RESEARCH PAPER

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Indoleamine 2,3-dioxygenase activity and clinical outcome following induction chemotherapy and concurrent chemoradiation in Stage III non-small cell lung cancer

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Keywords: indoleamine 2,3 dioxygenase, innate immunity, kynurenine, non-small cell lung cancer, tryptophan

Abbreviations: AJCC, American joint committee on cancer; AUC, area under the curve; BIN1, bridging integrator 1; CI, confidence interval; CT, computed tomography; CTLA-4, cytotoxic T-lymphocyte antigen 4; D-1MT, 1-methyl-*D*-tryptophan; ECOG, Eastern Cooperative Oncology Group; GCN-2, general control nonrepressed 2; HPLC, high performance liquid chromatography; HR, hazard ratio; IDO, indoleamine 2,3 dioxygenase; IFN, interferon; IHC, immunohistochemistry; ILT, immunoglobulin-like transcript; IPMN, intraductal papillary mucinous neoplasm; Kyn, kynurenine; MS, mass spectrometry; mTOR, mammalian target of rapamycin; NCT, national clinical trial; NSCLC, non-small cell lung cancer; OS, overall survival; PET, positron emission tomography; PFS, progression-free survival; PKCΘ, protein kinase C Θ; RECIST, response evaluation criteria in solid tumors; STAT1, signal transducer and activator of transcription 1; TGF, transforming growth factor; TIL, tumor-infiltrating lymphocyte; Trp, tryptophan

Indoleamine 2,3-dioxygenase (IDO) has recently been proposed to account for tumor-induced immunosuppression by influencing the conversion of tryptophan (Trp) into kynurenine (Kyn). The objective of our study was to correlate IDO activity with disease outcome in non-small cell lung cancer (NSCLC) patients treated with multimodal combination therapy. In a single-arm Phase II trial involving induction gemcitabine and carboplatin followed by concurrent paclitaxel, carboplatin and 74 Gy thoracic radiation in Stage III NSCLC patients, plasma was drawn at baseline, post-induction, and post-concurrent therapy. The mean plasma Kyn/Trp ratio was used as a surrogate indicator of IDO activity. The 33 participants were distributed as follows: 15 females, 18 males; median age = 62; median overall survival (OS) = 22.4 (95% CI 19.3–25.1) months; median progression-free survival (PFS) = 11.5 (95% CI 6.7–16.3) months. The mean Kyn/Trp ratio at baseline (4.5 ± 2.8) was higher than that of healthy controls (2.9 ± 1.9, p = 0.03) and increased after induction therapy (5.2 ± 3.2, p = 0.08) and chemoradiation (5.8 ± 3.9, p = 0.01). The post-treatment Kyn/Trp ratio and radiologic responses were not significantly associated at any time point. No significant correlation was found between baseline Kyn/Trp ratios and OS (HR = 1.1, 95% CI 0.45–2.5) or PFS (HR = 0.74, 95% CI 0.30–1.82). A post-induction chemotherapy increase in IDO activity portended worse OS (HR = 0.43, 95% CI 0.19–0.95, p = 0.037) and PFS (HR = 0.47, 95% CI 0.22–1.0, p = 0.055). This observed increase in IDO transcription may be a means for tumors to evade immunosurveillance.

Introduction

Indoleamine 2,3-dioxygenase (IDO) is a cytosolic protein that catalyzes the rate-limiting step of human tryptophan (Trp) metabolism. Trp depletion induces effector T cells to undergo a G_1 cell cycle arrest[1] and triggers an integrated stress response pathway that involves general control nonrepressed 2 (GCN2) signaling and eukaryotic initiation factor 2α (eIF2α) phosphorylation.[2,3] IDO also causes systemic anergy by inducing naive T cells to differentiate into $CD4^+CD25^+FOXP3^+$ regulatory T cells.[4] Dendritic cells respond to low Trp levels by upregulating inhibitory factors such as immunoglobulin-like transcript 3 (ILT3), ILT4 and transforming growth factor β1 (TGF-β1), thus becoming immunosuppressive.[5] Moreover, Trp metabolites such as kynurenine (Kyn), quinolinic acid, and picolinic acid exert cytotoxic effects on $CD8^+$ tumor-infiltrating lymphocytes (TILs) and $CD4^+$ T_H1 cells.[6,7] The IDO-mediated deprivation of Trp also inhibits the immunomodulatory kinases mammalian target

