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Influence of a B16/F10 melanoma variant on the Bcl-2 levels in mitochondria in various organs of female mice

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ABSTRACT

Aim. To study the Bcl-2 level in mitochondria of various organs in female mice with standard and stimulated growth of an experimental B16/F10 melanoma.

Materials and methods. The study included C57BL/6 female mice (n = 168). The experimental animals were divided into the following groups: an intact group (n = 21), a group with modelled chronic neuropathic pain (CNP) (n = 21), an M group with B16/F10 melanoma (n = 63), and a CNP + M group (n = 63). The Bcl-2 concentration (ng / mg protein) in mitochondrial samples was determined by ELISA (Thermo Fisher Scientific, Austria). Statistical analysis of the results was carried out using Statistica 10.0.

Results. Compared to the Bcl-2 levels in the intact animals, CNP decreased this parameter in the cardiac mitochondria by 1.3 times, while increasing it by 5.9 times in the skin mitochondria. In the dynamics of standard melanoma growth, the Bcl-2 content changed compared with the corresponding intact values in the mitochondria of the brain, heart, and skin, but did not change in the liver and kidneys. In the mitochondria in melanoma, the Bcl-2 levels were high throughout the entire period of standard tumor growth in comparison with the intact skin. The stimulated melanoma growth in CNP was involving more organs into the pathological process as the tumor was growing. Thus, in comparison with the values in the CNP group, the mitochondrial Bcl-2 levels changed in the heart at week 1; in the heart and skin – at week 2; in the heart, skin, and brain – at week 3. The Bcl-2 levels did not change in the liver and kidney mitochondria. In the mitochondria in the CNP-stimulated melanoma, the Bcl-2 levels were lower than in the skin mitochondria in CNP throughout the entire tumor growth period.

Conclusion. The liver and kidney mitochondria are somewhat Bcl-2 stable in both standard and stimulated tumor growth. It is assumed that different Bcl-2 dynamics in the mitochondria in melanoma depending on the variant of tumor development reflects the modulating effect of CNP and the ability to change the Bcl-2 levels according to the growth phase.

Key words: cell mitochondria, Bcl-2, chronic neuropathic pain, experimental B16/F10 melanoma, female mice.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. The study was approved by the Bioethics Committee for Working with Animals of the NMRCO of the Ministry of Health of the Russian Federation (Protocol No. 2 of 31.05.2018).

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Влияние варианта развития меланомы B16/F10 на содержание Bcl-2 в митохондриях клеток различных органов самок мышей

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РЕЗЮМЕ

Цель – изучить содержание Bcl-2 в митохондриях различных органов самок мышей при стандартном и стимулированном росте экспериментальной меланомы B16/F10.

Материалы и методы. Работа выполнена на самках мышей линии C57BL/6 (n = 168), которых разделили на группы: интактную (n = 21), группу с воспроизведением модели хронической нейрогенной боли (ХНБ) (n = 21), группу «М» – меланома B16/F10 (n = 63), группу «ХНБ + М» (n = 63). В митохондриальных образцах методом иммуноферментного анализа определяли концентрацию Bcl-2 в нг/мг белка (Thermo Fisher Scientific, Австрия). Статистическая обработка результатов проводилась с использованием пакета прикладных программ Statistica 10.0.

Результаты. По сравнению со значениями Bcl-2 у интактных животных, ХНБ способствовала снижению данного показателя в митохондриях сердца в 1,3 раза, а в митохондриях кожи, напротив, повышала в 5,9 раз. В динамике стандартного роста меланомы содержание Bcl-2 изменялось относительно соответствующих интактных величин в митохондриях мозга, сердца, кожи, при этом не менялось в печени и почках. В митохондриях меланомы уровень Bcl-2 по сравнению с интактной кожей был высоким на всем протяжении стандартного роста опухоли. Стимулированный рост меланомы при ХНБ вовлекал в патологический процесс органы, количество которых увеличивалось по мере развития опухоли. Так, по сравнению со значениями в группе ХНБ изменение уровня Bcl-2 на 1-й нед роста фиксировали в митохондриях сердца, на 2-й – в сердце и коже, на 3-й нед – в сердце, коже и мозге. Не изменялся показатель в митохондриях печени и почек. В митохондриях меланомы, стимулированной ХНБ, уровень Bcl-2 на протяжении всего роста опухоли был ниже, чем в митохондриях кожи при ХНБ.

