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**G. A. Hovhannisyan, F. P. Sarukhanyan, H. H. Zakaryan, N. H. Barkhudaryan**

# Antioxidative and Immunological Patterns of Cervical Cancer Progression

(Submitted by corresponding member of NAS RA E. S. Gevorgyan 2/IX 2019)

**Keywords:***calcineurin, interleukin-2, tumor necrosis factor alpha, glutathione, catalase, cervical cancer.*

Cervical cancer is the fourth most frequent female cancer in the world [1] and the second one in Armenia [2]. A large number of evidence indicates that infection with the human papillomavirus (HPV) is the primary risk factor for cervical cancer and plays a key role in cervical carcinogenesis [3]. However, few prospective studies suggest that HPV infection alone may not be sufficient to promote cervical carcinogenesis and a number of co-factors such as deficiency in antioxidants and inflammation are involved in the development of cancer [3]. Intriguingly, despite the availability of various studies, the interdependent nature of oxidative stress and inflammation is discussed relatively recently [4]. Selection of antioxidants that do not simultaneously inhibit both oxidative stress and inflammation or use of nonselective anti-inflammatory agents that block some of the oxidative and/or inflammatory pathways but exaggerate the others might be responsible for the failures of the antioxidant clinical trials [4]. Thus, to establish the validity of schemes involved in all stages of cancer progression and to increase the positive outcome from the future treatment it would be essential to quantify both redox and inflammatory status of cancer patients.

Calcineurin (CN) is a key enzyme leading to the activation of the immune system by participating in the synthesis of several cytokines (interleukin (IL)-2, tumor necrosis factor (TNF)-α, etc.) via dephosphorylation and activation of NFAT (nuclear factor of active T cells) transcription factors [5]. Being closely and widely involved in various signaling pathways related to immune response, inflammation and cancer pathophysiology [5-6], CN levels in plasma and tissue not only express the extent of the immune system activity, but the tumor driven inflammation as well. Moreover, redox sensitive nature of CN active center [7]

additionally makes CN being closely related to redox systems as well. Hence, in the terms of cervical cancer, taking into account its multifactor origin, the study of the activity of CN with its dualistic nature described previously [8], could serve as a complex estimator of the complicity of the interplay between host immune and antioxidant systems' and the tumor microenvironment. In this concept, to evaluate simultaneous contribution of host immunity, antioxidant defense system and tumor driven inflammation in pathogenesis of cervical cancer we considered advisable to study the parallel changes in the activity of CN as a major activator of the immune system, immunoregulatory cytokine IL-2, pro-inflammatory cytokine TNF-α, glutathione (GSH) as the most abundant intracellular non enzymatic antioxidant, and catalase (CAT) as one of the potent scavengers of the reactive oxygen species (ROS).

**Material and methods.** The blood and tissue samples from postoperative material of untreated patients with the I (n=15), II (n=5) and III (n=11) stages of cervical cancer were provided by the National Centre of Oncology after V.A. Fanarjyan (NCO MH RA). The plasma of healthy donors (n=6) and histologically checked healthy parts of remote tissue (n=8) were used as a control. Histological study of the postoperative material was conducted by the Laboratory of Clinical Pathomorphology at the NCO MH RA. The most cases of cervical cancer were diagnosed as a moderately and poorly differentiated squamous cell carcinoma. Age of patients ranged from 33-73, and the average age was 50 years. Among these patients women with the age of 35-65 (86,7%) were predominated.

Blood (1.5-2 ml) was collected into sodium citrate (3.2%)-coated vacutainer tubes and centrifugated at 1500 rpm for 10 min. Plasma was separated and stored at -32°C. Tissue samples were homogenized with 2.5 volumes of 50 mM Tris-HCI, pH 7.5 buffer, containing 0.05% Triton-X-100, 0.1 mM EDTA, 1 mM DTT, protease inhibitors, and centrifugated at 20000 ***g*** for 60 min at 4ºC. The supernatant was separated and stored at -32°C as well. The protein content in samples was determined by Bradford assay [9].

Calcineurin activity was measured by spectrofluorimetric assay using 4-methylumbelliferyl phosphate (4-MUP) as a substrate [10]. We have adapted the assay for our research as described before [11]. One unit of enzyme activity is defined as amount of enzyme that caused the formation of 0.1 nM of 4-methylumbelliferon (4-MU) at 32ºC for 1 h. The quantity of 4-MU was determined fluorimetrically using a Perkin-Elmer MPF-44A spectrofluorimeter (PerkinElmer Inc., USA). TNF-α and IL-2 levels in plasma and tumor tissue samples were determined using human TNFα ELISA MAX kit (BioLegend Inc., USA) and human IL-2 ELISA MAX™ kit (BioLegend Inc., USA), respectively, according to the manufacturer's recommendations. The optical density was measured in each well at the 450 nm using LABLine-022 microplate reader (LABLINE Diagnostics, Austria). The activity of GSH was studied using DTNB/GR enzyme recycling method [12] and the activity of CAT was determined using the spectrophotometric assay of hydrogen peroxide based on formation of its stable complex with ammonium molybdate [13].

