International Journal of Natural and Engineering Sciences
E-ISSN: 2146-0086 15(2): 57-66, 2021
Research Article

RELATIONSHIPS OF NO WITH NOSIP, MMP-2/9 AND TIMP-2 IN HUMAN COLORECTAL CANCER

Selda Kabadere[1,a,*], Aygul Cemre Sahin[1,b], Rumeysa Ozyurt[2,c], Gökhan Kus[3,d], Bugra Koca[4,e], Nilufer Erkasap[1,f]

[1]*Eskisehir Osmangazi University, Faculty of Medicine, Department of Physiology, Eskisehir, Turkey*
[2]*Kutahya Health Science University, School of Medicine, Department of Physiology, Kutahya, Turkey*
[3]*Anadolu University, Open Faculty, Department of Health Programme, Eskisehir, Turkey*
[4]*Afyon Kocatepe University, Faculty of Medicine, Department of Biochemistry, Afyon, Turkey*

**Corresponding Author:*
E-mail: sdorman@ogu.edu.tr

(Received 03th September 2020; accepted 15th December 2020)

a: ORCID 0000-0002-9589-0063, b: ORCID 0000 0002 0513 4986, c: ORCID 0000 0002 9887 2645,
d: ORCID 0000-0002-2424-2720, e: ORCID 0000-0002-5353-3228, f: ORCID 0000-0002-2742-3519

ABSTRACT. Background/aim: Gelatinases (matrix metalloproteinases/MMP-2 and -9) have a key role during progression of colorectal cancers (CRCs). The imbalance between tissue inhibitor metalloproteinases (TIMP-2) and MMPs results in pathological outcomes. Previous studies indicate that gelatinases and TIMP-2 are stimulated by nitric oxide (NO). NO has been reported to exert both tumoricidal and tumor promoting effects at different stages of many cancer types. Therefore, control of NO production may be important for progression of cancer. Nitric oxide synthase interacting protein (NOSIP), an inhibitor of eNOS and nNOS enzymes, leads to a tissue preservation process. Thus, we assume that NOSIP may play an important role in progression of CRC. Tumorous tissue, normal tissue and plasma were collected from 17 CRC patients, and normal plasma was collected from 17 healthy individuals. Tissue samples were used for q-RT-PCR assay and plasmas for nitrite/nitrate quantification and ELISA analysis. The levels of NO, MMP-2 and MMP-9 increased in the plasma of CRC patients, whereas level of NOSIP protein decreased and amount of TIMP-2 did not change. Gelatinase gene expressions were found to be higher and the levels of NOSIP gene expression lower, but TIMP-2 gene expressions did not differ in CRC samples. We suggested that NO may interfence activities of MMP-2 and -9 in CRC patients as a result of a reduction in NOSIP level. NOSIP protein and NOSIP gene expression levels were determined for the first time in human cancerous tissues.

Keywords: *Gelatinases, nitric oxide, nitric oxide synthase interacting protein, tissue inhibitor metalloproteinases*

INTRODUCTION

Colorectal cancers (CRC) are the third most common cancer types in the world [1]. About 250,000 new cases of colon cancer are diagnosed each year in Europe, and this constitutes about 9% of all malignancies [1, 2]. In previous cancer studies, nitric oxide (NO) has been reported to exert dichotomous effects on many types of cancer at different stages. NO may also have tumoricidal effects as opposed to tumor promoting effects. Therefore, the dual effect of NO in tumor biology leads to confusion in the development of novel therapy for many cancer types [3].

NO is a small gaseous molecule produced by L-arginine via NO synthases (NOSs) [4], has a half-life of several seconds, and its cellular effect is mediated by cyclic guanosine monophosphate (cGMP)-dependent pathways (classical pathways) and cGMP independent pathways (nonclassical pathways). In a cGMP-dependent pathway, NO binds at nanomolar levels to soluble guanylate cyclase (sGC) and triggers cGMP formation [5]. Pathways that are cGMP independent include posttranslational modifications of target proteins. The effects of NO posttranslational modification reactions leading to the formation of radical nitrogen species (RNS) are destructive and include lipid peroxidation and, DNA and protein denaturation [3, 6].