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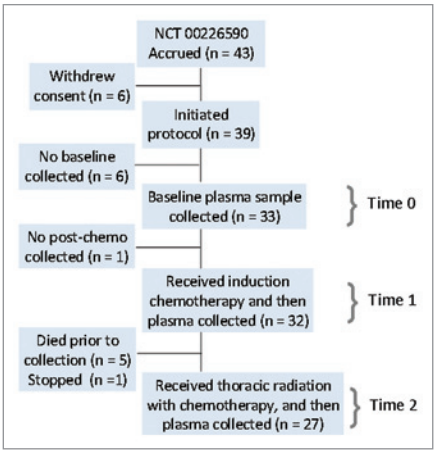

Figure 1. Trial flow diagram. Induction chemotherapy: gemcitabine and carboplatin. Thoracic radiation: 74 Gy conformal. Concurrent chemotherapy: paclitaxel and carboplatin.

of rapamycin (mTOR) and protein kinase C Θ (PKCΘ), thereby inhibiting autophagy.[8] In fact, IDO is overexpressed by many human cancers[9] and may serve as an escape mechanism from host immunity.[10] For example, in humans and animals, the induction of IDO limits the immune response to dendritic cell-based anticancer vaccines.[11,12] Cancer cells bearing mutations in the oncosuppressor gene *BIN1* are stimulated by interferon γ (IFNγ) to overexpress IDO, leading to increased intracellular levels of signal transducer and activator of transcription 1 (STAT1) and nuclear factor κB (NFκB).[13] Thus, IDO may be an important stimulator of immune tolerance in human cancer.

Non-small cell lung cancer (NSCLC) is an aggressive epithelial malignancy that often overexpresses the mRNA coding for IDO.[14] NSCLC has an annual incidence of 1.61 million people worldwide,[15,16] and 83 percent of NSCLC patients will eventually die of their cancer.[16] Locoregionally-advanced NSCLC comprises more than one-third of cases at presentation and is classified as Stage III.[17] Over three quarters of Stage III NSCLC patients are technically or medically inoperable.[18] Chemotherapy and concurrent radiation is the standard therapy for inoperable Stage III NSCLC, but the five year overall survival (OS) remains poor, that is, 20% and 8% for IIIA and IIIB disease, respectively.[19] Such an aggressive malignant phenotype has been hypothesized to stem from an innate resistance of NSCLC to chemotherapy[20,21] and to its ability to evade immunosurveillance.[22,23] However, the role of IDO in mediating immune tolerance in NSCLC is unclear.

Trp catabolism in lung cancer patients is associated with advanced disease stage.[24,25] This said, how IDO activity changes

Table 1. Demographics and baseline measurements

Characteristics	Patients n (%)	Kyn (μM) Mean ± SD	Trp (μM) Mean ± SD	Kyn/Trp (μM/μM) Mean ± SD
Total	**33**	**1.6 ± 0.8**	**37.9 ± 8.5**	**4.5 ± 2.8**
Age in years (Median ± SD)	62.4 ± 7.9			
Age < 65 y	19 (58)	1.6 ± 0.9	40.3 ± 8.2	4.0 ± 2.3
Age 65 y	14 (42)	1.6 ± 0.7	34.7 ± 8.1	5.1 ± 3.3
Race				
Caucasian	31 (94)	1.6 ± 0.8	37.9 ± 8.8	4.6 ± 2.8
Asian	1 (3)	0.9	36.5	2.5
Black	1 (3)	1.1	37.9	2.9
Gender				
Female	15 (45)	1.6 ± 0.8	36.3 ± 8.0	4.9 ± 2.3
Male	18 (55)	1.5 ± 0.8	38.9 ± 8.9	4.2 ± 1.9
ECOG* performance status				
PS 0	11 (33)	1.5 ± 1.0	37.4 ± 10.7	5.5 ± 2.3
PS 1	22 (66)	1.6 ± 0.7	38.0 ± 8.0	4.6 ± 1.6
Smoking status				
Active smoker	12 (36)	1.7 ± 0.8	38.6 ± 7.7	4.5 ± 3.1
Former smoker	21 (64)	1.5 ± 0.8	37.4 ± 9.3	4.4 ± 2.3
AJCC § clinical stage				
IIIA	16 (48)	1.6 ± 0.9	38.2 ± 9.2	4.7 ± 2.7
IIIB	17 (52)	1.6 ± 0.7	37.5 ± 8.0	4.3 ± 2.2
Histotype of NSCLC				
Adenocarcinoma	11 (33)	1.5 ± 0.7	37.4 ± 7.2	4.0 ± 1.3
Squamous cell	10 (30)	1.4 ± 0.8	39.7 ± 7.4	3.8 ± 3.3
Other ‡	12 (36)	1.8 ± 0.8	36.9 ± 10.7	5.3 ± 2.3

