Deregulation of purinergic ectoenzyme activity in head and neck cancer promotes immunosuppression

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Abstract
Background Head and neck cancer (HNC) comprises a spectrum of neoplasms that affect the upper aerodigestive tract and are the sixth most common cancers worldwide. Individuals with HNC exhibit various symptoms and metabolic changes, including immune alterations and alterations of the purinergic pathway, which may signal worse outcomes. Therefore, the purpose of this research was to measure the activity of purinergic ectoenzymes and interleukins in patients with HNC, oral cavity cancer, and larynx cancer.

Methods and Results We recruited 32 patients and 33 healthy control subjects and performed the laboratory analyses. We identified dysregulation in the purinergic signaling pathway characterized by an increase in adenosine triphosphate (ATP) and adenosine monophosphate (AMP) hydrolysis and a decrease in the deamination of adenosine to inosine in these cancers (p < 0.05). These alterations were likely caused by increased activity of the ectoenzymes E-NTPDase and ecto-5'-nucleotidase and reduced adenosine deaminase activity. This dysregulation was associated with immune alterations, increased levels of IL-10, and decreased myeloperoxidase activity (p < 0.05), suggesting immunosuppression in these patients and suggesting possible accumulation of adenosine in the extracellular environment.

Conclusions Adenosine is a potent immunosuppressive molecule associated with tumor progression and immune evasion. Our findings suggest a relationship between extracellular purines and the development and progression of the tumor microenvironment and poor outcomes. These findings increase the understanding of biological mechanisms related to HNC and demonstrate that these components are potential diagnostic markers and therapeutic targets for future management strategies and improvement in the quality of life.

Keywords Head and neck neoplasms · Oral cavity · Laryngeal neoplasms · Adenosine · Interleukin-10

Introduction

Head and neck cancer (HNC) comprises tumors that affect the upper aerodigestive tract, involving the nasal cavity, oral cavity, pharynx, and larynx. HNC is most frequent in the oral cavity and larynx [1], particularly in males, older individuals, smokers, those with low levels of education, and low socioeconomic level. These cancers comprise the sixth highest incidence worldwide; in Brazil, there are 41,000 new cases each year, representing a severe public health problem [2, 3].
HNC carries high mortality rates, low survival (about 5 years), and a high incidence of recurrences associated with complex and debilitating treatments, in addition to...
numerous side effects and functional impairments, and non-existence of early detection biomarkers [4]. The latter include complications related to biological functions such as breathing, swallowing, speaking, and other poor outcomes [1, 5]. In addition to functional changes, patients with HNC also exhibit metabolic and biochemical imbalances directly related to oncological outcomes, among which are changes in the purinergic signaling pathway [5, 6].

The purinergic signaling system consists of the adenine nucleotides, 5′-triphosphate (ATP), 5′-diphosphate (ADP), and 5′-monophosphate (AMP), and nucleoside adenosine (Ado), which act as extracellular flags [7, 8]. Signal transduction and effectiveness of biomolecular function depend on the coupling to surface receptors, P1 and P2 (P2X and P2Y) [9]. This signaling pathway is regulated by ectoenzymes, including ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs), ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, NTP-Dases or CD39), ecto-5′-nucleotidase (E-5′-nucleotidase or CD73), and adenosine deaminase (ADA), all of which prevent the excessive accumulation of these compounds in the extracellular environment, promoting the hydrolysis and deamination of these components into inosine [8, 10]. These ectoenzymes, receptors, nucleotides, and nucleosides are critical for the modulation of inflammatory and immunological responses, pain mechanisms, cell proliferation, differentiation, development, and death in several diseases, including cancer [7, 11–13].

ATP is associated with the pro-inflammatory response, and adenosine has anti-inflammatory and immunosuppressive effects. These purinergic components promote interactions with the tumor microenvironment promoting growth, invasion, metastasis, and immune evasion [13–17]. Previous studies showed changes in the purinergic system and its potential involvement in breast cancer, lung cancer [18], colorectal cancer [19], cervical intraepithelial neoplasia, uterine cancer [20], and cutaneous melanoma [21].

