Exp Oncol 2014
36, 3, 191–195

Experimental ONCOLOGY

THE PHOSPHO-c-JUN (SER63) CONTENT IN MUCOSA CELLS UNDER EXPERIMENTAL DEVELOPMENT OF GASTRIC CANCER

M.O. Tymoshenko, O.O. Kravchenko, O.V. Sokur, L.I. Ostapchenko*
Educational and Scientific Centre "Institute of Biology",
Taras Shevchenko National University, Kyiv 01601, Ukraine

Aim: **The aim of the study was to determine the phosphorylated c-Jun content and reduced and oxidized glutathione (GSH/GSSG) ratio in gastric mucosa cells during the process of gastric cancer development in rats.** ***Materials and Methods:*** **Gastric carcinogenesis was initiated in 80 white male rats by 10-week replacement of drinking water with 0.01% solution of N-methyl-N′-nitro-N-nitrosoguanidine, at the same time they were redefined on diet containing 5% NaCl. Then the animals were fed with standard vivarium diet till the end of 24th week. The gastric mucosa cells were examined at the end of 4th, 6th, 8th, 10th, 12th, 18th, and 24th weeks. Sandwich ELISA method was used to determine the content of phospho-c-Jun. The contents of GSH and GSSG were analyzed by spectrofluorymetric method with the use of orthophthalic aldehyde.** ***Results:*** **At the end of 6th, 8th, 10th weeks of MNNG and NaCl treatment the gastric mucosa cells were characterized by 4-, 6.3-, 1.9-fold higher content of phospho-c-Jun compared to the control, respectively, and 12, 18 and 24 weeks there was registered a stable increase of phospho-c-Jun content on the average at 3.6-fold compared to control values. Starting from 6th week of gastric cancer development an average decrease of GSH/GSSG was 3.4-fold compared with the control.** ***Conclusion:*** **During gastric carcinogenesis there was registered the decrease of GSH/GSSG ratio and increased level of phosphorylated c-Jun what points on MAP-kinase cascade activation in prooxidant conditions.**
Key Words: **c-Jun, reduce glutathione (GSH), oxidized glutathione (GSSG), gastric cancer.**

Gastric cancer is the second leading cause of cancer deaths worldwide. Several factors are thought to play a role in gastric carcinogenesis, including diet, exogenous chemicals, intragastric synthesis of carcinogens, genetic factors, infectious agents and pathological conditions in the stomach (such as gastritis) [1]. Smoked foods rich in nitrates, a high-salt diet, and *Helicobacter pylori* infection seem to be major environmental inducers of gastric cancer [2]. However, it remains unclear which molecular signals actually initiate the program of cell transformation.

Oxidative stress, nevertheless, appears to be an important factor in the induction of cancer [3, 4]. Reactive oxygen species (ROS) can directly produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. Persistent DNA damages can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, which are all seen in carcinogenesis [5]. Cellular oxidative stress can modify intercellular communication, protein kinase activity, membrane structure and function, and gene expression, and may result in modulation of cell growth [3].

At the same time a vast number of cellular processes is affected by the redox state, in which glutathione has a pivotal role. The most prevalent non-protein thiol in mammalian cells and the most abundant low molecular-weight peptide present in eukaryotic cells [6] — glutathione, exists in reduced (GSH) and disulfide/oxidized (GSSG) forms (GSH being predominant). The redox regulation affects numerous signalling pathways through protein phosphorylation and induces selectively a number of genes. Molecules activated in this way include transcription factor activator protein-1 (AP-1), responsible for the expression of a number of genes including many cytokines, TGF-β and collagenase, AP-2, c-Jun N-terminal kinase (JNK), protein kinase C and tyrosine kinase [7]. The overactivity of transcription factors that are activated directly by specific phosphorylation or that became activated following phosphorylation of other cellular proteins is an event in carcinogenesis that increases disregulation. Redox cycling of cysteinyl residues is one of several oxidant-dependent mechanisms that regulate the activity of many transcription factors, such as AP-1 [8] and particularly c-Jun, a potent transcriptional regulator is involved in cellular proliferation control [9, 10].