Заключение. Выявлено, что митохондрии клеток печени и почек обладают определенной стабильностью по Bcl-2 как при стандартном развитии опухолевого процесса, так и при стимулированном. Полагаем, что различная динамика Bcl-2 в митохондриях клеток меланомы в зависимости от варианта развития опухоли свидетельствует о модулирующем эффекте ХНБ и способности менять уровень показателя в зависимости от фазы роста.

Ключевые слова: митохондрии клеток, Bcl-2, хроническая нейрогенная боль, экспериментальная меланома B16/F10, самки мышей.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено в рамках государственного задания.

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INTRODUCTION

Resistance to apoptosis is a confirmed marker of cancer. Different sensitivity of tumors and healthy tissues to apoptosis is a more complex problem than simply the fact that cancer cells are more resistant to death. A deeper understanding of apoptosis, which has developed over the past decades, now opens up new opportunities for identifying agents capable of triggering apoptosis in carcinogenesis [1].

The concept of selective activation of cell death in malignant tumors has a long history. Programmed cell death can occur through a number of mechanisms,

including apoptosis and necroptosis regulated by various signaling pathways, for instance, the mitochondrial pathway of apoptosis. Several of these signaling pathways for apoptotic cell death have been used with varying success as potential therapeutic targets, which resulted in disappointing early clinical findings due to an incomplete understanding of the fundamentals of biology [2, 3]. The search for and use of agents with selective action on mitochondria is proceeding even more carefully, since it is necessary to take into account the specifics of rearrangement of both mitochondria and mitochondrial metabolism of a transformed cell, as well as (or) activation of energy metabolism by reprogrammed mitochondria [4].

The understanding that Bcl-2 overexpression promoted oncogenesis by inhibiting programmed triggering of cell death helped to identify a family of apoptosis regulators. Moreover, it proved evasion of apoptosis to be the main sign of cancer [5]. Currently, about 20 Bcl-2 family members with pro- and anti-apoptotic features have been found in vertebrates. It was also determined that many interactions between these proteins play a central role in a response of healthy and malignant cells to cytotoxic damage [6].

Apoptotic priming in healthy cells and tissues dynamically changes at several levels. This change can be observed between different tissues of an adult human, within the same tissue at different stages of development, between separate cells in the tissue, and in the same cell at different time, as a cell can be damaged or changed in terms of signaling. The existing fluctuations in the levels of pro- and anti-apoptotic proteins create certain difficulties when acting on neoplastic cells. Moreover, it is necessary to simultaneously minimize the damage to cells of healthy tissues [1].

When apoptosis is activated during embryonic development, changes in the Bcl-2 function are associated with different developmental periods. Thus, in the developing nervous system of mice, the neuronal Bcl-2 levels significantly reduce by the age of 5 months. The Bcl-2 family members, Bcl-XL and Mcl-1, play a key role in the survival of immature B cells during development, while Bcl-2 is necessary for the survival of mature B cells [7]. A recent study showed that minor changes in the balance of pro- and anti-apoptotic Bcl-2 proteins have profound effects during craniofacial development [8]. Many signaling pathways that control cell proliferation and the Bcl-2 protein function also determine heterogeneity of separate cells in their activity [9].

Therefore, it should not be surprising that apoptosis is dependent on intercellular heterogeneity. Separate cells can rapidly shift their apoptotic threshold in response to changes in internal and external signals [10]. One of the ways in which this can happen is related to the fact that Bcl-2 proteins continuously move between cytosol and mitochondria. Under stress, the transmitted signals result in cell buffering with the Bcl-2 protein above the permissible levels [10]. The apoptotic threshold of separate cells is dynamic; the cell population reaches a balance between sensitivity and resistance to proapoptotic stimuli [11]. The level of apoptotic signal stimulation varies considerably within and between cell populations [12]. Thus, affecting malignant cells through apoptosis by disrupting protein interactions of Bcl-2 in mitochondria is complex. Currently, the existing knowledge does not reveal all the intricacies of this interaction, which requires further research in this area.

The aim of this study was to determine the Bcl-2 content in mitochondria of various organs of female mice in standard and stimulated growth of an experimental B16/F10 melanoma.

