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Analysis of Kynurenine/Tryptophan ratio and expression of IDO1 and 2 mRNA in tumour tissue of cervical cancer patients

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ABSTRACT

Objectives: Indoleamine 2,3-Dioxygenase (IDO) catalyses the degradation of the essential amino acid tryptophan leading to the production of immunosuppressive Kynurenine. In the present study, we developed a modified method for measurement of Kynurenine/tryptophan (K/T) ratio in the cervical tissue using HPLC and investigated its relationship with the expression of IDO1 and 2 genes in the cervical tumour milieu.

Design and Methods: Cervical cancer punch biopsy samples of 27 women who presented at the Cancer Institute (WIA) were used for detection of K/T ratio by HPLC as well as expression of IDO1 and 2 at the mRNA level by Realtime PCR after obtaining Institutional ethical committee approval.

Results: The K/T ratio was elevated significantly in cancer cervix samples compared to normal cervix ($p < 0.05$). IDO1 mRNA levels were up-regulated whereas IDO2 mRNA levels were down-regulated in cancer cervix compared to the normal cervix. A positive correlation was observed between IDO1 and K/T ($p < 0.05$) indicating that IDO1 was responsible for the increase in K/T ratio.

Conclusion: Our preliminary data indicates that the K/T ratio at the tissue level may be investigated reliably using HPLC, as an indirect measure of IDO1 gene expression and activity.

1. Introduction

Cervical cancer is the second most common cancer in women worldwide and is now linked to Human papillomavirus (HPV) in almost all the cases. The peak age of incidence of cervical cancer is 55–59 years, and a considerable proportion of women report in the late stages (stage III and IV) of disease. Patients presenting at stage I and early IIA have an 80–90% five year survival; for those presenting at later stages, survival ranges from 65 to 20% or even less [1,2].

Tumour progression occurs as a result of continuous combat between tumour cells and the immune system. The tumour cells employ several immune evasion strategies in order to establish their tumour niche. One such strategy is the expression of immune suppressive factors like Indoleamine 2,3-Dioxygenase (IDO), IL-10, TGF-β, and VEGF [3]. The enzyme IDO catalyses the first and the rate limiting step of Tryptophan (Trp) catabolism that causes Trp depletion by breaking the amino acid down to its metabolite Kynurenine (Kyn). This metabolite is directly toxic to T lymphocytes and may convert dendritic cells to a tolerogenic phenotype [4]. IDO1 expression is generally found in tissues like placenta, lung, ovary etc. and is known to be induced by immunological signals, including interferon-gamma (IFN-γ), lipo-polysaccharide and the tumour necrosis factor.

In 2007, an isoform of IDO1 called IDO2 was reported by three independent groups [5–7]. The genes encoding IDO1 and IDO2 utilize multiple promoters to express transcripts of different lengths and generate both active and truncated proteins [5,7]. IDO2 is known to be expressed in a number of cancers and cell lines but its functional role is not clear [8].

A study by Lob et al. (2008) [8] reported that IDO2 mRNA levels were strongly induced by IFN-γ in HeLa cells whereas a similar study [9] reported no such change in IDO2 expression even though IDO1 mRNA was strongly induced.

The Serum Kyn/Trp ratio may serve as an indicator of IDO enzyme activity and immune suppression, and is known to be elevated in many cancers like prostate, lung, glioblastoma, endometrial, ovarian, vulvar and adult T cell leukemias [10–13]. Some studies reported that [14,15] the K/T ratio could also be used as a surrogate prognostic marker in some cancers. Earlier studies suggested that immune dysfunction via IDO mediated suppression of immune infiltrates also contributes to the progression of cervical neoplasia to advanced disease [16], making it necessary to assess the expression status of the enzymes IDO1 and 2 in the tumour milieu.

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High-pressure liquid chromatography (HPLC) is a simple qualitative and quantitative method for measuring tissue amino acids levels. Qualitative analysis in the aspect of identifying unknown peaks by comparing the retention factor (k) and response of the peak in the chromatogram of the standard solution and quantitative analysis is done with the availability of known standards. Simultaneous measurement of Trp and Kyn using HPLC [19] has been widely studied at the systemic level in plasma. However with respect to cervical cancer, a systemic level approach may not be helpful and will need to focus on the tumour milieu. To our knowledge this is the first study to determine the K/T ratio in the cervical cancer tissue and correlate the same with mRNA level expression of IDO1 and 2.

2. Objectives

a. To develop an effective method using HPLC for quantitating the K/T ratio in the cervical tissue and assess the mRNA level expression of the IDO1 and 2 genes in the same.

3. Design and methods

3.1. Chemicals and other materials

Trp and Kyn were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK) and were stored as directed by the manufacturer. Water and Acetonitrile (HPLC grade) were purchased from Fisher Scientific (UK). Perchloric acid (ACS reagent 70%) and sodium acetate were also purchased from Sigma-Aldrich and were of the purest commercially available grades and were made up in HPLC-grade water (Thermo Fisher Scientific Inc., USA).

