Prognostic impact of indoleamine 2,3-dioxygenase 1 (IDO1) mRNA expression on circulating tumour cells of patients with head and neck squamous cell carcinoma

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ABSTRACT

Background We sought to determine the prognostic role of indoleamine 2,3-dioxygenase 1 (IDO1) by evaluating IDO1 expression in circulating tumour cells (CTCs) at baseline and after completion of chemoradiotherapy in patients with locally advanced (LA) head and neck squamous cell carcinoma (HNSCC) treated with curative intent.

Methods In a prospective cohort of 113 patients with LA HNSCC, we evaluated expression of IDO1 in the EpCAM+ CTC fraction at baseline and after cisplatin chemoradiation. The prognostic value of combined programmed cell death ligand-1 (PDL-1) and IDO1 expression was assessed.

Results IDO1 was significantly overexpressed at baseline compared with the post-treatment counterparts (p=0.007). IDO1 messenger RNA (mRNA) expression at baseline was associated with better survival in terms of progression-free survival (PFS) (HR=0.19, p=0.017). Post-treatment IDO1 mRNA levels were correlated with unfavourable prognosis in terms of overall survival (OS) (HR=3.27, p=0.008).

Patients with combined decreased expression levels of PDL-1 and IDO1 after treatment exhibited superior PFS (p=0.043) and OS (p=0.021).

Conclusions Our results strongly suggest that IDO1 mRNA expression is an independent prognostic factor for clinical outcome. Our study provides useful information for future trials combining chemoradiation with immune checkpoint inhibitors and IDO1 inhibitors.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a malignancy with well-known contributing factors, such as tobacco and alcohol consumption; in addition, human papilloma virus is implicated in the pathogenesis of an increasing proportion of oropharyngeal cancers.1,2 Despite advances in multimodality treatment, the 5-year progression-free survival (PFS) rates of patients with locally advanced (LA) disease do not exceed 40%–50%, and survival rates in the recurrent or metastatic setting remain poor.3 The discovery of novel therapeutic agents aimed at minimising toxicity associated with chemotherapy and radiation and improving patient outcomes.

Analysis of tumour microenvironment in patients with a variety of solid tumours has revealed that cancer evolution and treatment response are both influenced by the interplay between malignant cells and cells of the immune system. More specifically, it has been demonstrated that detection of CD8+ T cells is an indicator of an effective antitumour immune response4,5 and correlates with the
upregulation of immune inhibitory mechanisms mediating immune suppression. Indoleamine 2,3-dioxygenase 1 (IDO1) is an enzyme that participates in the catabolism of the essential amino acid L-tryptophan, causing its depletion, and contributes to immune suppression and tolerance in the tumour microenvironment. These IDO1-inducing signals may be constitutively present in the inflammatory microenvironment of the tumour and may be stimulated by the dying cells and release of tumour antigens that is triggered by chemotherapy. However, it remains largely unknown to what degree IDO1 is produced after chemotherapy. Several studies suggest that IDO1 expression determines the choice between immunogenic and tolerogenic cell death in response to chemotherapy.

On the other hand, detection of circulating tumour cells (CTCs) is used for real-time monitoring of tumour status and has been shown to correlate with prognosis in several cancers. In addition, molecular characterisation of CTCs potentially provides valuable information for the development of novel drugs.

Based on these considerations, we sought to prospectively determine IDO1 messenger RNA (mRNA) expression in CTCs at baseline and after completion of cisplatin chemoradiation therapy (CRT) in a cohort of patients with LA HNSCC treated with curative intent. To achieve this, we first developed a highly sensitive, specific and reproducible real-time quantitative real-time reverse transcription PCR (RT-qPCR) assay for the quantification of IDO1 mRNA expression in CTCs. We demonstrate for the first time that high IDO1 mRNA expression at baseline is associated with favourable overall survival (OS), whereas high IDO1 mRNA expression at the end of treatment is associated with shorter OS.

