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In vitro COMPARABLE ANALYSIS OF CARCINOLYTIC ACTIVITY OF MUTANT PROTEIN PARASPORIN-2 ON THE HUMAN HEPATOCARCINOMA CELL MODEL

Abstract. The paper presents the results of the study on the effect of amino acid substitution in the receptor-binding and transmembrane domain of the carcinolytic bacterial protein parasporin-2 on cytotoxic activity against the Hep G2 (human hepatocarcinoma) tumor cell line. The substitution of amino acids tyrosine for alanine and serine for cysteine was carried out by introducing a point mutation into a recombinant plasmid DNA containing the sequence of the gene encoding the paraspirin-2 protein with a polyhistidine tag. 3 new mutant parasporins 2 were prepared by substituting amino acids in two protein domains. A tenfold reduction in the cytotoxic activity of parasporin-2 was observed when the amino acid tyrosine was substituted for alanine in the receptor-binding domain of the protein; serine to cysteine substitution had no significant effect on the carcinolytic activity. The resulting data and experimental samples could be used for searching targets on the surface of tumor cells by means of affinity chromatography.

Keywords: mutations, parasporins, cell lines, cytotoxicity.

Introduction. Parasporins are genealogically heterogeneous Cry proteins synthesized by the bacterial species *Bacillus thuringiensis*. A characteristic feature of parasporins is high cytotoxic activity against human cancer cells of various origins. Proteins exhibit cytotoxic activity only after proteolytic activation (Ohba M., 2009: 427).

Mizuki et al. studied for the first time the parasporal inclusion proteins isolated from a total of 1744 *B. thuringiensis* strains. They determined the cytotoxic activity against human leukaemia T cells and hemolytic activity against sheep erythrocytes. It was concluded that the *B. thuringiensis* protein inclusions could be used for medical purposes (E. Mizuki, 1999: 477).

Further studies of the unusual properties of the *B. thuringiensis* parasporal inclusions and their ability to recognize human leukaemia cells discovered a protein named parasporin, which is responsible for carcinolytic activity. This protein was subsequently cloned (Mizuki E., 2000: 625).

Different research groups later found new strains producing carcinolytic parasporal inclusions and characterized their parasporins (Ito A., 2004: 21282; Brown K., 1992: 549; Saitoh H., 2006: 2935; Nagamatsu 2010: 494; Okumura S. 2004: 89; Okumura S., 2013: 1889).

Parasporin-2 is a carcinolytic non-hemolytic and non-insecticidal protein toxin derived from the *B. thuringiensis* parasporal inclusion. This pore-forming protein has a mass of 37 kDa, consists of 338 amino acids, the length of the coding gene is 1014 bp. To manifest cytotoxic activity, it requires the presence of GPI-anchored proteins, which indicates the possibility of pore formation in raft domains of the tumor cell membranes (Lee D., 2000: 218).

The active form of recombinant parasporin-2 was crystallized in the presence of ethylene glycol and polyethylene glycol 8000 at neutral pH (Akiba T., 2004: 2355). Hayakawa et al. reported about a new Cry