MATERIALS AND METHODS

The experimental study was carried out on female mice of the C57BL/6 line (n = 168), aged 8 weeks and having an initial weight of 21–22 g. The animals were randomly divided into the following experimental groups: an intact group C (n = 21), a CNP group with a model of chronic neuropathic pain (CNP) (n = 21), an M group with a B16/F10 melanoma (n = 63) with standard subcutaneous transplantation of the B16/F10 melanoma, and a CNB + M group (n = 63), where the B16/F10 melanoma was transplanted 3 weeks after creating the CNP model.

The experimental animals were obtained from the Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency (Andreevka Branch, the Moscow Region). The strain of the B16/F10 murine melanoma was provided by N.N.Blokhin National Medical Research Center for Oncology of the Ministry of Health of the Russian Federation. Work with animals was carried out in accordance with the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Directive 86/609/EEC) and the Declaration of Helsinki. The researchers also adhered to the International Guiding Principles for Biomedical Research Involving Animals and the Order of the Ministry of

Health of the Russian Federation No. 267 "On approval of laboratory practice rules" of 19.06.2003.

The animals were kept under natural lighting with free access to water and food. The manipulations with animals were performed in an isolation room in compliance with the generally accepted rules of aseptics and antiseptics. The study was approved by the Bioethics Committee for Working with Animals of the NMRCO of the Ministry of Health of the Russian Federation (Protocol No. 2 of 31.05.2018).

The B16/F10 melanoma was transplanted into the animals by a standard subcutaneous injection of a 0.5 ml tumor cell suspension diluted 1 : 10 in saline under the right scapula. The model of chronic neuropathic pain (CNP) was reproduced by ligating the sciatic nerve on both sides under Zoletil – xylazine anesthesia [13]. The following anesthesia was used: Zoletil with xylazine administered 10 minutes before the main anesthesia; premedication: xylazine (the drug "Xila") intramuscularly at a dose of 0.05 ml / kg of body weight (according to the instructions), followed by Zoletil-50 at a dose of 10 mg per 100 g of body weight 10 minutes later.

The animals of the M group and the CNP + M group were decapitated by a guillotine after transplantation of the B16/F10 melanoma according to the following schedule: week 1 – day 7 of the melanoma growth, week 2 – day 14 of the melanoma growth, week 3 – day 21 of the melanoma growth. The animals of the CNP group were sacrificed 3 weeks after the CNP model reproduction, in parallel, the intact animals were decapitated. After decapitation, the skin and tumor were dissected from the animals, their brain, liver, kidneys, and heart were also removed. The apparently healthy skin was dissected at the maximum distance from the tumor node. The mitochondria were isolated according to the method proposed by M.V. Egorova and S.A Afanasyev [14] (using cooling agents and differential centrifugation in a high-speed refrigerated Avanti J-E centrifuge (Beckman Coulter Life Sciences, USA).

Before the analysis, the obtained mitochondrial samples (with the protein concentration of 4–6 g / l) were stored at –80 °C in the isolation medium. In the mitochondrial samples, the Bcl-2 concentration in ng / mg protein (Thermo Fisher Scientific, Austria) was determined using an ELISA analyzer (Infinite F50 Tecan, Austria), and the protein concentration in mg / ml was determined by the biuret method (Olvex Diagnosticum, Russia) using a ChemWell automatic analyzer (Awareness Technology Inc., USA).

Statistical analysis of the results was carried out using Statistica 10.0 software. The obtained data were analyzed for the correspondence to the normal distribution law using the Shapiro – Wilk test (for small samples). The comparison of the quantitative data in the groups (independent samples) was performed using the Kruskal – Wallis test (multiple comparisons). The data in the tables were presented as $M \pm m$, where M is the arithmetic mean, and m is the standard error of the mean. The results were considered statistically significant at $p < 0.05$. The obtained results were statistically processed in accordance with the general recommendations for medical studies.

RESULTS

The results of the experiment studying the Bcl-2 level in the mitochondria of various organs and the B16/F10 melanoma are presented in the table.