Recent studies demonstrated that nitric oxide synthase interacting protein (NOSIP) has an inhibitory effect on the activities of endothelial NOS (eNOS) and neuronal NOS (nNOS) enzymes [7, 8]. According to the limited data, NOSIP is located in the cytoplasmic area and-/or in the nucleus, depending on the cell type [9]. Previous studies demonstrating the presence of NOSIP revealed a protein-protein interaction between eNOS and NOSIP. NOSIP is active in the caveola region and translocates to the internal regions of the cell, thereby reducing enzyme activity and leading to a decrease in NO levels [7]. It has been also claimed that there is a similar interaction between nNOS and NOSIP [8].

NO promotes metastasis of tumor cells by increasing vascular permeability and up-regulating matrix metalloproteinases (MMPs) [10]. MMPs were shown to have an important role in tumor progression, including invasion, metastasis and angiogenesis [11]. These are proteinases that are responsible for the degradation of extracellular matrix molecules such as collagen, fibronectin, laminin and proteoglycan [12]. In particular, gelatinases (MMP2 and MMP9) have been proven to contribute to invasion and metastasis in various malignant tumors [13]. The maintenance of equilibrium between MMPs and their endogenous inhibitors is crucial for maintaining physiological balance. Tissue inhibitor metalloptoteinases (TIMPs) are among the best-known endogenous inhibitors of MMPs. Four types of TIMP isoforms are defined to date. The dynamic equality between MMP2 and TIMP plays a key role in the cellular processes of tumor cells such as proliferation, migration, adhesion and apoptosis [14].

We assume that NOSIP may play a crucial role in the development of CRC since NO has dose-dependent effects on cancer cells. Therefore, we aimed to first determine the possible relationship between NO and NOSIP, and second, to measure the levels of gelatinases and TIMP2 in CRC, because it is known that NO boosts MMPs and vascular permeability, both of which result in metastasis of tumor cells.

MATERIALS AND METHODS

Groups

The present study was approved by the Ethics Committee for Clinical Research of the Eskisehir Osmangazi University (80558721/216/2017). Tissue specimens from the tumors of 17 patients (8 females, 9 males) who underwent surgical resection for CRC and normal colon tissue samples (mucosal tissue 10 cm far away from the tumor) from these patients were collected at the Department of General Surgery, Hospital of Eskisehir Osmangazi University, Eskisehir, Turkey. The clinical characteristics of the patients are provided in the Table. Blood samples were also collected from the same CRC patients

and 17 (8 females, 9 males) healthy subjects with an average age of 28.7 (the youngest is 26 and the oldest is 39 years old) without any chronic disease and medication.

Table 1. *Clinical characteristics of patients*

Variable	Patients
Number	17
Men/Women	9/8
Mean Age (Range)	61.8 (The youngest is 34; the oldest is 79)
Mean Weight (kg)	71.6 (the weakest is 50; the fattest is 158)
Mean Height (cm)	166 (The shortest is 153; the longest is 185)
Tumor Localization	
Left Colon	6
Right Colon	4
Sigmoid Colon	6
Rectum	1
Pathological Type	
Tubular and papillary	15
adenocarcinoma	2
Mucinous and signet ring cell carcinoma	
Tumor Stage*	
T1	1
T2	1
T3	14
T4	1
Lymph Node Metastases	
N0	13
N1a	1
N1b	2
N2a	1
Distant Metastases	0

*: Tumor staging was done according to the TNM classification [15].

Tissue and Plasma Samples

Immediately after excision, the tissue samples were fixed in RNA-later solution (Invitrogene, USA) for quantitative Real-Time Polymerase Chain Reaction (q-RT PCR) assay. After they were collected from the patients and healthy people, the blood samples were centrifuged at 1000 g for 15 minutes at 2-8 °C to collect plasma, and stored at minus 80 °C for ELISA analysis.