The resident nuclear protein c-Jun is one of hundreds of nuclear proteins that are being targets of serine proteinkinase cascades that are initiated in the cytoplasm and lead to phosphorylation and activation of this protein [11]. c-Jun is a major component of AP-1 and forms homodimers, or heterodimers with other Jun, Fos or ATF proteins [12]. Although the repertoire of AP-1 complexes varies between different cell types, c-Jun is a dominant component in many cells [13]. It is believed, that c-Jun is a critical promoter of cellular proliferation and dysregulated expression and activation of its oncogene are frequently observed in many tumor types [14] including gastric cancer [2]. But there are a lot of evidences that c-Jun/AP-1 activation has been implicated in various, often opposing cellular responses. Although there is a considerable evidence that c-Jun activation can be a positive step in the events leading

Submitted: May 21, 2014.
*Correspondence: E-mail: maria.bulavka@gmail.com
Fax: +38 (044) 521-35-98

Abbreviations used: AP-1 – activator protein-1; GSH – reduce glutathione; GSSG – oxidized glutathione; JNK – c-Jun N-terminal kinase; MAP kinase – mitogen-activated protein kinase; MNNG – N-methyl-N'-nitro-N-nitrosoguanidine; ROS – reactive oxygen species.

a cell towards apoptosis, there are also many reports stating the opposite: that under certain circumstances c-Jun can inhibit apoptosis and promote proliferation or differentiation. It is clear that the effects of c-Jun on cellular responses depend strongly on the cell type and the context of other regulatory influences that the cell is receiving [15].

The aim of this study was to determine the changes in phosphorylated c-Jun content and the level of GSH/GSSG ratio in gastric mucosa cells during the process of experimental gastric carcinogenesis using the N-methyl-N´-nitro-N-nitrosoguanidine (MNNG) mediated gastric cancer model in rats.

MATERIALS AND METHODS

The experiments were conducted using white male rats (n = 80) with the initial weight of 100 ± 20 g.

Gastric carcinogenesis was initiated by 10-week replacement of drinking water by 0.01% solution of carcinogen MNNG while the rats were given the feed, containing sodium chloride (5% NaCl of dry weight). Then the animals were fed with standard vivarium diet till the end of the 24th week [16, 17]. The control group of animals was fed with the standard diet during the all experimental period. The samplings of experimental material were taken at the end of 4th, 6th, 8th, 10th, 12th, 18th, and 24th weeks. The gastric cancer development was verified visually and histologically.

The gastric mucosa cells were isolated by the method, based on the enzymatic disaggregation of cells using pronase [18]. This method presupposed turning the mucosa of the isolated stomachs outward, deligation, filling them with the pronase solution (1 mg/ml), incubating (30 min, 37 °C) at intense stirring in the medium, saturated with 95% O_2 and 5% CO_2, and harvesting the disintegrated cells. To cytosol obtaining the isolated cells were homogenized on ice in teflon Potter-Elvehjem homogenizer. 0.01 M formic acid was added to the homogenate (1:1) for precipitation of proteins [19] and the mixture was centrifuged at 20 000 *g* for 15 min (4 °C) at Sigma centrifuge (USA). Both GSSG and GSH contents were measured in the supernatant.

The GSH content was registered using ortho-phthalic aldehyde, the reaction of the latter with GSH resulted in the formation of highly fluorescent products. The final mixture for the analysis contained 100 µl of the supernatant, diluted tenfold with 0.1 M phosphate buffer with 5 mM EDTA (pH 8.0), 1.8 ml of phosphate-EDTA buffer and 100 µl of ortho-phthalic aldehyde (1 mg/ml in methanol). The fluorescence intensity was measured at 420 nm with the activation of 350 nm after 15 min incubation at room temperature. The GSH concentration was expressed as nmol/mg protein. The GSSG content was determined similarly to fluorometric method of GSH estimation with previous incubation of supernatant with 0.04 M N-ethylmaleimid and following substitution 0.1 M phosphate buffer with 5 mM EDTA (pH 8.0) for 0.1 N NaOH (pH = 12) [19].