Data were analyzed statistically by one-way ANOVA using Origin 6.1 software. Statistical significance – $p<0.05$. All data were expressed as mean±SEM.

**Results and discussion.** Data obtained revealed that CN activity in both plasma and tissue samples of patients with cervical cancer were changed in a parallel manner (Fig. 1a).It demonstrated a statistically significant 2.5 fold and 10.7 fold increase in tumor tissue and in plasma, respectively, for the I stage of disease compared with the control groups. In the II and III stages of disease CN activity was shown to be decreased 1.45 and 1.7 fold in tumor tissue, and 1.8 and 2.1 fold in plasma, respectively, compared with the I stage of disease. TNF-α has been shown to be decreased by 1.9 and 1.8 times in plasma and tumor tissue, respectively, in the I stage of disease compared with the control groups (Fig. 1b). In plasma it was shown to be increased significantly for the II (2.7 fold) and III (3.3 fold) stages of disease compared with the I stage. In contrary, in tumor tissue TNF-α demonstrated 2.2 fold and 1.3 fold decrease for the II and III stages, respectively, compared with the I stage. As one can see from Fig. 1c, IL-2 activity in plasma of patients with cervical cancer, demonstrated 2.48 fold increase for the I stage compared with the control group, and 1.4 fold and 1.39 fold decrease for the II and III stages, respectively, compared with the I stage of disease. In tumor tissue IL-2 level demonstrated significant increase for the I (2 fold) and II (1.6 fold) stages compared with the control and the I stage, respectively. In the III stage IL-2 was shown to be decreased significantly (5.2 fold) compared with the I stage.

Results obtained have also shown 1.14 fold (not statistically significant) and 1.5 fold decrease in both tissue and plasma GSH activity, respectively, for the I stage compared with the control (Fig. 2a). However, the decrease in GSH activity was more marked in the tumor tissue in advanced stages of cancer (1.9 fold and 5.9 fold, respectively, for the II and III stages compared with the I stage). In plasma GSH activity demonstrated 1.6 fold and 4 fold increase in the II and III stages of cancer compared with the I stage.

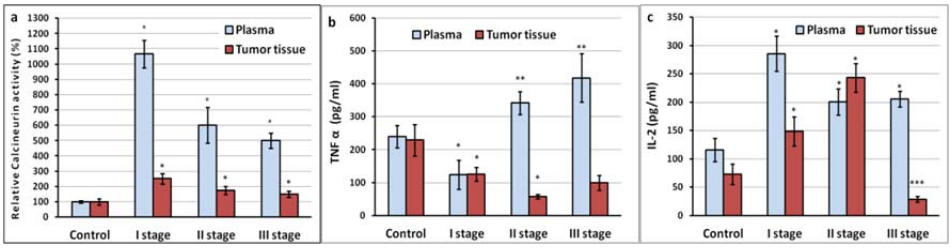


Fig. 1. Calcineurin activity (**a**), TNF-α (**b**) and IL-2 (**c**) levels in plasma (grey) and tumor tissue (black) of the primary cervical cancer patients with the I, II and III stages of disease. Control for calcineurin activity considered as 100% and data expressed as % of control. * P<0,05 for the I stage compared with control, as well as for the II and III stages compared with the I stage; ** P<0.01 for the II and III stages compared with the I stage (**b**); *** P<0,001 for the III stage compared with the I stage (**c**).

Concerning the changes in CAT activity, we have revealed modest, but statistically significant 1.35 fold decrease in tumor tissue for the I stage of cancer compared with the control group and 1.5 fold and 2.4 fold increase for the II and III stages, respectively, compared with the I stage (Fig. 2b). In contrary, in plasma we have found 1.6 fold increase for the I stage compared with the control and 1.5 fold and 1.6 fold decrease for the II and III stages, respectively, compared with the I stage.

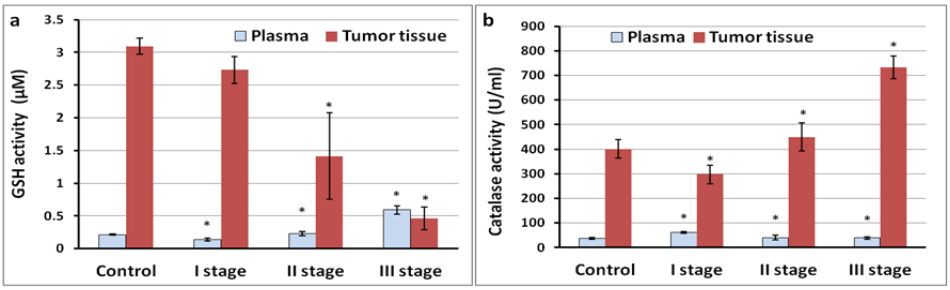


Fig. 2. Glutathione (**a**) and catalase (**b**) activity in plasma (grey) and tumor tissue (black) of the primary cervical cancer patients with the I, II and III stages of disease. *p<0,05 for the I stage compared with control, and for the II and III stages compared with the I stage.