Colorimetric Nitrite/Nitrate Assay

The NO level of the plasma was determined using colorimetric nitrite/nitrate assay kit (Cayman Chemical, USA). In this assay total nitrite (nitrites/nitrates) was analyzed as an indicator of NO production. A mixture of 40 µl plasma, 40 µl assay buffer and 10 µl nitrate reductase was incubated at room temperature for 1 hour. Then, 50 µl of Griess reagent was added to each well and incubated at room temperature for another hour. Absorbance was detected spectrophotometrically at 540-550 nm.

ELISA Analysis

Plasma NOSIP (Sunredbio, Shanghai), gelatinases and TIMP2 (Elabscience, Texas) levels were determined using the sandwich ELISA method. First, order wells were filled with standard working solution samples and 100 µl plasma samples were placed into other wells for incubation for 90 minutes at 37 °C. Then, the liquid from each well was removed and 100 µL of biotinylated detection Ab working solution was added to each well for one hour at 37 °C. After this incubation, all wells were washed with 350 µl of washing buffer. A 100 µl horseradish peroxidase conjugate working solution was added to each well, incubated at 37 °C for 30 minutes and the washing step repeated with washing buffer. A substrate reagent was added to all wells and incubated about 15 minutes. Finally, a stop solution was added to each well and then the optical density (OD) was determined at 450 nm.

q-RT PCR Assay

The mRNA expressions of gelatinases (Roche Diagnostics Germany, MMP2 product number: 0000024192; MMP9 product number: 0000024195), TIMP2 (Roche Diagnostics Germany, product number: 0000024193) and NOSIP (Roche Diagnostics Germany, product number: 0000024196) in relation to the housekeeping gene (β-actin, Thermo Fisher Scientific, USA, 401846) were analyzed using q-RT-PCR with TaqMan probes (Roche Diagnostics Germany, product number: 0453526001) in CRC tissue and normal tissue. Total RNA was extracted using a Gene JET RNA Purification Kit (Thermo Fisher Scientific, USA) and stored at minus 80 °C until use. Total RNA concentrations were measured with a Nanodrop 1000 (Thermo Fisher Scientific, USA) at the absorbance of 260 nm. From each sample, 500 ng of RNA was used for the production of complementary DNA (cDNA). cDNA was synthesized by using a transcription high-fidelity cDNA synthesis kit (Roche Diagnostics, Germany) and stored at minus 20 °C. The q-RT PCR thermal cycling conditions were as follows: 10 minutes at 95 °C, 20 seconds at 95 °C, 30 seconds at 55 °C and 20 seconds at 72 °C incubations for 50 cycles. Data was collected from a LightCycler® Nano Software (Roche Diagnostics, Germany) detection system. Cycle threshold values were identified using automated threshold analysis, and final calculations performed using the 2-ΔΔCt relative quantification method. All experiments were carried out in duplicate.

Statistical Analysis

The GraphPad Prism 6 program was used for statistical evaluations. The groups were analyzed according to whether they showed normal distribution in the Shapiro-Wilk Normality test. Differences between the groups were assessed using the Tukey test, after evaluating the normal distribution with a one-way ANOVA test. Data showing non-normal distribution was studied using the Mann-Whitney U test and Dunn's test, post-

hoc. Results were pointed out as being means ± standard deviation (SD). Refusal of the null hypothesis was set at the p value which was less than 0.05.

RESULTS AND DISCUSSION

Colorimetric Nitrite/Nitrate Assay

Colorimetric nitrite/nitrate assay experiments are based on determining the levels of the final products (such as nitrite and nitrate) of NO in biological fluids. Present findings clearly show that total nitrite/nitrate levels in the plasma of CRC patients is about 10 μM higher ($p=0.0077$) than the plasma levels of healthy individuals (Fig 1, $p<0.01$).

