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ADAPTOR PROTEIN Ruk/CIN85 MODULATES RESISTANCE TO DOXORUBICIN OF MURINE 4T1 BREAST CANCER CELLS

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The acquisition of chemoresistance in the course of tumor progression includes activation of membrane ABC transporters, detoxification enzymes, cell cycle deceleration and activation of specific signaling pathways such as Akt/mTOR, MAPK, NF-κB. Adaptor proteins play an essential role in the assembly of supramolecular signaling complexes, maintaining and directing the intracellular signaling. One of such proteins, called Ruk/CIN85, is strongly associated with malignant transformation and metastasis. In present study we investigated the Ruk/CIN85 effect of up/down-regulation on the transforming potential and doxorubicin resistance of highly aggressive mouse breast adenocarcinoma 4T1 cells. It was demonstrated that 4T1 cells overexpressing Ruk/CIN85 possessed increased resistance to doxorubicin (in the range of concentrations 0.1–10.0 μM) while knockdown cells were the most sensitive. Also, high levels of Ruk/CIN85 in 4T1 cells positively correlated with their ability to form colonies in semi-solid agar. Ruk/CIN85-overexpressing cells formed four times more colonies in comparison with Ruk/CIN85 nockdown cells, the growth of which revealed higher resistance to doxorubicin action.

K e y w o r d s: Breast cancer, chemoresistance, adaptor proteins, Ruk/CIN85.

Cancer treatment technics evolved a lot during last decades, and such approaches as personalized medicine [1], immunotherapy [2], cancer stem cells-directed therapy [3], miRNA-directed therapy [4], as well as various ways of targeted drugs delivery [5] have become widespread. However, the development of resistance to the action of antitumor drugs in the course of cancer progression remains the most challenging. Currently, this phenomenon is explained by the heterogeneity of tumor cells including the presence of a pool of cancer stem cells in tumor mass as well as the activation of molecular mechanisms underlying cell plasticity and enabling tumor to develop new options in drugs resistance [6, 7]. Modern studies are focused on targeting the molecular mechanisms, which provide acquired therapeutic resistance of tumor cells: growth factor receptor (RTK) signaling, cell survival regulation, membrane transporters and detoxification enzymes, EMT- and stemness-maintaining signaling [8, 9]. Adaptor/scaffold proteins consisting of domains and motifs involved in intermolecular interactions function as organizers of multimolecular signaling complexes that possess the ability to regulate their composition in time and space thereby directing intracellular signaling. A member of (Ruk/CIN85)/(CD2AP/CMS) family of adaptor proteins, Ruk (regulator of ubiquitous kinase) in rodents and CIN85 (Cbl-interacting protein with MW 85 kDa) in human (thereafter, Ruk/CIN85) [10], consists of three SH3 domains on the N-terminus, proline- and serine-rich domains, and C-terminal coiled-coil region [11, 12]. Due to interaction with numerous binding partners, Ruk/CIN85 is involved in such processes as vesicle-mediated endocytosis and sorting of activated RTKs [13-15], apoptosis [16], cell adhesion, motility and invasiveness [17-19]. Also, the previous studies demonstrated that Ruk/CIN85 is overexpressed in various cancers and may be associated with increased metastatic potential [20-22].

The aim of present study was to investigate the effect of Ruk/CIN85 overexpression and down-regulation on doxorubicin resistance of mouse breast adenocarcinoma 4T1 cells and their transforming potential.

Materials and Methods

Cell culture and generation of 4T1 sublines with overexpression and down-regulation of Ruk/CIN85. Murine breast adenocarcinoma 4T1 cells with different levels of adaptor protein Ruk/CIN85 content were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), in a humidified atmosphere containing 5% CO_2 at 37 °C. Ruk/CIN85-overexpressing subline RukUp and corresponding control subline Mock were obtained by Ca-phosphate transfection of 4T1 cells with pRc/CMV2-Rukl or empty vector, respectively [23]. Ruk/CIN85 expression was suppressed in subline called RukDown using lentiviral construction pLKO.1-shRuk/CIN85 R22 [24] and for generation of control subline (Scr) nontargeting vector was used. Transfected/infected cells were subcloned and selected with 1mg/ml geneticin (G418) or 10 μg/ml puromycin, respectively.