protein that exhibited strong cytotoxicity against human leukaemia T cells, which was cloned from the *B. thuringiensis* Tth-E6 strain. The protein designated as parasporin-2Ab (PS2Ab) is a polypeptide composed of 304 amino acid residues with a molecular weight of 33,017. The deduced amino acid sequence of PS2Ab showed significant homology (84% identity) with parasporin-2Aa (PS2Aa) from the *B. thuringiensis* strain A1547. Upon processing of PS2Ab with proteinase K, the active form of 29 kDa was produced. The activated PS2Ab showed potent cytotoxicity against MOLT-4 and Jurkat cells, and the EC_{50} values were estimated as 0.545 and 0.745 ng/mL respectively. The cytotoxicity of PS2Ab was significantly higher than that of PS2Aa. Although both cytotoxins were structurally related, it was believed that the detected minor differences in amino acid sequence were responsible for the different degrees of cytotoxicity of PS2Ab and PS2Aa (Hayakawa T., 2007: 278). Cytological and biochemical observations on PS2Aa showed that the protein is a pore-forming toxin. To confirm this hypothesis, Akiba et al. have determined the crystalline structure of its active form with a resolution of 2.38 Å. The protein is unusually elongated and consisted mainly of long β-pleated sheets aligned along its long axis. It is similar to the aerolysin-type β-pore-forming toxins, the similarity with which confirms the pore formation hypothesis. The molecule can be divided into three domains: Domain 1, comprising small β-pleated sheets flanked by short α-helices, is probably the receptor-binding domain. The other two are both "beta-sandwich" domains, which are thought to be involved in oligomerization and pore formation. Domain 2 has a putative channel-forming β-hairpin, characteristic of the aerolysin-type toxins. The surface of the protein has an extensive track of exposed side chains of serine and threonine residues. This might orient the molecule on the cell membrane when Domain 1 binds to the target until oligomerization and pore formation are initiated. The β-hairpin has such a tight structure that it seems unlikely to be oligomerized, as postulated in a recent model of pore formation developed for aerolysin-type toxins. The spontaneous oligomerization lock model is proposed as an inactivation mechanism by the N-terminal inhibitory segment (Akiba T., 2009: 121).

Studies on the cytotoxic effect of parasporin-2 have showed that unlike parasporin-1, it increases the permeability of the plasma membranes of tumor cells (Ohba M., 2009: 427; Petit L., 1997: 6480). Cytoplasmic lactate dehydrogenase flows out of the treated HepG2 cells, while extracellular propidium iodide enters the cytoplasm. The initial stage of the cytotoxic effect of parasporin-2 is the specific binding of the toxin to a putative receptor protein, not yet identified, which is located in a lipid raft of the plasma membrane of tumor cells susceptible to this protein. This is followed by the formation of oligomers of parasporin-2 in the plasma membranes, which leads to the pore formation and cell lysis (Petit L., 1997: 6480). Oligomerization occurs in the presence of membrane proteins, a lipid bilayer, and cholesterol. It should be noted that substantial homology exists in amino acid sequences between PS2Aa1 and *Clostridium perfringens* epsilon-toxin, whose cell action mechanism involves the toxin oligomerization in lipid rafts and pores formation in the plasma membrane (Petit L., 1997: 6480).

Abe et al. (Abe Y., 2005: 113) examined the mechanism of action of parasporin-2. They found that the toxin binds to the surface of target cells and increases the permeability of the plasma membrane. Subcellular fractionation and immunoblotting of the cells treated with the toxin showed that the toxin is associated with lipid rafts and forms SDS-resistant oligomers. The binding and oligomerization of the toxin was inhibited by treating the cells with phosphatidylinositol-specific phospholipase C. The interaction of parasporin-2 with glycosylphosphatidylinositol proteins was therefore required to form an oligomeric toxin that could penetrate the plasma membrane (Abe Y., 2005: 113). Abe et al. (Abe Y., 2008: 269) examined the mechanism of action of parasporin-2 on the human HepG2 (hepatomocarcinoma) cell line and showed that this Cry toxin targets lipid rafts and is assembled into oligomeric complexes in the tumor cell membrane. The authors concluded that this protein is a pore-forming toxin that accumulates in lipid rafts of tumor cells. Recently, Bokori-Brown et al. (Bokori-Brown M., 2011: 4589) showed that the ε-toxin produced by *Clostridium perfringens* (the etiological agent of dysentery in newborn lambs, enteritis and enterotoxicity in goats, calves and foals) forms heptameric pores in the membranes of the target cells in the same way as parasporin-2.

