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DETECTION OF ANTIBODIES AGAINST BENZO[A]PYRENE IN BLOOD SERA OF LUNG CANCER PATIENTS BY ELISA USING HUMAN RECOMBINANT IDIOTYPIC AND ANTI-IDIOTYPIC ANTIBODIES

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Aim **of this study was to develop and apply an ELISA method for assessment of the levels of endogenous antibodies against benzo[a] pyrene (Bp) in blood sera of lung cancer (LC) patients. *Materials and Methods*: ELISA was developed and applied for the detection of endogenous idiotypic and anti-idiotypic antibodies against Bp in human blood sera using recombinant human idiotypic and anti-idiotypic antibodies against Bp. *Results*: Serum samples of LC patients (n = 22) and healthy donors (n = 24) were analyzed by the new method. Statistical analysis showed that in sera of LC patients the levels of endogenous idiotypic and anti-idiotypic antibodies against Bp were significantly higher than in healthy donors. A logistic regression model for the LC detection utilizing such predictors as the serum levels of idiotypic and anti-idiotypic antibodies against Bp, smoking status, and age, identified LC patients with 83% specificity and 82% sensitivity. *Conclusion*: The proposed method could be further developed as additional lung cancer screening tool.**
Key Words: **polycyclic aromatic hydrocarbons, anti-idiotypic antibodies, idiotypic antibodies, IgG, ELISA, lung cancer.**

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Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more aromatic rings, which are synthesized by result of high-temperature reactions such as pyrolysis of fossil fuels and incomplete combustion of organic materials. Sixteen PAHs are listed as priority environmental pollutants by the US Environmental Protection Agency [1]. Benzo[a]pyrene (Bp) is ubiquitously widespread. Bp is considered to be an indicator of the PAHs [2–3]. The pathogenic strength of the impact of Bp on an individual person depends on the duration of human exposure to PAHs [4], lifestyle [5], profession [6–9], places of residence [10].

When PAHs enter the human body, they induce production of antibodies (Abs) [11]. It has been shown that Abs against PAHs can affect the formation of cancer in the human due to their binding to PAHs and PAHs adducts [12]. According to the hypothesis of the immunological imbalance in carcinogenesis, Abs against PAHs, progesterone, and estradiol can stimulate or suppress carcinogenesis depending on their levels and combinations in sera [13, 14].

It was demonstrated that immunization of mouse with the Bp-protein conjugate increased the level of idiotypic (Ab1) and anti-idiotypic (Ab2) Abs against Bp in mouse serum [15]. The ratio of anti-Bp Ab2/Ab1 in mouse serum after immunization coincided with the ratio of anti-Bp Ab2/Ab1 in sera of lung cancer (LC) patients. In our previous paper, we suggested to use the levels of Ab1 and Ab2 against PAHs as well as Ab2/Ab1 ratios in serum as immunological markers of LC for identifying LC risk groups in healthy population [16]. However, these previous studies were performed using heterogeneous enzyme-linked immunosorbent assay (ELISA). The Bp-bovine serum albumin (BSA) conjugate was used for detection of endogenous Ab1 against PAHs in sera [16]. The rabbit polyclonal Abs against Bp-BSA or mouse single-chain variable fragment (scFv) against Bp were used for detection of endogenous Ab2 against PAHs in sera [15, 16]. Here we offered a homogeneous ELISA method for detecting the levels of endogenous Abs against Bp in serum samples based on the use of human scFv Ab1 and human scFv Ab2. The proposed detection system is not inferior to the previously described detection system and even surpasses it.

MATERIALS AND METHODS

Synthesis of Bp-BSA conjugate. The Bp-BSA conjugate was synthesized by covalently linking the aldehyde group of the hapten with the amino groups of the BSA [17]. 1.5 ml of pyridine was added dropwise to 0.1 g of BSA in 1 ml of 0.1 N NaOH with stirring and cooling, followed by addition of 16 mg benzo[a]pyrene-6-carboxaldehyde and stirring for 3 h at 25 °C. 50 mg of blocking compound acrylamide and 10 mg of the reducing agent sodium borohydride in 1 ml of water were added. After 30 min of stirring, 4 drops of glacial acetic acid were added and the conjugate was precipitated with 8 ml of acetone. After 15 min, the precipitate was collected

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Abbreviations used: Abs – antibodies; Ab1 – idiotypic antibodies; Ab2 – anti-idiotypic antibodies; Bp – benzo[a]pyrene; BSA – bovine serum albumin; CBD – cellulose-binding domain; ELISA – enzyme-linked immunosorbent assay; IgG – immunoglobulin G; LB – Luria–Bertani liquid medium; LC – lung cancer; OD – optical density; PAHs – polycyclic aromatic hydrocarbons; scFv – single-chain variable fragment.

by centrifugation and washed 5 times with 8 ml of acetone. After drying in vacuum, the conjugate was dissolved in 10 ml of 0.01 N NaOH. The conjugate was stored in a soluble form at pH 7.2–7.4. No insoluble polymer products were formed when the conjugate was stored at a temperature of 5 °C for at least six months.