MTT assay and doxorubicin IC_{50} determination. 4T1 sublines viability after doxorubicin treatment was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyl tetrazolium bromide) reduction assay. Cells were plated on 96-well plate (10^4 cells per well), allowed them to adhere for 12 h and then treated for 24 hours with 0, 0.1, 1, and 10 μM of doxorubicin. Formation of MTT-formazane was measured spectrophotometrically at wavelength of 570/630 nm using an absorbance microplate reader μQuant (BioTEK). The graph of survival dependence on doxorubicin concentration was built in order to determine doxorubicin IC_{50} for each 4T1 subline.

Soft agar colony formation assay. 4T1 cells ($1x10^3$) were suspended in 150 μl top agar (RPMI supplied with 0.4% agar) with 0.01, 0.05, 0.1 μM of doxorubicin or without it, and layered over 100 μl bottom agar (RPMI supplied with 0.8% agar) in the wells of 96-well plate. After 3 weeks, the cells were photographed with magnification 40x and 200x and the number of colonies was counted. Independent experiments were performed in triplicates.

Statistical analysis. All the experiments were independently repeated at least three times, the results were presented as mean ± SEM. Statistical analysis was performed using Statistica software. Data were analysed using factorial ANOVA with Newman-Keuls post-hoc correction for multiple comparisons. Pairwise comparisons were then analyzed by Student's *t*-test for unequal variances and difference between groups was decided to be significant at $P < 0.05$.

Results and Discussion.

In order to study the effect of adaptor protein Ruk/CIN85 on chemoresistance of mouse breast adenocarcinoma 4T1 cells we generated Ruk/CIN85-overexpressing subline RukUp (and corresponding control subline named Mock) using calcium-phosphate transfection as well as Ruk/CIN85-nockdown subline (RukDown) by infection of 4T1 cells with Ruk/CIN85-specific shRNA lentiviral particles (and a control Scr subline, infected with non-targeted virus). As additional control we also used WT 4T1 cells.

Survival of 4T1 sublines with different levels of Ruk/CIN85 expression in the presence of increasing concentrations of doxorubicin (Dox) was evaluated by MTT-test. It was demonstrated, that overexpression of adaptor protein Ruk/CIN85 was associated with significantly increased survival of 4T1 cells at 0.1 μM and 1 μM Dox compared to Mock cells, while down-regulation of Ruk/CIN85- resulted in significantly lower survival rate at 0.1, 1 and 10 μM of Dox in comparison to Scr subline (Fig. 1, *A*). Then, we evaluated Dox IC_{50} for each of the analyzed 4T1 cells sublines and found that the highest value of IC_{50} (0.678884 ± 0.006931 μM of Dox) was characteristic of RukUp subline, whereas IC_{50} for RukDown cells was the lowest (0.443734 ± 0.02338 μM of Dox) (Fig. 1, *B*). These results demonstrate that adaptor protein Ruk/CIN85 regulates resistance of 4T1 cells to doxorubicin in concentration-dependent manner: increases resistance in the case of overexpression and attenuates in the case of down-regulation.

Colony formation in soft agar reflects the anchorage-independent growth ability and is widely used as a test for malignant transformation of tumor cells [25]. We analyzed the colony formation potential of 4T1 cells with up- or down-regulation of Ruk/CIN85 in the presence of 0.01, 0.05, and 0.1 μM doxorubicin. It was demonstrated that under the control conditions (without Dox) RukUp cells formed significantly higher number of colonies in semi-solid agar in comparison to Mock cells. In contrary, down-regulation of Ruk/CIN85 led to supression of colony