MATERIALS AND METHODS

Study design

In a single-institution study, 113 patients with LA HNSCC participated in this analysis. Written informed consent was obtained from all patients. For this population of patients, our group has previously published results regarding expression of immunogenic cell death (ICD) biomarkers. Inclusion criteria have been previously described; patients with newly diagnosed, histologically confirmed squamous cell carcinoma of the oral cavity, oropharynx, larynx or hypopharynx were included. Patients had tumours not amenable to surgical treatment or wished to preserve their larynx. Exclusion criteria have been previously described. Determination of disease stage was done using the TNM classification by performing a CT scan of the head and neck, thorax and abdomen. All patients underwent cisplatin chemoradiation, and registration was done before the initiation of treatment. All patients received high-dose cisplatin (100 mg/m² every 21 days) in combination with radiotherapy. Eighty-five per cent of patients received >200 mg/m² cisplatin. All patients received 66 Gy in 30 daily fractions over 6 weeks to the primary tumour site and involved nodes. Sample collection occurred at two timepoints: at baseline and at the end of CRT (a week after treatment was stopped). All patients were subjected to standard follow-up, which was CT of the head and neck and evaluation by an ear, nose and throat physician every 3 months for the first 2 years and every 6 months after the period of 2 years. The first assessment of treatment response was done 12 weeks post-CRT.

We evaluated the expression of IDO1 in the EpCAM+ CTC fraction at baseline and after cisplatin-based CRT.

Isolation of EpCAM(+) CTCs

For the isolation of EpCAM(+) CTCs from peripheral blood (30 mL), we followed our previously described protocols.

RNA extraction

The mirNeasy micro kit (QIAGEN, Germany) was used for the isolation of total RNA from the EpCAM(+) CTC fraction, according to the manufacturer’s instructions. cDNA synthesis cDNA synthesis was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies, USA) according to manufacturer’s protocol, using 7 µL of isolated total RNA as starting template.

RT-qPCR assay for the quantification of IDO mRNA

Primer and probe design

We designed in silico the primers and hydrolysis probes (TaMMan) for IDO1 and B2-microglobulin (B2M, used as a reference gene) using Primer Premier V.5.0 software (Premier Biosoft, California, USA). Our primers and probes were carefully designed to completely avoid primer–dimer formation, false priming sites, formation of hairpin structures and hybridisation to genomic DNA, while amplifying specifically only the genes of interest. The sequences of primers and probes are available on request.

qPCR

Expression analysis of genes was conducted using the TaqMan chemistry, in a Rotor Gene Q Real-Time PCR machine (Qiagen), after thorough optimisation of the methodology (data not shown).

Regarding IDO1, each cDNA was amplified in a 10 µL reaction containing 2.5 µL of the PCR synthesis buffer (5×), 1.6 µL MgCl₂ (25 mM), 0.2 µL deoxyribonucleotide triphosphates (dNTPs) (10 mM), 0.1 µL Taq DNA polymerase (5 U/µL), 1 µL of forward and reverse primers (2.5 µM), 0.83 µL TaqMan probe (3 µM) and H₂O to the final volume. The 10 µL reaction mixture for B2M contained 1 µL of PCR synthesis buffer (5×), 1.2 µL MgCl₂ (25 mM), 0.15 µL dNTPs (10 mM), 0.3 µL Bovine Serum Albumin Solution (BSA) (10 µg/µL), 0.1 µL Taq DNA polymerase 5 U/µL, 0.25 µL of forward and reverse primers (10 µM), 0.83 µL TaqMan probe (3 µM) and H₂O to the final volume. The thermal cycling protocol for IDO1 consisted of an initial 2 min polymerase activation step at 95°C and...
50 cycles of 95°C for 10 s for template denaturation, 60°C for 20 s for primer annealing and 72°C for 20 s for extension. Finally, the cycling conditions for B2M were 95°C for 2 min, 45 cycles of 95°C for 10 s, annealing at 58°C for 20 s and extension at 72°C for 20 s.

