sures may include the following: otolaryngology training should be standardized for all of these countries, and a common board examination should be administered; training programs should be developed in countries that lack audiologist and speech therapists; and countries with existing training programs should either develop programs in countries lacking such programs or open their doors to foreigners.

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Огляд отоларингічних послуг у Центральній Америці: Потреба у комплексному втручанні

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Резюме. У світі, що розвивається, існує дефіцит у послугах отоларингологів і нестача закладів, що їх готують, а також спеціалістів з аудіології та мовної терапії. Це підтверджується різницею у якості надання медичної допомоги між країнами з високим рівнем заробітку і країнами з низьким рівнем заробітку. За допомогою анкет, ми досліджували країни Центральної Америки (за винятком держави Беліз) і виявили наступні проблеми: наявність послуг та обладнання з отоларингології, аудіології і мовної терапії; наявність цих послуг у сільських місцевостях. Анкети були розповсюджені електронною поштою і людьми. На конгресі з отоларингології у Центральній Америці у 2011 році в Сан Сальвадорі, Ель Сальвадорі їх отримали отоларингологи, аудіологи і мовні терапевти. Ми не здивувались, коли дізнались про недостачу у послугах, які надаються представниками цих трьох професій, а також про недостачу навчальних закладів, що готують цих фахівців. Дані, зібрані й представлені у цьому дослідженні, стануть основою для впровадження подальших змін.

Ключові слова: отоларингологія, ВГН, аудіологія, мовна терапія, країни, що розвиваються, Центральна Америка.

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Purification of Ku70, Ku80, Rad51 and Rad52 proteins in Arabidopsis thaliana and generation of anti-Ku70 and anti-Rad51 antibodies

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Summary. Successful techniques of genetic engineering and gene targeting depend on high activity of homologous recombination repair. Strand breaks in plant and animal somatic cells are primarily repaired by non-homologous end joining, a competitor of homologous recombination pathway. The objective of this study was to shift the naturally occurring balance of double strand break DNA repair in cells away from non-homologous end joining and towards homologous recombination. We hypothesized that this will improve the current methods for genetic engineering and ultimately practical applications like gene therapy. We amplified full cDNA copies of the proteins Ku70, Ku80, Rad51 and Rad54 using cDNA from Arabidopsis. The cDNA were overexpressed in Rosetta cells and the proteins were sent away to GenScript to obtain polyclonal antibodies. Generated antibodies were tested for recognition of Arabidopsis Ku70 and Rad51 proteins. We hypothesized that introducing the anti-Ku70 antibodies into cells would deplete the amount of Ku70 and this would result in the shift of strand break repair towards homologous recombination. In the future, we will use cell-penetrating peptide to deliver the antibody and to test the level of homologous recombination.

Key words: Ku70, Ku80, Rad51 and Rad52 proteins, generation of anti-Ku70 and anti-Rad51 antibodies.

Introduction. Altering the balance between non-homologous end joining (NHEJ) frequency and homologous recombination (HR) frequency has been the focused direction of this work. NHEJ involves among other proteins Ku70 and Ku80, while homologous recombination utilizes Rad51 and Rad54 [7]. In *S. cerevisiae* Ku70 and Ku80 work in conjunction with ligase IV and XRCC4 to ensure proper DNA repair via the NHEJ pathway [7]. In vertebrate cells Ku70 and Ku80 form a heterodimer that has a strong penchant for DNA ends and consequently binds with the damaged DNA [3]. The DNA bound Ku70/Ku80 heterodimer associates with, stabilizes, and activates the DNA-PKcs a catalytic subunit to form DNA-PK (DNA-dependent protein kinase), a necessary component of the non-homologous end joining pathway [9]. In studies investigating cell lines deficient in Ku proteins, little if any DNA-PK activity is seen indicating that the cell requires the presence of the Ku70/Ku80 for proper DNA-PK functioning and therefore also non-homologous end joining [15]. The role of Ku proteins has been linked to the detection of damaged DNA in all eukaryotic cells. Rad51 and Rad54 are also conserved in eukaryotic cells and necessary elements of the homologous recombination DNA repair pathway [2]. Rad51 is very important for the HR DNA repair process because it catalyses the exchange of DNA between homologous strands by encouraging the association of the damaged DNA strand to its respective complementary, template strand [7]. Rad51 requires interaction with Rad54 for optimal efficiency and proper functioning [11]. Animal studies have shown that inactivation of Rad51 through artificial means results in early death of developing embryos while knockout studies of Rad54 show only an increased sensitivity to agents that induce double stranded breaks in DNA which emphasizes the essentialness of Rad51 and the complementary actions of Rad54 [4].