Isolation of scFvs containing a cellulose-binding domain (CBD) by affinity chromatography on amorphous cellulose. All scFvs used in this work were fusions with a CBD for easily purification by affinity chromatography on amorphous cellulose, prepared as described in early papers [18–20].

A colony of *E. coli* strain M15 cells, transformed with a plasmid encoding scFvs against Bp (human Ab1 T72 and Ab2 A4, mouse Ab1 pSh), was placed on agar with Luria–Bertani (LB) liquid medium containing 150 µg/ml ampicillin and 50 µg/ml kanamycin. Plated colonies were grown overnight at 37 °C. Then the colonies were inoculated into 2 ml of LB containing the same antibiotics and grown at 37 °C overnight with stirring at 120 rpm. An overnight bacterial culture was inoculated into flasks with 250 ml of LB medium with the same antibiotics and incubated at 37 °C with stirring at 130 rpm until optical density (OD) at 600 nm was 0.4–0.6. Then IPTG (isopropyl β-d-1-thiogalactopyranoside) was added at a final concentration of 1 mM for Abs expression. Bacterial cells were grown for 4 h under the same conditions and pelleted by centrifugation at 1000 g for 30 min at 4 °C. Cell pellets were dissolved in 6 ml of lysis buffer (20 mM Tris-HCl pH 8.8, 200 mM NaCl, 0.1% Triton X-100) and sonicated on ice using Sonics Vibracell device (Sonics & Materials, Inc., USA). The resulting cell lysate was pelleted by centrifugation at 14000 rpm (45 Ti rotor, Optima L-90K centrifuge, Beckman Coulter, USA). The pellet of inclusion bodies was dissolved in a buffer: 50 mM Tris-HCl pH 8.8, 750 mM NaCl, 0.75% Triton X-100, 14 mM β-mercaptoethanol, 8 M urea followed by centrifugation under the same conditions. The supernatant was taken and diluted with a buffer: 50 mM Tris-HCl pH 8.8, 750 mM NaCl, 0.75% Triton X-100 to final urea concentration of 2 M. 1 ml of amorphous cellulose solution was added and incubated at 4 °C overnight with stirring. Washing cellulose from unabsorbed protein was carried out with 0.05% Triton X-100 in PBS. The absorbed scFvs on cellulose were eluted with 1 ml of buffer (20 mM Tris pH 8.0, 10 mM EDTA, 50 mM NaCl, 4 M guanidine chloride), followed by dialysis against PBS. The quality of purified Abs was checked by SDS electrophoresis in PAAG [21].

ELISA. The study of the binding of scFv to antigens was performed using a non-competitive ELISA in our own modification [22]. 50 µl of Bp-BSA at a concentration of 5 ng/µl was added to the wells of the immunological polystyrene plate at 25 °C overnight following 250 µl of a blocking solution containing 0.5% BSA and 0.05% Tween-20 in PBS to all wells and incubation for an hour at 37 °C with shaking. Then 50 µL of T72-CBD at a concentration of 20 ng/µl or pSh-CBD at a concentration of 20 ng/µl were added to the wells with the absorbed Bp-BSA followed by 1.5-fold serial dilution of Abs with PBS. The plate was incubated for an hour at 37 °C on a shaker. After each incubation step, the plate was washed 3 times with PBS with 0.05% Tween-20. For the registration, rabbit anti-CBD at a concentration of 10.8 ng/µl was added to the wells and the immunological plate was incubated for 1 h at 37 °C. Then anti-rabbit Abs labeled with horseradish peroxidase were added and the plate was incubated at 37 °C for an hour with stirring. The signal was recorded following addition of 50 µl of 3,3',5,5'-tetramethylbenzidine. The staining was stopped by adding 50 µl of 2N hydrochloric acid to the wells. The signal was recorded in an iMark plate reader (Bio-Rad, USA) at a wavelength of 450 nm.