Because the purinergic enzyme chain plays a critical role in promoting inflammation and tumorigenesis, the objective of this study was to measure the activity of purinergic ectoenzymes and inflammatory parameters, to understand the role of adenine nucleotides and adenosine (Ado) in HNC, oral cavity cancer, and laryngeal cancer.

Materials and methods

Chemicals and equipments

Nucleotides, Coomassie Blue G-250, and Ficoll-Histopaque reagent were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and the highest purity. Aqueous solutions were prepared using distilled water. The centrifuge used was a model Sigma 3-16KL, and the microplate spectrophotometer was a Thermo Scientific Multiskan GO (Waltham, MA, USA).

Experimental design and patients

Patients were diagnosed with HNC at the West Regional Hospital (Chapecó, SC, Brazil). According to the Classification of Malignant Tumors for the Union for International Cancer Control, patients were in stages II and III. This group consisted of 32 individuals. No patient had been undergoing drug treatment at the time of sample collection. The control group was composed of 33 healthy volunteers free from chronic diseases that could compromise the research data; the gender and age distribution were similar to the HNC patients. All subjects gave written informed consent. The Human Ethics Committee of the Federal University of Fronteira Sul (Chapecó, SC, Brazil) approved the study under the ethical appraisal number (CAAE) 03057018.6.0000.5564 and approval protocol number 3.105.374, following the Declaration of Helsinki. All analyses were performed in triplicate to ensure reliability.

Lymphocyte separation and protein determination

Mononuclear leukocytes were isolated from human blood collected in EDTA-containing tubes and separated on Ficoll-Histopaque density gradients after centrifugation at 561 x g for 30 min and washing with saline solution, according to Böyum [22] with adaptations. The separation method employed allowed the isolation of peripheral blood mononuclear cells (PBMCs), with a high incidence of lymphocytes (95%) in these samples, and the number of monocytes is insignificant. For this reason, we considered the samples to contain only lymphocytes. The protein concentration was standardized from the isolated lymphocytes, and purinergic ectoenzymes’ activity was subsequently measured.

According to Bradford [23], protein concentrations were measured with bovine serum albumin as the standard, using Coomassie Blue G-250 reagent. Absorbance was measured at 595 nm. For the determination of ectonucleotidase activities, concentrations were adjusted to 0.1–0.2 mg/mL.

E-NTPDase and ecto-5′-nucleotidase assays

To measure the activity of ectoenzymes of the purinergic system involved in ATP, ADP, and AMP hydrolysis, we measured the activities of E-NTPDase and ecto-5′-nucleotidase, respectively, as the amount of inorganic phosphate released during a colorimetric in a spectrophotometer reading at 630 nm, according to Pilla et al. [24] and adapted...
factor-alpha (TNF-α) were determined using Human Interleukin Uncoated ELISA Kits from Invitrogen (Carlsbad, CA, USA), according to the manufacturer’s instructions. The results were expressed as picograms per milliliter (pg/mL). These analyses were performed on serum samples.

MPO activity was analyzed according to Metcalf et al. [27]. The reactions were performed in the presence of 1.7 mM hydrogen peroxide as an oxidizing agent. MPO catalyzed the oxidative coupling of phenol and 2.5 mM aminoantipyrine, and absorbance was measured at 492 nm. The results were expressed as mM of quinoneimine produced in 30 min. This analysis was performed on serum samples.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Normality was tested using the Shapiro–Wilk test. Outliers were excluded using the Grubbs test. Differences between the groups concerning the studied variables were evaluated using the unpaired Student’s t-test and analysis of variance (ANOVA) with the Tukey post hoc test. The results were presented as mean and standard deviation. Differences in the probability of rejection of the null hypothesis of < 5% (p < 0.05) were considered statistically significant.

Adenosine deaminase (ADA) assay

According to Giusti and Galanti [26], adenosine deaminase activity was determined based on the direct measurement of ammonia produced by the enzyme during the deamination of adenosine to inosine measured at 620 nm. Briefly, 50 µL of sample reacted with 21 mmol/L of adenosine, pH 6.5, incubated at 37 °C for 60 min. Subsequently, the reaction was stopped by adding a solution of 106.2 mM phenol and 167.8 mM sodium nitroprusside, and a hypochlorite solution. The results were expressed as units per liter (U/L). These analyses were performed on lymphocyte samples.