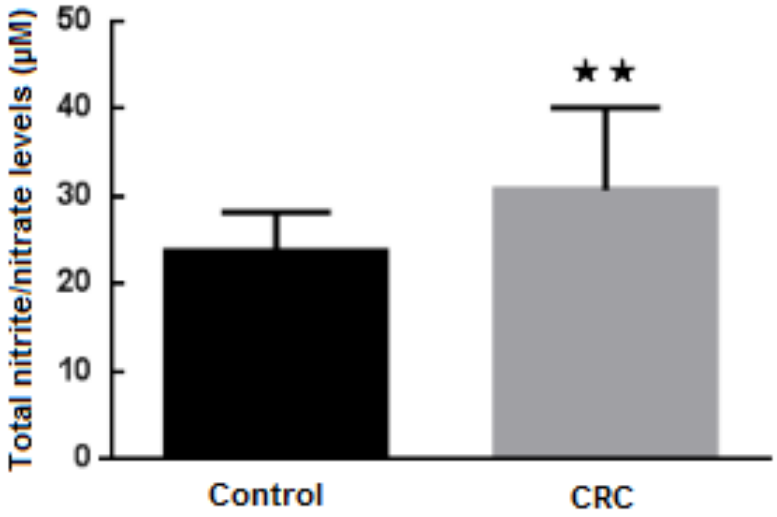

Fig. 1. *Total nitrite/nitrate levels of the CRC and control groups ($p<0.01$)*

ELISA Analysis

The results obtained from ELISA analysis indicate that the NOSIP levels significantly decreased in the plasma of CRC patients compared to the levels of healthy individuals (Figure 2A, $p=0.0421$). Furthermore, we found that levels of gelatinases in the plasma of CRC patients were higher than in the control group (Figure 2B and 2C, $p<0.005$). Conversely, there was no statistical difference in TIMP2 levels between the plasma of the patients and the plasma of the healthy people (Figure 2D, $p=0.9713$). However, dynamic equality between gelatinases and TIMP2 is impaired (Figure 2B, 2C and 2D).