For ELISA using human serum samples, scFvs (A4-CBD or T72-CBD or pSh-CBD at 50 ng/µl of each) or 10 ng/µl Bp-BSA conjugate were added to the wells of the immunological polystyrene plate. The plate was incubated for 1 h at 37 °C with shaking. Then a solution of 1% BSA with 0.05% Tween-20 in PBS was added to the wells of the immunological plate followed by incubation for 1 h at 37 °C with shaking. After that, serum samples were applied at a 1:100 dilution with PBS. The plate was incubated for 1 h at 37 °C with shaking, then washed 3 times with 0.05% Tween-20 in PBS. The anti-human immunoglobulin G (IgG) labeled with horseradish peroxidase was added at a 1:5000 dilution with PBS. Then the results were developed and recorded as above.

Cohort information. The present study was approved by the Ethical Evaluation Committee of Regional Clinical Oncology Center and all subjects provided written informed consent form. The blood samples of LC patients were obtained from the State Budgetary Institution of Healthcare, the Regional Clinical Oncological Dispensary of the Kemerovo Region (22 men) and blood of healthy donors obtained from the State Treasury Institution of Healthcare, the Kemerovo Regional Blood Center of the Kemerovo Region (24 men). LC was diagnosed by histological examination. All blood samples of LC patients were drawn at the time of initial diagnosis, before they received any treatment. Details of patients age, gender, outcome, date of diagnosis and disease substratification were restricted by the agreement with the donating clinic and details of healthy blood donors age, gender, and smoking was limited by the agreement with the Regional Blood Center. Serum specimens were recorded on the registration form, and frozen at –80 °C in a refrigerator. Repeated freezing and thawing was avoided before testing.

Statistical analysis. Statistica 10.0 and Excel programs (Microsoft Office, USA) were used for statistical data processing. The ELISA data were checked for normal distribution using the modified

Shapiro — Wilk test. Outlier points, which were greater than three standard deviations, were removed. The Mann — Whitney U-test was used to determine the differences between the ELISA grouped data. The Pearson Chi-square was used for relationship health with smoking status. The correlation was determined by the Spearman method. The predictive significance of Ab1 and Ab2 levels in serums, age, and smoking status was tested by logistic regression analysis for LC detection. The sensitivity, specificity, and area under the receiver operating characteristic curve (AUC) with 95% confidence interval were all calculated by receiver operating characteristic curve analysis. The difference was considered statistically significant if $p < 0.05$.

RESULTS

Binding characteristics of scFvs. The human scFv Ab1 T72 against Bp was in details described in our previous paper [19], the human scFv Ab2 A4 against Bp in [20], and the mouse scFv Ab1 pSh against Bp in [18].

Since pSh (mouse scFv against Bp) and T72 (human scFv against Bp) are both Ab1, but these Abs possess different structures and origins, we compared their binding to Bp as common antigen for both Abs. The binding of pSh and T72 to Bp was assessed by ELISA. At the first step, 50 µl of Bp-BSA conjugate at a concentration of 5 ng/µl was added to a 96-well polystyrene plate. Then, 50 µl of T72-CBD and pSh-CBD were added to the wells with dilution in the concentration range from 1.24 ng/µl to 22 ng/µl. As can be seen from Fig. 1, the binding of T72 and pSh to Bp-BSA was dose-dependent and the differences in binding were insignificant. Thus, selected from the naïve combinatorial library of human scFv T72 bound Bp in a manner similar to the binding of mouse pSh scFv to Bp.

Comparison of binding serum Abs with absorbed antigens Bp-BSA or A4 and T72 or pSh. The selection of ELISA conditions for the determination of endogenous Ab1 and Ab2 in sera using Bp and A4 or T72 and pSh as antigens was carried out as follows. The optimal conditions for the sorption of antigens on flat-bottom 96-well immunological plates were checked by adding 50 µL with the concentrations: Bp-BSA (10 ng/µl), A4 (50 ng/µl), T72 (50 ng/µl) or pSh (50 ng/µl) followed by serial 1.5-fold Abs dilution with PBS. The optimal sorption for A4, T72, and pSh were at 37 °C for 1 h and for sorption of Bp-BSA — 12 h at RT.