The phospho-c-Jun content was revealed in gastric mucosa cell lysate prepared with the use of Cell Lysis Buffer (Cell Signaling Technology, USA). The phospho-c-Jun content was measured by Sandwich ELISA method using the assay kit PathScan Phospho-c-Jun (Ser63) (Cell Signaling Technology, USA) and represented in conventional unit of absorbancy (λ = 450 nm) on mg of the protein.

The protein concentration was registered by Bradford's method [20].

The experimental data were processed by the common methods of the variance analysis with 7 repeats. The reliability of discrepancies between two samplings was determined using Student's criterion. The results are presented in the values of the arithmetic mean and standard error mean, M ± S.E.m [21].

The investigations were carried out in accordance with the main requirements to keeping and working with laboratory animals and to the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) as well as with the ethic norms specified in the Ukrainian legislation.

RESULTS AND DISCUSSION

Histological investigations were conducted for establishment of gastric cancer progression and estimation of gastric mucosa state under the gastric cancer development. The inflammation signs, the extension and plethora of vessels, the desquamation of epithelial cells, and the increase in the production of mucus at the end of the 6th week of experimental gastric carcinogenesis were shown [22]. After the effect of MNNG for 8 weeks the gastric mucosa demonstrated the atrophic changes. Metaplastic modifications, sites with hyperplastic impairments, and some cells with atypia were observed after 10 weeks of MNNG treatment. Some animals had adenomas and one adenocarcinoma at the end of the 12th week of the experiment. Such neoplasms were visualized in stomach pyloric region in 70% rats at the end of 18th week of the experimental gastric carcinogenesis.

The role of c-Jun in cellular transformation has been defined previously in rodent and avian model systems. Deregulated expression of c-Jun can lead to malignant transformation of immortalized rat fibroblasts while transformation of primary rat embryo cells required co-expression of an activated c-Ha-*ras*. In chicken embryo fibroblasts, c-Jun can induce cellular transformation by itself. Stable expression of a trans-activation suppressing deletion mutant of *c-jun* in malignant mouse epidermal cell lines inhibited tumor formation in nude mice. In human cancer, the role of *c-jun*/AP-1 is less clear. Increased constitutive levels of *c-jun* and *c-fos* mRNA and AP-1 levels have been reported for drug-resistant cells (such as etoposide resistant human leukemia K562 cells) compared to drug-sensitive parental lines. In the human breast adenocarcinoma cell line MCF-7, mitogenic stimulation by insulin or other insulin-like growth factors leads to increased *c-jun* or *c-fos* expression and AP-1 activity. In a study of non-

small cell lung primary and metastatic tumors, c-Jun is found to be overexpressed in 31% of the cases [23].

However, c-Jun role and even its activated form phospho-c-Jun in gastric tumorigenesis remain unknown. The submitted studies showed the control reference of phospho-c-Jun content at the end of 4th week of MNNG and NaCl consumption (Fig. 1) and morpho-histological changes at the same timepoint weren't observed. The gastric mucosa cells were characterized by increased content of phospho-c-Jun at 4.0- and 6.3-fold over the control at the end of 6th and 8th weeks, respectively, of the MNNG and NaCl treatment. Also it was established that the MNNG and NaCl treatment for 10 weeks caused 1.9-fold increase in phospho-c-Jun content compared to the control. At the terminal stages (12th, 18th and 24th weeks) of the gastric carcinogenesis study, that were characterized by adenocarcinomas in pyloric region, there was a stable increase of phospho-c-Jun content on the average at 3.6-fold in comparison with reference values.