*Eastern Cooperative Oncology Group. §American Joint Committee on Cancer. ‡Includes bronchoalveolar, large cell and "not otherwise specified." No statistically significant mean difference was detected for any comparison of the groups presented ($p < 0.05$, Wilcoxon rank-sum test).

after chemotherapy or radiation in NSCLC patients is unclear. The abrogation of *BIN1* causes IDO overexpression and promotes NSCLC in animals.[26] NSCLC-infiltrating lymphocytes are often anergic and hypoproliferative,[27] and the overexpression of IDO in the NSCLC peritumoral stroma is associated with poor prognosis.[28] Based on the these observations, we hypothesized that IDO activity may increase in Stage III NSCLC patients undergoing conventional multimodal therapy. Since Kyn is the primary metabolite of Trp metabolism, the plasma Kyn/Trp ratio has been used as a surrogate indicator of IDO activity.[29,30] Our objective was to prospectively correlate serial changes in IDO activity, as measured by the plasma Kyn/Trp ratio, with radiologic response and survival in NSCLC patients treated with multimodal therapy.

Results

Forty-three participants were enrolled from December 2003 to February 2006. Of 39 participants initiated on treatment, 33 had plasma drawn at Time 0 evaluable for mass spectrometry (**Fig. 1**). We observed no trends between baseline plasma metabolites and demographic features such as age, race, gender, and performance status (**Table 1**). Moreover, we observed no correlations between baseline plasma markers and tumor characteristics such as histology or clinical stage. However, these correlative studies were limited by a relatively low statistical power. To assess the presence of outliers, we used a formal outlier test, the extreme studentized deviate (ESD) method with $p < 0.05$, which identified one outlier at time 0 (14.5 with $z = 3.6$) and no outliers at Time 1 and Time 2. To further assess the influence of outliers, we examined the clinicopathologic features of the six participants displaying the highest Kyn/Trp ratio at Time 0 and Time 1 compared with the rest the cohort. No statistically significant differences in age, survival, tumor burden, chemotherapy response, white blood count and serum albumin was observed.

Compared with a sample of 24 healthy controls (2.9 ± 1.9), participants had higher mean Kyn/Trp ratio at baseline (4.5 ± 2.8, $p = 0.03$) (**Fig. 2**). Mean plasma Kyn/Trp ratios increased after induction chemotherapy (Time 0 to Time 1, 5.2 ± 3.2, $p = 0.08$), and after concurrent definitive chemoradiation (Time 1 to Time 2, 5.8 ± 3.9, $p = 0.01$). No correlation was observed between baseline plasma Kyn/Trp ratios and primary tumor cross-sectional area ($r = 0.10$, 95% CI −0.27–0.43) or total cross-sectional area of all target lesions on CT (CT) ($r = 0.02$, 95% CI −0.33–0.37) (**Fig. 3A**). Likewise, no appreciable correlation was observed between changes in plasma Kyn/Trp ratios from Time 0 to Time 1 (ΔIDO_1) and changes in primary tumor cross-sectional area ($r = -0.25$, 95% CI −0.56–0.12) or changes in total cross-sectional area of all target lesions on CT ($r = -0.17$, 95% CI −0.50–0.20) (**Fig. 3B**).

After induction chemotherapy, we observed one complete response, 11 partial responses and 21 patients with stable disease. Using response evaluation criteria in solid tumors (RECIST), a non-statistically significant association was observed between improved radiologic responses and plasma Kyn/Trp ratios at Time 1, with $p = 0.34$ (**Fig. 4A**). A similar non-significant trend was observed between radiologic responses and plasma Kyn/Trp ratios at Time 2, with $p = 0.19$ (**Fig. 4B**). To index the shift in IDO activity after chemotherapy, the differences in Kyn/Trp ratios between Time 0 and Time 1 (ΔIDO_1) and between Time 1 and Time 2 (ΔIDO_2) were calculated. Participants were subdivided into two groups based on the median ΔIDO value. Using RECIST to define the percentage of response, a non-significant trend was observed between radiologic responses at Time 1 and ΔIDO_1 (**Fig. 5**).

Median OS was 22.4 mo (95% CI 19.3–25.1) and median progression-free survival (PFS) 11.5 mo (95% CI 6.7–16.3) (**Table 2**).