The success of genetic engineering and gene targeting in particular depends directly upon the activity of homologous re-

combination repair pathway [6]. Currently concerns exist surrounding the actual process of genetic modification and gene therapy. Part of this worry relates to the imbalance between the occurrence of homologous recombination and non-homologous end joining in cell DNA repair. The insertion of the transgene into the genome typically occurs through available breaks in the genomic DNA. These breaks are often the results of mistakes in replication and transcription as well as direct damage to DNA via free radicals and other toxic metabolites. The cell has two predominant ways to repair strand breaks – HR and NHEJ [10]. HR is quite specific because it makes use of its partner chromosome as a template. In contrast, NHEJ ligates the broken ends together without the use of such a homologous template [10]. When the ends can not be ligated directly, the modification of the broken ends occurs, followed by microhomology search and subsequent rejoining via ligation.The repair via end joining thus leads to insertions, deletions, and translocations. In vertebrates, the breaks are repaired predominantly via NHEJ, thus reducing the possibility of using somatic cells for efficient gene targeting and gene therapy [16].

Taking into consideration all the mentioned above, it can be hypothesized that a decrease in the amount of proteins required for NHEJ would result in a decrease in the frequency with which cells utilize NHEJ. This would in turn lead to a compensatory increase in the frequency of usage of HR repair mechanism. Similarly the overexpression of the proteins required for HR would help fuel the compensatory increase in HR activity.

We hypothesized that delivery of antibodies against Ku70 or Ku80 proteins into cells used for gene targeting may decrease the activity of NHEJ by binding and precipitating Ku70/Ku80 proteins. We further hypothesized that by limiting the availability of resources required for proper completion of NHEJ, the cell will compensate by relying more on homologous recombination and shift the balance in favor of HR when repairing breaks in the cell's DNA. The use of antibodies would make this shift temporary so only a limited upset in this balance would occur. Research by Pierce et al. (2001) [12]. has recently shown that knockouts of either Ku70, XRCC4, or DNA-PK_{CS} in mammalian cells resulted in the increase of the frequency of occurrence of HR, supporting our prediction that a compensatory shift can occur [12]. As the first step in the aforementioned technique of increasing gene targeting rate we attempted to purify Ku70 and Rad51 proteins and to generate antibodies against them. We report successful purification of these proteins and generation of the anti-Ku70 and ant-Rad51 antibodies.

Methods

Primers used for the experiments

The Vector NTI program was used to design forward and a reverse primers specific for Ku70, Ku80, Rad51 and Rad54 based on previously determined sequences in *Arabidopsis thaliana* (see Table 1). These were used together with PCR to amplify the genes from a cDNA library. They were designed with consideration for the availability of convenient restriction sites and melting temperature.

Table 1. The sequence of the primers designed for all four genes: Ku70, Ku80, Rad51, and Rad54

Gene	Primer name	Sequence
Ku70	Ku70.for	ATGGAATTGGACCCAGATGATG
Ku70	Ku70.rev	TTATTTACCAATGTGAGTCAGAATCCG
Ku80	KU80.for	ATGGCACGAAATCGGGAGG
Ku80	Ku80.rev	TTAGCTCTCGAGCATTGACTCTTGTT
Rad51	Rad51.for	AAAGGTACCATGACCACCATGGAACAG CGCC
Rad51	Rad51.rev	AAAGGTACCCTATCAGTCCTTGCAGTCG GTCACG
Rad54	Rad54.for	AAAGGTACCATGGCCAGGCGCAGGCTC
Rad54	Rad54.rev	AAAGGTACCCTATCAGTGAGAGATGTA CTGGAAGGCGAAG