The odtained experimental data of high phospho-c-Jun content in gastric mucosa cells are in agreement with the results of the immunohistochemical analysis that revealed activation of JNK in human gastric cancer tissue. Also mice lacking JNK1, a major JNK isozyme, exhibited a marked decrease in gastric carcinogenesis induced by N-methyl-N-nitrosourea relative to their wild-type counterparts [2].

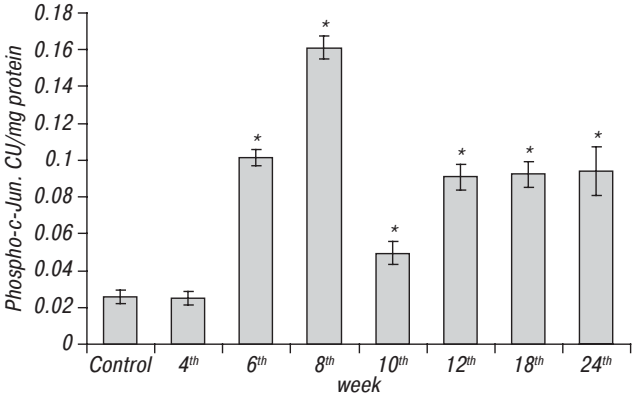

Fig. 1. The phospho-c-Jun content in gastric mucosa cells under gastric cancer development. *The difference is significant as compared to appropriate control ($p < 0.05$)

c-Jun is unique in its ability to positively regulate cell proliferation through repression of tumor suppressor gene expression and function, and induction of cyclin D1 transcription [9]. c-Jun, a specific target of JNK [13], is phosphorylated at serines 63 and 73 and threonines 91 and 93 within the transactivation domain [12]. Phosphorylation is the most important regulation of c-Jun and influences the activity of a protein by affecting the DNA-binding, stability, ability to interact with other proteins, and transactivation potential [12]. This phosphorylation event was suggested to stimulate c-Jun transcriptional activity, leading to the autoregulatory induction of *c-jun*, and, consequently, other genes [24]. JNK-mediated phosphorylation of Ser63/73 was demonstrated to inhibit both ubiquitination and degradation of c-Jun, leading to accumulation of the protein and increased transcriptional activity. Phosphorylation of c-Jun stimulates transcriptional activity by recruiting co-activator CREB-binding protein (CBP). CBP binds to the N-terminal activation domain of c-Jun and connects the phosphorylated activation domain to the basal transcriptional machinery.

Also c-Jun is phosphorylated at threonines 231 and 239 and serines 243 and 249 located proximal to the DNA-binding domain in the C-terminus. These sites are dephosphorylated during c-Jun activation and represent inhibitory phosphate groups. Activation of c-Jun requires phosphorylation of serines 63 and 73, as well as dephosphorylation of at least one of the C-terminal sites [12]. JNKs are also subject to inactivation. This is mediated by a specialized group of phosphatases, called MAP Kinase Phosphatases [13].

Thus, the growth of the phosphorylated form of c-Jun content from 6th to 24th weeks of gastric cancer development was caused by its high expression, amplification of JNK-mediated phosphorylation or inactivation of the phoshpatases. Like other Protein Tyrosine Phosphatases, MAP Kinase Phosphatases contain a highly reactive cysteine that mediates their inactivation, offering a way to regulate MAP kinase activity in response to production of ROS or exposure to thiol-reactive compounds [13]. Besides, it has been reported that N-nitroso compounds, which are important gastric carcinogens and potent inducers of cellular stress, leading to chromosomal aberrations, point mutations, cell death, and DNA damage, induce a specific cellular response program, which includes the activation of JNKs [25]. ROS-dependent redox cycling of cysteinyl thiols is also critical for the establishment of the protein-protein and protein-DNA interactions that determine many aspects of a signal transduction pathway. There is growing evidence that the reversible formation of mixed disulfides between GSH and low-pKa cysteinyl residues of proteins (*S*-glutathionylation) is an important mechanism for dynamic, posttranslational regulation of a variety of regulatory, structural, and metabolic proteins, and for the regulation of signaling and metabolic pathways in intact cell systems [26]. The c-Jun protein belongs to such molecules whose activity depends on the glutathione redox state.