Normalisation of qPCR data in clinical samples
qPCR data for IDO1 expression were normalised in respect to B2M expression in the same cDNAs, using the 2^ΔΔCq approach. CTCs isolated through positive immunomagnetic enrichment are partly contaminated, since ‘contamination’ of peripheral blood mononuclear cells in the EpCAM(+) CTC fraction could affect IDO assay specificity, we assessed this contamination by analysing peripheral blood samples from 20 healthy individuals in exactly the same way as patients. We estimated a cut-off based on IDO1 normalised expression in respect to B2M expression in this control group. Using this approach, we defined a sample as IDO1 overexpressed based on the fold change of each target gene expression in the EpCAM(+) CTC fraction in respect to the corresponding EpCAM(+) fraction in the group of 20 healthy individuals.

Statistical analysis
The Wilcoxon statistical test was performed to analyse the differential expression of IDO1 before and after treatment. The non-parametric Mann-Whitney U, Jonckheere-Terpstra and Kruskal-Wallis tests were used appropriately in order to assess the associations of IDO1 expression levels with patients’ clinicopathological parameters. Independent associations between baseline IDO1 levels and tumour characteristics were assessed by linear regression. The association between IDO1 levels and response to treatment was evaluated by logistic regression.

Our survival analysis included the construction of Kaplan-Meier survival curves, and the long-rank statistical test was performed for the assessment of any differences in the Kaplan-Meier curves and the estimation of the p value. The prognostic value of IDO1 expression for PFS and OS was evaluated by univariate and multivariate bootstrap Cox proportional hazards regression models, where the 95% CI was calculated using the bias corrected and accelerated approach, and the resulting p values were evaluated by the test for trend approach. PFS was defined as the time from registration to the date of tumour progression or death from other causes or censored at the time of the last contact. OS was defined as the time from registration to the study to death from any cause or censored at the time of the last contact. Statistical analyses were performed using the SPSS Statistics V.22.0 software. Two-tailed tests were used and p values of <0.05 were considered statistically significant.

RESULTS
Patient population
We collected samples from 113 patients with LA HNSCC at two timepoints: at baseline and after completion of CRT. Baseline patient characteristics have been described in our previously published paper.

IDO1 levels at baseline and post-treatment
In our cohort, 73 patients had evaluable specimens for CTC gene expression analysis. Forty patients were not included in the analysis because of low-quality RNA. Wilcoxon signed-rank test analysis revealed a significant overexpression of IDO1 at baseline compared with the post-treatment counterpart (p=0.007, online supplementary figure S1).

Association of IDO1 levels with tumour characteristics
According to our statistical analysis, IDO1 at baseline exhibited increased expression levels in male patients (p=0.030, online supplementary table S1). Moreover, a significant IDO1 upregulation was observed in heavy and ex-heavy smokers not only at baseline (p<0.001, online supplementary table S1) but also postchemoradiation (p=0.029, online supplementary table S2).

Association with clinical outcome
Median follow-up was 27.16 months (range 2.3–69.3), during which 24 patients progressed and 34 died. In our cohort, IDO1 levels were evaluated for association with PFS and OS. According to Kaplan-Meier analysis for IDO1 expression levels at baseline, patients stratified as IDO1 positive unequivocally demonstrated (p=0.010) longer PFS intervals compared with IDO1-negative ones (figure 1A). Furthermore, the cumulative probability of 3-year PFS for IDO1-positive patients was 0.85, whereas the corresponding probability for IDO1-negative ones was 0.55. Bootstrap univariate Cox proportional hazard regression analysis corroborated the favourable prognostic value of IDO1 at baseline, since IDO1 overexpression was associated with better survival in terms of PFS (HR=0.23, p=0.018; table 1), and patients categorised as IDO1 positive were 4.35 times less likely to relapse compared with IDO1-negative ones. Interestingly, post-treatment IDO1 mRNA levels seem to constitute a significant marker of unfavourable prognosis in terms of OS since both Kaplan-Meier analysis (p=0.007, figure 2B) and bootstrap univariate Cox proportional hazard regression analysis (HR=2.92, p=0.011; table 1) demonstrated that patients categorised as IDO1 positive are characterised by inferior OS intervals compared with those belonging to the IDO1-negative group. An also interesting finding was that post-treatment IDO1 expression levels retained their significant prognostic value as a continuous variable (HR=2.45, p=0.019; table 1).