Table 2. PCR Reaction components for Ku70, Ku80, Rad51 and Rad54

	Ku70 and Ku80	Rad51 and Rad54
HF 5X Buffer (50 mM)	10 μl	10 μl
dNTPs (2 mM)	5 μl	5 μl
Forward Primer (10 μM)	1 μl	1 μl
Reverse Primer (10 μM)	1 μl	1 μl
DNA	8 μl	0.5 μl
Phusion HF Polymerase (2 u/μl)	0.5 μl	0.5 μl
Gibco Pure Water	24.5 μl	32 μl
Total Volume	50 μl	50 μl
No. of Cycles	35	32
Denaturation Temperature (°C)	98	98
Denaturation Time (s)	10	30
Annealing Temperature (°C)	58	65
Annealing Time (s)	30	30
Elongation Temperature (°C)	72	72

RNA purification and cDNA synthesis

RNA was extracted from frozen ground in liquid nitrogen leaf tissues (~ 100 mg) of *A. thaliana* using Trizol reagent (Molecular Research Center, Inc.). The concentration of the isolated RNA was measured using the Amersham Pharmacia Biotech Ultraspec 1100pro UV/Visible spectrophotometer. For cDNA synthesis the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) was used. For our purposes, Oligo $(dT)_{18}$ (100μM, 0.5μg/μl (15 A_{260} u/ml)) was used. The primers and the RNA (5-15 μg) were incubated at 70ЂC for five minutes to denature any double-stranded RNA pieces. Next the 5X Reaction Buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50mM DTT) was used to provide the appropriate chemical condition. Nucleotides (10 mM) and a ribonuclease inhibitor (RiboLock Ribonuclease Inhibitor; 20 u/μL) were added to the mixture and incubated at 37ЂC for 2 min. Reverse transcriptase (RevertAid H Minus M-MulV RT; 200 u/μL) finished the procedure by constructing strands of DNA using the mRNA as a template resulting in a single stranded cDNA library.

Isolation of Ku70, Ku80, Rad51, and Rad54

Arabidopsis cDNA was used as the template in the PCR reactions for amplification of *Ku70* (1866 bp) and *Ku80* (2043 bp) genes. Amplification of *Rad51* (1029 bp) was done from the plasmid pDEST::AtRad51, whereas amplification of *Rad54* (2733 bp) – from plasmid pDEST::AtRad54. The PCR reactions also included a 1x Phusion GC Buffer (1.5 mM $MgCl_2$) to provide optimal conditions for the PCR reaction, dNTPs (2 mM), the appropriate forward and reverse primers (10 mM) (Table 1) and Phusion HF polymerase (2 u/ml) (New England Biolabs, Inc.) and ddH2O to the final volume of 50 ml. See PCR reaction details in Tables 2. PCR products were excised from the gel and purified using the QIAquick PCR Purification Kit (QIAGEN) and used to clone into protein overexpression vector pET45b.

Cloning into pET45b plasmid

pET45b plasmid was selected for protein overexpression because it utilizes strong bacteriophage T7 signals for transcription and translation which allow for high expression of the cloned gene. PCR fragments with genes of interest (*Ku70*, *Ku80*, *Rad51* and *Rad54*) were cloned into pET45b plasmids, generating pET45b::Ku70, pET45b::Ku80, pET45b::Rad51, and pET45b::Rad54 plasmids. Cloning of intact gene fragments was confirmed by sequencing.

Induction and isolation of proteins

Rosetta 2 cells were transformed with pET45b::Ku70, pET45b::Ku80, pET45b::Rad51, and pET45b::Rad54 plasmids. Gene expression was induced using 1 mM IPTG in 10 mL of $LB/Carb_{100}/Cm_{35}$. The transformed Rosetta 2 cells were incubated at 37°C with the IPTG enriched media at constant shaking (200 rpm) for five hours. The protein was purified using "Protocol 18: FPLC Purification of 6x His-tagged Proteins using Ni-NTA Superflow under Denaturing Conditions" and "Protocol