Since little is known about the receptor molecules that bind parasporins and the mechanism of antitumor activity, Krishnan et al. (Krishnan K., 2010: 86) conducted a study with the Malaysian isolate *B. thuringiensis* 18. It produces a parasporal protein that exhibits predominant cytotoxic activity against human leukaemia T cells (CEM-SS), but is non-cytotoxic against normal T cells and other cancer cell lines, such as human cervical cancer (HeLa), human breast cancer (MCF-7) and colon cancer (HT-29),

showing properties similar to those of parasporins. The study was aimed at identifying a binding protein for the *B. thuringiensis* 18 parasporin in human leukemic tissues. The protein was separated using the Mono Q ion-exchange column in HPLC system using antibodies against the purified 68-kDa parasporal protein. A receptor binding assay was used to determine the binding protein for the Bt18 paraspore protein in CEM-SS cells, the identified protein was sent for N-terminal sequencing. Double immunofluorescence staining was applied to localize *B. thuringiensis* 18 and the binding protein on the surface of CEM-SS cells. The findings of this study showed that ion-exchange separation of Bt18 parasporal proteins yielded a 68-kDa parasporal protein with cytotoxic activity. Polyclonal IgG (anti-B. thuringiensis 18) for the 68-kDa parasporal protein was successfully prepared and purified. An analysis of receptor-binding showed that the *B. thuringiensis* 18 parasporal protein is bound to a 36-kDa protein from the CEM-SS cell lysate. N-terminal amino acid sequence of the 36-kDa protein was GKVKVGVNGFGRIGG, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found to be a binding protein. Double immunofluorescence staining showed co-localization of the *B. thuringiensis* 18 parasporin and GAPDH on the plasma membrane of the CEM-SS cells. GAPDH has been well known as a glycolytic enzyme, but it was recently discovered that GAPDH has a role in apoptosis and carcinogenesis. Pre-incubation of the anti-GAPDH antibody with CEM-SS cells decreases binding of Bt18 parasporin to the susceptible cells. Based on a qualitative analysis of the immunoblotting and immunofluorescence results, GAPDH was identified as a binding protein located on the plasma membrane of CEM-SS cells for the Bt18 parasporal protein (Krishnan K., 2010: 86).

The purpose of the study was to determine the effect of amino acid substitution in the transmembrane and receptor-binding domains in parasporin-2 on *in vitro* carcinolytic activity.

Materials and Methods. *Plasmids, bacteria and culture conditions.* The recombinant pET-23a (+) plasmid carrying the parasporin-2 gene was used (Okumura S., 2005: 6313). DH5α competent E. coli cells were used for intermediate transformation, and *E. coli* BL21-AI was used as a producer strain to prepare mutant parasporins 2. Bacteria were cultured in agar and liquid LB culture medium in the presence of 0.1% ampicillin. Plasmids were isolated using the WizardTMPlus SV Minipreps DNA Purification System adsorption kit (Promega) (Poornima K., 2010: 348).

Tumor cell line and culture conditions. Hep G2 (hepatocarcinoma) tumor cell line was grown in RPMI medium supplemented with 10% FBS in the presence of ampicillin (100 μg/mL), at 37 °C, 5% CO_2 (Kitada S., 2006: 26350).

DNA manipulations. PCR conditions: 1 cycle at 95^{0}C for 30 seconds, 16 cycles at 95^{0}C for 30 seconds, 1 minute at 55^{0}C, and a final incubation at 68 ^{0}C for 15 min (Okumura S., 2005: 6313).

DNA electrophoresis was performed in 6% polyacrylamide gel prepared with 0.5 X TBE buffer of the following composition: 0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA. In order to allow gel to polymerize, 300 μl of 10% ammonium persulfate and 30 μl of TEMED (Kitada S., 2009: 80) were added to 50 ml of the solution.

DNA sequencing. The obtained PCR products were analyzed on the Beckman CEQ™ 8000 Genetic Analysis System capillary sequencer. DNA sequences were compared with the original sequence encoding parasporin-2 using the BLAST NCBI service (http://blast.ncbi.nlm.nih.gov).