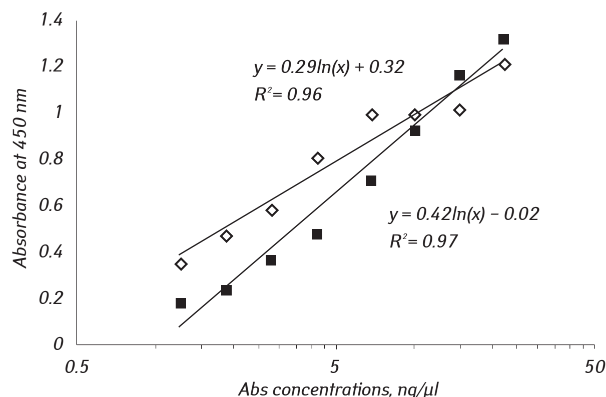

Fig. 1. The binding of pSh (squares) and T72 (diamonds) to absorbed Bp-BSA in ELISA

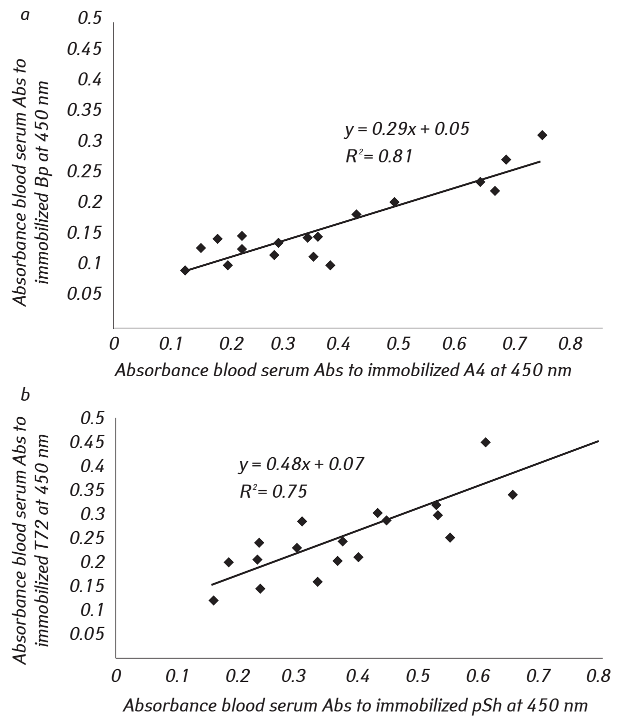

Fig. 2. Comparing interactions of serum Abs with absorbed antigens: Bp-BSA and A4 (*a*) or T72 and pSh (*b*)

A4 is a human anti-idiotypic scFv against Bp. Consequently, the active center of A4 has a BP image. Thus, A4 binds similar pool of serum Ab1 to the pool of serum Ab1 that Bp binds. The binding of serum Ab1 was compared with absorbed Bp-BSA and A4 in a 96-well immunoassay plate. Fig. 2, *a* shows a correlations graph of the same serum responses to different absorbed antigens. As can be seen, the binding of Ab1 from sera to Bp and A4 was quite similar.

By analogy with A4 and Bp, the binding of serum Ab2 to absorbed T72 (human scFv Ab1 against Bp) and pSh (mouse scFv Ab1 against Bp) was compared (Fig. 2, *b*). The same similar binding of serum Ab2 was observed for the T72-pSh pair as for the A4-Bp pair.

Comparison with previously published method. The reproducibility and sensitivity of two ELISA methods were determined by several measurements of interactions of serum Ab1 and Ab2 with absorbed Bp-BSA and pSh or A4 and T72 (Table 1). The sensitivity of the new proposed method was calculated using a calibration curve plotted from the ELISA results of direct binding of purified human scFv (A4 and T72)

Table 1. Reproducibility and sensitivity of the proposed method in comparison with the previously suggested method

	Reproducibility, % of errors		Sensitivity	
	Ab1	Ab2	Ab1	Ab2
Suggested method	21.81	27.57	300 pg/ml	1 ng/ml
Previously described method	43.3	38.61	1230 pg/ml	5 ng/ml

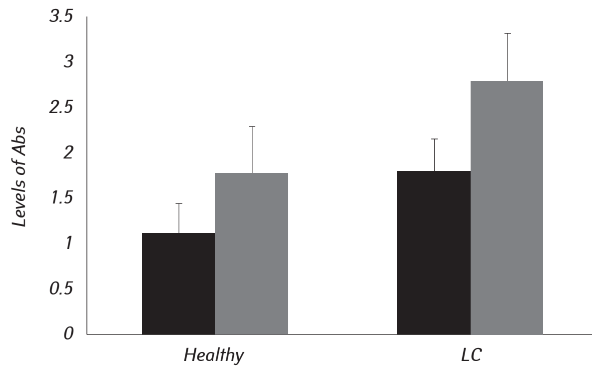

Fig. 3. The mean values of Ab1 and Ab2 levels in sera of healthy blood donors and LC patients. Black and gray bars are Ab1 and Ab2 levels, respectively. Standard deviations are indicated

to absorbed Bp-BSA. The suggested method for the determination of Abs against Bp in serums is more sensitive and reproducible in comparison with the previously published one.