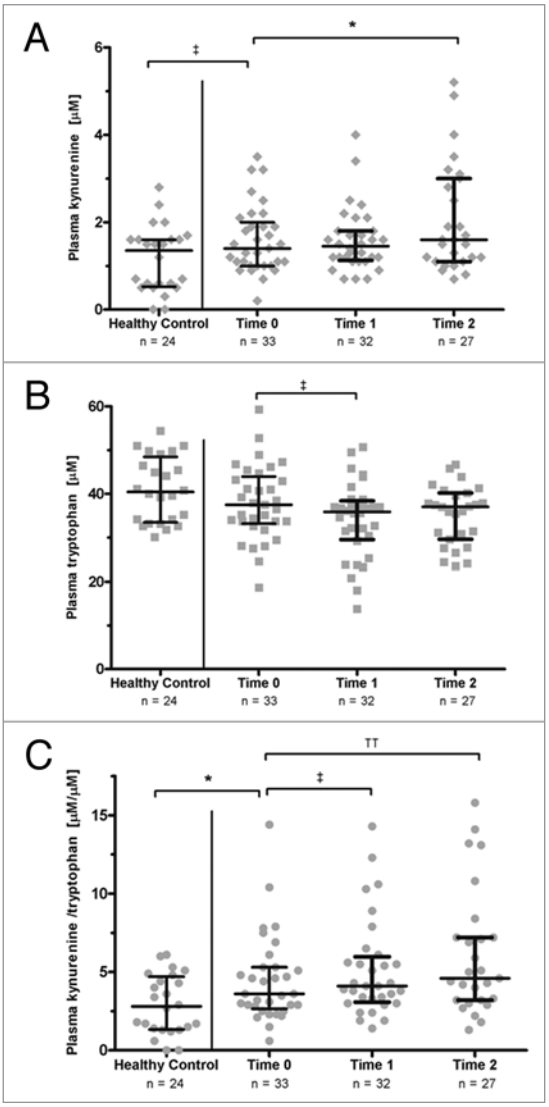

Figure 2. Plasma metabolites in Stage III non-small cell lung cancer patients. (**A**) Plasma kynurenine (Kyn) concentration. (**B**) Plasma tryptophan (Trp) concentration. (**C**) Plasma Kyn/Trp ratios, a surrogate for indoleamine 2,3 dioxygenase (IDO) activity. Plasma IDO activity was higher in non-small cell lung cancer (NSCLC) patients at baseline (Time 0) than in healthy individuals. Plasma IDO activity increased after induction carboplatin/gemcitabine (Time 1) and after concurrent carboplatin/paclitaxel with thoracic radiation (Time 2). Bars: Median with interquartile range. For healthy individuals vs. Time 0: Wilcoxon rank-sum test. For Time 0 vs. Time 1 and Time 1 vs. Time 2: Wilcoxon signed-rank test. ‡$p < 0.10$; *$p < 0.05$; ††$p < 0.01$.

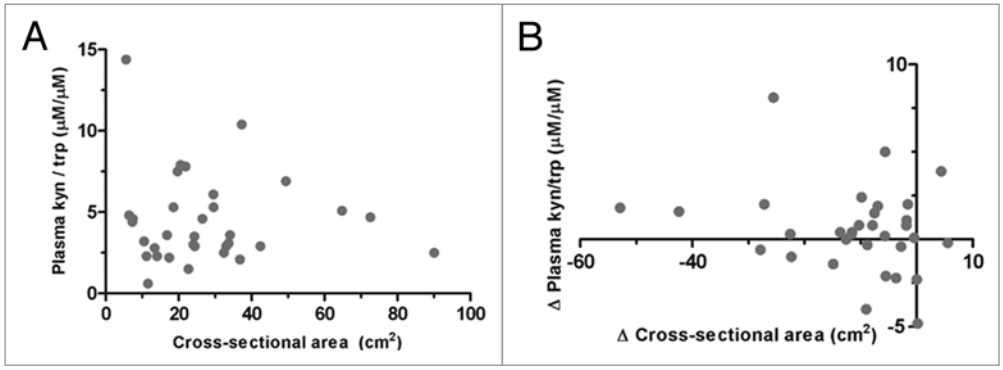

Figure 3. Correlation between tumor burden and indoleamine 2,3 dioxygenase activity. (**A**) Correlation between cross-sectional tumor burden and plasma kynurenine/tryptophan (Kyn/Trp) ratio. n = 33, *r* = 0.02 (95% CI −0.33–0.37). (**B**) Correlation between changes in cross-sectional tumor burden and changes in plasma Kyn/Trp ratios after induction carboplatin/gemcitabine (Time 1 – Time 0). n = 32, *r* = −0.17 (95% CI −0.50–0.20). Total burden represents the sum of cross-sectional areas for primary tumor and all target lymph nodes. Cross-sectional area represents the product of the longest tumor diameter and its perpendicular axis. *r* = Spearman's rank correlation coefficient.