Cytokine and myeloperoxidase (MPO) serum levels

The cytokines interleukin 1-beta (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), and tumor necrosis factor-alpha (TNF-α) were determined using Human Interleukin Uncoated ELISA Kits from Invitrogen (Carlsbad, CA, USA), according to the manufacturer’s instructions. The results were expressed as picograms per milliliter (pg/mL). These analyses were performed on serum samples.

Fig. 1 Activity of purinergic ectoenzymes E-NTPDase, ecto-5’-nucleotidase, and ADA in lymphocyte samples in patients with HNC and healthy control subjects, and HNC pre- and post-treatment. (a, e) Hydrolysis of ATP, (b, f) Hydrolysis of ADP, (c, g) Hydrolysis of AMP, (d, h) Deamination of Ado. The data are expressed as mean ± standard deviation. The sample consisted of 32 patients with HNC (pre- and post-treatment, n = 6 and 26, respectively) and 33 healthy individuals in the control group (a, b, c and d: Unpaired T-Student test, *p < 0.05; e, f, g, and h: one-way ANOVA, with Tukey as the post hoc test, different letters indicate p < 0.05)
Results

Clinical characteristics

We evaluated the clinical characteristics of the groups using an interview with each participant and analysis of outpatient medical records.

The average ages were similar between the control and HNC groups (61.4 ± 9.1 and 61.2 ± 9.4 years, respectively). There was a higher incidence of HNC in men (87.5%) than in women (12.5%), and the primary tumor sites were the oral cavity (50%) and the larynx (31.3%). Patients had been diagnosed for less than 2 years, and 81.3% underwent surgical removal and drug treatment.

The patients with HNC had lower socioeconomic status and lower schooling, with 84.4% not completing primary school. Smoking was the primary risk factor for HNC development (75%).

Alteration on purinergic ectoenzymes activity

The evaluation of purinergic enzymes in HNC indicated greater hydrolysis levels of ATP and AMP (p < 0.05) in HNC patients than in control patients. There were no significant differences (p > 0.05) between groups in terms of hydrolysis of ADP. ADA activity in lymphocytes was significantly lower (p < 0.05) in HNC patients than in controls (Fig. 1a-d).

We then measured the activity of purinergic ectoenzymes, comparing individuals who underwent surgical excision of the tumor (n = 26) with those with active tumors (n = 6). The results were identical to those seen without the inclusion of the variable “treatment” (Fig. 1e-h). Significantly greater E-NTPDase activity with the ATP substrate was observed in pre-treatment and post-treatment HNC patients (p < 0.05). Regarding the ADP substrate, there was no significant difference between groups (p > 0.05). For ecto-5'-nucleotidase, significantly greater activity was observed in the HNC group before and after treatment than in the control group (p < 0.05), suggesting more significant transformation of AMP into Ado; and significantly lower ADA activity was observed, suggesting decreased Ado deamination to inosine in the post-treatment HNC group (p < 0.05). These data suggest accumulation of Ado in the extracellular environment, even after the removal of the tumors.

The HNC group was categorized according to the most frequent tumors, and we assessed the activity of purinergic ectoenzymes in cancer of the oral cavity and larynx (Fig. 2a-d and e-h, respectively). There was significantly greater ATP hydrolysis (p < 0.05). The hydrolysis of AMP (p < 0.05) in lymphocytes was significantly greater in laryngeal cancer patients than in the control group. There were no significant differences (p > 0.05) between groups in terms of hydrolysis of ADP. In both the oral cavity and laryngeal cancer groups, ADA activity was significantly lower than in the control group (p < 0.05).

These results suggest an accumulation of large extracellular Ado concentrations in patients with HNC, oral cavity cancer, and laryngeal cancer, possibly promoting immunosuppression and pro-tumor effects, even after tumor removal. However, despite methodological advances, the assessment of extracellular levels of Ado in the tumor microenvironment (TME) remains challenging because of fluctuating concentrations and the short half-life of this metabolite; therefore, we measured IL-10 and MPO as markers of immunosuppression and indirect indicators of increased levels of extracellular Ado [28].