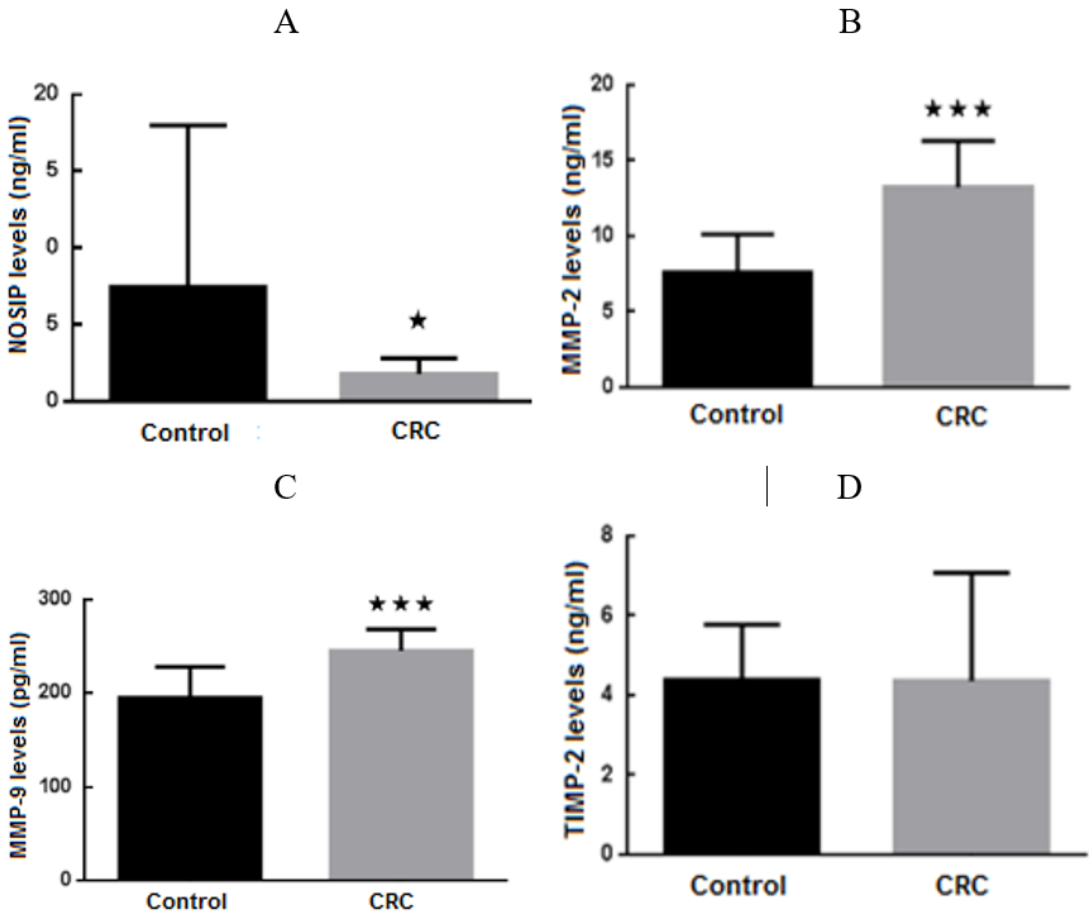

Fig. 2. *Plasma levels of NOSIP (A. p<0.05), MMP2 (B. p<0.005), MMP9 (C. p<0.005) and TIMP2 (D. p>0.05)*

q-RT PCR Assay

The results from the qRT PCR assay indicated that the gene expression level of NOSIP is slightly lower in the CRC tissue as compared to the normal surrounding tissue removed 10 cm far from the tumor. However, this difference is not statistically significant (Figure 3A, p=0.3003). mRNA expression levels of MMP2 (Figure 3B, p=0.0226) and MMP9 (Figure 3C, p=0.0412) were increased in CRC tissue (p<0.005). Similar to the findings obtained from ELISA analysis, no statistically significant difference between CRC and the normal tissue (Figure 3D, p=0.4169) was calculated in terms of mRNA levels of TIMP2.