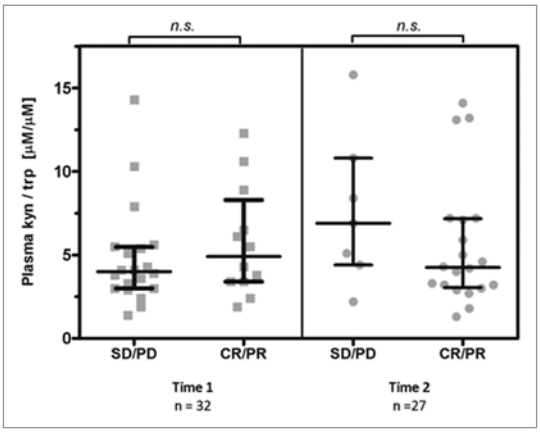

Figure 4. Radiologic response and plasma kynurenine/tryptophan ratio after induction therapy (Time 1) and after concurrent carboplatin/paclitaxel therapy coupled to thoracic radiation (Time 2). Bars: median, interquartile range. *n.s.* = non significant (Wilcoxon rank-sum). CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

Table 2. Summary of results

Characteristics	Time 0	Time 1	Time 2
N	**33**	**32**	**27**
Analytes (mean ± SD)			
Kyn (μM)	1.59 ± 0.77	1.58 ± 0.73	2.05 ± 1.25
Trp (μM)	37.9 ± 8.5	34.1 ± 8.7	35.2 ± 6.7
Kyn/Trp (μM/μM)	4.5 ± 2.8	5.15 ± 3.12	6.11 ± 4.02
Radiology			
Primary tumor area (cm²)	18.7 ± 17.9	11.0 ± 10.4	5.5 ± 4.7
Total tumor area (cm²)	27.6 ± 19.2	16.1 ± 12.0	7.4 ± 5.5
Survival	**Median**	**95% CI**	**Events**
PFS (mo)	11.5	(6.7–16.3)	29/33
OS (mo)	22.2	(19.2–25.1)	27/33

PFS, progression-free survival; OS, overall survival; CI, confidence interval; SD, standard deviation; Mo, months.

Baseline Kyn/Trp ratios were not significantly associated with OS (HR = 0.88, 95% CI 0.41–1.35). After induction chemotherapy at Time 1, patients with high ΔIDO_1 had worse OS (p = 0.037) and a trend toward worse PFS (p = 0.055) as compared with individuals with low ΔIDO_1 (**Fig. 6**). However, no association was found after chemoradiation between ΔIDO_2 and OS (HR = 1.1, 95% CI 0.45–2.5) or PFS (HR = 0.74, 95% CI 0.30–1.82).

Discussion

To our knowledge, this is the first report to serially explore changes in plasma IDO activity during the treatment of Stage III NSCLC patients, as measured by Kyn/Trp ratios. We found that IDO activity was higher in cancer patients than in healthy individuals, consistent with previous observations in lung cancer,[24,25,31] bladder cancer, hepatocellular carcinoma, renal cell carcinoma, melanoma,[32] colorectal carcinoma,[33] esophageal cancer,[34,35] thyroid carcinoma,[36] acute myelogenous leukemia,[37] breast cancer[38] and gynecologic cancer[29] patients. This observation lends credence to the hypothesis that cancer cells may stimulate dendritic cells to express IDO or may directly overexpress the enzyme. Indeed, NSCLCs exhibit high Trp metabolism as monitored by α-methyl-tryptophan positron emission tomography (PET) imaging as compared with normal tissues.[39] We could not observe a strong correlation between baseline tumor sizes and Kyn/Trp ratios, or a strong correlation between changes in tumor size and changes in Kyn/Trp ratios. This suggests that Kyn/Trp ratios are unlikely to be determined by the tumor burden alone.