3.2. Sample collection

Institutional ethical committee approval was obtained prior to initiating the study. Paired tumour punch biopsy samples were collected (one for routine histopathology; one for this study) from cervical cancer patients presenting at the Cancer Institute (WIA) outpatient facility after informed consent. Punch biopsy samples of ~60 mg were snap frozen and processed when required for HPLC while the rest were stored in RNA later (Ambion, Thermo Fisher Scientific, Inc., USA). Gross normalization was done by adjusting the volume of perchloric acid according to tissue weight, measured prior to homogenization. Frozen ectocervix samples histopathologically found to be normal, from women who underwent hysterectomy for non-cancerous conditions were used in the study as normal cervix controls after obtaining their informed consent.

3.3. Buffer preparation

Sodium acetate (15 mM) was prepared in HPLC grade water; the pH was adjusted to 4.7 with glacial acetic acid and filtered sterile using a 0.45 μm size membrane filter (Thermo Fisher Scientific, Inc., USA). 40 ml of Acetonitrile (4% w/v) was added to 1 l of the buffer for peak stabilization. The buffer was degassed in a sonicator (Grant instruments, Cambridge, UK).

3.4. Preparation of standard solutions

Trp and Kyn stocks (1 mg/ml) were prepared as individual solutions using HPLC grade water. From the individual solutions, mixtures of the two components were prepared at a primary working concentration of 2.5 μg/ml using 6% $HClO_4$. The rest of the standards were prepared by serial dilution of the primary working concentration using 6% $HClO_4$. Eight different standards ranging from 2.5 μg/ml–0.019 μg/ml (12 μmol/l–0.09 μmol/l) were finally run along with the samples.

3.5. Sample preparation

Approximately 60 mg of the snap frozen tissue was homogenized using the fast prep® homogenizer (MP Biomedicals CA, USA) with 120 μl of 9% (w/v) perchloric acid for 2 min at 4 °C and centrifuged for 10 min at 10000 g. The volume of supernatants was adjusted with HPLC grade water to a final concentration of 6% (w/v) perchloric acid. Normal cervix tissue samples were also processed as above.

3.6. HPLC conditions

HPLC analysis was carried out using a Dionex Ultimate 3000 UHPLC system (Dionex Corporation, CA, USA). Chromeleon® v6.8 software was used for system control and data acquisition and integration. Analysis was carried out at 30 °C using an Agilent Zorbax SB (Agilent technologies, CA, USA) C-18 column (4.6 mm × 250 mm, 5 μm particle size) protected by an Acclaim® (Dionex Corporation, CA, USA) 120 guard C18 2 mm × 10 mm column. Extracted samples (5 μl) were delivered into the column at a flow rate of 1 ml/min using a mobile phase composed of 15 μM Sodium acetate at pH 4.7.

3.7. Quantitative expression of IDO1 and 2

RNA was extracted using Trizol (Invitrogen, CA, USA), according to the manufacturer's instructions, and was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen, Netherlands). Quantitative PCR was performed with Sybr Green Master Mix chemistry (Eurogentec, Belgium), according to the manufacturer's instructions, with Quantstudio™12K flex (Applied Biosystems,CA, USA). The amounts of transcripts were normalized to that of two housekeeping genes β-actin and GAPDH in order to minimize errors due to variation among different tissues. Melting curves were determined to ensure the amplification of a single product. The primer sequences used for IDO detection were the same as those used in previous studies [17] and are mentioned in Table 1. The thermal cycling conditions were 10 min at 95 °C, 40 cycles at 95 °C for 15 s and annealing at 59 °C for 1 min. The Ct values obtained were normalized with the Ct values of the housekeeping genes beta actin and GAPDH. Fold change was calculated from the ΔΔCt values which were obtained by subtracting the ΔCt value of cancer cervix and normal cervix samples as reported previously [18].

3.8. Statistical analysis

Statistical analyses were performed using Prism version 6.00 for Windows, GraphPad Software (La Jolla CA, USA) and *p* values less than 0.05 were considered statistically significant. Non-parametric

Table 1
Sequence of primers used for q-PCR.[a]a

Gene	Direction	Primer sequence	Product size (bp)
GAPDH	Forward	GTGAAGGTCGGAGTCAAG	112
	Reverse	TGAGGTCAATGAAGGGGTC	
Beta actin	Forward	AGACAGTGTTGTGGGTGTAGG	125
	Reverse	TGGGATGGGGAGTCTGTTCA	
Full IDO1	Forward	GGCTTTGCTCTGCCAAATCC	119
(Exon1–2)	Reverse	TTCTCAACTCTTTCTCGAAGCTG	
Partial IDO1	Forward	GATGTCCGTAAGGTCTTGCCA	187
(Exon 4–6)	Reverse	TGCAGTCTCCATCACGAAATG	
Full IDO2	Forward	AAATGCACTGCCAGTTGAAA	174
(Exon1)	Reverse	CTGGTGGGTGAAGTGTCAAG	
Partial IDO2	Forward	CTGATCACTGCTTAACGGCA	281
(Exon 10)	Reverse	TGCCACCAACTCAACACATT	

[a] Reference: Croitoru-Lamoury et al. [17].