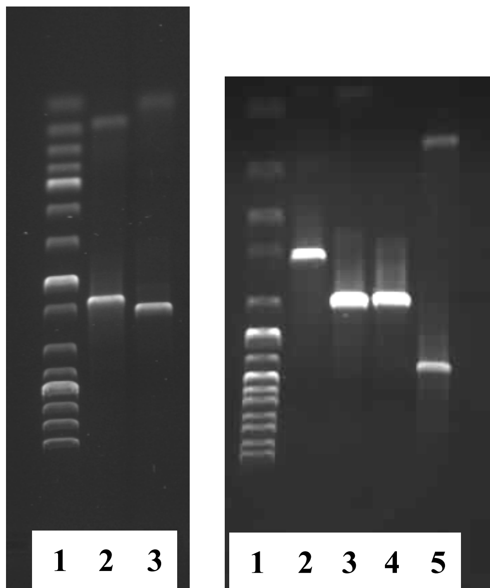
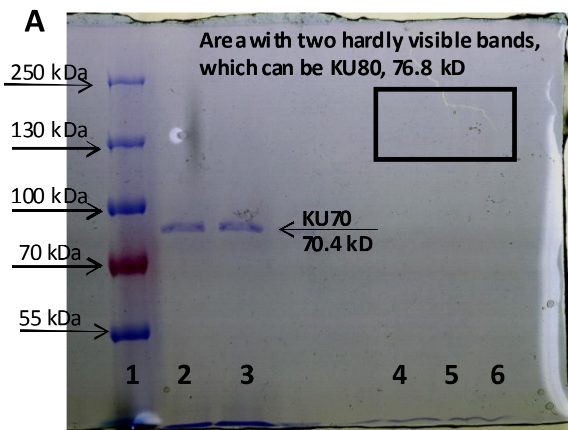


Figure 1. PCR amplification of Ku70, Ku80, Rad51, Rad52 and Rad54 cDNA

A. 1% agarose gel. Lane 1 - 1kb DNA Ladder Plus; Lane 2 - Ku70 PCR product; Lane 3 - Ku80 PCR product.

B. 1% agarose gel. Lane 1 - 1kb DNA Ladder Plus; Lane 2 - Rad51 PCR product; Lane 3 and 4 - Rad52 PCR product; Lane 5 - Rad54 PCR product



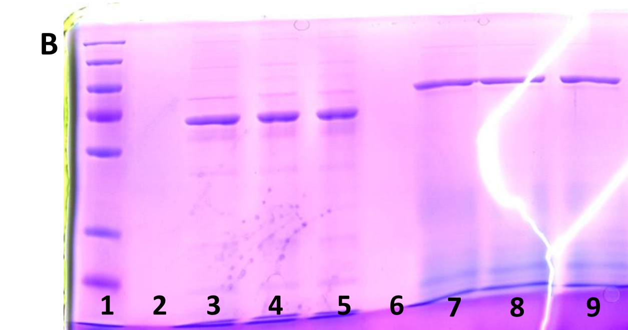


Figure 3. Overexpression of Ku70, Ku80 and Rad52 proteins in Rosetta 2 cells

A. 10% Coomassie-stained PAGE gel. Lane 1 - PageRuler Plus Prestained Protein Ladder; Lane 2 and 3 - Ku70; Lane 4 - Empty; Lane 5 and 6 - Ku80.

10% Coomassie-stained PAGE gel. Lane 1 - PageRuler Plus Prestained Protein Ladder; Lane 2 - empty; Lane 3 to 5 - Rad52; Lane 6 - empty; Lane 7 to 9 – Ku70

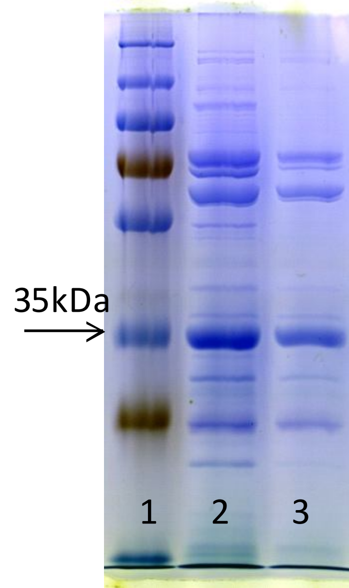


Figure 2. Purification of Rad51 protein from Rosetta 2 cells

12% Coomassie-Stained PAGE gel. Lane 1 - PageRuler Plus Prestained Protein Ladder; Lane 2 – Rad51; Lane 3 – Re-purified Rad51

