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Influence of some biologically active substances on amount of MGMT and MARP proteins in human cells *in vitro*

K. V. Kotsarenko[1], V. V. Lylo[1], L. L. Macewicz[1], T. P. Ruban[1],

Yu. S. Luchakivska [2], M. V. Kuchuk[2], L. L. Lukash[1]

[1]Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680

[2]Institute of Cell Biology and Genetic Engineering, NAS of Ukraine
148, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680

lukash@imbg.org.ua

Aim. To investigate an effect of biologically active compounds IFN-α2b, EMAPII, Card medium, fibronectin on the amount of MGMT (O⁶-methylguanine-DNA methyltransferase) and MARP (anti-Methyltransferase Antibody Recognizable Protein) proteins in human cells in vitro. **Methods***. The human cells of 4BL, Hep-2 and A102 lines were treated with growth factors and cytokines. Changes in the amount of MGMT and MARP proteins were studied by Western blot analysis with anti-MGMT mAbs.* **Results***. The treatment of A102 cells with EMAPII, fibronectin, Laferon and Card medium led to a decreased level of the MGMT protein, whereas the amount of MARP was highly increased in these cells. The treatment with the recombinant protein IFN-α2b increased the amount of MGMT and MARP proteins in Hep-2 cells. The treatment with extracts of transgenic plants,containing human IFN-α2b, caused a significant decrease in the content of both proteins in Hep-2 cells and MARP in 4BL cells.* **Conclusions***. Both MGMT and MARP are highly inducible proteins. Their amount in cells can be changed by some growth factors (Card medium, fibronectin), cytokine (IFN-α2b), cytokine-like (EMAPII) or cytokine-containing substances (Laferon and IFN-α2b in plant extracts). This regulation depended not only on the type of biologically active substances but on the cell line used in this study as well.*

Keywords: human cell lines, repair enzyme MGMT, MARP, cytokines, growth factors, Western blot analysis.

Introduction. The repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) removes alkyl adducts from the O^6-position of guanine in DNA. It is the major defense factor against the mutagenic, carcinogenic and cytotoxic effects of alkylating agents [1, 2]. MGMT protects both normal cells from endogenous and exogenous carcinogens and tumor cells from chemotherapeutic alkylating compounds. Therefore, this enzyme is considered to be one of the targets to regulate antitumor efficacy of the alkylating agents [3].

According to the literature data the expression of *MGMT* gene may be affected by various factors: the al-

kylating agents, single-strand DNA breaks, transcription factors, activators of protein kinase C, the *MGMT* gene promoter hypermethylation, p53 protein *etc*. [4, 5]. There are some works where cytokines have been also shown to be able to influence the *MGMT* gene expression. For example, IFN-β decreased the *MGMT* gene expression in human glioma and neuroblastoma cells *in vitro* and increased the sensitivity of these cells to the antitumor alkylating agent temozolomide [6, 7]. IL-24 also reduced the level of the *MGMT* gene expression in the human melanoma cells in dose-dependent manner [8]. A combined action of two cytokines IL-1β and IFN-γ increased the level of this gene expression in the rat β-cells *in vitro* [9]. Nowadays, application of regulation

of the *MGMT* gene expression with some cytokines is launched in clinical practice. Complex treatment of the patients, who had newly diagnosed primary glioblastoma multiforme, with cytokine IFN-β and temozolomide has resulted in favorable outcome, particularly in cases when the tumor cells contained the unmethylated *MGMT* promoter [10]. So the combined action of cytokines, which reduced the level of *MGMT* gene expression, and antitumor alkylating agents improves their efficiency in both cell lines and humans.

The aim of our study was to investigate the effect of biologically active compounds such as IFN-α2b, EMAP II, Card medium, fibronectin, on the amount of MGMT and MARP proteins in the human cells *in vitro*.

Recombinant interferon α2b (IFN-α2b) is widely used in oncology due to its well-known antitumor activity [11]. Endothelial-monocyte activating polypeptide II (EMAP II), whose properties are not completely understood, also exhibits the anticancer activity [12]. Since the *MGMT* gene expression is known to change during cell differentiation [13], such factors as Card medium, ATP and fibronectin, which take part in the process of cell differentiation, were also investigated in this study.