Protein expression, isolation, and analysis. 2.5 ml of an overnight transformed *E. coli* BL21-AI culture was added to 250 ml of fresh LB medium comprising 0.1% ampicillin, and then cultured for 3-4 hours until an OD600nm of 0.6-0.8 was reached. The expression of target protein was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 μM. Protein purification was carried out on the HisTrap FF (GE) 5 ml chromatography column; the concentration was measured by the Lowry method at 750 nm. The compliance of the samples with the molecular mass of parasporin-2 was determined by polyacrylamide gel electrophoresis (Akiba T., 2009: 121).

Determination of cytotoxic activity. LD_{50} was determined by analyzing the amount of formazan (MTS test) in cell culture at a wavelength of 490 nm in 96-well plates with Cell Titer 96 Proliferation assay kit (Promega). The number of cells per well in 90 μl was 2×10^4. Triton X-100 was used as a positive control, and physiological saline served as a negative control (Brown K., 1992: 549).

Results and Discussion. Parasporin-2 was chosen as the object of study, since it is the most effective antitumor parasporin possessing low-level cytotoxicity against normal human cells (table 1) (Okumura S., 2013: 1889; Lee DW., 2000: 218; Okumura S., 2005: 6313; Poornima K., 2010: 348; Namba A., 2003: 29; Uemori A., 2005: 122; Yasutake K., 2005: 124; Kitada S., 2009: 121; Akiba T., 2009: 121).

Table 1 – Cytotoxic activity of various parasporins against tumor and normal human cells (Okumura S. et al.)

Cell line	Characteristics of cells	LD_{50} (μg/mL)			
		Parasporin-1			Parasporin-1
MOLT-4	T-cell leukaemia	2,2	0.022	>10	0.472
Jurkat	T-cell leukaemia	>10	0.018	>10	>2
HL-60	T-cell leukaemia	0,32	0.019	1,32	0.725
T cell	Normal T cells	>10	–	>10	>2
HepG2	Hepatocarcinoma	3,0	0.019	2,8	1,90
HC	Normal hepatocytes	>10	1.1	>10	>2
HeLa	Cervical cancer	0,12	2.5	>10	>2
Sawano	Uterus cancer	>10	0.0017	>10	0.245
TCS	Cervical cancer	–	7.8	>10	0.719
UtSMC	Normal uterus cells	>10	2.5	>10	>2
Caco-2	Colon cancer	>10	0.013	>10	0.124

Two main functional domains can be identified in parasporin-2: transmembrane and receptor-binding. The recognition domain is represented by beta-pleated regions and short alpha-helices, and in contrast to the transmembrane domain, has a greater number of aromatic amino acids in absolute value (Kitada S., 2009: 80).

Table 2 – Mutant parasporins obtained upon amino acid substitutions

SN	Amino acids in the original parasporin-2 subjected to substitution and their position in the sequence	Amino acids in the modified parasporins 2 and their position in the sequence	Functional Domain
1	Serine 297	Cysteine 297	Transmembrane
2	Serine 297, Cysteine 111	Cysteine 297, Serine 111	Transmembrane
3	Serine 297, Cysteine 111, Tryptophan 81	Cystein e297, Serine 111, Alanine 81	Receptor-binding

Substitution of semantic triplets was carried out using the QuikChangeTMSite-Direct Mutagenesis Kit (Stratagene) with the following primers to replace the corresponding amino acids:

S297C-forward (5'-TCATCTAGTAAACAGCGTGCTGGTTGCAC-3'),
S297C-reverse (5'-GCAACCAGCAGCGTGTTTACTAGATGAAC-3'),
C111S-forward (5'-TTTTTATAGCTATAGTATCCATATTGAGC-3'),
C111S-reverse (5'-TATGGATACTATAGCTATAAAAATGTTGG-3'),
Y81A-forward (5'-GGGATTAGTACCAGCAATAGAGGAAAATCTAG-3'),
Y81A-reverse (5'-TCCTCTATTGCTGGTACTAATCCCGATGG-3').