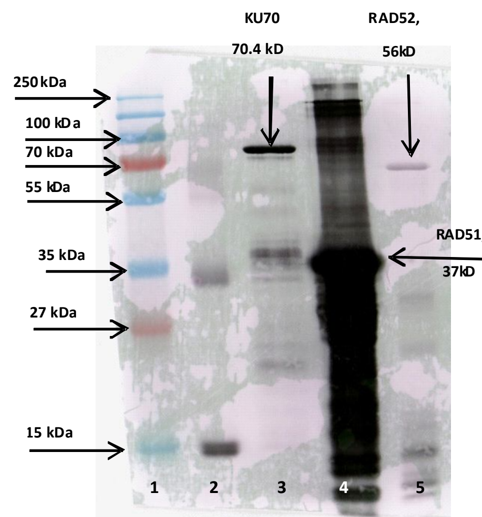


Figure 4. Western Blot using anti-his-tag antibodies

Lane 1 - PageRuler Plus Prestained Protein Ladder; Lane 2 – marker for 15, 30, 55 and 70 kDA; Lane 3 - Ku70; Lane 4 - Rad51; Lane 5 - Rad54

19: 6X His-tagged Protein Minipreps under Denaturing Conditions” from the QIAexpressionist (QIAGEN) kit. Polyacrylamide gel electrophoresis (PAGE) using a 10% gel was used to identify the purified protein samples. Western Blots were run to further confirm the identity of the bands from the PAGE gels. The isolated and purified Rad51 and Ku70 proteins were sent to GenScript for antibody production. Polyclonal rabbit antibodies obtained from GenScript were used to detect purified Rad51 and Ku70 proteins.

Results and Discussion

As the first step in the attempt to regulate the NHEJ and HR activity in the cells, we decided to amplify cDNA and overexpress main NHEJ and HR repair proteins. As a next step, we have attempted to prepare antibodies against these proteins.

Ku70, *Ku80*, *Rad51*, *Rad54* and *Rad52* genes were successfully amplified from *A. thaliana* cDNA (Figure 1). The PCR fragments were used for overexpression in pET45b plasmid. Ku70, Ku80, Rad51 and Rad52 were successfully overexpressed in Rosetta 2 cells, although Ku80 concentration was very low

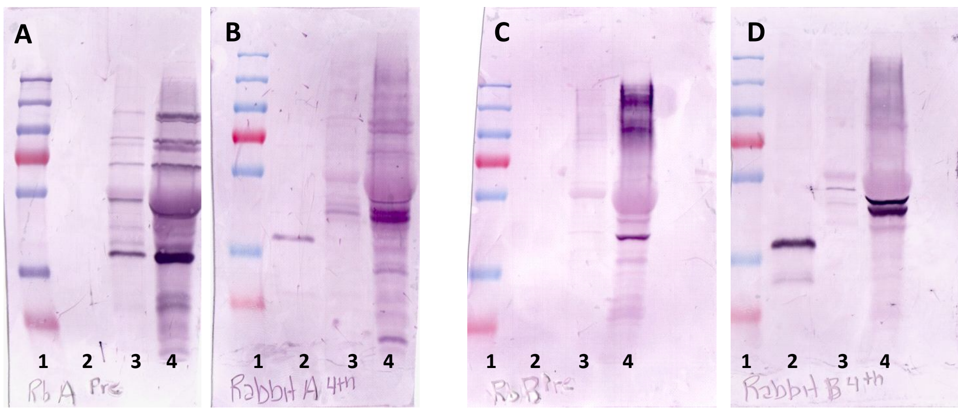


Figure 5. Western Blot analysis using GenScript anti-Rad51 antibodies

A. Reconstituted Pre-immune serum from Rabbit A. From left to right: Lane 1 – PageRuler Plus Prestained Protein Ladder; Lane 2 - purified Rad51; Lane 3 – 1:10 dilution of Arabidopsis plant extract; Lane 4 - undiluted Arabidopsis plant extract.

B. Reconstituted Antiserum after 4th Immunization from Rabbit A. From left to right: Lane 1 – PageRuler Plus Prestained Protein Ladder; Lane 2 - purified Rad51; Lane 3 – 1:10 dilution of Arabidopsis plant extract; Lane 4 - undiluted Arabidopsis plant extract.

C. Reconstituted Pre-immune serum from Rabbit B. From left to right: Lane 1 – PageRuler Plus Prestained Protein Ladder; Lane 2 - purified Rad51; Lane 3 – 1:10 dilution of Arabidopsis plant extract; Lane 4 - undiluted Arabidopsis plant extract.