The major marker of intracellular redox state is the ratio of GSH to glutathione disulfide so it has been suggested to regulate the activation of redox-sensitive transcriptional factors [27].

The 4th week of MNNG-stimulated carcinogenesis was characterised by increased GSH/GSSG ratio (Table). Such change was the consequence of more than twofold increased GSH content compared with the control values while the GSSG remained unchanged (Fig. 2). So the first carcinogenesis period was accompanied by compensatory activation of antioxidant cell state and there was no change registered in phospho-c-Jun content. The GSH content remained elevated but the GSSG was increased

(more than 7-fold) at 6th week of MNNG treatment. A decrease of GSH/GSSG on the average at 3.4-fold compared with reference value was revealed since 6th week of gastric cancer development, which proved the prooxidant state in the gastric mucosa cells. These indicators were caused by marked growth of GSSG content at 6th, 8th, 10th, 12th weeks and reduction of GSH at 8th, 12th, 18th and 24th weeks. The minimum values of GSH/GSSG ratio and severe prooxidant state were observed during 10th and 12th weeks of experimental gastrocarcinogenesis. These timepoints of experiment were characterised by the increase of phospho-c-Jun content but the peak of this parameter was observed at 6th and 8th weeks, when the GSH/GSSG ratio exceed the refence value at 3.8- and 3.5-fold respectively. Thus, there was the crucial value of intracellular redox state when phospho-c-Jun quantity extremely grew and then despite of amplification of prooxidant state the phospho-c-Jun content remained consistently high (from 12th to 24th week of gastrocarcinogenesis).

Table. The GSH/GSSG ratio in gastric mucosa cells under gastric cancer development

Control	Observation period, week						
	4th	6th	8th	10th	12th	18th	24th
16.1±2.6	24.4±4.3*	4.2±0.*	4.6±0.2*	1.4±0.1*	1.1±0.2*	8.2±2.0*	8.7±1.3*

Note: * the difference is significant as compared to appropriate control ($p < 0.05$).

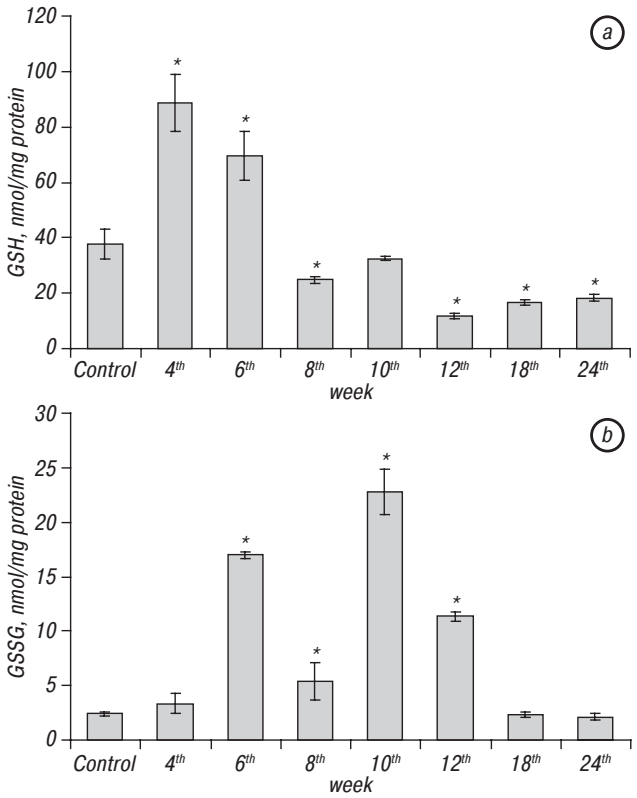

Fig. 2. The GSH (*a*) and GSSG (*b*) contents in gastric mucosa cells under gastric cancer development. *The difference is significant as compared to appropriate control ($p < 0.05$)