A multivariate Cox regression analysis, adjusted for important and established prognostic parameters, such as smoking status, alcohol consumption, TNM stage and gender, revealed that IDO1 expression levels before treatment correlate with favourable prognosis for patients with HNSCC (HR=0.19, p=0.017) (table 2). Next, we evaluated the independence of IDO1 expression levels after treatment in predicting the probability of OS in patients with
HNSCC by developing the same Cox multivariate proportional hazard regression model. According to this model, IDO1 expression levels after treatment are associated with unfavourable prognosis in terms of OS for those patients (HR=3.27, p=0.008) (table 2).

Moreover, given that we have previously assessed PDL-1 expression levels in CTCs after treatment at the same patient cohort, we evaluated the prognostic value of IDO1 and PDL-1 expression levels after categorisation of patients into PDL-1 and IDO1 negative, PDL-1 or IDO1 negative and PD-L1 and IDO1 positive. Therefore, according to Kaplan-Meier analysis, we found that patients with decreased expression levels of PDL-1 and IDO1 after treatment exhibited superior PFS (p=0.043) and OS (p=0.021) compared with the patients belonging to the other groups (figure 3).

**Table 1** Univariate Cox proportional hazard regression analysis showing the correlation of IDO levels before and after treatment with PFS and OS

| Variable                      | HR*  | 95% CI† | P value |
|-------------------------------|------|---------|---------|
| PFS                           |      |         |         |
| Log IDO1 (before treatment)   | 0.66 | 0.23 to 1.07 | 0.26   |
| IDO1 (before treatment)       | 1.00 |         |         |
| Negative                      | 0.23 | 0.02 to 0.50 | 0.018  |
| Positive                      | 1.19 | 0.49 to 2.89 | 0.60   |
| Log IDO (after treatment)     | 1.19 | 0.49 to 2.89 | 0.60   |
| IDO (after treatment)         | 1.00 |         |         |
| Negative                      | 1.20 | 0.25 to 3.23 | 0.75   |
| Positive                      | 2.92 | 1.10 to 7.70 | 0.011  |

| Variable                      | HR*  | 95% CI† | P value |
|-------------------------------|------|---------|---------|
| OS                            |      |         |         |
| Log IDO1 (before treatment)   | 1.35 | 0.51 to 3.31 | 0.39   |
| IDO1 (before treatment)       | 1.00 |         |         |
| Negative                      | 0.57 | 0.19 to 1.36 | 0.21   |
| Positive                      | 2.45 | 1.17 to 7.60 | 0.019  |

**DISCUSSION**

The classical form of cell death induced by chemotherapy is apoptosis, which triggers production of immunosuppressive transforming growth factor-β by the macrophages that phagocytose the debris, leading to immune suppression and tolerance. With the majority of chemotherapy agents, a complex state of a combination of these events is observed, with much immunosuppressive apoptosis occurring side-by-side with more immunogenic forms of cell death. Thus, the main question remains which signal dominates on the local immune system. In vivo studies in apoptotic cells have revealed a potent regulatory role for IDO1 in controlling the choice between tolerance and immunity to dying cell. In this study, we demonstrate that in a cohort of patients with LA HNSCC, IDO1...
is significantly overexpressed at baseline compared with post-treatment counterparts. Most importantly, we show a significant association between IDO1 expression and clinical outcome. More specifically, IDO1-positive patients at baseline were found to have significantly longer PFS and OS compared with IDO1-negative counterparts, whereas IDO1-positive patients post-treatment were characterised by inferior OS; indeed, both baseline and post-treatment IDO1 expression levels remained a strong and independent prognostic factor in multivariate analysis.