Biological properties of the substances mentioned above have been known for a long time, but their possible role in regulation of the *MGMT* gene expression has not been studied yet.

Materials and methods. The following human cells were used in this study: 4BL – fibroblast-like cell line derived in our laboratory [14], standard Hep-2 cell line (laryngeal cancer) and A102 cell line (skin fibroblasts), kindly provided by Prof. McCormick (Michigan State University, The United States).

The cells were cultivated in standard DMEM («PAA», USA) with 10 % FBS («Sigma», USA) and antibiotics penicillin (0.02 %) and streptomycin (0.02 %) at 37 °C with 4 % CO_2.

Different commercial and non-commercial biologically active substances were used. IFN-α2b was used as purified recombinant protein («Interpharmbiotek», Ukraine), in the form of commercial Laferon preparation («Interpharmbiotek») and as a component of the crude extract of transgenic carrot plants (*Daucus carota* L., Nantskaya and Perfektsiya varieties). The transgenic carrot plants expressing IFN-α2b were obtained *via*

Agrobacterium tumefaciens-mediated transformation using two vector constructs containing: i) the sequences encoding interferon gene fused with *Nicotiana plumbagenifolia* calreticulin apoplast targeting signal driven by 35S CaMV promoter and selective neomycin phosphotransferase II (*nptII*) gene in order to obtain the transgenic carrot plants able to accumulate human IFN-α2b protein; ii) the sequence encoding *gusA* gene driven by 35S CaMV promoter and the selective *nptII* gene for obtaining the transgenic carrot plants that we used as control ones in our study – so-called «empty» vector. According to the previous studies the transgenic carrot plants were characterized by high level of the recombinant human IFN-α2b protein accumulation. The protein extracts of these plants were characterized by a high antiviral activity [15].

The purified recombinant protein EMAPII was kindly provided by Prof. A. I. Korneluk (IMBG, Ukraine). «Card differentiating» medium supplemented with growth factors of different origin was developed and used in our laboratory for the differentiation of stem cells into cardiomyocytes [16]. The commercial preparations Fibronectin and ATP were received from «Sigma».

We used the chemical agents 5-azacytidine and mitomycin C. The conditions of cell treatment with the biologically active substances in the serum-free culture medium were described previously [17].

Cell lysates were prepared according to [18]. SDS-PAGE (12 % gel) was performed using Laemmli method [19]. The concentration of total protein in cell lysates was measured colorimetrically according to Bradford method [20].

Monoclonal anti-MGMT antibodies (clone 23.2, isotype Ig G2b) were obtained from Novus Biologicals (USA), secondary antibodies conjugated with horseradish peroxidase were obtained from «Sigma». The procedure of MGMT identification in the samples was performed by Western blot analysis according to the methodological instructions of the manufacturer of monoclonal antibodies (http://www.novusbio.com/ support/ protocols/protocol-specific-for-mgmt-antibody-nb100-168.html). Densitometry of stained membranes by Scion-Image 4.0.2 and Origin 8.1 programs was used as loading control [21, 22].

Results and discussion. The human MGMT protein was shown to have molecular weight of 22–24 kDa
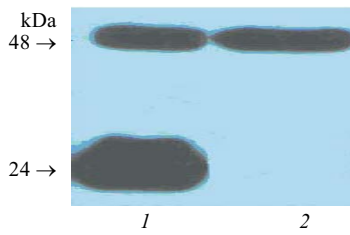
Fig. 1. Western blot analysis of *MGMT* gene expression in cells Hep-2 (*1*) and 4BL (*2*)
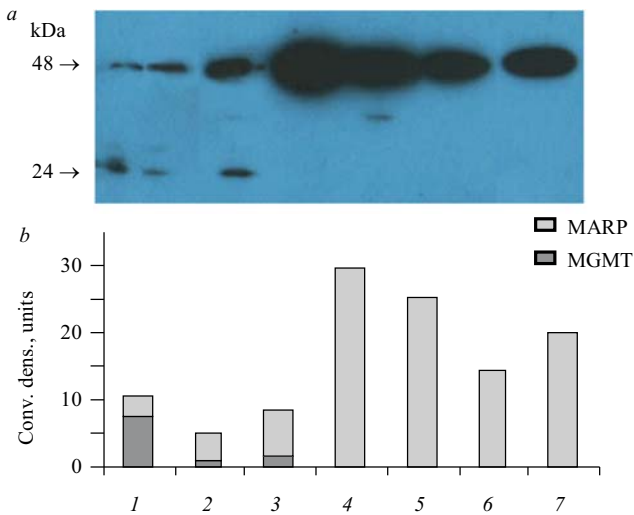