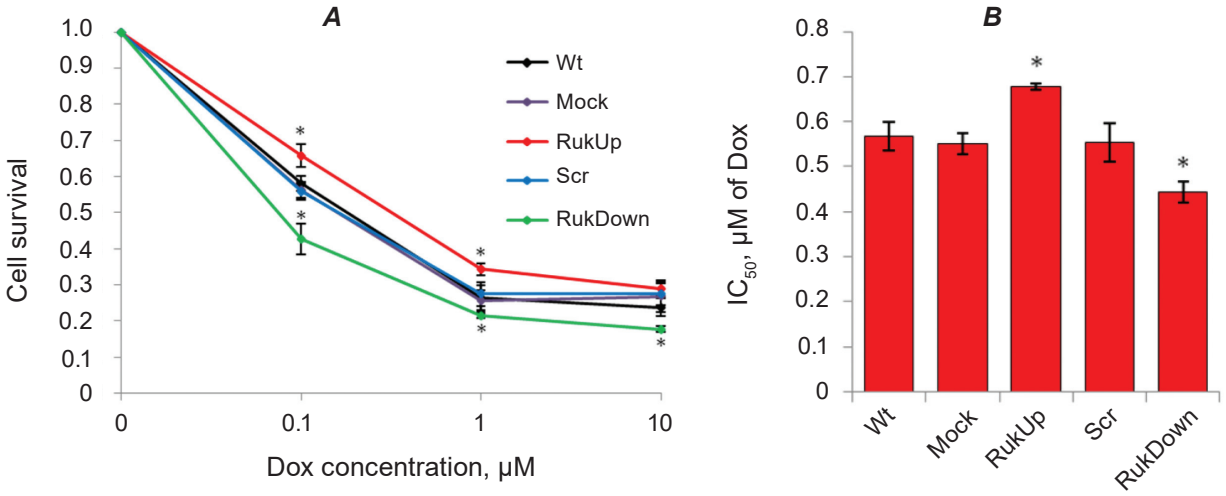

Fig. 1. High Ruk/CIN85 expression level in 4T1 cells positively correlates with doxorubicin resistance. ***A*** *– Doxorubicin survival of mouse breast adenocarcinoma 4T1 cells with different levels of Ruk/CIN85 expression (Wt – wild-type 4T1 cells; Mock – 4T1 cells transfected with empty pRc/CMV2 plasmid; RukUp – 4T1cells, stably transfected with pRc/CMV2 plasmid encoding full-length form of Ruk/CIN85; Scr – 4T1 cells infected with irrelevant lentivirus; RukDown – 4T1 cells stably infected with Ruk/CIN85-specific shRNA lentivirus).* ***B*** *– Ruk/CIN85 effect on doxorubicin IC_{50} value of 4T1 cells.* $P < 0.05$ in comparison to corresponding control*

formation ability. In the presence of Dox the overall number of colonies formed by each subline was decreased in comparison to control without Dox. Importantly, at each Dox concentration point we found significantly higher number of colonies for RukUp subline in comparison to Mock cells, and substantial decrease of colonies number formed by RukDown cells in comparison to Scr control (Fig. 2, *A*). The representative images of colonies characteristic of each 4T1 subline are presented at Fig. 2, *B*. It should be noticed that RukDown cells formed big, spherical colonies with smooth edges, whereas colonies formed by control cells had also spherical shape but were something smaller in size. In contrast, Ruk/CIN85-overexpressing cells gave colonies of smaller size with irregular edges and wherein small colonies usually surrounded bigger one. Such features in the morphology of soft agar colonies suggest that up-regulation of Ruk/CIN85 in 4T1 cells drive the development of a more malignant cellular phenotype potentially associated with increased metastatic potential.

Chemoresistance is an essential feature of cancer stem cells (CSCs) – small subpopulation of tumor cells with self-renewal and tumorigenic potential that allow survival and spreading of the tumor [26]. We demonstrated that expression level of Ruk/CIN85 correlates with the ability of 4T1 cells to form colonies in soft agar and the ability of these colonies to survive in the presence of doxorubicine. Taking into account that Ruk/CIN85 overexpression lead to the increased malignant properties also on other cell types [28, 28], and its ability to affect cell cycle progression and proliferation [17, 22], it may be concluded, that Ruk/CIN85-overexpressing cells are enriched with subpopulation with the properties of CSCs.

Currently, it is known that different mechanisms could be responsible for the development of drug resistance including those involved in the control of cell cycle progression and DNA damage response, providing evasion of apoptosis and increased drug efflux (such as ATP-binding cassette (ABC) membrane transporters) or detoxification of drugs (aldehyde dehydrogenase ALDH) [29]. In addition, acquisition of drug resistant phenotype requires activation of PI3K/Akt/mTOR, NF-κB, p53 pathways [30-32]. Previous studies demonstrated, that overexpression of adaptor protein Ruk/CIN85 in another breast cancer model (human breast adenocarcinoma MCF-7 cells) was accompanied by increased resistance to cisplatin and etoposide along with increased rhodamine 123 efflux and ALDH activity [32], that is consistent with our results on 4T1 breast cancer cells. Moreover, Ruk/CIN85-overexpressing MCF-7 cells were also demonstrated to have constitutively activated Akt kinase [22] that plays major role in the chemoresistance mechanisms.