![Activity of purinergic ectoenzymes E-NTPDase, ecto-5'-nucleotidase, and ADA in lymphocyte samples in patients with oral cavity cancer (a-d), laryngeal cancer (e-h), and healthy control subjects. (a, c) Hydrolysis of ATP, (b, f) Hydrolysis of ADP, (c, g) Hydrolysis of AMP, and (d, h) deamination of Ado. The data are expressed as mean ± standard deviation. The sample consisted of 16 patients with oral cavity cancer, ten patients with laryngeal cancer, and respective control groups (unpaired Student’s T-test, *p < 0.05)
Our findings regarding the associations of age, sex, tumor location, and presence of risk factors, including smoking, corroborate those of previous reports [5]. Smoking and indicators of low socioeconomic status (low levels of education) in HNC found in this study are relevant epidemiological factors for understanding these patients.

Understanding the biochemical mechanisms involved in developing these tumors, including purinergic signaling mechanisms and immunological mechanisms, are critical for better understanding the pathophysiology of this group of tumors. Our findings might lead to proposals for therapeutic strategies and appropriate clinical management.

The dysregulation in the metabolism and activity of purinergic ectoenzymes in HNC, oral cavity cancer, and laryngeal cancer represent modifications of the TME, tumor progression, disordered cell proliferation, angiogenesis, deregulation of apoptosis, metastases, and recurrences. This pathophysiology has been described in melanoma, lung cancer, breast cancer, and cervical cancer [20, 29].

It is noteworthy that HNC, as indicated by Theodoraki et al., comprises a group of carcinomas with strongly immunosuppressive characteristics. Patients with more advanced stages of the disease, metastases, and recurrences, display systemic immunological defects, with a high degree of immunological suppression correlated with poor outcome [30, 31]. For this reason, it is essential to identify the mechanisms associated with the development of this

**Serum cytokine and MPO levels**

Figure 3a-d displays IL-10 levels in samples from patients with HNC, oral cavity cancer, and laryngeal cancer, as well as evaluating pre- and post-treatment HNC. Significantly higher levels were observed in the cancer groups (p<0.05). There were no changes in IL-10 regarding treatment (p>0.05). Figure 3e-h displays MPO activity in samples from patients with cancer and evaluating pre- and post-treatment HNC. Significantly decreased levels of this marker were observed in all cases (p<0.05). These results suggest the promotion of an anti-inflammatory and immunosuppressive TME.

Regarding pro-inflammatory interleukins, IL-6 levels are represented in Fig. 4a-d, demonstrating decreased levels in patients with cancer and the HNC group before treatment (p<0.05). IL-1β and TNF-α levels did not show a significant difference between groups (p>0.05) (Fig. 4e-h, and 4i-l, respectively).

**Discussion**

We uncovered an essential correlation between dysregulation of purinergic signaling metabolism and several tumors. This signaling pathway acts powerfully in HNC, oral cavity cancer, and laryngeal cancer and is associated with poor outcome (Fig. 5 represents the graphic abstract of this study).
We detected increased activity of E-NTPDase on the ATP substrate in the evaluated tumors, suggesting more immunosuppressive environment to design adequate treatments and management.

We detected increased activity of E-NTPDase on the ATP substrate in the evaluated tumors, suggesting more
The high activity and expression of CD73 in cancer cells have been associated with low overall survival, presence of metastases, and recurrence in several tumors [14, 35, 36]. These data suggest that the findings of our study, i.e., increased activity of this ectoenzyme, represent an essential factor associated with tumor recurrences, even after surgical removal.

The accumulation of Ado in the TME is a critical mechanism for immune evasion. It stimulates angiogenesis in solid hypoxic tumors, resulting in the proliferation of human endothelial cells, expression of vascular endothelial growth factor, metastasis, dysregulation of apoptosis, stimulation of immunosuppressive cells such as regulatory T cells and myeloid-derived cells, suppression of antigen-presenting cells, and promotion of survival in cancer cells [14, 28, 35–38]. Adenosine decreases the function of antitumor T cells by inhibiting a series of T-cell responses. This molecule can suppress natural killer cell and lymphokine-activated killer cell function, leading to failure of an effective antitumor immune response [14].