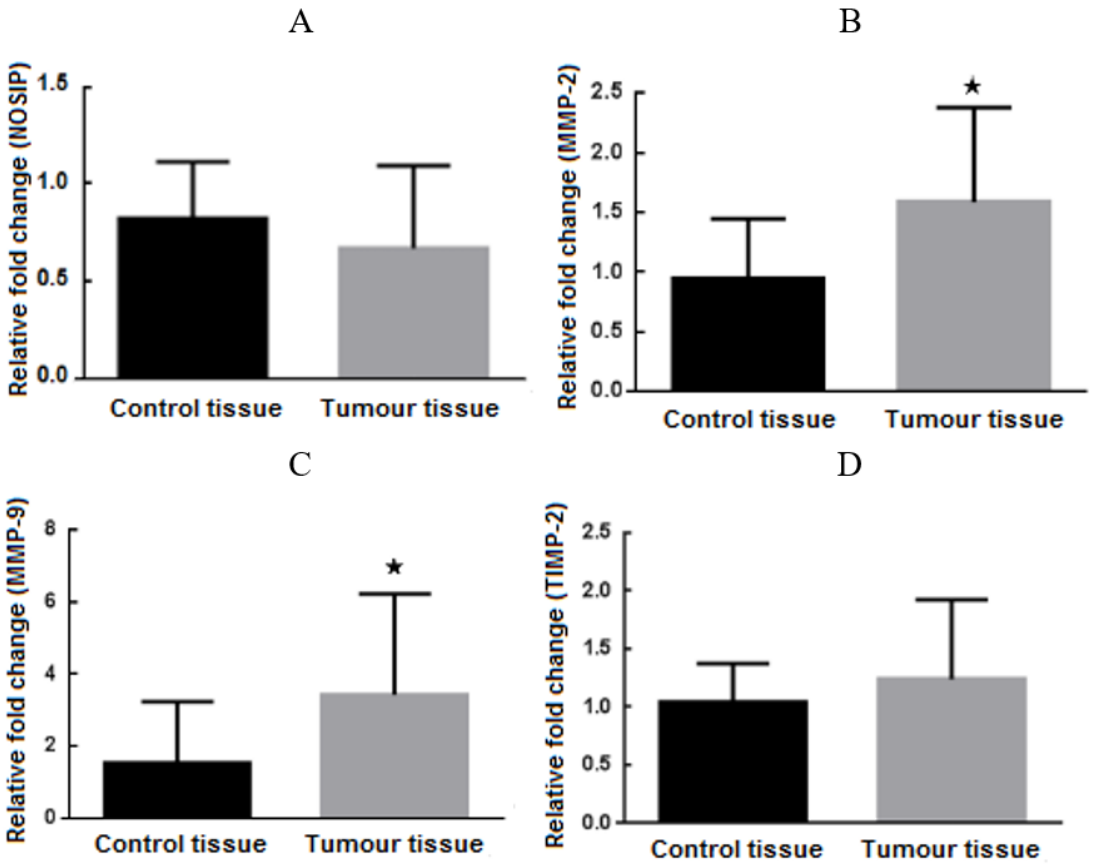

Fig. 3. *A. NOSIP mRNA levels, B. MMP2 gene expression levels ($p<0.05$), C. MMP9 gene expression levels ($p<0.05$) and D. TIMP2 gene expression levels*

The results of our experiments show that there is more NO in the plasma of CRC patients than in the plasma of healthy individuals. According to previous information, NO induces the proliferation of tumors, but also has a tumoricidal effect through direct and indirect mechanisms [16]. Therefore, the dual effect of NO in tumor biology leads to confusion when developing novel therapies for many cancer types. Supporting our results, Ying and Hofseth mentioned that chronic inflammation causes production of activated inflammatory cells that can release RNS, reactive oxygen species, and cytokines. These events cause activation of NOS enzymes. NO production is one of the important biomarkers in the pathogenesis of CRC. NO plays key role in modulating tumour progression from a colorectal adenoma to a colorectal carcinoma and metastases [17]. Additionally, NO can stimulate tumor growth by triggering angiogenesis and increasing blood flow to the tumor tissue [4]. Additionally, Oláh et al found that increased expression of inducible NOS, nNOS was detected in colon cancer tissues, when compared with surrounding colon tissues. In the same article, inhibitor of NOS, at lower concentrations, slightly stimulated HCT116 cell proliferation, but inhibited proliferation at higher concentrations. Donors of NO inhibited HCT116 proliferation in a concentration-dependent manner, in vitro [18]. The pro-apoptotic effect of NO leads to the formation of RNS through cGMP-independent pathways which can lead to the formation of peroxynitrite, disrupts carbohydrates, initiates lipid peroxidation and causes DNA degradation [3]. Furthermore, Choudhari et al. [3] mentioned that the tumoricidal effect of high dose NO is not proven in cancer patients, however this tumoricidal effect

has been seen in most in vitro experiments. When this information is compared with our results, we can conclude that a high level of NO in the plasma of cancer patients may not have a tumoricidal effect.

The NOSIP molecule, which has an inhibitory effect on eNOS and nNOS enzymes, suppresses NO production, causing a decrease in its level [7, 8]. Schleicher et al. emphasized that NOSIP accumulates in cytoplasm and leads to the translocation of eNOS into actin skeleton. NO level is reduced during the G2 phase of the cell cycle. Schleicher et al. thought that this could play an important role in apoptosis and cell proliferation [9]. For this reason, it is thought that NOSIP is able to develop a protective mechanism against the cell-damaging effects of NO [19]. In the present study, NOSIP gene expression and protein levels were measured for the first time in human CRC tissue and plasma, and compared with normal tissue and plasma of healthy individuals. We found that levels of both NOSIP gene expression and NOSIP protein decreased, weakly supporting our findings of NO rise in CRC. Determining the relationship between NO and NOSIP might be important information regarding mechanisms of cancer progression.