Fig. 2. Effect of different biologically active substances on the amount of MGMT and MARP proteins in A102 cells: *a* – Western blot analysis (*1* – Hep-2, positive control; *2* – A102– control; *3* – A102 + ATP (20 μg/ml); *4* – A102 + Card medium; *5* – A102 + EMAP II (10 μg/ml); *6* – A102 + Laferon (20 IU/ml); *7* – A102 + fibronectin (10 μg/ml)); *b* – results of densitometry. The vertical line represents the level of MGMT and MARP proteins amount in conventional densitometry units

[1]. However, the Western blot analysis with monoclonal anti-MGMT antibodies (clone 23.2) revealed two highly specific immunoreactive bands – 24 kDa (classic MGMT protein) and 48 kDa (anti-Methyltransferase Antibody Recognizable Protein or MARP) (Fig. 1). It should be noted that in our previous works MARP was named as a modified form of MGMT, while the classic 24 kDa protein was named as an unmodified form of MGMT [17, 23]. We proposed several hypotheses about the nature of MARP, namely, post-translation modifications, dimerization of MGMT, *etc.*, which have been discussed in the mentioned articles. So far there is an open question regarding the nature of the 48 kDa protein.

In this work we have compared the effect of various exogenous biologically active substances on the amount of both MGMT and MARP proteins in the human cell lines. The results of the A102 cells treatment with Card medium, EMAPII, Laferon, fibronectin and ATP are shown in the Fig. 2. The treatment of A102 cells with different exogenous factors led to the dramatic changes of the amount of MGMT and MARP proteins. Hep-2 cells were used as positive control in Western blots, because they express both types of proteins (Fig. 1).

Almost all of the studied factors (except ATP) decreased the level of MGMT protein amount in the A102 cells (lanes *4–7*) compared with the control A102 cells (lane *2*). The treatment with ATP and to a greater extent with Card medium (lane 4), EMAP II (lane *5*), Laferon (lane *6*) and fibronectin (lane *7*) led to an increased amount of MARP.

In this work we studied the influence of purified recombinant protein IFN-α2b and the extract of transgenic carrot tissues, expressing human IFN-α2b, on the levels of MGMT and MARP proteins in Hep-2 and 4BL cell lines.

As shown in Fig. 3, the treatment of Hep-2 cells with the human recombinant protein IFN-α2b led to slight increase of the amount of MGMT and MARP proteins (lanes *2* and *3*). No significant changes in the amount of MARP were detected in 4BL cells. In contrast to these data, the results presented in Fig. 4 show a significant decrease of the MGMT and MARP proteins amount in Hep-2 cells as well as the MARP amount in 4BL cells after their treatment with the extracts of transgenic plant tissues, expressing human IFN-α2b. On the other hand the amount of MGMT in Hep-2 cells and MARP in both cell lines did not significantly change after the treatment with the extract of plants without interferon gene.

We suppose that different effects of the purified recombinant protein IFN-α2b and the interferon-containing plant extract on the MGMT and MARP proteins amount may be caused by the influence of some plant extract components on the action of interferon.