Such marked increase in the level of phospho-c-Jun at the 8th week of MNNG-stimulated gastrocarcinogenesis accompanied with a moderate decrease of GSH and increase of GSSG is a probable consequence of redox disturbances in cell cytoplasma under ROS. It is known that ROS activate MAPK, but the response of distinct regulatory chains in different cell types is unequal. There are several mechanisms of MAPK activation under ROS: activation of redox-dependent ASK-1 as a consequence of thioredoxin oxidation and dissociation of JNK from the complex with GST Pi. Also inactivation of JNK-specific phosphatase by H_2O_2 may couse permanent JNK activation [28]. Similar activation by ROS determined in our study might lead to the growth of the phosphorylated c-Jun amount.

But if the level of oxidative stress is excessive, there will be a direct impact of ROS on Cys residues of DNA-binding proteins. The oxidation of SH-groups in these residues results to reversible or irreversible (according to the ratio ROS/antioxidants) inactivation of AP-1. Since the promoter region of c-Jun gene has several zones of binding of AP-1, phosphorylation and subsequent activation of factor c-Jun leads to the induction of its own gene [28]. Thus the high level of GSSG with the phospho-c-Jun amount decrease at the 10th week may indicate to acute prooxidant conditions that affected genome and led to an inhibition of c-Jun expression. In spite of this the level of phospho-c-Jun remained above the control values but less than at the previous 8th week.

The 18th and 24th weeks were characterized by the shift of redox-station to reduced state with the recovery of c-Jun expression and following its phosphorylation.

Experimental evidences support an important role of ROS in the cancer process. Oxidative stress can occur through overproduction of reactive oxygen and nitrogen species with the shift of GSH/GSSG ratio to prooxidative state. Disruption of this ratio is involved in several cellular reactions involved in signal transduction and cell cycle regulation under conditions of oxidative stress. The unregulated or prolonged production of cellular oxidants has been linked to mutation, as well as modification of gene expression. But the effects of ROS and oxidative stress within cells appear to be cell specific and dependent upon the form as well as the intercellular concentration of ROS [3]. In particular, MAPK/AP-1 signal transduction pathway, including c-Jun activation by phosphorylation, is known to be activated by ROS, and leads to the transcription of genes involved in cell growth regulatory pathways. The cellular concentration of ROS appears to influence the selective activation of this transcription factor and therefore may help explain the observation that either cell death or cell proliferation may result from exposure to ROS. A common effect of AP-1 activation is an increased cell proliferation and the several lines of evidence have demonstrated that c-fos and c-jun are positive regulators of cell proliferation [9].

The acute oxidative injury may produce selective cell death and a compensatory increase in cell proliferation. This stimulus may result in the formation of newly initiated preneoplastic cells and/or enhance the selective clonal expansion of latent initiated preneoplastic cells. Similarly, sublethal acute oxidative injury may produce unrepaired DNA damage and result in the formation of new mutations and, potentially, new initiated cells. In contrast, sustained chronic oxidative

injury may lead to a nonlethal modification of normal cellular growth control mechanisms [3].

In conclusion, the decrease of GSH/GSSG ratio and simultaneously increased level of active phosphorylated form of c-Jun during long period (from 6th to 24th week of histological established progression of gastric cancer) point on possible MAP kinase cascade activation in prooxidant conditions.

REFERENCES

1. Ilhan N, Ilhan N, Ilhan Y, ***et al.*** C-reactive protein, procalcitonin, interleukin-6, vascular endothelial growth factor and oxidative metabolites in diagnosis of infection and staging in patients with gastric cancer. World J Gastroenterol 2004; **10**: 1115–20.

2. Shibata W, Maeda S, Hikiba Y, ***et al.*** c-Jun NH_2-terminal kinase 1 is a critical regulator for the development of gastric cancer in mice. Cancer Res 2008; **68**: 5031–9.

