Indoleamine-2,3-dioxygenase (IDO) metabolic activity is detrimental for cervical cancer patient survival

Debbie M Ferns1, Ido P Kema2, Marrije R Buist3, Hans W Nijman4, Gemma G Kenter1,3, and Ekaterina S Jordanova1,*

1Centre for Gynaecological Oncology Amsterdam; Free University Medical Centre; Amsterdam, The Netherlands; 2Department of Laboratory Medicine; University Medical Centre Groningen; The Netherlands; 3Centre for Gynaecological Oncology Amsterdam; Academic Medical Centre; Amsterdam, The Netherlands; 4Department of Gynaecological Oncology; University Medical Centre Groningen; The Netherlands

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Abbreviations: IDO, indoleamine-2,3-dioxygenase; IFNγ, interferon γ; Kyn/Trp ratio, kynurenine/tryptophan ratio; FIGO, International Federation of Gynaecologists and Obstetricians; Gy, Gray; HPV, human papillomavirus; M0, no metastasis; NK, natural killer; SCC, squamous cell carcinoma; TDO, tryptophan-2,3-dioxygenase; TLR, toll-like receptor; Tregs, regulatory T cells; XLC-MS/MS- extraction: liquid chromatographic tandem mass spectrometry.

The expression of the immunomodulating enzyme indoleamine-2,3-dioxygenase (IDO) suppresses T-lymphocyte function, thus correlating with poor survival in a variety of cancer patients. IDO degrades the essential amino acid tryptophan leading to immunosuppressive kynurenines production. In the present study, concentrations of tryptophan, 3-hydroxykynurenine, and kynurenine were measured in pre-treatment serum samples of 251 cervical cancer patients by a mass-spectrometric method (XLC-MS/MS) and IDO activity determined by the kynurenine/tryptophan (Kyn/Trp) ratio. A low concentration of tryptophan was found to be significantly associated with tumors greater than 4 cm and lymph node metastatic spread. Furthermore, significant positive correlations were found between high concentrations of the tryptophan metabolites kynurenine and 3-hydroxykynurenine and advanced disease stage (FIGO >IIA) and lymph node metastases. High levels of kynurenine were further associated with parametrial invasion and tumor size. A high Kyn/Trp ratio was related to lymph node metastasis, FIGO stage, tumor size, parametrial invasion and poor disease-specific survival. These results suggest that IDO activation is linked to poor clinicopathological parameters and worse survival in cervical cancer, warranting the use of IDO inhibitors in future clinical trials.

Introduction

Cervical cancer is the fourth most common cancer in women worldwide and is typically caused by a persistent human papillomavirus (HPV) infection.1,2 Although 80% of women will be infected by HPV, the majority are able to clear the infection, indicating a crucial role for the immune system in cervical carcinogenesis and progression.3 Not surprisingly, various mechanisms are employed by malignant and premalignant cells to escape recognition and destruction by the immune system and to modulate the tumor microenvironment allowing tumor development and growth.4,5

The intracellular enzyme indoleamine-2,3-dioxygenase (IDO) plays a central role in tumor-induced immunosuppression.6,7 IDO is induced in many cell types like trophoblastic cells, dendritic cells, macrophages and tumor cells by Toll-like receptor (TLR) ligands, endotoxin and most efficiently by the T helper type 1 (Th1) cytokine, interferon γ (IFNγ).8-12 IDO provokes immune tolerance through catalysing degradation of the essential amino acid L-tryptophan. Tryptophan is used for protein and indole synthesis and is metabolized along the kynurenine pathway.13 Kynurenines, which include L-kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid, have been reported to block T-cell proliferation, resulting in growth arrest of alloreactive T cells and natural killer (NK) cells.13,14

In serum, IDO activity can be estimated by the kynurenine-to-tryptophan (Kyn/Trp) ratio, which represents the quotient of the first product of the IDO pathway (kynurenine), divided by the substrate tryptophan.15,16 In cancer patients, lower serum concentrations of tryptophan, higher kynurenine concentrations, and a higher Kyn/Trp ratio have been reported by us and others.8,16-18 Furthermore, in lung cancer patients and patients with B-cell lymphoma, a high Kyn/Trp ratio has been found to be associated with advanced disease.16,19 In addition, IDO activity has been reported to correlate with tumor progression20-22 and may facilitate tumor metastasis.23 IDO expression has also been surmised to be involved in regulatory T cell (Treg) activity.24,25