Consistent with previous observations in NSCLC patients,[24] we did not find a correlation between baseline IDO activity and survival. Several authors have examined the relationship between IDO activity and survival in other cancers (**Table 3**). Of interest, studies based on the immunohistochemical detection of IDO more often reported an unfavorable role for IDO than studies based on the detection of *IDO* mRNA levels (16:1 vs 1:6,

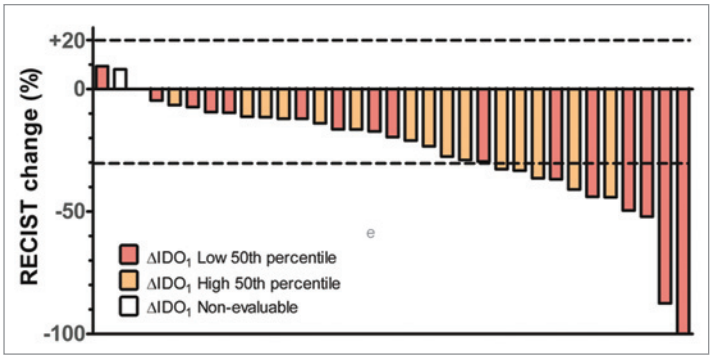

Figure 5. Waterfall plot of response after induction chemotherapy. Participants with reduced surges in indoleamine 2,3 dioxygenase (IDO) activity after induction chemotherapy (ΔIDO_1) had non-significant trend toward improved radiologic responses. The median difference in the kynurenine/tryptophan (Kyn/Trp) ratio was taken as pre-specified cut-point. Evaluable patients, n = 32; non-evaluable patients, n = 1.

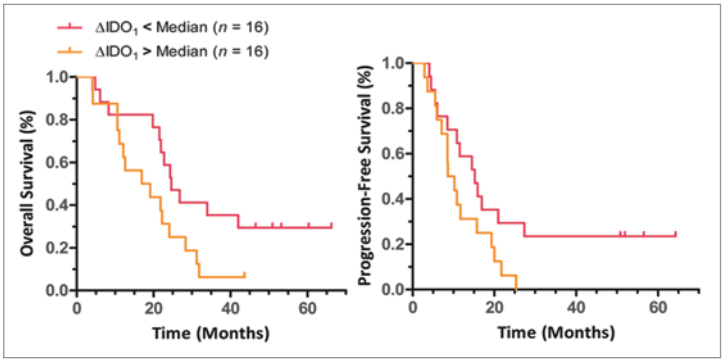

Figure 6. Relationship between changes in indoleamine 2,3 dioxygenase activity and non-small cell lung cancer patients survival. Participants with a consistent increase in indoleamine 2,3 dioxygenase (IDO) activity from baseline (Time 0) to induction chemotherapy (Time 1) had shorter overall survival (OS) and a trend toward shortened progression-free survival (PFS). Median OS = 24.6 vs. 18.0 mo, HR = 0.43 (95% CI 0.19–0.95), *p = 0.037 (Log-rank test) Median PFS: 15.2 vs. 9.4 mo, HR = 0.47 (95% CI 0.22–1.02), *p = 0.055 (Log-rank test). Median difference in kynurenine/tryptophan (Kyn/Trp) (ΔIDO_1) was taken as pre-specified cut-point.

Fischer's exact p = 0.0003). This difference may reflect a post-transcriptional regulation of IDO. Our study also examined the relationship between changes in IDO activity and disease outcome. Our results suggest that a high increase in IDO activity after chemotherapy is associated with a higher risk of mortality. A possible explanation for our findings is that the expression of IDO by tumor cells may mediate—at least in part—resistance to chemotherapy.[40] Along these lines, it has recently been reported that a subset of NSCLC patients with favorable radiologic responses after chemotherapy exhibit a significant decrease in IDO activity within monocytes as compared with baseline conditions.[41] Tumors may generate a hostile microenvironment that impedes host antitumor immune response. Interestingly, no changes in the expression of IDO by tumor cells was observed in melanoma biopsies at baseline and during regression in response to tremelimumab treatment,[42] suggesting that IDO activity may not be influenced by cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibition.