Characterization of blood donor groups by the new method. ELISA was performed using absorbed T72 and A4 in the wells of a 96-well polystyrene plate. Diluted serum samples were then added to the wells after blocking solution incubation. The mean Ab1 and Ab2 levels in sera were calculated for groups of healthy donors and LC patients separately (Fig. 3).

The data obtained in ELISA were analyzed for normal distribution using the Kolmogorov–Smirnov and Shapiro — Wilk tests. The analysis showed that the ELISA data for the group of healthy people had a unimodal distribution with positive asymmetry, but the distribution of the data for the group of LC patients was normal. The Mann — Whitney U-test was used for calculating the difference in Abs levels between the groups of LC patients and healthy people (Table 2). The analysis showed that the levels of Ab1 and Ab2 in sera were significantly differed for the groups of healthy and LC patients. It was higher in LC patients.

In order to incorporate the smoking factor into the calculations, the studied groups of people were analyzed by the Pearson Chi-square depending on the presence of LC and smoking status. The association between factorial and effective characteristics was statistically significant, $p < 0.001$ (data not shown).

Table 2. The maen values of Ab1 and Ab2 levels in sera of LC patients and healthy people calculated by the Mann – Whitney U-test

Abs	Medians values of Abs in sera of healthy donors	Medians values of Abs in sera of LC patients	Mann – Whitney U-test, *p*
Ab1	1.42 (0.55:2.08)	2.1 (1.34:3)	0.01
Ab2	1.82 (0.82:2.86)	2.83 (2:3.57)	0.003

Table 3. Correlations between blood donor subgroups by serum Ab1 and Ab2 levels, smoking status, age, and health status using Spearman method

	Ab1	Ab2	Smoking	Age	Health status
Ab1	**1.00**	–	–	–	–
Ab2	–	**1.00**	–	0.34	0.44
Smoking	–	–	**1.00**	-0.34	-0.53
Age	–	0.34	-0.34	**1.00**	0.59
Health status	–	0.44	-0.53	0.59	**1.00**

The Spearman method was used to calculate the correlation between Ab1 and Ab2 levels in sera, age, smoking status, and health status (Table 3). The correlation analysis between subgroups showed that the level of Ab2 correlated with age (ρ = 0.34) and health status (ρ = 0.44). There was no correlation between Ab2 and smoking status and no correlation was found between Ab1 and other predictors.

Logistic regression model for determining LC. The logistic regression method was used to establish a model for determining the likelihood of LC in tested sera of healthy donors. For calculations, 4 predictors were used: the level of Ab1 in sera, the level of Ab2 in sera, smoking status, and age. The coefficients for the predictors obtained from the logistic regression method are: for Ab2 0.87, $p = 0.034$, CI 0.23; for smoking –3.09, $p < 0.001$, CI 0.72.

The sensitivity and specificity of the logistic regression model were 82% and 83%, respectively. The AUC was 0.89 (Fig. 4). This logistic regression model has good specifications in LC predicting since sensitivity and specificity are consistent with other published before systems for determining LC.

DISCUSSION

The results of determining endogenous Ab1 and Ab2 against Bp in sera using human scFvs correlated with the results obtained using the conjugate of Bp-BSA and mouse scFv pSh. The suggested new ELISA method was more reproduced and sensitive (Table 1).

The current work used the simultaneous measurement of the levels of Ab1 and Ab2 against Bp in sera to assess the changes in the human immune system during carcinogenesis. The lower levels of Abs in sera of healthy donors can be explained by the participation of Abs against Bp in the development of LC or Abs against Bp reflect changes in human immune system of patients.

