Research Article

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Circulating concentrations of citrulline, neopterin, kynurenine, and tryptophan during chemoradiation in patients with cervical carcinoma

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Abstract: The aim of this study was to investigate the changes in circulating concentrations of citrulline, neopterin, kynurenine, and tryptophan during the course of chemoradiation in patients with cervical cancer. Sixteen patients with histologically confirmed carcinoma of the uterine cervix, aged 53 ± 15 years (range 29–76 years), were included in this study. Plasma neopterin, kynurenine, and tryptophan were determined with an enzyme-linked immunosorbent assay. Plasma citrulline was measured with high-performance liquid chromatography. Compared to baseline, citrulline concentration was markedly and statistically significantly decreased at visits 2, 3, and 4, while returning to pretreatment concentrations at visit 5. A significant increase in serum neopterin concentrations was observed at visits 4 and 5. With the exception of decreased kynurenine/tryptophan ratio at visit 3, no significant changes were observed in the concentrations of kynurenine, tryptophan, and kynurenine/tryptophan ratio throughout the course of the treatment. In conclusion, present data demonstrate that citrulline concentrations decrease early and neopterin concentrations

increase late during the course of chemoradiation in patients with cervical carcinoma. Citrulline represents a biomarker of intestinal toxicity in this population.

Keywords: cervical carcinoma, chemoradiation, citrulline, kynurenine, neopterin, tryptophan

1 Introduction

Cervical cancer represents a major cause of cancer mortality in women [1]. Despite the availability of screening programs, many patients present at an advanced stage. For more than two decades, chemoradiation remains the treatment of choice for patients with locally advanced cervical carcinoma [2,3]. This aggressive combination treatment results in a cure in the majority of patients at a price of considerable toxicity that may be life threatening in some cases [4,5].

Prominent among the adverse events associated with chemoradiation is gastrointestinal toxicity. Along with hematological toxicity, gastrointestinal toxicity is the second most common side effect of chemotherapy and radiation. However, unlike hematological toxicity, which can easily be followed by the measurements of peripheral blood cell count, the assessment of gastrointestinal toxicity still mostly relies on the symptoms reported by the patient, which is inherently prone to bias [4,5]. A biomarker or set of biomarkers that would allow for repeat objective assessment of gut toxicity remains an unmet medical need.

Circulating citrulline concentrations have been shown to reflect the bowel mass. In different studies, circulating citrulline concentrations have been associated with the gut toxicity of chemotherapy or radiation [6,7,9–13]. In an earlier study, an inverse association was observed between the concentrations of citrulline and neopterin [7], a biomarker of immune response [14–21].

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The aim of this study was to investigate the changes in circulating concentrations of citrulline, neopterin, kynurenine, and tryptophan during the course of chemoradiation in patients with cervical cancer.

2 Patients and methods

Sixteen patients with histologically verified carcinoma of the uterine cervix aged (mean $\pm$ standard deviation) 53 ± 15 years (range 29–76 years) who underwent chemoradiation with curative intent in the Department of Oncology of the Tomáš Baťa Regional Hospital in Zlín, Czech Republic were enrolled in the study. The initial staging was based on clinical, radiological, and endoscopy investigations that included complete physical examination, X-ray of the chest, computed tomography of the abdomen and pelvis, rectoscopy, and cystoscopy. Fourteen patients had squamous cell carcinoma, while mucinous carcinoma and adenosquamous carcinoma were represented by one case each. Tumor stage according to the Fédération Internationale de Gynécologie et d'Obstétrique stage IIB was in 11 patients and IIIB in 5 patients. The tumors were p16 positive in seven patients and p16 negative in nine patients. The tumor grade was 2 in nine patients, grade 3 in six patients, and grade 1 in a single patient.

Intensity-modulated radiotherapy and three-dimensional (3D) conformal radiotherapy were used to deliver external radiation at a dose of 45 Gy in 25 fractions to regional lymph node basins and a boost of 6 Gy in three fractions to parametria followed by high-dose-rate brachytherapy with ^{192}Ir uterovaginal application of five times 6 Gy or four times 7 Gy. Concomitant cisplatin was administered weekly at a dose of 40 mg/m² that was substituted by carboplatin (area under-the-curve 2) in case of renal dysfunction.