Several aspects of our report necessitate a more detailed investigation before firm conclusions can be drawn. Due to a small sample size, our trial was statistically underpowered to detect any significant association between Kyn/Trp ratios and demographic characteristics. Likewise, our patient sample was too small to establish a stepwise dose-effect relationship between IDO activity and clinical outcome. Next, tissues were not available for the immunohistochemical detection of IDO, so we used plasma Kyn/Trp ratios as a convenient surrogate marker for IDO activity. Other studies have used plasma Kyn/Trp ratios as an indirect measure for IDO activity (**Table 3**) and these correlate well with immunohistochemical data.[43] However, plasma Kyn/Trp ratios are not a direct measure of IDO activity. In particular, other Trp catabolizing enzymes, including tryptophan 2,3-dioxygenase and the IDO splice variant known as IDO2 contribute to Kyn production as well. Moreover, our study did not mandate uniform timing for blood draws. Since plasma Trp levels may vary by as much as 20% in the same subject depending on the distance of the last meal and the diurnal cycle,[44] we acknowledge this as a potential confounder. Nonetheless, plasma Kyn has only minor diurnal variation,[43] and the changes that we observed in Kyn/Trp ratios appeared predominantly attributable to Kyn (**Fig. 2**). Another drawback of the present study is that correlative immunologic markers, such as FOXP3 expression, CD3 activity, and IFN production were not measured. Additional markers may help to substantiate the putative role of IDO in this patient population. Moreover, conclusions cannot be drawn about the effect of individual chemotherapy agents, which are known to have specific immunomodulatory properties.[45,46] Finally, our calculations were performed without multiple comparison rules.[47] Thus, the possibility exists that the observed associations could stem from family-wise errors. In view of all these limitations, our results should be strictly viewed as exploratory.

In summary, our findings may serve as a rationale for larger studies correlating changes in the Kyn/Trp ratio with clinical parameters during standard multimodal therapy against NSCLC. As IDO may contribute to the resistance of NSCLC to therapy, this clinical setting may be a fertile area for the investigation of the antineoplastic effects of IDO inhibitors, such as1-methyl-*D*-tryptophan (D-1MT).[48]

Materials and Methods

Patients. The design of the clinical trial has been previously described.[49] Briefly, our single-arm trial used induction gemcitabine (1 g/m^2) on day 1 and day 8 and carboplatin (AUC = 5) on day 1 given at 4-week intervals for two cycles. This was followed by weekly carboplatin (AUC = 2) and paclitaxel (50 mg/m^2) with concurrent conformal radiation (dose = 74 Gy ± 5%) administered over 40 daily fractions. Plasma was collected at baseline (Time 0), after induction at week 9 (Time 1), and after

Table 3. Select studies of indoleamine 2,3 dioxygenase IDO in human cancers

Neoplasm type	Stage	Tissue source	Sample size (n) for survival	Prognostic for survival	Reference
Lung	All	Serum kyn/trp	123	Null	24
Breast	I-III	Serum kyn/trp	33	n/a	38
Ovarian, serous	III-IV	Tumor IHC	24	Unfavorable	40
Ovarian, epithelial	IC-IV	Tumor IHC	60	Unfavorable	51
Renal cell	IV	Tumor mRNA	107	Favorable	52
Hepatocellular	All	Tumor IHC	138	Unfavorable	53
Ovarian	All	Serum kyn/trp	28	n/a	29
Endometrial			41		
Vulvar			40		
Ovarian	III-IV	Tumor IHC	122	Unfavorable	54
Lung	All	Tumor IHC	28	n/a	14
Breast	All	Tumor mRNA	1059	Null	55
Breast, basal-like			209	Favorable	
Oral squamous	All	Tumor IHC	88	Unfavorable	56
Breast	All	Tumor mRNA	30	Null	57
Colorectal	All	Tumor IHC	143	Unfavorable	58
Colorectal	All	Tumor IHC	265	Unfavorable	59
Endometrial	All	Tumor IHC	80	Unfavorable	60
Pancreatic	All	Tumor IHC	17	n/a	61
Colorectal	II-IV	Tumor IHC	69	n/a	62
Osteosarcoma	All	Tumor IHC	47	Unfavorable	63
Esophageal, squamous	I-III	Tumor IHC	45	n/a	34
Acute myelogenous leukemia	All	Tumor mRNA	262	Unfavorable	37
Melanoma, uveal	–	Tumor IHC	7	n/a	64
Pancreatic IPMNs*	–	Tumor IHC	39	n/a	65
Endometrial	All	Tumor IHC	355	Unfavorable	66
Vulvar	All	Tumor IHC	286	Null	67
Melanoma	III	Tumor IHC	25	Unfavorable	68
Melanoma	I-III	Tumor IHC	26	n/a	69
Cervical	n/a	Tumor IHC	17	n/a	70
Hepatocellular	All	Tumor mRNA	21	Favorable	71
Colorectal	All	Tumor IHC	71	Unfavorable	72
Lymphoma	All	Tumor IHC	119	Unfavorable	73
Melanoma	All	Serum kyn/trp	87	Unfavorable	32
Endometrial	All	Tumor IHC	65	Unfavorable	74
Esophageal, squamous	All	Tumor mRNA	30	Unfavorable	35
Cervical	IB-IIB	Tumor IHC	112	Unfavorable	75
Thyroid	All	Tumor mRNA	20	Null	36
Melanoma	All	Tumor IHC	116	Unfavorable	76
Lung, Non-small cell	n/a	Serum kyn/trp	36	n/a	25