At present, we are not aware of the extent to which IDO1 is modified following chemotherapy treatment. In some tumours, IDO1 is constitutively expressed by the tumour cells themselves. This may serve as an immune-escape mechanism or may confer some non-immune survival advantage on the tumours. Several studies in

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**Table 2** Multivariate Cox regression analysis showing the correlation of IDO expression before and after treatment with PFS and OS

|         | Variable (before treatment) | PFS | | Variable (after treatment) | OS |
|---------|-----------------------------|-----|---|-----------------------------|----|
| PFS     |                             |     |   |                             |    |
|         | IDO1 (before treatment)      |     |   | IDO1 (after treatment)      |    |
|         | Negative                    | 1.00|   | Negative                   | 1.00|
|         | Positive                    | 0.19|0.03 to 0.46 | Positive                   | 3.27|
|         |                             |     |   |                             |    |
|         | Smoke                       |     |   |                             |    |
|         | Light/never                 | 1.00|   | Light/Never                | 1.00|
|         | Heavy                       | 0.60|0.09 to 3.35 | Heavy                     | 0.66|
|         |                             |     |   |                             |    |
|         | Ethanol                     |     |   |                             |    |
|         | Social/no                   | 1.00|   | Social/no                  | 1.00|
|         | Heavy                       | 3.26|0.58 to 3.78 | Heavy                     | 2.14|
|         |                             |     |   |                             |    |
|         | TNM stage‡                  |     |   |                             |    |
|         | I–III                       | 1.00|   | I–III                      | 1.00|
|         | IV                          | 2.33|0.30 to 9.45 | IV                         | 2.12|
|         |                             |     |   |                             |    |
|         | Sex                         |     |   |                             |    |
|         | Male                        | 1.00|   | Male                       | 1.00|
|         | Female                      | 0.80|0.27 to 2.41 | Female                    | 0.77|

Bold values indicate statistical significance.

*HR estimated from Cox proportional hazard regression model.
†CI of the estimated HR. Results are based on 1000 bootstrap samples and obtained after the bias corrected and accelerated approach.
‡TNM staging system.
OS, overall survival; PFS, progression-free survival.
hepatocellular carcinoma, pancreatic cancer, and endometrial and ovarian cancers have shown that high IDO1 expression is correlated with dismal prognosis. Furthermore, high IDO1 expression has been shown to significantly correlate with poor survival in tumours of the larynx, oral cavity and nasopharynx. Nevertheless, all these studies have evaluated IDO1 in pretreatment biopsies. This is useful for identifying which tumours constitutively express or stimulate the production of IDO1 as part of their underlying biology, but it gives no information about how much reactive IDO1 may have been induced in response to cell death and inflammation; this would have required treatment biopsies. Using a novel approach, we estimated for the first time IDO1 mRNA expression on CTCs derived for patients with HNSCC undergoing CRT, both at baseline and post-treatment, allowing real-time monitoring of IDO1 expression status.

In our study, 36% of patients had IDO1 overexpression post-treatment compared with 58% of patients at baseline. Interestingly, IDO1-positive patients at baseline were shown to have a significant improvement in PFS and OS compared with IDO1-negative patients. Upregulation of IDO1 is observed in tumours with T cell-inflamed phenotype, which is characterised by the presence of activated cytotoxic CD8+ T cells both within the tumour and the peritumoural stroma. Indeed, there is a growing body of evidence suggesting that these tumours show high expression of IDO1 and PDL-1, which is stimulated by interferon-γ produced by CD8+ T cells in vivo. These defined immune-system inhibitory pathways blunt the function of T cells and eventually allow tumour outgrowth. In this situation, upregulation of IDO may be a surrogate for a more robust spontaneous antitumour immune response and thus may be associated with a more favourable prognosis. Therefore, in our study, high IDO1 expression at baseline might reflect the presence of a T cell-inflamed phenotype, which is associated with good prognosis and potentially response to immunotherapy and chemotherapy. Of note, in a retrospective study by Saloura et al, 33%–47% of 558 HNSCC tumours demonstrated a T cell-inflamed phenotype similar to melanoma based on a gene expression signature.