A peripheral blood sample was obtained at weekly intervals before and during the course of the treatment. The samples were immediately centrifuged to remove plasma. Plasma aliquots were kept at -80°C until analysis. Plasma neopterin was determined by enzyme-linked immunoassay (ELISA) using commercial kits as described [7]. Plasma kynurenine and tryptophan were determined using commercial kits (ImmuSmol SAS, Talence, France) according to the instructions of the manufacturer. Plasma citrulline was determined as described [7]. Briefly, 10 μL of plasma samples were mixed with 100 μL of the internal standard mixture (amino acids and acylcarnitines, nonderivatized kit, Chromsystems, Munich, Germany), vortexed, and centrifuged (568 g, 10 min at 4°C). Then, 80 μL of supernatant

was transferred to a 96-well plate and used for direct injection mass spectrometry analysis on API 4000 (AB Sciex, Framingham, MA, USA) by flow injection mass spectrometry direct analysis infusion. Data were processed by software Chemoview 2.0 (AB Sciex).

The toxicity was evaluated at weekly visits by Radiation Therapy Oncology Group Common Toxicity Criteria (hematological, gastrointestinal, and genitourinary toxicity) [22].

Differences between subgroups of patients were studied with the Mann–Whitney *U*-test. Differences before and during the therapy were compared by the Wilcoxon signed-rank test. Correlations were analyzed using Spearman's rank correlation coefficient. The decision on statistical significance was based on $p = 0.05$ level. The analyses were performed using NCSS software (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration and has been approved by the authors' institutional review board or equivalent committee.

3 Results

Compared to pretreatment (baseline) values, citrulline concentration was markedly and statistically significantly decreased at visits 2 (2 weeks), 3 (4 weeks), and 4 (6 weeks), while returning to pretreatment concentrations at visit 5 (Table 1). On the other hand, a significant increase in plasma neopterin concentrations was observed at visits 4 and 5, with an increase of borderline significance at visit 2. With the exception of decreased kynurenine/tryptophan ratio at visit 3, no significant changes were observed in the concentrations of kynurenine, tryptophan, and kynurenine/tryptophan ratio throughout the course of the treatment. No differences were observed in the concentrations of the biomarkers examined between patient subgroups according to the stage or p16 status at baseline and throughout the course of the treatment (data not shown).

Citrulline concentrations did not correlate significantly with age (data not shown), but as indicated in Table 2, significant correlations were observed between age and neopterin concentrations at baseline and during the course of the treatment while the correlations between age and kynurenine concentrations as well as kynurenine/tryptophan ratio

Table 1: Changes of citrulline, neopterin, kynurenine, and tryptophan concentrations during the course of the treatment

Visit (week)	0	2	4	6	8
Interval from radiotherapy start (days)	-2 ± 3	15 ± 6	29 ± 8	43 ± 8	58 ± 10
Citrulline ($\mu\text{mol/L}$)	30.0 ± 8.4	$18.2 \pm 5.1^*$	$19.8 \pm 5.9^*$	$22.3 \pm 8.8^{**}$	31.6 ± 8.2
Neopterin ($\mu\text{g/L}$)	2.9 ± 1.6	$3.6 \pm 1.9^{\S}$	3.1 ± 1.8	$4.8 \pm 4.6^{**}$	$5.3 \pm 3.7^{***}$
Tryptophan ($\mu\text{mol/L}$)	76.4 ± 23.0	77.4 ± 23.0	81.8 ± 30.4	74.4 ± 27.4	71.0 ± 21.1
Kynurenine ($\mu\text{mol/L}$)	4.3 ± 2.2	4.2 ± 2.7	3.3 ± 1.8	4.5 ± 3.0	4.7 ± 2.8
Kynurenine/tryptophan ratio (mmol/mol)	61 ± 29	59 ± 42	$43 \pm 24^{**}$	76 ± 87	76 ± 59

* $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$; $^{\S}p = 0.05$ compared to visit (week) 0, all other differences were not statistically significant compared to visit (week) 0.

Table 2: Correlation (Spearman's rank correlation coefficient) between age and serum neopterin and kynurenine concentrations, and kynurenine/tryptophan ratio before and during the course of the treatment (p values in parentheses)

Visit (week)	0	2	4	6	8
Neopterin	0.738 (0.001)	0.712 (0.002)	0.560 (0.030)	0.646 (0.009)	0.733 (0.004)
Kynurenine	0.339 (0.199)	0.464 (0.070)	0.406 (0.133)	0.623 (0.013)	0.782 (0.016)
Kynurenine/tryptophan ratio	0.424 (0.101)	0.550 (0.027)	0.553 (0.032)	0.568 (0.027)	0.736 (0.004)

reached statistical significance only during the course of the treatment. Significant correlations were observed between neopterin concentrations, and serum kynurenine concentrations and kynurenine/tryptophan ratio before and during the course of the treatment (Table 3). No significant correlations were observed between neopterin and citrulline concentrations (data not shown), and a negative correlation between citrulline concentrations and kynurenine and kynurenine/tryptophan ratio reached statistical significance only at visit 2 (Table 4).