*Correspondence to: Ekaterina S Jordanova; Email: e.jordanova@vumc.nl
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Few studies have investigated the expression of IDO in cervical cancer.26–30 Two such studies found higher levels of IDO expression in cervical cancer lesions as compared to normal cervical cells.28,29 Fotopoulou et al., described less IDO activity in primary cervical cancer in comparison to samples from healthy individuals, but this study included only 20 patients.30 A single study reported on correlation of increased IDO expression with clinical stage, lymph node metastasis, lympho-vascular space invasion and reduced disease-specific and disease-free survival.26

Here, we report for the first time on IDO metabolic activity in a large cohort of 251 cervical cancer patients by measuring tryptophan, kynurenine, 3-hydroxykynurenine and the Kyn/Trp ratio in pre-treatment serum samples, examining potential correlations between serum concentrations, clinicopathological characteristics and patient survival.

**Results**

The main clinicopathological characteristics of the 251 cervical cancer patients analysed in this study are summarized in Table 1. All additional data on the treatment is given in the Materials and Methods section.

In 107 patients (43%) parametrium invasion was found and in 174 (69%) vaso-invasion was present. In one patient the operation was not complete and of 2 patients data was not found. The surgical margins of the removed tissue were negative in all patients, but 4 patients had only a margin of 1 mm, nevertheless, these 4 patients did not have disease recurrence within 5 years. Of the 90 patients who received chemoradiation, 35 (39%) had recurrent disease within 5 years, 30% of the whole cohort had disease recurrence within 5 years.

**Association between tryptophan, kynurenine, 3-hydroxykynurenine, Kyn/Trp ratio and clinicopathological parameters**

The interquartile concentration ranges of tryptophan, kynurenine, 3-hydroxykynurenine and the Kyn/Trp ratio are presented in Table 2.

The Mann-Whitney U test was used to compare the distribution of IDO metabolite concentrations and associations with clinicopathological data. Low concentration of tryptophan was found between high kynurenine concentration and lymph node metastases (p = 0.003). A significant positive association was found between high kynurenine concentration and tumor size (p = 0.037), advanced stage of disease (FIGO > IIA) (p < 0.001), parametrical invasion (p < 0.0001) and lymph node metastases (p = 0.045). High concentration of 3-hydroxykynurenine was associated with advanced stage of disease (FIGO > IIA) (p = 0.009) and lymph node metastases (p = 0.012). A high Kyn/Trp ratio was associated with tumor size (p < 0.001; Fig. 1A), advanced stage of disease (FIGO > IIA) (p < 0.001; Fig. 1B), parametrical invasion (p < 0.001; Fig. 1C), and lymph node metastases (p < 0.001; Fig. 1D).

**Table 1. Clinicopathological characteristics**

| Clinicopathological parameter* | Category | Number of patients (%) |
|-------------------------------|----------|------------------------|
| Histopathology                | SCC      | 190 (76)               |
|                               | A(S)CC   | 55 (22)                |
| Age                           | Median   | 50                     |
|                               | Range    | 23-90                  |
| FIGO stage†                   | IAI      | 2 (1)                  |
|                               | IIAI     | 6 (2)                  |
|                               | IBI      | 114 (45)               |
|                               | IIIB     | 19 (8)                 |
|                               | IIA      | 25 (10)                |
|                               | IIB      | 40 (16)                |
|                               | IIIA     | 5 (2)                  |
|                               | IIIB     | 21 (8)                 |
|                               | IVA      | 6 (2)                  |
|                               | IVB      | 12 (5)                 |
| Lymph nodes                   | Negative | 165 (66)               |
|                               | Positive | 86 (34)                |
| Tumor size (mm)               | < 40 mm  | 166 (66)               |
|                               | ≥ 40 mm  | 82 (33)                |
| Vasoinvasion                  | Absent   | 72 (29)                |
|                               | Present  | 174 (69)               |
| Parametrium invasion          | Absent   | 140 (56)               |
|                               | Present  | 107 (43)               |
| Type of treatment             | None     | 3 (1)                  |
|                               | Surgery  | 79 (32)                |
|                               | Surgery + adjuvant (CRT) | 47 (19) |
|                               | Chemoradiation | 90 (36) |
|                               | Radiotherapy | 27 (11) |
|                               | Chemotherapy | 3 (1) |
|                               | NA CRT* | 2 (1)                  |