Thus, the results presented in Fig. 2–4 show that the effect of interferon preparations may differ depending on the composition of these preparations and the type of cell lines. There are several hypotheses about the mechanisms of modulating the *MGMT* gene expression by cytokines. According to one of them the p53 protein is involved in regulation of the MGMT transcription by cy-
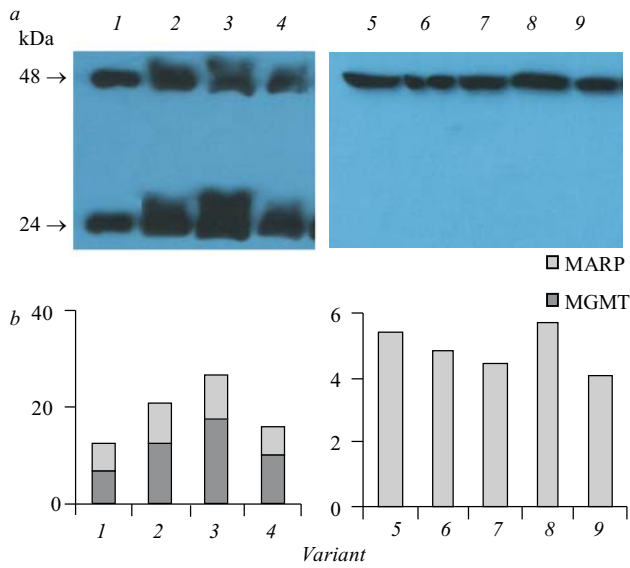
Fig. 3. The effect of purified recombinant protein IFN-a2b on the MGMT and MARP proteins amount in the human cell lines: *a* – Western blot analysis (*1* – Hep-2, control; *2* – Hep-2 + IFN-a2b (2000 IU/ml); *3* – Hep-2 + IFN-a2b (200 IU/ml); *4* – Hep-2 + IFN-a2b (2 IU/ml); *5* – 4BL, 182 p., control; *6* – 4BL + IFN-a2b (2000 IU/ml); *7* – 4BL + IFN-a2b (200 IU/ml); *8* – 4BL + IFN-a2b (20 IU/ml); *9* – 4BL + IFN-a2b (2 IU/ml)); *b* – results of densitometry. The vertical line represents the amount of MGMT and MARP proteins in conventional densitometry units
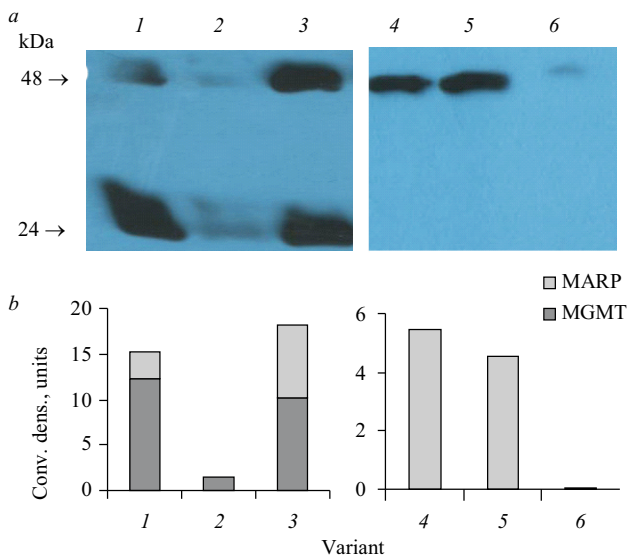

Fig. 4. The effect of extract of transgenic carrot on MGMT and MARP proteins amount in the human cell lines: *a* – Western blot analysis (Hep-2: *1* – control; *2* – extract of transgenic carrot plants, containing human IFN-a2b (600 IU/ml); *3* – extract of transgenic carrot cells transfected with an «empty» vector; 4BL: *4* – 182 p., control; *5* – extract of transgenic carrot cells transfected with an «empty» vector; *6* – extract of transgenic carrot plants, containing human IFN-a2b (600 IU/ml)); *b* – results of densitometry. The vertical line represents the level of MGMT and MARP proteins in conventional densitometry units
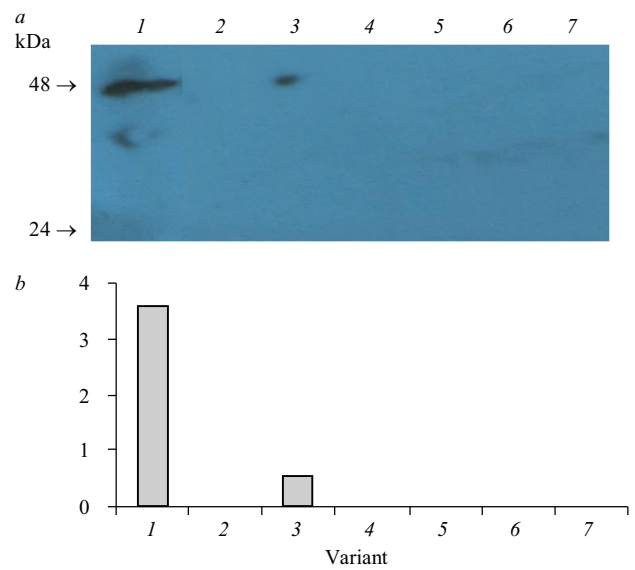