In general, negative correlations were observed between the grade of toxicity and neopterin, kynurenine,

and kynurenine/tryptophan ratio, but these correlations reached statistical significance only at some visits (Table 5).

4 Discussion

As expected, citrulline concentrations decreased significantly during the course of the treatment returning to pre-treatment values at the last visit, in agreement with prior studies on chemoradiation [6–13]. In contrast, serum neopterin concentrations increased later during the course of the treatment.

Table 3: Correlation (Spearman's rank correlation coefficient) of serum neopterin concentrations, and serum kynurenine concentrations and kynurenine/tryptophan ratio before and during the course of the treatment

Visit (week)	0	2	4	6	8
Kynurenine	0.547 (0.028)	0.532 (0.034)	0.268 (0.334)	0.604 (0.017)	0.714 (0.006)
Kynurenine/tryptophan ratio	0.600 (0.014)	0.706 (0.002)	0.532 (0.041)	0.761 (0.001)	0.835 (0.0004)

Table 4: Correlation (Spearman's rank correlation coefficient) of citrulline concentrations, and serum kynurenine concentrations and kynurenine/tryptophan ratio before and during the course of the treatment

Visit (week)	0	2	4	6	8
Kynurenine	-0.182 (0.499)	-0.725 (0.002)	-0.235 (0.398)	-0.318 (0.248)	0.247 (0.415)
Kynurenine/tryptophan ratio	-0.247 (0.356)	-0.689 (0.004)	-0.482 (0.069)	-0.404 (0.136)	0.385 (0.194)

Table 5: Correlation (Spearman's rank correlation coefficient) between toxicity grade and serum neopterin and kynurenine concentrations, and kynurenine/tryptophan ratio before and during the course of the treatment

Visit (week)	0	2	4	6	8
Neopterin	−0.428 (0.098)	−0.456 (0.076)	−0.560 (0.030)	−0.322 (0.241)	−0.648 (0.0167)
Kynurenine	−0.519 (0.040)	−0.331 (0.211)	−0.420 (0.120)	−0.376 (0.167)	−0.380 (0.200)
Kynurenine/tryptophan ratio	−0.583 (0.018)	−0.454 (0.078)	−0.425 (0.115)	−0.381 (0.161)	−0.607 (0.028)

The correlations of circulating concentrations of neopterin with age, kynurenine concentrations and kynurenine/tryptophan ratios are expected and have been documented in numerous prior studies [14–21,23–34]. In contrast to a prior study in patients with rectal cancer, no correlation was evident between neopterin and citrulline concentrations, but a significant negative correlation was observed between citrulline and kynurenine and kynurenine/tryptophan ratio immediately after the start of the treatment (after 2 weeks) [7,17,32]. Significant correlations of neopterin with kynurenine concentrations and kynurenine/tryptophan ratios were evident throughout the course of observation. Contrary to expectations, a trend of inverse correlation was observed between the toxicity grade and concentrations of inflammatory biomarkers neopterin, kynurenine, and kynurenine/tryptophan ratio that reached significance at some visits. This may suggest that the toxicity of the therapy could also result in inhibition of the immune response (e.g., by suppressing T lymphocytes), but an exact mechanism remains speculative. An inverse correlation observed between kynurenine concentrations and kynurenine/tryptophan ratio at the start of treatment and subsequent toxicity could indicate that immune activation could even be protective against subsequent toxicity, but, again, this remains a speculation. Further studies should confirm and evaluate in more detail this seemingly surprising finding.

Citrulline certainly represents an interesting biomarker of gastrointestinal toxicity. A decrease in circulating citrulline concentrations has been documented in patients treated with cytotoxic chemotherapy or radiation [7,9,11,13]. The assessment of intestinal toxicity still largely depends on data obtained by patient interrogation that are prone to subjective error. Citrulline measurement may represent a simple test that would allow the objective assessment of toxicity and the effect of interventions [35].

This study has obvious weaknesses. First, the number of patients was limited, and it is possible that some of the trends of biomarker changes or correlations would have been significant with a higher number of patients examined. This is also true for the correlations with clinical parameters like toxicity grade that may have been affected

by changes observed in a few individuals. All patients in this study responded to the treatment, and it was therefore not possible to evaluate whether the investigated biomarkers could serve as predictors of response.

In conclusion, present data demonstrate that citrulline concentrations decrease early and neopterin concentrations increase late during the course of chemoradiation in patients with cervical carcinoma. Citrulline represents a biomarker of intestinal toxicity in this population.

Conflict of interest: Bohuslav Melichar is a member of Pteridines' Editorial Board. Other authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during this study are available from the corresponding author on reasonable request.

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