* For some variables, data were not available for all patients (n = 251).
† FIGO, International Federation of Gynecologists and Obstetricians.
‡ SCC: squamous and A(S)CC: adeno(squamous) cervical cancer
≡ (CRT): (Chemo)Radiotherapy.
¶ NA CRT: Neo-adjuvant chemoradiation.

**Disease Specific Survival in Association with IDO Metabolite Concentrations**

The median follow-up time was 76 months (range, 1-118) for all patients and 88 months (range, 39-118) for patients alive at the time of data collection. Of the 84 patients who died during the follow-up period, 67 deaths could be attributed to cervical cancer and 4 patients died of another cause. For 13 patients the cause of death was unknown and for the remaining 3 patients it was unknown whether they were alive or not at time of data collection.

There was a trend towards worse survival for patients with the lowest quartile of tryptophan concentrations compared to higher amounts of tryptophan (p = 0.057; Fig. 2A), while there was a significant difference when solely squamous cell carcinomas (SCCs) were analyzed (p = 0.011; Fig. 2B). Patients with low amounts of kynurenine had a significantly better 5-year survival rate compared to patients with high kynurenine concentration (p = 0.015; Fig. 2C). This was also the case for squamous tumors...
alone (p = 0.021; Fig. 2D). There was no difference in survival rates for the total group of patients or for the SCC cases when expression of 3-hydroxykynurenine was considered (p = 0.38 and p = 0.20, respectively) (data not shown). In the complete cohort, the highest quartile of the Kyn/Trp ratio was associated with poor survival (p = 0.007; Fig. 2E) and this was also observed in the squamous cell carcinomas (p = 0.032; Fig. 2F).

Because of the strong associations found with clinicopathological parameters such as FIGO stage and lymph node metastasis, the amount of the studied IDO metabolites was, although significantly correlated with survival in a univariate Cox analysis (HR 2.1, 95% CI: 1.20-3.52, p = 0.008), not an independent prognostic factor (Table 3).

**Discussion**

In this study, we showed for the first time that tryptophan degradation along the kynurenine pathway (the presence of active IDO) in pre-treatment serum, as measured by the Kyn/Trp ratio, is associated with detrimental clinicopathological parameters and poor survival in a large cohort of cervical cancer patients. Because of the strong associations found with FIGO stage and lymph node metastasis (p < 0.001), the Kyn/Trp ratio was, although being significantly correlated with survival in a univariate Cox proportional hazards analysis (HR 2.1, 95% CI: 1.20-3.52, p = 0.008), not an independent prognostic factor. However, the

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**Table 2.** The concentration of the IDO pathway metabolites in cervical cancer patient serum samples as measured by the XLC-MS/MS method

| Quartile    | Tryptophan μmol/L | Kynurenine μmol/L | 3-Hydroxykynurenine μmol/L | Kyn/Trp ratio μmol/L |
|-------------|-------------------|-------------------|-----------------------------|----------------------|
| 1st         | 10.86-41.55       | 0.22-1.33         | 3.20-26.50                  | 16.20-26.53          |
| 2nd         | 41.56-49.26       | 1.34-1.64         | 26.51-33.90                 | 26.54-33.81          |
| 3rd         | 49.27-55.05       | 1.65-2.03         | 33.91-47.10                 | 33.82-43.54          |
| 4th         | 55.06-80.97       | 2.04-5.77         | 47.11-240.40                | 43.55-123.54         |