Table

Dynamics of the Bcl-2 level in organ mitochondria of female mice with standard and stimulated growth of the B16/F10 melanoma, ng / mg protein, $M \pm m$

Parameter	Organ					Tumor
	brain	liver	heart	kidneys	skin	
Intact group (C)	82.937 ± 2.455	96.335 ± 4.561	62.00 ± 2.85	103.079 ± 4.033	5.56 ± 0.203	–
CNP	107.391 ± 3.738[1] $p^1 = 0.00014$	107.348 ± 3.806	46.66 ± 3.87[1] $p^1 = 0.00008$	103.573 ± 3.712	32.69 ± 1.37[1] $p^1 = 0.00000$	–
M, week 1	107.392 ± 3.787[1] $p^1 = 0.00015$	112.189 ± 4.405	36.85 ± 1.92[1] $p^1 = 0.00000$	108.786 ± 4.136	49.61 ± 1.71[1] $p^1 = 0.00000$	24.22 ± 1.51[1] $p^1 = 0.00000$
M, week 2	49.476 ± 2.689[1, 3] $p^1 = 0.00000$ $p^3 = 0.00000$	103.349 ± 3.766	21.38 ± 1.56[1, 3] $p^1 = 0.00000$ $p^3 = 0.00004$	109.584 ± 4.129	15.46 ± 1.41[1,3] $p^1 = 0.00001$ $p^3 = 0.00000$	28.58 ± 1.95[1] $p^1 = 0.00000$
M, week 3	31.688 ± 3.172[1, 3] $p^1 = 0.00000$ $p^3 = 0.00107$	103.384 ± 3.413	21.6 ± 1.55[1] $p^1 = 0.00000$	113.318 ± 5.042	1.35 ± 0.11[1,3] $p^1 = 0.00000$ $p^3 = 0.00000$	13.88 ± 1.36[1,3] $p^1 = 0.00005$ $p^3 = 0.00004$
CNP + M, week 1	104.265 ± 3.849	102.353 ± 3.805	80.99 ± 1.6[2] $p^2 = 0.00000$	113.928 ± 4.982	27.28 ± 1.77	11.02 ± 1.18[2] $p^2 = 0.00000$

Table (continued)

Parameter	Organ					umor
	brain	liver	heart	kidneys	skin	
CNP + M, week 2	98.069 ± 5.023	98.077 ± 3.501	24.03 ± 1.38[2, 3] $p^2 = 0.00000$ $p^3 = 0.00000$	86.933 ± 13.574	19.08 ± 1.38[2] $p^2 = 0.00001$	20.83 ± 1.42[2,3] $p^2 = 0.00006$ $p^3 = 0.00018$
CNP + M, week 3	44.994 ± 3.833[2, 3] $p^2 = 0.00000$ $p^3 = 0.00000$	89.653 ± 10.933	19.35 ± 1.09[2, 3] $p^2 = 0.00000$ $p^3 = 0.00207$	103.412 ± 3.715	14.92 ± 1.38[2] $p^2 = 0.00000$	21.65 ± 1.68[2] $p^2 = 0.00026$

Note: C – intact group; CNP – group with the CNP model; M – group with the B16/F10 melanoma; CNP + M – group with the B16/F10 melanoma which was transplanted 3 weeks after the reproduction of the CNP model.
Statistically significant compared with the parameters: [1] – in the intact group; [2] – in the corresponding control group (CNP group); [3] – in the previous study period.

When studying Bcl-2 in the organ mitochondria of the intact mice, it was revealed that the skin mitochondria showed the minimum Bcl-2 level. The same parameter was 11.2 times higher in the heart mitochondria than in the skin mitochondria. The Bcl-2 levels in the mitochondria of the brain, liver, and kidneys were approximately equal and on average 16.9 times higher than in the skin mitochondria.

It was determined that CNP in female mice did not have a significant effect on the change in the Bcl-2 level in the liver and kidney mitochondria in contrast to the corresponding values in the organs of the intact animals (the C group). At the same time, CNP caused a 1.3-fold decrease in Bcl-2 level in the heart mitochondria. In the brain and skin mitochondria, on the contrary, there was a 1.3-fold ($p < 0.05$) and 5.9-fold increase, respectively.