The validity of the mutations was confirmed during the comparison (alignment) of DNA sequences obtained as a result of performed sequencing:

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Query  43
ATGAGAGGATCGCATCACCATCACCATCACGACGTTATTCGAGAATATCTTATGTTTAAT  102
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1
ATGAGAGGATCGCATCACCATCACCATCACGACGTTATTCGAGAATATCTTATGTTTAAT  60

Query  103
GAGTTATCAGCATTAAGTTCAAGTCCAGAAAGTGTAAGATCTAGATTTTCCTCTATTTCT  162
       |||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  61
GAGTTATCAGCATTAAGTTCAAGTCCAGAAAGTGTAAGATCTAGATTTTCCTCTATTTAT  120
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Query 163
GGTACTAATCCCGATGGTATTGCATTAAATAATGAAACGTATTTTAACGCCGTAAAACCG 222
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121
GGTACTAATCCCGATGGTATTGCATTAAATAATGAAACGTATTTTAACGCCGTAAAACCG 180

Query 223
CCTATTACTGCTCAATATGGATACTATAGCTATAAAAATGTTGGGACTGTTCAGTACGTA 282
              |||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct 181
CCTATTACTGCTCAATATGGATACTATTGCTATAAAAATGTTGGGACTGTTCAGTACGTA 240

Query 283
AATAGACCTACTGATATTAACCCAAACGTTATTCTTGCTCAAGACACATTAACAAATAAT 342
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241
AATAGACCTACTGATATTAACCCAAACGTTATTCTTGCTCAAGACACATTAACAAATAAT 300

Query 343
ACTAATGAACCATTTACTACAACTATCACTATAACTGGGTCTTTTACCAACACGTCTACT 402
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301
ACTAATGAACCATTTACTACAACTATCACTATAACTGGGTCTTTTACCAACACGTCTACT 360

Query 403
GTGACATCTAGTACAACAACAGGCTTTAAATTTACTAGTAAACTATCAATTAAAAAAGTC 462
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361
GTGACATCTAGTACAACAACAGGCTTTAAATTTACTAGTAAACTATCAATTAAAAAAGTC 420

Query 463
TTTGAAATTGGTGGAGAAGTTTCATTCTCTACTACAATTGGAACATCTGAAACAACTACA 522
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421
TTTGAAATTGGTGGAGAAGTTTCATTCTCTACTACAATTGGAACATCTGAAACAACTACA 480

Query 523
GAAACAATTACTGTATCTAAATCCGTTACGGTTACGGTTCCAGCTCAAAGTAGAAGAACT 582
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 481
GAAACAATTACTGTATCTAAATCCGTTACGGTTACGGTTCCAGCTCAAAGTAGAAGAACT 540