**Figure 1.** IDO metabolites correlate with clinicopathological features of cervical cancer patients. Association between IDO activity as measured by Kyn/Trp ratio and clinicopathological parameters in cervical cancer (n = 251*). The kynurenine and tryptophan concentrations were determined by an automated online solid-phase XLC-MS/MS method with deuterated internal standards. Association of IDO activity (high Kyn/Trp ratio) and tumor size (A), FIGO stage (B), parametrium invasion (C), and lymph node metastasis (D). The non-parametric Mann-Whitney U test was used, p < 0.05 was considered to be statistically significant. A high Kyn/Trp ratio was associated with tumor size (p < 0.001), advanced stage of disease (FIGO > IIA) (p < 0.001), parametrial invasion (p < 0.001) and lymph node metastases (p < 0.001). CI, confidence interval; FIGO, International Federation of Gynaecologists and Obstetricians. * For some variables, data were not available for all patients.
main goal of this study was to ascertain the rational for using IDO inhibitors in cervical cancer treatment; in contrast, the use of IDO activity as a prognostic marker should be analysed in comparison with serum markers already in use, such as prognostic serum biomarkers in SCC.31

Our results are in accordance with earlier studies that have shown decreased tryptophan levels, increased kynurenine levels and a high Kyn/Trp ratio in the serum of patients with various cancer types.16-19,32 In addition, we have previously described the same for endometrial, ovarian and vulvar cancer.8 These results substantiate that tryptophan catabolism can be monitored in serum samples from cancer patients and could have prognostic significance. Only in one other study has the presence of IDO metabolites in cervical cancer been analysed, but these results were unclear as a very limited number of samples was included (n = 20).30 Plasma/serum tryptophan status and the tryptophan kynurenine ratio are influenced by both IDO and the liver enzyme tryptophan 2,3-dioxygenase (TDO); however, we did not analyze the potential involvement of the latter enzyme in cervical cancer in the present study.33 IDO expression in the tumor microenvironment leads to specific in situ depletion of tryptophan and production of immune inhibitory metabolites, which subsequently inhibit T-cell proliferation and T-cell apoptosis.34 In line with these data, the numbers of CD3+ and CD8+ T cells have been found to decrease with IDO activity increase.10,35,36 Kynurenine has also been implicated in the direct inhibition of T-cell function.37 Furthermore, IDO has been shown to induce immune tolerance through modulating inhibitory dendritic cells in mouse models and cancer patients38 and by suppressing TCR-mediated activation of T cells via blocking multiple downstream kinases, such as the mitogen activated protein kinase family members ERK and MAPK, and the nuclear factor kB inhibitory enzyme IκB.39,40 In mouse models of lung and breast carcinoma, it has been previously reported that IDO deficiency reduces tumor burden and improves survival. Interestingly, during tumor and metastasis outgrowth, interleukin (IL)-6 induction was found to be significantly impaired in IDO deficient mice, causing a substantial diminishing of pro-tumorigenic...
myeloid-derived suppressor cells (MDSCs). These findings have been substantiated by a study in human breast cancer, showing that IDO is linked to higher MDSC numbers and signal transducer and activator of transcription 3 (STAT3)-dependent IDO expression mediates the immunosuppressive effects of MDSCs.

Moreover, suppressive action of IDO can also be exerted by the induction and expansion of Tregs by an IDO-dependent mechanism. In cervical cancer, we have previously shown that the presence of high intraepithelial numbers of CD4+FoxP3+ Tregs is associated with poor survival. It will be of interest to further evaluate additional links between the IDO pathway and other important immunological parameters in the tumor microenvironment in cervical cancer patients.

As we have shown, the activity of IDO can be easily measured in pre-treatment serum of cancer patients. This will allow the selection of patients who might benefit from treatment with IDO inhibitors. Various IDO inhibitors have been described and have already gone through Phase I cancer clinical trials, including 1-Methyl-D-tryptophan (Indoximod). Further studies with IDO inhibitors INCBO24360, Indoximod and NLG919 are on-going and the use such inhibitors as sole treatment or in combination with chemotherapy in various advanced tumours is promising.