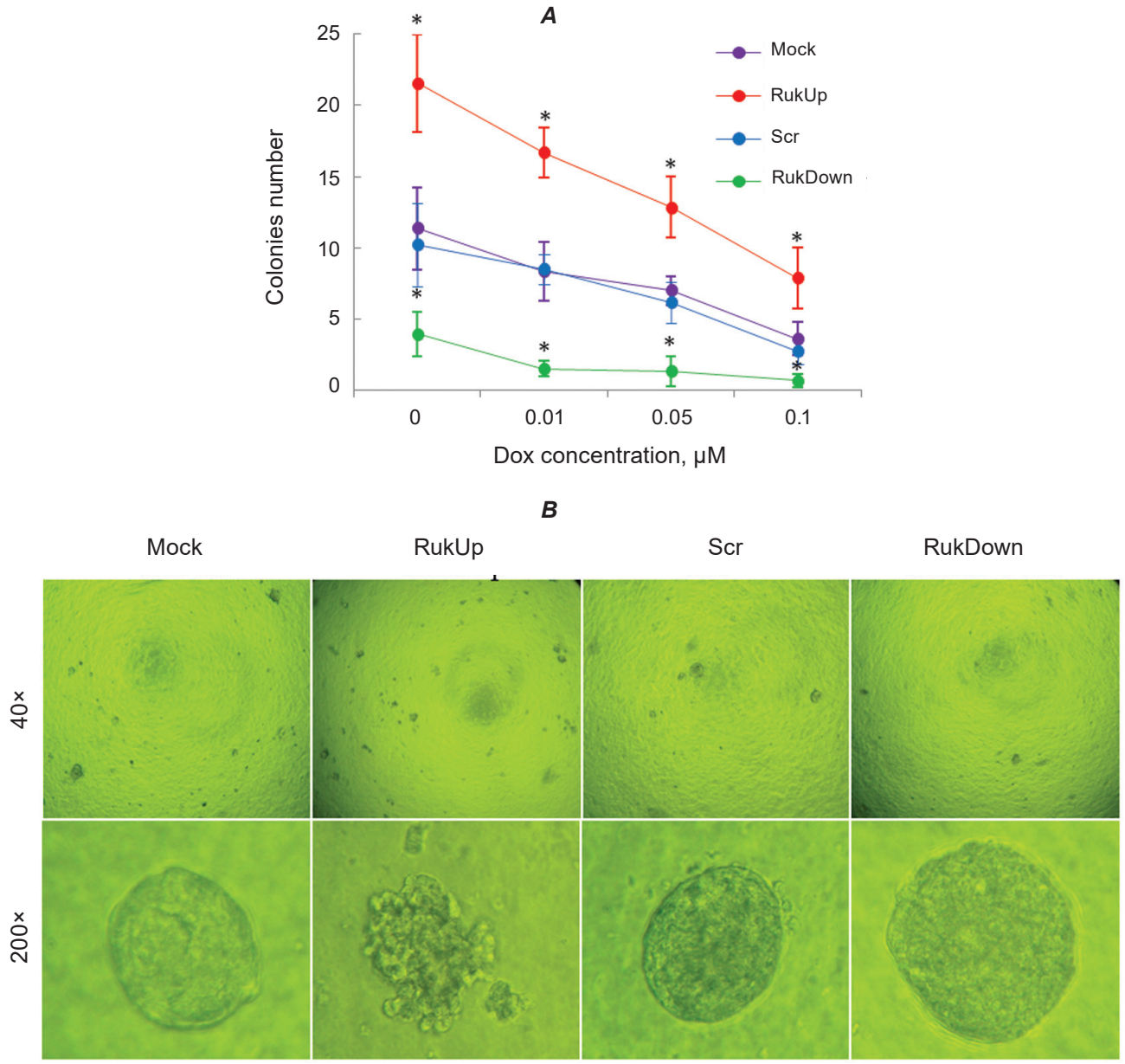

Fig. 2. Adaptor protein Ruk/CIN85 modulates the transforming potential of 4T1 cells in a manner dependent on its expression levels. ***A*** *– Soft agar colonies formation ability of 4T1 cells with different levels of Ruk/CIN85 expression levels in the presence or absence of doxorubicin (Wt – wild-type 4T1 cells; Mock – 4T1 cells transfected with empty pRc/CMV2 plasmid; RukUp – 4T1cells, stably transfected with pRc/CMV2 plasmid encoding full-length form of Ruk/CIN85; Scr – 4T1 cells infected with irrelevant lentivirus; RukDown – 4T1 cells stably infected with Ruk/CIN85-specific shRNA lentivirus). * P < 0.05 in comparison to corresponding control.* ***B*** *– Representative pictures of soft agar colonies formed by 4T1 cells with different levels of Ruk/CIN85 expression*