Fig. 5. The effect of 5-azacytidine on the MARP protein amount in the 4BL cells (180 p.): *a* – Western blot analysis (*1* – control; *2* – mitomycin C + 5-aza (10 µg/ml), 8 h; *3* – 5-aza (1 µg/ml), 11 days; *4* – 5-aza (10 µg/ml), 6 days; *5* – 5-aza (10 µg/ml), 11 days; *6* – mitomycin C + 5-aza (1 µg/ml), 6 days; *7* – mitomycin C + 5-aza (10 µg/ml), 6 days); *b* – results of densitometry. The vertical line represents the MGMT and MARP proteins amount in conventional densitometry units

tokines [6, 8]. Another one suggests that the transcriptional factor NF-κB is activated by cytokines and affects the transcription of target genes including MGMT [24, 25]. We plan to continue our research of involving these transcriptional factors in regulation of the *MGMT* gene expression under the influence of the studied cytokines.

We presume that the gene encoding MARP protein does not belong to the housekeeping genes because its expression can be regulated by various exogenous factors. The Hep-2 cells usually have both proteins (Fig. 1, lane *1*) as well as the 4BL cells in early passages [26]. However, after prolonged cultivation (more than 130 passages) the 4BL cells have lost the conventional MGMT protein (Fig. 1, lane *2*). According to the literature data, hypermethylation of the *MGMT* gene promoter commonly occurs causing a gene silencing in both different cell lines and human cells *in vivo* [1, 5, 10]. Therefore in our experiments 5-azacytidine was selected as a demethylating agent that can affect the *MGMT* gene expression in the MGMT-deficient 4BL cells (Fig. 5). 5-azacytidine was the first identified demethylating agent that inhibits DNA methyltransferases and reverses DNA hypermethylation, restoring the expression of silenced genes [27]. The mechanisms involved in the 5-azacyti-

dine cytotoxic effects include inhibiting DNA, RNA and protein synthesis, drug incorporation into DNA and RNA, as well as the activation of DNA damage pathways. Since the non-proliferating cells are practically insensitive to azacytidine [28], mitomycin C was used in this study as a cytostatic agent for the inhibition of cell proliferation [29].

As shown in Fig. 5, the treatment of 4BL cells with 5-azacytidine alone or in the combination with mitomycin C did not influence the MGMT protein amount. These results may indicate that the absence of the conventional MGMT protein in the 4BL cells is not associated with the promotor hypermethylation. However, the MARP amount significantly changed under the 5-azacytidine alone or in combination with mitomycin C treatment. This fact requires further investigation.

Conclusions. Monoclonal anti-MGMT antibodies (clone 23.2) recognize both the MGMT protein (M. w. ~ 24 kDa) and the unknown protein (M. w. ~ 48 kDa), named as MARP, in Western blot analysis. These proteins are highly inducible and their amount can be changed by some growth factors (Card medium, fibronectin), cytokine (IFN-α2b), cytokine-like (EMAP II) or cytokine-containing substances (Laferon and IFN-α2b in plant extracts). However, this regulation depends not only on the type of biologically active substances but on the type of cell lines. The absence of MGMT protein in 4BL cells is unlikely due to the *MGMT* gene promotor hypermethylation. The mechanisms of regulation of the *MGMT* gene expression by growth factors and cytokines require further studies.