Reconstituted Antiserum after 4th Immunization from Rabbit B. From left to right: Lane 1 – PageRuler Plus Prestained Protein Ladder; Lane 2 - purified Rad51; Lane 3 – 1:10 dilution of Arabidopsis plant extract; Lane 4 - undiluted Arabidopsis plant extract

and the bands were hardly visible on the gel (Figure 2,3). After being run through a 12% PAGE gels, the purified Rad51 protein samples showed bands near the 35 kDa band of the PageRuler Plus Prestained Protein Ladder (Fermentas), very close to the size of native Arabidopsis Rad51 protein of 37 kDa (see Figure 2). The purified Ku70 protein samples showed bands between the 70 kDa and 100 kDa bands of the PageRuler Plus Prestained Protein Ladder (Fermentas) in a 10% PAGE gel, with the size of native protein in Arabidopsis being 70.4 kDa (Figure 3).

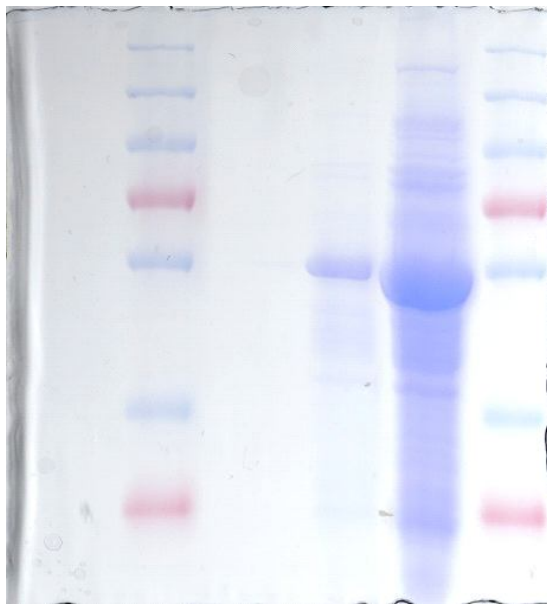


Figure 6. Western Blot analysis using GenScript anti-Rad51 antibodies against purified Arabidopsis Rad51 protein

Reconstituted Antiserum after 4th Immunization from Rabbit B was used. From left to right: Lane 1 – PageRuler Plus Prestained Protein Ladder; Lane 2 - purified Rad51;

Lane 3 – 1:10 dilution of Arabidopsis plant extract; Lane 4 - undiluted Arabidopsis plant extract;

Lane 5 – PageRuler Plus Prestained Protein Ladder

To further confirm that the products visualized on Commassie gel carried the his-tag, we performed the Western blot with the antibodies against the his-tag. We were able to identify Ku70, Rad51 and Rad52 proteins, with corresponding sizes of 70.4, 37 and 56 kDa, respectively (Figure 4).

We then used the purified Rad51 proteins to generate polyclonal antibodies that can be in the future used for delivering into mammalian cells. The polyclonal anti-Rad51 antibodies from Genscript when tested on *A. thaliana* plant extract displayed a great degree of nonspecific binding (Figure 5). However, when using a purified Rad51 protein solution instead of the plant extract it was clear that the polyclonal antibody serums detected Rad51 while the pre-immune serums did not (Figure 6).

It is not clear why the polyclonal anti-Rad51 antibodies were not able to recognize plant Rad51. There are four different homologs of Rad51 in Arabidopsis, including Rad51B, Rad51C, Rad51D and AtXRCC3 [1]. Although we used purified Rad51, the antibodies may recognize several homologs, including truncated and modified proteins, thus preventing us from identification of a single protein band. As mentioned in the introduction, the frequency of successful gene targeting events depends on the activity of homologous recombination in the cell. That is, the higher is the HR frequency, the higher is the chance of integration of the transgene at a specific genomic location. This is one of the reasons the gene targeting is more efficient in the cells in which HR repair is more frequent, such as dividing cells, including embryonic stem cells [8]. Also, gene targeting and gene replacing is much easier in the organisms with high frequency of HR, such as yeasts [5] and *Physcomitrella patens* [14]. If our hypothesis is correct, then downregulation of key HR proteins would decrease the gene targeting frequency in the cells. In the future, we will test this hypothesis by delivering purified anti-Rad51 antibodies into cells, followed by the analysis of HR frequency and gene targeting efficiency. In addition, we will test whether delivery of anti-Ku70 antibodies into cells may increase the HR frequency as well as gene targeting events.

