.05.002.
19. *Jin B., Robertson K.D.*: DNA methyltransferases, DNA damage repair, and cancer. Adv Exp Med Biol. 2013; 754: 3-29. doi:10.1007/978-1-4419-9967-2_1.
20. *Kostka G., Urbanek K., Ludwicki J.K.*: The effect of Phenobarbital on the methylation level of the p16 promoter region in rat liver. Toxicology 2007; 239(1-2): 127-35. doi:10.1016/j.tox.2007.07.003.
21. *Kostka G., Urbanek-Olejnik K., Wiadrowska B.*: Di-butyl phthalate-induced hypomethylation of the c-myc gene in rat liver. Toxicol Ind Health 2010; 26(7): 407-16. doi:10.1177/0748233710369124.
22. *Koturbas I., Beland F.A., Pogribny I.P.*: Role of epigenetic events in chemical carcinogenesis: a justification for incorporating epigenetic evaluations in cancer risk assessment. Toxicol Mech Methods 2011; 21(4): 289-97. doi:10.3109/15376516.2011.557881.
23. *Kutanzi K.R., Koturbash I., Bronson R.T., Pogribny I.P., Kovalchuk O.*: Imbalance between apoptosis and cell proliferation during early stages of mammary gland carcinogenesis in ACI rats. Mutat Res 2010; 694(1-2): 1-6. doi:10.1016/j.mrfmmm.2010.07.006.
24. *Lempiäinen H., Müller A., Brasa S., Teo S.S., Roloff T.C., Morawiec L., Zamurovic N., Vicart A., Funhoff E., Couttet P., Schübeler D., Grenet O., Marlowe J., Moggs J., Terranova R.*: Phenobarbital Mediates an Epigenetic Switch at the Constitutive Androstane Receptor (CAR) Target Gene *Cyp2b10* in the Liver of B6C3F1 Mice. PLoS ONE. 2011; 6:e18216. doi:10.1371/journal.pone.0018216.
25. *Luisier R., Lempiäinen H., Scherbichler N., Braeuning A., Geissler M., Dubost V., Müller A., Scheer N., Chibout S.D., Hara H., Picard F., Theil D., Couttet P., Vitobello A., Grenet O., Grasl-Kraupp B., Ellinger-Ziegelbauer H., Thomson J.P., Meehan R.R., Elcombe C.R., Henderson C.J., Wolf C.R., Schwarz M., Moulin P., Terranova R., Moggs J.G.*: Phenobarbital induces cell cycle transcriptional responses in mouse liver humanized for constitutive androstane and pregnane x receptors Toxicol Sci 2014; 139(2): 501-11. doi:10.1093/toxsci/kfu038.
26. *Miousse IR., Murphy L.A., Lin H., Schisler M.R., Sun J., Chalbot M.G., Sura R., Johnson K., LeBaron M.J., Kavouras I.G., Schnackenberg L.K., Beger R.D., Rasoulpour R.J., Koturbash I.*: Dose-response analysis of epigenetic, metabolic, and apical endpoints after short-term exposure to experimental hepatotoxicants. Food Chem Toxicol 2017;109(Pt 1):690-702. doi:10.1016/j.fct.2017.05.013.

27. *Miousse I.R., Currie R., Datta K., Ellinger-Ziegelbauer H., French J.E., Harrill A.H., Koturbash I., Lawton M., Mann D., Meehan R.R., Moggs J.G., O'Lone R., Rasoulpour R.J., Pera R.A., Thompson K.*: Importance of investigating epigenetic alterations for industry and regulators: An appraisal of current efforts by the Health and Environmental Sciences Institute. Toxicology 2015; 335: 11-9. doi:10.1016/j.tox.2015.06.009.
28. *Mohan K.N., Chaillet J.R.*: Cell and molecular biology of DNA methyltransferase 1. Int Rev Cell Mol Biol 2013; 306: 1-42. doi: 10.1016/B978-0-12-407694-5.00001-8.
29. *Nishida N., Kudo M.*: Alteration of Epigenetic Profile in Human Hepatocellular Carcinoma and Its Clinical Implications. Liver Cancer 2014; 3(3-4): 417-27. doi:10.1159/000343860.
30. *Ozden S., Turgut Kara N., Sezerman O.U., Durasi I.M., Chen T., Demirel G., Alpertunga B., Chipman J.K., Mally A.*: Assessment of global and gene-specific DNA methylation in rat liver and kidney in response to non-genotoxic carcinogen exposure. Toxicol Appl Pharmacol 2015; 289(2): 203-12. doi:10.1016/j.taap.2015.09.023.
31. *Pfaffl M.W., Horgan G.W., Dempfle L.*: Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002; 30: e36.
32. *Pfaffl M.W.* A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29: e45.
33. *Phillips J.M., Goodman J.I.*: Identification of genes that may play critical roles in phenobarbital (PB)-induced liver tumorigenesis due to altered DNA methylation. Toxicol Sci 2008; 104(1): 86-99. doi:10.1093/toxsci/kfn063.
34. *Phillips J.M., Yamamoto Y., Negishi M., Maronpot R.R., Goodman J.I.*: Orphan nuclear receptor constitutive active/androstane receptor-mediated alternations in DNA methylation during phenobarbital promotion of liver tumorigenesis. Toxicol Sci 2007; 96(1): 72-82. doi:10.1093/toxsci/kfl188.