In the same time, doxorubicin-resistant breast cancer MCF-7 cells have considerably repressed TOP2A gene, the product of which is the main target of doxorubicin, as well as up-regulated expression of ABC membrane transporters, cell cycle and proliferation regulators [33]. Along with the known information regarding the complexity and diversity of chemoresistance mechanisms, our data suggest that adaptor protein Ruk/CIN85 may function as important component of regulatory networks need to acquire drug resistant phenotype by breast cancer cells. This means that Ruk/CIN85-overexpressing breast cancer

cells acquire drug resistant phenotype, provided by various molecular mechanisms.

In this study we analysed the effect of overexpression/down-regulation of adaptor protein Ruk/CIN85 on the survival and soft agar colonies formation ability of mouse breast adenocarcinoma 4T1 cells in the presence of doxorubicin. We found that increased expression level of Ruk/CIN85 positively correlates with doxorubicin resistance and transforming potential of 4T1 cells.

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АДАПТЕРНИЙ ПРОТЕЇН Ruk/CIN85 МОДУЛЮЄ РЕЗИСТЕНТНІСТЬ КЛІТИН АДЕНОКАРЦИНОМИ МОЛОЧНОЇ ЗАЛОЗИ МИШІ ЛІНІЇ 4T1 ДО ДОКСОРУБІЦИНУ

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Набуття пухлинними клітинами хіміорезистентності відбувається завдяки активації мембранних ABC-транспортерів, ензимів деградації ксенобіотиків, затримці клітинного циклу та активації специфічних сигнальних шляхів, таких як Akt/mTOR, MAPK, NF-κB. Адаптерні протеїни відіграють важливу роль у збиранні надмолекулярних сигнальних комплексів, підтриманні та спрямуванні внутрішньоклітинного сигналювання. Один з таких протеїнів, Ruk/CIN85, залучений до процесів злоякісної трансформації та метастазування. В цій роботі досліджено вплив адаптерного протеїну Ruk/CIN85 на трансформувальний потенціал та резистентність до доксорубіцину клітин аденокарциноми молочної залози миші лінії 4T1. Продемонстровано, що Ruk/CIN85 модулює резистентність клітин 4T1 до доксорубіцину в концентрації 0,1–10,0 μM. Також виявлено позитивний зв'язок між вмістом Ruk/CIN85 у клітинах лінії 4T1 та здатністю до формування колоній у напіврідкому агарі, в тому числі в присутності 0,01–0,1 μM доксорубіцину.

Ключові слова: рак молочної залози, хіміорезистентність, адаптерні протеїни, Ruk/CIN85.

АДАПТЕРНЫЙ ПРОТЕИН Ruk/CIN85 МОДУЛИРУЕТ РЕЗИСТЕНТНОСТЬ КЛЕТОК АДЕНОКАРЦИНОМЫ МОЛОЧНОЙ ЖЕЛЕЗЫ МЫШИ ЛИНИИ 4T1 К ДОКСОРУБИЦИНУ

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Приобретение опухолевыми клетками химиорезистентности происходит за счет активации мембранных ABC-транспортеров, энзимов деградации ксенобиотиков, задержки клеточного цикла и активации специфических сигнальных путей, таких как Akt/mTOR, MAPK, NF-κB. Адаптерные протеины играют важную роль в сборке надмолекулярных сигнальных комплексов, поддержке и направленности внутриклеточной сигнализации. Один из таких протеинов, Ruk/CIN85, вовлечен в процессы злокачественной трансформации и метастазирования. В данной работе исследовано влияние адаптерного протеина Ruk/CIN85 на трансформирующий потенциал и резистентность к доксорубицину клеток аденокарциномы молочной железы мыши линии 4T1. Продемонстрировано, что Ruk/CIN85 модулирует резистентность клеток 4T1 к доксорубицину в концентрации 0,1–10,0 μM. Также выявлена положительная связь между содержанием Ruk/CIN85 в клетках линии 4T1 и способностью к формированию колоний в полужидком агаре, в том числе в присутствии 0,01–0,1 μM доксорубицина.

Ключевые слова: рак молочной железы, химиорезистентность, адаптерные протеины, Ruk/CIN85.

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