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Очищення білків Ku70, Ku80, Rad51 і Rad52 арабідопсису та покоління антитіл анти-Ku70 і анти-Rad51

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Резюме. Успішні техніки генної інженерії та генного таргетингу залежать від високої активності репарування гомологічної рекомбінації. Розриви нитки рослинних і тваринних соматичних клітин головним чином репаруються негомологічним з'єднанням кінців, шляхом протилежним до гомологічної рекомбінації. Метою даного дослідження було змінити натурально виникаючий баланс репарування подвійного розриву нитки ДНК від негомологічного з'єднання кінців до гомологічної рекомбінації. Ми зробили припущення, що це дозволить поліпшити сучасні методи генної інженерії та, врешті-решт, практичні застосування, такі як генна терапія. Ми посилили повні копії кДНК білків Ku70, Ku80, Rad51і Rad54 використовуючи кДНК арабідопсису. Дані кДНК були надлишково виражені в клітинах Rosetta і відіслані на GenScript, щоб отримати поліклональні антитіла. Генеровані антитіла були протестовані на наявність білків Ku70 і Rad51. Ми припустили, що введення антитіл анти-Ku70 в клітини вичерпає кількість Ku70 і це призведе до зміни репарування розриву нитки до гомологічної рекомбінації. В подальшому ми використаємо клітинно-проникні пептиди, щоб доставити антитіла і протестувати рівень гомологічної рекомбінації.

Ключові слова: білки Ku70, Ku80, Rad51 і Rad52, покоління антитіл анти-Ku70 і анти- Rad51.

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Хромосомна нестабільність спадкового апарату у дітей раннього віку, хворих на ускладнену пневмонію у поєднанні із залізодефіцитною анемією

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Резюме. Вивчено цитогенетичні зміни спадкового апарату у дітей раннього віку, хворих на ускладнену пневмонію у поєднанні із залізодефіцитною анемією. Порівняльним аналізом змін генетичного апарату дітей, які хворіли на ускладнену пневмонію у поєднанні із залізодефіцитною анемією, було доведено їх залежність від ступеня тяжкості анемії. Виявлено збільшення частоти асоціацій акроцентричних хромосом та хромосомних аберацій у дітей з ускладненою пневмонією, порівняно з контролем, та залежність цих показників від ступеня тяжкості залізодефіцитної анемії. Встановлено, що найбільшу здатність до утворення асоціацій мають хромосоми 21, 15 і 14, найменшу – 12 і 13 у кожній групі хворих дітей. Серед типів хромосомних аберацій ідентифіковано хроматидні (одиночні фрагменти), хромосомні (парні фрагменти, поодинокі дицентрики і аномальні моноцентрики), делеції та розриви хромосом. У спектрі хромосомних аберацій доведено перевагу делецій довгого плеча четвертої та короткого плеча п'ятої і шостої хромосом.

Ключові слова: діти, пневмонія, анемія, асоціації акроцентричних хромосом.

Постановка проблеми і аналіз останніх досліджень: Однією з актуальних проблем дитячої пульмонології, що пов'язано з високим рівнем розповсюдженості та смертності, є пневмонії. Особливої уваги заслуговують випадки перебігу пневмонії на тлі обтяженого преморбідного фону чи супутньої патології [1, 2]. Серед багатьох причин, що можуть ускладнювати перебіг пневмонії, є дефіцит заліза. В останні роки спостерігається збільшення числа хворих на пневмонію серед дітей раннього віку, що протікає у поєднанні із залізодефіцитною анемією (ЗДА) [2]. Одним з найменш освітлених аспектів розглянутої проблеми залишаються порушення цитогенетичного гомеостазу при вищевказаній поєднаній патології. В даний час встановлений зв'язок між змінами хромосомного апарату клітин і запаленням. [5, 6, 9]. Зокрема, в літературі описано підвищення рівня хромосомних перебудов при таких запальних захворюваннях бронхолегеневої системи, як хронічний бронхіт, бронхіальна астма, гостра пневмонія [5, 11]. Серед чинників, які порушують генетичну стабільність при запаленні бронхів і легень, виділяють наступні: вплив інфекції, включаючи пряму цитогенетичну дію вірусів, а також непрямі ефекти бактеріальних і вірусних агентів; підвищений викид медіаторів запалення; кількісну та функціональну недостатність клітинної ланки імунітету.