35. *Pogribny I.P., Beland F.A.*: DNA methylome alterations in chemical carcinogenesis. Cancer Lett 2013; 334(1): 39-45. doi:10.1016/j.canlet.2012.09.010.
36. *Pogribny I.P., Rusyn I.*: Role of epigenetic aberrations in the development and progression of human hepatocellular carcinoma. Cancer Lett 2014; 342(2): 223-30. doi: 10.1016/j.canlet.2012.01.038.
37. *Pogribny I.P, Rusyn I.*: Environmental Toxicants, Epigenetics, and Cancer. In: Karpf A. (eds) Epigenetic Alterations in Oncogenesis. Advances in Experimental Medicine and Biology 2013; vol 754. Springer, New York, NY.
38. *Pogribny I.P., Tryndyak V.P., Boureiko A., Melnyk S., Bagnyukova T.V., Montgomery B., Rusyn I.*: Mechanisms of peroxisome proliferator-induced DNA hypomethylation in rat liver. Mutat Res 2008; 644(1-2): 17-23. doi:10.1016/j.mrfmmm.2008.06.009.
39. *Pogribny I.P., Tryndyak V.P., Woods C.C., Witt S.E., Rusyn I.*: Epigenetic effects of the continuous exposure to peroxisome proliferator Wy-14,643 in mouse liver are dependent upon peroxisome proliferator-activated receptor alpha. Mutat Res 2007; 625(1-2): 62-71. doi:10.1016/j.mrfmmm.2007.05.004.
40. *Stefanska B., Huang J., Bhattacharyya B., Suderman M., Hallett M., Han Z.G., Szyf M.*: Definition of the Landscape of Promoter DNA Hypomethylation in Liver Cancer. Cancer Res 2011; 71(17): 5891-903. doi: 10.1158/0008-5472doi:10.1158/0008-5472.CAN-10-3823.
41. *Stine, Z.E., Walton Z.E., Altman B.J., Hsieh A.L., Dang C.V.*: MYC, Metabolism, and Cancer. Cancer Discov 2015; 5(10): 1024-39. doi:10.1158/2159-8290.CD-15-0507.
42. *Szyf M, McGowan P., Meaney M.J.*: The Social Environment and the Epigenome. Environ Mol Mut 2008; 49(1): 46-60. doi:10.1002/em.20357.
43. *Tao L., Wang W., Li Y., Kramer P.M., Pereira M.A.:* Effect of dibromoacetic acid on DNA methylation, glycogen accumulation, and peroxisome proliferation in mouse and rat liver. Toxicol Sci 2004; 82(1): 62-69. doi:10.1093/toxsci/kfh266.
44. *Tasaki M., Umemura, Suzuki T. Y., Hibi D., Inoue T., Okamura T., Ishii Y., Maruyama S., Nohmi T., Nishikawa A.*: Oxidative DNA damage and reporter gene mutation in the livers of gpt delta rats given non-genotoxic hepatocarcinogens with cytochrome P450-inducible potency. Cancer Sci 2010; 101(12): 2525-30. doi:10.1111/j.1349-7006.2010.01705.x.
45. *Tien Y.C., Liu K., Pope C., Wang P., Ma X., Zhong X.B.*: Dose of Phenobarbital and Age of Treatment at Early Life are Two Key Factors for the Persistent Induction of Cytochrome P450 Enzymes in Adult Mouse Liver. Drug Metab Dispos 2015; 43(12): 1938-45. doi:10.1124/dmd.115.066316.
46. *Uehara T., Minowa Y., Morikawa Y., Kondo C., Maruyama T., Kato I., Nakatsu N., Igarashi Y., Ono A., Hayashi H., Mitsumori K., Yamada H., Ohno Y., Urushidani T.*: Prediction model of potential hepatocarcinogenicity of rat hepatocarcinogens using a large-scale toxicogenomics database. Toxicol Appl Pharmacol 2011; 255(3): 297-306. doi:10.1016/j.taap.2011.07.001.
47. *Urbanek-Olejnik K., Liszewska M., Kostka G.*: The effect of phenobarbital on gene expression levels of p53 and Dnmt1 in the liver of Wistar rats. Rocz Panstw Zakl Hig 2014; 65(1): 199-203.
48. *Waterman C.I., Currie R.A., Cottrell L.A., Dow J., Wright J., Waterfield C.J., Griffin J.L.*: An integrated functional genomic study of acute Phenobarbital exposure in the rat. BMC Genomic 2010; 11: 1-17. doi:10.1186/1471-2164-11-9.
49. *Zhang Y.*: Detection of epigenetic aberrations in the development of hepatocellular carcinoma. Methods Mol Biol 2015; 1238: 709-31. doi:10.1007/978-1-4939-1804-1_37.
50. *Ziech D., Franco R., Pappa A., Panayiotidis M.I.*: 2011. Reactive oxygen species (ROS)-induced genetic and epigenetic alterations in human carcinogenesis. Mutat Res 2011; 71(1-2): 167-73. doi:10.1016/j.mrfmmm.2011.02.015.

Received: 20.05.2018
Accepted: 10.07.2018