We believe the data presented here underscore the clinical potential of applying IDO inhibitors as a treatment modality for cervical cancer in the future.

### Material and Methods

**Patients**

For measuring IDO activity, available pre-surgical treatment serum samples from all patients treated for cervical cancer between 2003 and 2008 at the Academic Medical Centre (AMC), Centre for Gynaecologic Oncology Amsterdam, The Netherlands were included (n = 251). None of the patients received chemo(radio)therapy before the serum was obtained. The main clinicopathological characteristics of the study cohort are summarized in Table 1.

Patient with early stage cervical cancer had radical hysterectomy plus lymph node resection by Okabayashi’s modification of the Wertheim procedure, with complete intent. Patients with extended ‘inoperable’ cervical cancer (≥bulky stage IIA or IIB, but M0), received external beam radiotherapy to the pelvis (46 Gray (Gy) in fractions of 2.0 Gy) or extended ‘chimney’ fields included the para-aortic lymph node region (50.4 Gy in 1.8 Gy fractions) plus weekly concurrent chemotherapy (cisplatin, 40 mg/m2/week).

Patients with parametrial tumor invasion and/or lymph node metastases received an external radiotherapy boost up to 60 Gy.

Patients samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

### Measuring IDO Activity in Serum Samples

The concentration of tryptophan, kynurenine and 3-hydroxykynurenine was determined by an automated online solid-phase extraction-liquid chromatographic tandem mass spectrometric (XLC-MS/MS) method with deuterated internal standards exactly as described previously. Briefly, 50 µL plasma equivalent was pre-purified by automated on-line solid-phase extraction, using strong cation exchange (PRS, propylsulphonic) cartridges. Chromatographic separation of the analytes and deuterated analogues occurred by C18 reversed phase chromatography. Mass spectrometric detection was performed in the multiple reaction-monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionization. Serum samples from 251 patients were analyzed. The Kyn/Trp ratio was calculated by dividing the amounts of the two metabolites. The concentrations of the different metabolites were measured in µmol/L.

**Statistics**

To determine whether serum concentrations of tryptophan, kynurenine, 3-hydroxykynurenine and the Kyn/Trp ratio were significantly different between groups, the non-parametric Mann-Whitney U test was used. Disease specific survival was defined as death by cervical cancer, while disease-free survival was defined as the time until disease recurrence. Patient survival rates were analyzed by Kaplan-Meier curves and the log-rank test and disease specific survival was analyzed by Kaplan-Meier curves and the log-rank test and univariate/multivariate Cox proportional hazards models.

All tests were 2-sided, and p < 0.05 was considered to be statistically significant. Analyses were performed using SPSS, version 21.0 for Windows (SPSS, Inc, Chicago, IL).

**Disclosure of Potential Conflicts of Interests**

No potential conflicts of interest were disclosed.

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**Table 3. Univariate and multivariate Cox regression analysis IDO metabolites in serum (n = 251)**

|                      | Univariate analysis | Multivariate analysis |
|----------------------|--------------------|-----------------------|
|                      | HR     | 95% CI* | p value | HR     | 95% CI* | p value |
| Tumor size           | 2.40   | 1.44–3.98 | 0.001 | 0.88   | 0.51–1.54 | 0.66   |
| Parametral invasion  | 4.46   | 2.51–7.92 | <0.001 | 1.59   | 0.76–3.36 | 0.22   |
| FIGO stage           | 5.96   | 3.45–10.28 | <0.001 | 3.46   | 1.67–7.20 | 0.001  |
| Lymph node metastasis| 3.82   | 2.27–6.43 | <0.001 | 2.65   | 1.53–4.61 | 0.001  |
| Kyn/Trp ratio ±      | 2.06   | 1.20–3.52 | 0.008 | 1.16   | 0.66–2.05 | 0.61   |

*For some variables, data were not available for all patients.† FIGO. International Federation of Gynecologists and Obstetricians.

§Highest quartile vs. rest.

∞Hazard ratio.

*Confidence interval.

**Material and Methods**

**Patients**

For measuring IDO activity, available pre-surgical treatment serum samples from all patients treated for cervical cancer...
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