Extracellular Ado acts on A2A and A2B type receptors, promoting the activation of immune cells. The activation of these receptors limits the production of pro-inflammatory cytokines and increases the release of anti-inflammatory cytokines such as IL-10, as noted in this study. Increased levels of this extracellular nucleoside may also be related to the development of drug resistance and a more aggressive course of active tumors [28, 33].

IL-10 is an anti-inflammatory cytokine and immunosuppressive [39]. The increase in IL-10 in tumor cells is associated with modulation of the TME, promoting proliferation, metastasis, and immune escape of the tumor [40]. Zhao et al., in a meta-analysis, verified the impact of IL-10 on outcome in several tumors, indicating that elevated serum levels of IL-10 have correlated with poor prognosis and low survival, suggesting that this comprises a promising biomarker in the evaluation of progression tumor and survival time in solid and hematological tumors [41]. Torres-Pineda et al. reported that cervical cancer cells produce high concentrations of Ado and increased levels of IL-10, promoting immune evasion and tumor progression [42].

The decrease in MPO activity is also used as an immunosuppressive marker. MPO is produced primarily by neutrophils, and Ado has an inhibitory effect on these blood cells, decreasing MPO secretion [28, 33, 43]. The MPO produced by neutrophils has potential tumoricidal activity; conversely, decreases in its activity are associated with tumor progression. The presence of healthy neutrophils in the TME, secreting toxic substances such as MPO, can prevent metastases. In an animal model, the administration of healthy neutrophils reduced tumor growth and prolonged the animal’s survival, which was also observed in breast cancer cells in the lungs of mice [44]. These findings suggest the critical importance of MPO to immunosuppression in cancer.

The immunosuppressive mechanisms promoted by the purinergic pathway suggest that these compounds can be used as cancer therapies [45]. Currently, the targeting of the E-NTPDase and ecto-5′-nucleotidase pathways have been used as a strategy in several tumors, with some medications in clinical trials, particularly inhibitors of molecules directed at ectoenzymes, compounds that promote directional manipulation of ATP in TME, and compounds targeting intracellular metabolism of Ado [32, 35, 36, 38, 46].

High levels of Ado in tumors are essential for the maintenance of a chronic immunosuppressive environment mediated by pro-tumor effects, causing the progression of tumors and recurrences. The purinergic signaling pathway that promotes the sequential breakdown of nucleotides in Ado and inosine in the tumor environment, especially the role of ecto-5′-nucleotidase, indicates this pathway’s targeting direct inhibition of ectoenzyme activity has compelling pharmacological applications against these tumors. If it were possible to decrease extracellular Ado levels, it would be possible to reduce immunosuppression by inhibiting the...
effects of this molecule, consequently decreasing recurrences and generating better outcomes [15, 16].

Conclusions

Ectoenzymes promote the extracellular accumulation of Ado, a potent immunosuppressive molecule that helps maintain an immunosuppressive microenvironment; this effect may influence metastases and tumor progression and recurrence. The inhibition of ecto-5 ’-nucleotidase may serve as a pharmacological strategy for the immuno-oncological treatment of these tumors. This correlation points the way toward developing therapeutic strategies for the management of patients with HNC, oral cavity cancer, and laryngeal cancer during treatment and after tumor removal.

Limitation of the study

Due to the pandemic caused by SARS-CoV-2 and increased restrictions on access to hospital environments, it was not possible to monitor the progression of treated patients.

Authors’ contributions All authors contributed to the study conception and design. Material preparation and, data collection and biological samples were performed by FM, BSRB, SRD, GCK, and, HFB. FM, BSRB and, SRD performed laboratory analysis; and interleukin analysis were performed by KM and MMZ. FM, AR and MDB performed data analysis and manuscript writing. All authors read and approved the final manuscript.

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Availability of data and material All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Human Ethics Committee of the Federal University of Fronteira Sul (Chapecó, SC, Brazil), with the number CAAE 03057018.6.0000.5564 and approval protocol number 3.105.374.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Patient consent for publication Not applicable.

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