It is well established that MMP2 and MMP9 molecules play a crucial role in the progression of tumors in CRC, and NO contributes to this development by triggering these enzymes [10]. MMP9 and MMP2, members of the endopeptidase family, were found to be high in the presence of many cancers, including those of the breast [20], stomach [21] and colon [22]. Both in gene expression level measurements and in ELISA quantitative assay of plasma samples, we also found that MMP2 and MMP9 levels were higher in CRC patients than in healthy individuals. This confirms again that MMPs might play an important role in tumor progression, including invasion, metastasis and angiogenesis. In a very recent study Gao et al demonstrated that the NOS inhibitors 1400W and L-NIO decrease NO production and suppress CRC cell proliferation and migration, which is associated with systematic inhibition of angiogenesis-related gene transcription and protein expression, including MMP2/9. Combining of 1400W or L-NIO with 5-FU showed greatly enhanced anti-proliferative effects on CRC cell proliferation and cell migration [23]. Their results support our findings from ELISA and qRT-PCR analysis.

TIMP2, an endogenous inhibitor of MMP2 and MMP9, binds to MMPs in a 1:1 stoichiometric relationship, directly affecting the activity of MMPs [14]. We found no significant difference between patients and healthy individuals in terms of TIMP2 gene expression and plasma level. However, we observed that the relationships between gelatinases (MMP2 and MMP9) and TIMP2 levels were unbalanced. Similarly, L-NIO, a NOS inhibitor that inhibits eNOS and nNOS, inhibits gene transcription of TIMP3, but it doesn't suppress TIMP-1 and TIMP2 on HT 29 cells [23].

CONCLUSION

It has been demonstrated that NOSIP has an inhibitory effect on the activities of nNOS and eNOS [7, 8]. In the present study, the level of NOSIP was found to have decreased significantly in relation to the increased level of NO in the CRC plasma. So far, no relationship has been reported between cancer and NOSIP. We believe that this is the first study describing an association between NO, NOSIP and other parameters, such as MMP2, MMP-9 and TIMP2 that are known to play an important role in cancer biology. Further studies are needed to understand these mechanisms more clearly.

Acknowledgement. This study was funded by Anadolu University Scientific Research Projects Commission (grant number 1508s596).

REFERENCES

[1] Migliore, L., Migheli, F., Spisni, R. & Coppedè, F. (2011): Genetics, cytogenetics, and epigenetics of colorectal cancer. Journal of Biomedicine and Biotechnology, 792362.

[2] Labianca, R., Beretta, G.D., Kildani, B., Milesi, L., Merlin, F., Mosconi, S., Pessi, M.A., Prochio, T., Quadri, A., Gatta, G., Braud, F. & Wils, J. (2010): Colon cancer. Critical Reviews in Oncology/Hematology, 74, 106-133.

[3] Choudhari, S.K., Chaudhry, M., Bagde, S., Gadbail, A.R. & Joshi, V. (2013): Nitric oxide and cancer: a review. World Journal of Surgical Oncology, 11, 118.

[4] Burke, A.J., Sullivan, F.J., Giles, F.J. & Glynn, S.A. (2013): The yin and yang of nitric oxide in cancer pogression. Carcinogenesis, 34(3), 503-512.

[5] Martinez-Ruiz, A., Araujo, I.M., Izquierdo-Alvarez, A., Hernansanz-Agustin, P., Lamas, S. & Serrador, J.M. (2013): Specificity in S-nitosylation: a short-range mechanism for NO signaling? Antioxidants and Redox Signaling,19(11), 1220-1235.

[6] Ruiz, A.M., Cadenas, S. & Lamas, S. (2011): Nitric oxide signaling: classical, less classical and nonclassical mechanisms. Free Radical Biology Medicine, 51, 17-29.

[7] Dedio, J., König, P., Wohlfart, P., Schroeder, C., Kummer, W. & Müller-Esterl, W. (2001): NOSIP, a novel modulator of endothelial nitric oxide synthase activity. FASEB Journal, 15, 79-89.

[8] Dreyer, J., Schleicher, M., Tappe, A., Schilling, K., Kuner, T., Kusumawidijaja, G., Müller-Esterl, W., Oess, S. & Kuner, R. (2004): Nitric oxide synthase (NOS)-interacting protein interacts with neuronal NOS and regulates its distribution and activity. The Journal of Neuroscience, 24(46), 10454-10465.