Query 583
ATTCAGTTAACAGCTGAAATAGCAAAAGAATCTGCAGACTTTAGTGCTCCTATT 635
              |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 541
ATTCAGTTAACAGCTAAAATAGCAAAAGAATCTGCAGACTTTAGTGCTCCTATT 594
```

The compliance of molecular mass of the pET-23a(+) plasmid vector encoding mutant parasporins was determined by means of agarose gel electrophoresis (aigure 1).

Cytotoxic concentrations of mutant parasporins (LD_{50}) showed an almost tenfold decrease in carcinolytic activity when an aromatic amino acid tryptophan was substituted to an aliphatic amino acid alanine in the receptor-binding domain in the absence of significant changes in cytotoxic concentrations in the case of serine to cysteine substitution in transmembrane regions of parasporin-2 (figure 2).

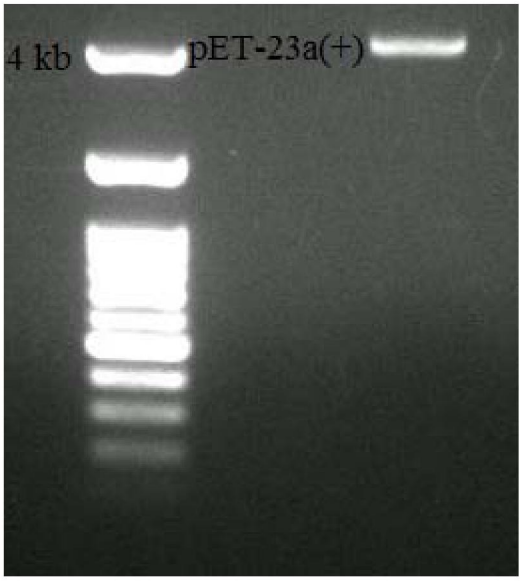

Figure 1 – Electrophoregram of pET-23a(+) plasmid

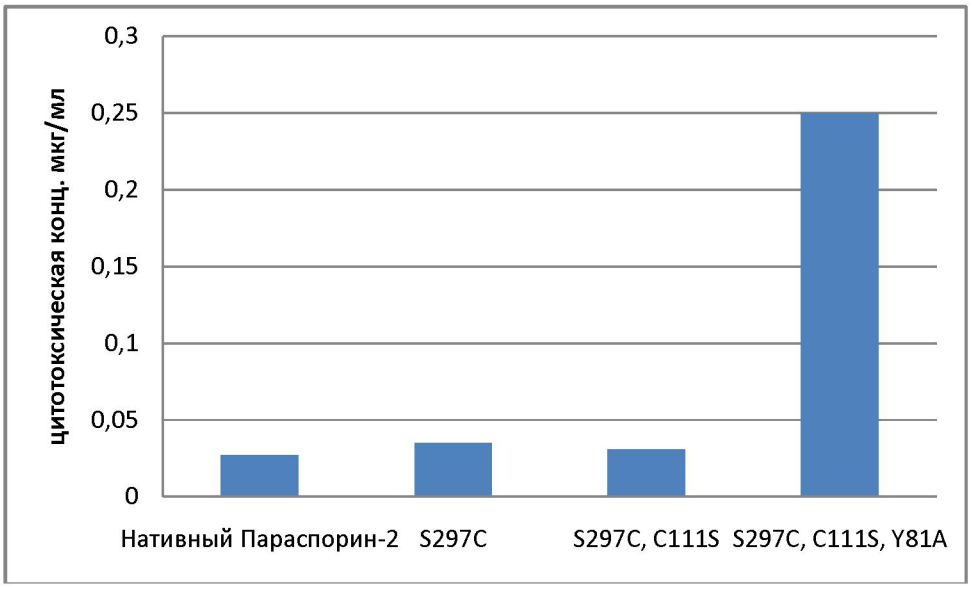

Figure 2 – Cytotoxic concentration (CTC_{50}) of mutant parasporins and native parasporin-2 in HepG2 (human hepatocarcinoma) cell line. Native PS-2 - 0.027 μg/mL; mutant parasporins S297C - 0.035 μg/mL; S297C, C111S - 0.031 μg/mL; S297C, C111S, Y81A - 0.25 μg/mL.

Conclusion. The findings of this study showed the effect of amino acid (serine, alanine, and tryptophan) substitutions on the carcinolytic activity of parasporin-2. It was found that the substitution of tryptophan to alanine in the receptor-binding domain of the protein had the greatest effect, which suggests the localization of the epitope in this region, which is responsible for affinity for the potential receptor on the surface of tumor cells for this particular anticancer protein agent. We believe that the resulting mutant parasporin-2 can be used as a control upon conjugation with potential targets of tumor cells to exclude molecules that enter into non-specific interaction, for example, in Western blotting.

Serine to cysteine and cysteine to serine substitutions in the transmembrane domain of parasporin-2 did not significantly affect the cytotoxic activity. This makes it possible to use this mutant parasporin-2 for chemical crosslinking with a high molecular weight carrier (gel, chromatography column, etc.)

through the sulfhydryl group of cysteine in order to determine surface targets by the way of their conjugation with the parasporin-carrier complex.

Establishment of the mechanism of action and targets for antitumor parasporins will determine the surface antigenic determinants of cancer cells; studies on their structures make it possible to develop more effective synthetic drugs. Determination of the most significant amino acids and epitopes in the receptor-recognizing part of parasporins enables *in silico* selection of a target from a database of known tumor antigens, as well as a development of a less effective parasporin, which can be used as a negative control when conjugating with potential surface targets of tumor cells in order to exclude molecules entering into interaction in a non-specific way, such as hydrophobic interaction.