The proposed method as well as the previously published one [16] shows an increase in serum Abs levels in LC patients compared to healthy people. The small difference between the data presented and those previously published can be explained by the different spectrum of serum Abs that interact with the antigens absorbed onto the immunological plate or by the difference in the number of tested

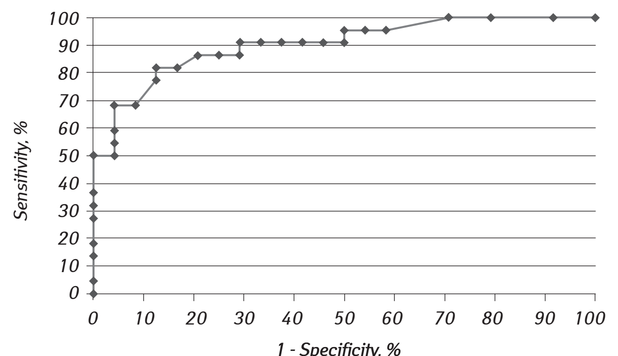

Fig. 4. Receiver operating characteristic curve of a logistic regression model for defining LC

serum samples. It can be assumed that both ELISA methods determine endogenous pool of Ab1 and Ab2 against PAHs in sera. Both ELISA methods determine endogenous pool of Ab1 and Ab2 against PAHs in serum. Interestingly, despite these minor differences, the ratio of Ab2 to Ab1 is very similar for both methods. In healthy blood donors, the ratio was about 2. The ratio in LC patients drops to about 1.

Spearman's correlation was calculated between the parameters of blood donors: levels of Ab1 and Ab2 in sera, age, smoking status, and health status (Table 3). A significant relationship was found between the level of Ab2 and age and health status. Both found correlations were weak. Based on the logistic regression model, only two predictors were significant — smoking and Ab2 level in serum.

The sensitivity and specificity of LC prediction by the logistic model were 86 and 95%, respectively. AUC was 0.89 (Fig. 4) with 95% confidence internal. These rates were comparable to other LC detection systems. For example, multiplex analysis system using a panel of 6 auto-Abs against: p53, NY-ESO-1, CAGE, GBU4-5, Annexin 1 and SOX2 had 38% sensitivity and 89% specificity [23]. In the system using a panel of 7 auto-Abs against: p53, CAGE, NY-ESO-1, GBU4-5, SOX2, MAGE A4 and Hu-D, the sensitivity was 47%, the specificity was 90%, and AUC was 0.9. Also, the new method was comparable to previously published one where Bp-BSA and mouse scFv against Bp used for detection of endogenous Ab1 and Ab2 levels against Bp in sera, which gave AUC = 0.9 at logistic regression model [16].

The designed logistic regression model confirms the difference in the mean levels of Ab1 and Ab2 against Bp in sera of LC patients and healthy people. Such a logistic regression method is often used to detect cancer, LC in particular, using the influence of various cancer markers [24–25]. Since the logistic regression model constructed in current work had high characteristic specifications, it seems reasonable to assess its possible usefulness to determine the risk of LC development.

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ВИЯВЛЕННЯ АНТИТІЛ ПРОТИ БЕНЗО[А]ПІРЕНУ В СИРОВАТЦІ КРОВІ ХВОРИХ НА РАК ЛЕГЕНІ ЗА ДОПОМОГОЮ ІМУНОФЕРМЕНТНОГО МЕТОДУ З ВИКОРИСТАННЯМ РЕКОМБІНАНТНИХ ІДІОТИПОВИХ ТА АНТИІДІОТИПОВИХ АНТИТІЛ

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Мета: Розробити та застосувати метод імуноферментного аналізу для визначення ендогенних антитіл проти бензо[а]пірену в сироватці крові хворих на рак легені. ***Матеріали та методи:*** Було розроблено метод імуноферментного аналізу ідіотипових та антиідіотипових антитіл у сироватці крові з використанням рекомбінантних людських ідіотипових та антиідіотипових антитіл проти бензо[а]пірену. ***Результати:*** У хворих на рак легені рівні ендогенних ідіотипових та антиідіотипових антитіл до бензо[а]пірену в сироватці крові були значно вищими, ніж у здорових людей. Модель логістичної регресії для визначення раку легені, яка включала як предиктори рівні ідіотипових та антиідіотипових антитіл проти бензо[а]пірену в сироватці крові, статус куріння та вік, дозволила ідентифікувати хворих на рак легені зі специфічністю 83% та чутливістю 82%. ***Висновок:*** Запропонований метод може в подальшому застосовуватися як додатковий засіб скринінгу для виявлення раку легені.

Ключові слова: поліциклічні ароматичні вуглеводні, антиідіотипові антитіла, ідіотипові антитіла, IgG, ELISA, рак легені.