On the contrary, we demonstrated that patients characterised as IDO1 positive post-treatment show a statistically significant inferior OS compared with their IDO1-negative counterparts. In the multivariate analysis, post-treatment IDO1 expression levels retained their prognostic significance. Similarly, patients with combined decreased expression of PDL-1 and IDO1 had a statistically significant improvement in OS. Our patients were treated with radical chemoradiotherapy; accumulating evidence suggests that a decisive contribution to the long-term successful elimination of cancer by radiation and several chemotherapy drugs is made by notifying the immune system for the presence of dying cancer cells. However, dying tumour cells represent a rich source of antigens that are potentially immunogenic, but which cannot become actually immunogenic unless the relevant inhibitory pathways in the tumour, such as IDO1, are blocked. Indeed, IDO1 and its related downstream pathways may help create an undesirable tolerogenic milieu, which precludes the immune system from responding to antigens released from dying tumour cells. Thus, high expression of IDO1 post-treatment might prevent the induction of ICD, block the efficacy of chemoradiotherapy and result in decreased OS.

A relevant question is how to harness the immunogenic potential of chemotherapy or chemoradiotherapy to increase its anticancer efficacy. First, IDO1 is emerging as a mechanism that influences the crucial choice of whether dying cells will eventually become...
tolerogenic or immunogenic. Therefore, if the tolerogenic IDO1 pathway can be blocked, then conventional chemotherapy may be more spontaneously immunogenic. Indeed, preclinical mouse models show that IDO1 inhibitor drugs are synergistic with a variety of chemotherapeutic agents in several tumour models, and clinical trials are currently evaluating potential combinations of IDO1 inhibitors and chemotherapy agents. In addition, early data demonstrated antitumour activity in bladder cancer with an IDO1 small molecule inhibitor combined with anti-programmed cell death (PD-1) therapy.

Immune checkpoints modulate signalling and either inhibit or stimulate T-cell response. Cytotoxic T-lymphyocyte antigen 4 (CTLA-4) and PD-1 are distinct examples of coinhibitory molecules. Although intriguing phase II clinical trial data of IDO1/PD-1 doublets drove rapid clinical development of IDO1 inhibitors, producing tainting clinical efficacy data, results of the phase III KEYNOTE-252/ECHO-301 clinical trial that evaluated the combination of IDO1 inhibitor epacadostat and anti-PD-1 antibody pembrolizumab in unresectable melanoma were disappointing.

Of note, using CellSearch system in CTCs expressing PDL-1 in the same cohort of patients, we have previously shown that the percentage of CTCs expressing a molecular marker is not directly correlated with the total number of CTCs, since CTCs are highly heterogeneous, and therefore, it is not expected that they are all overexpressing IDO-1. Thus, since our RT-qPCR assay does not enumerate CTCs, our findings are directly related to IDO1 overexpression rather than the total number of CTCs. Therefore, IDO1 expression levels at the end of treatment do not reflect disease burden (minimal residual disease).

A major limitation of our study is that it is a single-institution cohort and our results need to be validated in large cohorts with longer follow-up.

In conclusion, IDO1 is one of the regulatory mechanisms that trigger immune suppression and tolerance in the tumour microenvironment. Our study strongly suggests that IDO1 mRNA expression is an independent prognostic factor for clinical outcome; high IDO1 mRNA expression is associated with favourable outcomes at baseline and poor survival after treatment. Despite disappointing results in a phase III trial, interest in therapeutic application of IDO1 inhibition are constantly increasing, particularly since the clinical implementation of immunotherapeutic approaches.

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