К. В. Коцаренко, В. В. Лило, Л. Л. Мацевич, Т. П. Рубан, Ю. С. Лучаківська, М. В. Кучук, Л. Л. Лукаш

Вплив деяких біологічно активних речовин на вміст білків MGMT і MARP у клітинах людини *in vitro*

Резюме

Мета. Дослідження впливу біологічно активних сполук IFN-α2b, ЕМАРII, середовища Card і фібронектину на вміст білків MGMT (О⁶-метилгуанін-ДНК метилтрансфераза) і MARP (білок, що розпізнається анти-MGMT антитілами) у клітинах людини in vitro. Методи. Клітини людини ліній 4BL, Hep-2 і А102 обробляли ростовими факторами і цитокінами. Зміни в кількості білків MGMT і MARP досліджували з використанням Вестерн блот аналізу і моноклональних анти-MGMT антитіл. Результати. Обробка клітин А102 препаратами ЕМАРII, фібронектину і Лаферону призводить до зниження кількості білка MGMT на фоні значного зростання кількості білка MARP у цих клітинах. Обробка рекомбінантним білком IFN-α2b підвищує кількість білків MGMT і MARP у

клітинах Hep-2, а екстрактами трансгенних рослин, які містять IFN-α2b людини, – зменшує кількість обох білків у клітинах Hep-2 та білка MARP у клітинах 4BL. Висновки. MGMT і MARP є високоіндуцибельними білками. Їхня кількість може варіювати під дією деяких ростових факторів (середовище Card і фібронектин), цитокіну (IFN-α2b), цитокіноподібного (ЕМАРII) та цитокіновмісних (Лаферон і IFN-α2b у композиції з рослинними екстрактами) препаратів. Виявлена регуляція залежить не лише від типу біологічно активних речовин, але й від клітинних ліній, використаних в експериментах.

Ключові слова: лінії клітин людини, репаративний фермент MGMT, MARP, цитокіни, ростові фактори, Вестерн-блот аналіз.

Е. В. Коцаренко, В. В. Лыло, Л. Л. Мацевич, Т. А. Рубан, Ю. С. Лучаковская, Н. В. Кучук, Л. Л. Лукаш

Влияние некоторых биологически активных веществ на содержание белков MGMT и MARP в клетках человека *in vitro*

Резюме

Цель. Исследовать влияние биологически активных соединений IFN-α2b, ЕМАР II, среды Card и фибронектина на содержание белков MGMT (О⁶-метилгуанин-ДНК метилтрансфераза) и MARP (белок, распознаваемый анти-MGMT антителами) в клетках человека in vitro. Методы. Клетки человека линий 4BL, Hep-2 и А102 обрабатывали ростовыми факторами и цитокинами. Изменения в количестве белков MGMT и MARP исследовали с использованием Вестерн блот анализа и моноклональных анти-MGMT антител. Результаты. Обработка клеток А102 препаратами ЕМАРII, фибронектина, Лаферона и среды Card снижает количество белка MGMT на фоне значительного возрастания количества белка MARP в этих клетках. Обработка рекомбинантным белком IFN-α2b увеличивает количество белков MGMT и MARP в клетках Hep-2, а экстрактами трансгенных растений, содержащих человеческий IFN-α2b, – существенно снижает количество обоих белков в клетках Hep-2 и MARP в клетках 4BL. Выводы. MGMT и MARP являются высокоиндуцибельными белками. Их количество может варьировать под действием некоторых ростовых факторов (среда Card и фибронектин), цитокина (IFN-α2b) цитокиноподобного (ЕМАР II) и цитокинсодержащих (Лаферон и IFN-α2b в композиции с растительными экстрактами) препаратов. Выявленная регуляция зависит не только от типа биологически активных веществ, но и от клеточных линий, использованных в экспериментах.

Ключевые слова: линии клеток человека, репаративный фермент MGMT, MARP, цитокины, ростовые факторы, Вестерн-блот анализ.

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