After week 1 of melanoma growth (the M group), the mitochondria of the heart, brain, and skin demonstrated changes in the Bcl-2 level. In the heart mitochondria, the parameter decreased by 1.7 times ($p < 0.05$), while the brain and skin mitochondria showed a 1.3-fold ($p < 0.05$) and 8.9-fold increase compared with the values in the mitochondria of the corresponding organs in the intact animals (the C group). No statistically significant changes were found in the liver and kidney mitochondria. After 2 weeks of tumor growth, the brain mitochondria showed a 1.7-fold decrease ($p < 0.05$) in the Bcl-2 level in contrast to the corresponding parameter in the intact animals and a 2.2-fold decrease compared to the previous study period.

During this period of the study, a 1.7-fold decrease ($p < 0.05$) and a 3.2-fold decrease in the Bcl-2 level in the heart and skin mitochondria were registered in contrast to the corresponding values in the previous study period. No statistically significant changes in the liver and kidney mitochondria were observed. After 3 weeks of melanoma growth, the Bcl-2 concentration in the brain mitochondria decreased by 1.5 times ($p < 0.05$) compared with the previous study period and was 2.6 times lower than in the intact mice. In this study period, the Bcl-2 level in the heart mitochondria did not differ from the values of the previous study period and was 2.9 times lower than the values in the intact animals. A drastic drop in the content of Bcl-2 compared with the previous study period was registered in the skin mitochondria after 3 weeks of tumor growth. The Bcl-2 level decreased by 11.5 times and became 4.1 times lower than that in the intact mice. The parameter in the liver and kidney mitochondria did not change.

In the melanoma mitochondria of this group, the Bcl-2 concentration during weeks 1, 2, and 3 was, respectively, 4.4 times, 5.1 times, and 2.5 times higher than in the skin mitochondria of the intact mice. After 1 week of melanoma growth, the mice with CNP (the CNP + M group) demonstrated changes in the Bcl-2 level in the heart mitochondria only – a 1.7-fold increase ($p < 0.05$) in contrast to the corresponding control value (the CNP group). During this study period, no changes in the Bcl-2 level were detected in the brain, liver, kidney, and skin mitochondria. After 2 weeks of melanoma growth in CNP, the Bcl-2 content became 1.9 times lower in the heart mitochondria and 1.7 times ($p < 0.05$) lower in the skin mitochondria than the corresponding values in the CNP group.

Compared with the previous study period, the heart mitochondria demonstrated a 3.4-fold decrease. After 3 weeks of melanoma growth in CNP, the Bcl-2 level decreased by 2.2 times in the brain mitochondria and by 1.24 times ($p < 0.05$) in the heart mitochondria compared with the previous study period. No changes were detected in the Bcl-2 level in the liver and kidney mitochondria. In the mitochondria of the melanoma growing in CNP, the Bcl-2 level after 1 week of tumor

development was 3 times lower than the one in the skin mitochondria of the control CNP mice, after 2 weeks and 3 weeks, it was on average 1.5 times lower than the control values.

Thus, it was shown that CNP, the independent growth of melanoma, and the growth of melanoma in CNP did not affect the Bcl-2 level in the liver and kidney mitochondria of the female mice. At the same time, significant changes were found in the Bcl-2 content in the mitochondria of the brain, heart, non-malignant skin, and melanoma.

DISCUSSION

The Bcl-2 family proteins determine sensitivity of cells to apoptosis, an ancient cell suicide program being essential for body development, tissue homeostasis, and immunity. Low apoptosis rates can lead to cancer and autoimmune diseases, while high apoptosis rates can exacerbate degenerative states in organs and even lead to neurodegeneration. The studies using transgenic mice have elucidated the functions of many Bcl-2 family proteins in both normal physiology and various pathological conditions. There is no doubt that these proteins control survival of all mammalian cells.

Some biochemical findings allow to suggest that members of the Bcl-2 family are also involved in non-apoptotic processes, including mitochondrial fission and fusion, as well as autophagy [15], but the physiological evidence remains limited. It was detected that proteins of the Bcl-2 family regulate permeability of the outer mitochondrial membrane, which allows for release of proapoptotic proteins, including cytochrome c, from the intermembrane space into the cytoplasm [16].

In traditional tumor transplantation, a decrease in the Bcl-2 level in the brain mitochondria was revealed after 2 weeks of tumor growth, and in the melanoma development in CNP – after 3 weeks only. In the heart mitochondria, in case of traditional tumor growth, no increase in the Bcl-2 level was registered throughout the study. On the contrary, its content in the heart mitochondria progressively decreased along with tumor growth progression. In melanoma growth in CNP, the Bcl-2 level increased 1 week after transplantation and then decreased along with tumor growth progression.