Favorable, improved survival with higher IDO; unfavorable, worse survival with higher IDO; null, no difference observed; n/a, not tested; IHC, Immunohistochemistry. *Intraductal papillary mucinous neoplasm.

concurrent therapy at week 25–26 (Time 2). All participants enrolled had previously untreated clinical Stage IIIA or "dry" IIIB NSCLC, as determined by American Joint Committee on Cancer (AJCC) criteria v. 6. None of these patients had received previous chemotherapy or radiation.

All participants had measurable disease by RECIST v. 1.0 criteria. Both CT and PET scans were performed at Time 0, Time 1 and Time 2. Radiographic responses were quantified by the percentage of changes in the sum of all greatest tumor diameters between post- and pre-treatment scans. Institutional radiologists

evaluated all scans. OS was defined as the interval between the date of treatment and the date of death. PFS was defined as the interval between the date of treatment and the date of progression or death from any cause. Participants without an event were censored as of the date of last follow-up, which occurred between August and September 2009.

All procedures were approved by the Institution Review Board (IRB) from the University of South Florida (FWA00001669) and were in accordance with the Helsinki Declaration of 1975. Participants prospectively provided informed consent for the use of their tissue in research.

Measurement of plasma markers. Heparinized blood samples were drawn and centrifuged at 800 g for 10 min to extract plasma and then stored at −80°C. HPLC/MS/MS was performed with gradient elution on a C18 column (Thermo Betabasic, 150 mm × 2.1 mm, 5 μm particles) with matching guard column at a flow rate of 500 μL/min using heated electrospray ionization in the positive ion mode.[50] Gradient solvents were 0.1% formic acid in water and acetonitrile with 0.1% formic acid (HPLC grade, Fisher-Scientific). The mass spectrometer (TSQ Quantum Ultra, Thermo) was operated in selected reaction monitoring mode (SRM). Calibration standards for Trp and Kyn (Fisher-Scientific) were prepared in albumin (Sigma) in the ranges of 10–2,000 ng/mL for Kyn and 100–20,000 ng/mL for Trp. Quality control samples were prepared in albumin at 10, 100, 1000 ng/mL for Kyn and 100, 1,000, 10,000 ng/mL for Trp. 150 μL of internal standard solution containing Trp-d3 at 4,000 ng/mL and Kyn-d6 at 400 ng/mL (Cambridge Isotopes) were added and mixed to 150 μL of calibration standard, quality control, and clinical specimens. For protein precipitation, 30 μL of 2.4 M perchloric acid (Fisher-Scientific) were added, samples were vortexed and cooled for 5 min, then centrifuged at 20,400 g for 20 min. The supernatant was transferred to a filter tube (0.44 μm) and re-centrifuged. The filtered sample was transferred to a glass vial with 400 μL insert and 10 μL was injected onto the column. Each sample was performed in triplicate, and individual mean concentrations were recorded. The intraassay and day-to-day analytic coefficients of variation were less than 5% for each analyte (**Table S1**).

Statistics. The plasma concentrations of Kyn and Trp, as well as Kyn/Trp ratios approximated a parametric distribution. Therefore, data were presented as means ± SD. Due to the small sample size, the associations between serum biomarker levels and clinicopathologic variables were evaluated with Wilcoxon rank-sum and Kruskal-Wallis tests. Wilcoxon signed rank was used to compare between time points. Correlation was estimated using Spearman's rho. Survival curves were calculated according to the Kaplan-Meier method. To address the experimental hypothesis, the statistical population was equally divided into "hig" and "low" patients using the median Kyn/Trp value as pre-specified cut-point. The effects of biomarkers on PFS and OS were evaluated using log-rank (Mantel-Cox) test and Cox proportional-hazards regression models. All tests performed were two-tailed. Statistical analysis was performed using the SAS software (version 8.0). Figures and survival plots were generated using GraphPad Prism® (version 5.04).

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to disclose.

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Supplemental Material

Supplemental materials may be found here:
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