[9] Schleicher, M., Brundin, F., Gross, S., Müller-Esterl, W. & Oess, S. (2005): Cell cycle-regulated inactivation of endothelial NO synthase through NOSIP-dependent targeting to the cytoskleton. Molecular and cellular biology, 25(18), 8251-8258.

[10] Ziche, M. & Morbidelli, L. (2009): Molecular regulation of tumor angiogenesis by nitric oxide. European Cytokine Network, 20(4), 164-70.

[11] Damodharan, U., Ganesan, R. & Radhakrishan, U.C. (2011): Expression of MMP2 and MMP 9 (gelatinases A and B) in human colon cancer cells. Applied Biochemistry and Biotechnology, 165(5-6), 1245-1252.

[12] Bode, W., Fernandez, C.C., Tschesche, H., Grams, F., Nagase, H. & Maskos, K. (1999): Structural properties of matrix metalloproteinases. Cellular and Molecular Life Sciences, 55(4), 639–652.

[13] Yoon, S.O., Park, S.J., Yoon, S.Y., Yun, C.H. & Chung, A.S. (2002): Sustained production of H_2O_2 activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-κB pathway. Journal of Biological Chemistry, 277(33), 30271-30282.

[14] Peng, W., Yan, J., Wan, Y., Wang, B., Tao, J.H., Yang, G.J., Pan, H.F. & Wang, J. (2012): Matrix metalloproteinases: a review of their structure and role in systemic sclerosis. Journal of Clinical Immunology, 32, 1409-1414.

[15] Amin, M.B., Greene, F.L., Edge, S.B., Compton, C.C., Gershenwald, J.E., Brookland, R.K., Meyer, L., Gress, D.M., Byrd, D.R. & Winchester, D.P. (2017): The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. CA: A Cancer Journal for Clinicians, 67(2), 93-99.

[16] Lala, P.K. & Orucevic, A. (1998): Role of nitric oxide in tumor progression: lessons from experimental tumors. Cancer Metastasis Review,17, 91–106.

[17] Ying, L. & Hofseth, L.J. (2007): An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. Cancer Research, 67(4), 1407-1410.

[18] Oláh, G., Módis, K., Törö, G., Hellmich, M.R., Szczesn, B. & Szabo, C. (2018): Role of endogenous and exogenous nitric oxide, carbon monoxide and hydrogen sulfide in HCT116 colon cancer cell proliferation. Biochemical Pharmacology, 149, 186-204.

[19] Yu, X., Zhong, Y., Zhu, Z., Wu, T., Shen, A. & Huang, Y. (2012): Increased expression of nitric oxide synthase interacting protein (NOSIP) following traumatic spinal cord injury in rats. Journal of Molecular Histology, 43, 661-668.

[20] Li, H., Qiu, Z., Li, F. & Wang, C. (2017): The relationship between MMP2 and MMP9 expression levels with breast cancer incidence and prognosis. Oncology Letters, 14(5), 5865-5870.

[21] Emara, M., Cheung, P.Y., Grabowski, K., Sawicki, G. & Wozniak, M. (2009): Serum levels of matrix metalloproteinase-2 and -9 and conventional tumor markers (CEA and CA 19-9) in patients with colorectal and gastric cancers. Clinical Chemistry and Laboratory Medicine, 47(8), 993-1000.

[22] Liabakk, N.B., Talbot, I., Smith, R.A., Wilkinson, K. & Balkwill, F. (1996): Matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase-9 (MMP9) type IV collagenases in colorectal cancer. Cancer Research, 56(1), 190-196.

[23] Gao, Y., Zhoub, S., Xuc, Y., Shengb, S., Qian, S.Y. & Huo, X. (2019): Nitric oxide synthase inhibitors 1400W and L-NIO inhibit angiogenesis pathway of colorectal cancer. Nitric Oxide, 83, 33-39.