Perhaps, this fact can be considered as control over outer mitochondrial membrane permeability to regulate the level of calcium and activate mitochondria following stress caused by tumor growth, since it is the brain and heart that are the organs most susceptible to stress [17, 18]. It is assumed that along with changes in the outer mitochondrial membrane permeability, Bcl-2 mediates stress signals [19]. It was revealed that various intracellular stress signals act through the Bcl-2 family proteins, causing effector caspase activation [20].

At the same time, no changes were found in the Bcl-2 level in the liver and kidney mitochondria. In the mitochondria of the skin not affected by the malignant process, during independent and CNP-induced melanoma progression, the Bcl-2 level had both common and distinctive features. Thus, at the initial and logarithmic stages of tumor development (weeks 1–2), the skin mitochondria in the independent tumor growth had an increased level of the studied parameter in contrast to the corresponding control. In the stimulated growth, on the contrary, the first period (week 1) did not show significant differences from the control values. Later (week 2), a decrease in the parameter was detected, compared with the corresponding control. And only week 3 (the terminal stage of tumor growth) was characterized by a decrease in the parameter, i.e. unidirectional changes, however, the Bcl-2 values were significantly lower in the standard variant of tumor growth.

In multicellular organisms, cell growth, division, and death are regulated by a variety of signaling pathways that integrate the state and functions of the cell. In healthy tissues, there is a balance between these processes, which allows for existence of tissue homeostasis [21]. The Bcl-2 protein family combines signals that trigger either cell survival or apoptosis. The balance between these processes is important for tissue development and homeostasis. Impaired apoptosis contributes to the development of several pathologies, including cancer. In addition to apoptotic and anti-apoptotic functions, members of the Bcl-2 family play a non-apoptotic role in the regulation of cell migration and invasion through various signaling pathways [22].

The Bcl-2 family controls the integrity of the outer mitochondrial membrane (OMM) and is functionally divided into anti- and pro-apoptotic proteins. Anti-apoptotic Bcl-2 proteins (e.g. Bcl-2/Bcl-xL/MCL-1) maintain the OMM integrity through direct binding of pro-apoptotic proteins that interact and form pores in OMM. The OMM regulation is complex due to many proteins and pathways converging in the Bcl-2 family. Moreover, there are specific patterns of expression and functionality depending on the type of cells and the state of differentiation [21].

In the mitochondria of a tumor growing independently or in CNP, slightly different progression

was noted than in the skin not affected by the malignant process. In the mitochondria of melanoma cells, which grew in a standard manner, the level of Bcl-2 throughout the entire study period was significantly higher than the parameters of the skin in the controls. On the contrary, in the mitochondria of the melanoma cells stimulated by CNP, this level was lower than in the corresponding controls. Thus, CNP promotes changes in the Bcl-2 storage capacity of mitochondria both in cells of the tumor-bearing organ and the tumor itself.

CONCLUSION

Analyzing the obtained results in general, it should be concluded that not only the mitochondria of melanoma cells respond to concomitant chronic neuropathic pain, but also the ones in the skin that is not affected by the malignant process, as it is the target organ for malignant growth. The detected differences in the Bcl-2 level confirm this fact.

We believe that different Bcl-2 level dynamics in the melanoma cell mitochondria, depending on the variant of tumor development, indicates the modulating effect of CNP and the ability to change the parameter level according to the growth phase. Among all the examined internal organs, the mitochondria of the brain and heart cells reacted most vividly to the standard and stimulated growth of the experimental melanoma. At the same time, it was revealed that the mitochondria of two organs, the liver and the kidneys, had certain Bcl-2 stability both in standard and stimulated tumor development.

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Authors contribution

Kit O.I. – final approval of the manuscript for publication. Frantsiyants E.M. – conception and design, analysis and interpretation of data, final approval of the manuscript for publication. Neskubina I.V., Nemashkalova L.A. – analysis and interpretation of data. Cheryarina N.D., Surikova E.I., Kaplieva I.V. – critical revision of the manuscript for important intellectual content. Shikhlyarova A.I. – substantiation of the manuscript.

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