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АДАМНЫҢ ГЕПАТОКАРЦИНОМАСЫНЫҢ ЖАСУШАЛЫҚ ҮЛГІСІНДЕГІ ПАРАСПОРИН-2 МУТАНТТЫ ПРОТЕИНІНІҢ САЛЫСТЫРМАЛЫ ТАЛДАУЫ

Аннотация. Мақалада Парпринпин-2 бактериясының карцинолитикалық бактериялық ақуызының рецепторлы-байланыстырушы және трансмембраналық аймағында амин қышқылының алмастырылу әсерін зерттеп, нәтижелері бойынша Нер G2 жасушаларының (адамның гепатокарциномасы) ісік жасушакларына қарсы цитотоксикалық белсенділікке әсерін зерттеу нәтижелері келтірілген. Тирозин аминқышқылдарын аланин,серин және цистеинге алмастыру, параспорин-2 протеинін полиистидин тегімен кодтайтын геннің дәйектілігі бар рекомбинантты плазмидтік ДНҚ-ға нүктелік мутация енгізу жолымен жүзеге асырылды. Аминқышқылдарын екі ақуыз аймағына аусуымен 3 жаңа мутанты параспорина-2 алынды. Параспирин-2 цитотоксикалық белсенділігінің он есе төмендеуі аминқышқыл тирозині белоктың рецепторлы-байланыстыру аймағында аланинмен алмастырылған кезде байқалды, бұл цистеинмен серинді алмастыру карцинолитикалық белсенділігіне айтарлықтай әсер етпеді. Алынған мәліметтер мен сынама үлгілері ісік жасушаларының бетінде мақсатты хроматография арқылы іздеуге болады.

Түйін сөздер: мутациялар, параспориндер, клеткалық қыралар, цитоулылығы.

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In vitro СРАВНИТЕЛЬНЫЙ АНАЛИЗ КАНЦЕРОЛИТИЧЕСКОЙ АКТИВНОСТИ МУТАНТНОГО БЕЛКА ПАРАСПОРИНА-2 НА КЛЕТОЧНОЙ МОДЕЛИ ГЕПАТОКАРЦИНОМЫ ЧЕЛОВЕКА

Аннотация. В статье представлены результаты исследования влияния замены аминокислот в рецептор-связывающем и трансмембранном домене канцеролитического бактериального белка параспорин-2 на цитотоксическую активность против опухолевой культуры клеток Нер G2 (гепатокарцинома человека). Замена аминокислот тирозина на аланин и серина на цистеин осуществлялась посредством введения точечной мутации в рекомбинантную плазмидную ДНК, содержащую последовательность гена, кодирующую белок параспорин-2 с полигистидиновым тагом. Получено 3 новых мутантных параспорина-2 с заменой аминокислот в двух доменах белка. Было установлено десятикратное снижение цитотоксической активности параспорина-2 при замене аминокислоты тирозина на аланин в рецептор-связывающем домене белка, замена серина на цистеин не оказало значительного влияния на канцеролитическую активность. Полученные данные и опытные образцы могут быть использованы для поиска мишеней на поверхности опухолевых клеток посредством аффинной хроматографии.

Ключевые слова: мутации, параспорины, клеточные линии, цитотоксичность.

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