The involvement of the host immune and antioxidant systems in the control of cervical cancer progression was suspected but remained inconclusive for many years due to the lack of convincing evidences. Earlier, Padma et al. have shown downregulation of CN activity in both serum and biopsy samples of the cervical cancer patients [14]. In contrary, we have found increased CN activity in both plasma and tumor tissue of the cervical cancer patients. This could be due to the difference in studied specimens as well as different methods used. The significant increase in CN activity in the I stage of cervical cancer indicates on similar increase in the level of proinflammatory cytokines, such as TNF-α and IL-2, because, CN participates in the synthesis of these cytokines via activation of NFAT [5]. Indeed, we have found the increase of IL-2 level in the I stage of cervical cancer. This could be considered as the very first and rapid response of the host immune system to malignant transformation since the organism uses the inflammation to fight against the neoplasms. Actually, the role of IL-2 in the activation of the immune system against tumor cells has been established [15]. However, there are numerous mechanisms through which the tumor can escape from the immune system. For example, the presence of functional IL-2 receptor (IL-2R) expression on cervical cancer cell lines has been determined, although, normal cervical cells do not express IL-2R [16]. It was shown that exogenous IL-2 induces the proliferation of these cells. By blocking IL-2R using specific antibodies, the proliferation of these cancer cells had been inhibited. Furthermore, these cells not only express IL-2R, but also produce and secrete IL-2. Intriguingly, the amount of IL-2R expressed by

cervical cancer cells in vivo was shown to be increased along with the tumor stage [17]. Indeed, in contrary to the decline of CN activity in the II stage of disease, we have found the continuous increase of IL-2 level in tumor tissue in the same stage. Similarly, TNF-α, the main pro-inflammatory cytokine of the organism, also demonstrates both tumorigenic and antitumor properties. It has been demonstrated that the HPV E6 and E7 proteins suppress the protective effect of TNF-α, as E6 induces resistance to TNF-α-mediated apoptosis and E7 inhibits the antiproliferative effect of this cytokine [18]. This suggests that acquisition of resistance to TNF-α may be an important step in HPV-induced carcinogenesis. This data are in accordance with our finding that the level of TNF-α in tumor tissue is decreased depending on the stage of disease compared with the control. However, there is also evidence that, under certain conditions, TNF-α can act as a tumor promoter [18]. TNF-α level has been found to be increased in local tissue samples from cervical cancer and, hence, blocking antibodies have found therapeutic application. Very interestingly, TNF-α gene polymorphism can increase or decrease the susceptibility to cervical cancer depending on whether it is TNF-α-238A allele or TNF-α-308G>A which is altered [19]. Thus, decreased level of tumor tissue TNF-α and increased one for plasma, as well as levels of IL-2 opposite to TNF-α in our study are underlying the whole complexity of the interactions established between the broad spectrum of cytokines produced during the inflammatory response, in which certain cytokines have effects and functions that are occasionally contradictory, depending on the context in which they operate. Concerning the divergence in tumor tissue and plasma levels of IL-2 and TNF-α, this is most probably indicating the difference in modulation of local and systemic responses to active growth of neoplastic tissue.To explain the finding that changes in TNF-α levels in plasma and tumor tissue were not entirely consistent with changes in CN activity, it's worth to mention that while NFAT is a major target of CN, the study of Padma et al. rules out the involvement of this transcriptional factor indicating presence of a NFAT independent calcineurin pathway atleast in cervical cancer [14]. Moreover, in our study the simultaneous detection of the levels of IL-2 and TNF-α, as the constituent chains of $Ca^{2+}$/CaM/CN/NFAT pathway, generally have not shown changes parallel to CN activity. This indirectly points the existence of posttranslational modifications and/or CN independent pathways of NFAT regulation in cervical cancer cells.