3. Klaunig JE, Xu Y, lsenberg JS, ***et al.*** The role of oxidative stress in chemical carcinogenesis. Environ Health Perspect 1998; **106**: 289–95.

4. Schumacker PT. Reactive oxygen species in cancer cells: Live by the sword, die by the sword. Cancer Cell 2006; **10**: 175–6.

5. Klaunig JE, Kamendulis LM. The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 2004; **44**: 239–67.

6. Estrela JM, Ortega A, Obrador E. Glutathione in cancer biology and therapy. Crit Rev Clin Lab Sci 2006; **43**: 143–81.

7. Balendiran GK, Dabur R, Fraser D. The role of glutathione in cancer. Cell Biochem Funct 2004; **22**: 343–52.

8. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 1999; **39**: 67–101.

9. Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene 2001; **20**: 2390–400.

10. Mialon A. Role and function of c-Jun protein complex in cancer cell behaviour. Turku: Turun Yliopisto, 2008. 84 p.

11. Darnell JE. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002; **2**: 740–9.

12. Eriksson M. AP-1 transcription factor in cell differentiation and survival: dissertation. Helsinki: HBGS, 2005. 68 p.

13. Catherine D, Wiltshirea C, MacLarena A, ***et al.*** Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. Cell Signal 2002; **14**: 585–93.

14. Taira N, Mimoto R, Kurata M, ***et al.*** DYRK2 priming phosphorylation of c-Jun and c-Myc modulates cell cycle progression in human cancer cells. J Clin Invest 2012; **122:** 859–72.

15. Leppa S, Bohmann D. Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. Oncogene 1999; **18**: 6158–62.

16. Kuroiwa Y, Ishii Y, Umemura T, ***et al.*** Combined treatment with green tea catechins and sodium nitrite selectively promotes rat forestomach carcinogenesis after initiation with N-methyl-N′-nitro-N-nitrosoguanidine. Cancer Science 2007; **98**: 949–57.

17. Takahashi M, Nishikawa A, Furukawa F, ***et al.*** Dose-dependent promoting effects of sodium chloride (NaCl) on rat glandular stomach carcinogenesis initiated with N-methyl-N′-nitro-N-nitrosoguanidine. Carcinogenesis 1994; **15**: 1429–32.

18. Tairov M, Bersimbaev R, Argutinskaya S, ***et al.*** Cellular localization of adenylate cyclases, stimulated by histamine and prostaglandin E2 in the gastric mucosa of rats and their role in the regulation of gastric secretion. Biochemistry 1983; **48**: 1035–41.

19. Hissin P, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976; **74**: 214–26.

20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; **72**: 248–54.

21. Brandt Z. Statistical methods for analysis of observations. Moscow: Mir, 1975. 312 p.

22. Tymoshenko MO, Kravchenko OO, Gaida LM, ***et al.*** Glutathione transferase activity and reduce glutathione content in the cytosol of rat gastric mucosa cells under carcinogen N-methyl-N′-nitro-N-nitrosoguanidine treatment. Biopolym Cell 2012; **28**: 374–80.

23. Smith LM, Wise SC, Hendricks DT, ***et al.*** cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. Oncogene 1999; **18**: 6063–70.

24. Karin M, Gallagher E. From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB Life 2005; **57**: 283–95.

25. Uehara N, Miki K, Tsukamoto R, ***et al.*** Nicotinamide blocks N-methyl-N-nitrosoureainduced photoreceptor cell apoptosis in rats through poly (ADP-ribose) polymerase activity and Jun N terminal kinase/activator protein-1 pathway inhibition. Exp Eye Res 2006; **82**: 488–95.

26. Ballatori N, Krance SM, Notenboom S, ***et al.*** Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem 2009; **390**: 191–214.

27. Rokutan K, Techima S, Miyoshi M, ***et al***. Glutathione depletion inhibits oxidant-induced activation of nuclear factor-kappa B, AP-1, and c-Jun/ATF-2 in cultured guinea-pig gastric epithelial cells. J Gastroenterol 1998; **33**: 646–55.