Inflammatory processes are closely related to oxidative stress. Pro-inflammatory cytokines, including TNF-α, contributes the production of ROS which in turn can activate redox-sensitive transcription factors, inducing an additional synthesis of inflammatory cytokines. Although oxidative stress is a primary stimulus for the induction of antioxidative enzymes, however, their induction in cancer cells and systematically most commonly undergoes different modulation [3]. In this regard, gradually increasing CAT activity in cervical cancer tissue samples, could be the reflection of cancer cells adaptation to exacerbating oxidative stress, meanwhile decrease in plasma for the II and III stages of disease (compared to the I stage) is most probably indicating the growing imbalance between CAT reserves and $H_2O_2$ level. It is known that GSH is responsible for recycling of redox-sensitive proteins. However, high intracellular GSH levels are important contributors to pathologies such as,

cellular transformation and resistance to radiation and antineoplastic treatmentsin cancer cells [20]. Current study has shown reduction in both tumor tissue and plasma GSH activity for the I stage of disease compared to healthy counterparts. These data are in full compliance with other study showing a significant reduction in the GSH and GSH-peroxidase content compared to normal controls [21]. Similar to our data for the tumor tissue, Ahmed et al. also have demonstrated that the decrease for GSH level was more relevant for the advanced stages (III, IV) than for early stages (I, II) [22]. However, at early stages, GSH activity showed no substantial changes compared to control (healthy women). Changes in GSH activity can be affected by metabolic enzymes that use tripeptide as a substrate [23]. GSH is known to protect disulfide bonds of CN from ROS attacks via reduction of oxidized ones and, thus, protect CN from inactivation [24]. There is need for additional studies to explain the finding that changes in CN and GSH activities in cervical cancer were not parallel.

Thus, although this study has generated a lot of questions that need to be solved with the help of additional and detailed researches, however, data obtained reveal the light on the stage dependent changes in activity of calcineurin, IL-2, TNF-α, GSH and CAT in the pathophysiology of cervical cancer. Further studies aiming at understanding how immune and antioxidative defense systems are regulated in tumor context would most probably determine how they could be involved in effective anticancer therapy for cervical cancer as well.

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H. Buniatian Institute of Biochemistry of NAS RA
e-mail: gayane90.30@gmail.com

**G. A. Hovhannisyan, F. P. Sarukhanyan, H. H. Zakaryan, N. H. Barkhudaryan**

**Antioxidative and Immunological Patterns of Cervical Cancer Progression**

The changes in activity of mutually related immune and antioxidant factors: calcineurin, IL-2, TNF-α, glutathione and catalase have been investigated in plasma and tumor tissue samples of untreated patients with primary cervical cancer with the I, II and III stages. Results obtained demonstrated that activities of pro-inflammatory cytokines, antioxidants, as well as immunomodulating enzyme calcineurin in pathophysiology of cervical cancer changes in a stage-dependent manner. This study expands the knowledge about the complicity of the stage dependent interplay between host immunity, inflammation, antioxidants and cervical cancer.

Գ. Ա. Հովհաննիսյան, Ֆ. Պ. Սարուխանյան, Հ. Հ. Զաքարյան,
Ն. Հ. Բարխուդարյան

## Արգանդի վզիկի քաղցկեղի զարգացման հակաօքսիդանտային և իմունաբանական առանձնահատկությունները

Ուսումնասիրվել է փոխկապակցված իմունային և հակաօքսիդանտային գործոնների՝ կալցինեյրինի, IL-2-ի, TNF-α-ի, գլուտաթիոնի և կատալազի ակտիվության փոփոխությունն առաջնային արգանդի վզիկի քաղցկեղի I, II և III փուլերում գտնվող, բուժում չստացած հիվանդների պլազմայի և ուռուցքային նմուշներում: Արդյունքները ցույց են տվել, որ արգանդի վզիկի քաղցկեղի ժամանակ նախաբորբոքային ցիտոկինների, հակաօքսիդանտների, ինչպես նաև իմունակարգավորիչ կալցինեյրին ֆերմենտի ակտիվությունը փոփոխվում է հիվանդության փուլից կախված: Այս տվյալներն ընդլայնում են գիտելիքներն իմունիտետի, բորբոքման, հակաօքսիդանտների և արգանդի վզիկի քաղցկեղի միջև փուլից կախված բարդ փոխգործակցության մասին:

**Г. А. Оганнисян, Ф. П. Саруханян, Э. А. Закарян, Н. А. Бархударян**

## Антиоксидантные и иммунологические характеристики прогрессирования рака шейки матки

Исследованы изменения активности взаимосвязанных иммунных и антиоксидантных факторов: кальцинейрина, IL-2, TNF-α, глутатиона и каталазы, в образцах плазмы и опухолевой ткани нелеченных пациентов с первичным раком шейки матки в I, II и III стадий заболевания. Показано, что активность провоспалительных цитокинов, антиоксидантов, а также иммуномодулирующего фермента кальцинейрина при раке шейки матки изменяется в зависимости от стадии. Эти данные расширяют знания о сложности стадия-зависимых взаимодействий между иммунитетом хозяина, воспалением, антиоксидантами и раком шейки матки.

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