

ORIGINAL PAPER

THE PROGNOSTIC SIGNIFICANCE OF INDOLEAMINE-2,3-DIOXYGENASE AND THE RECEPTORS FOR TRANSFORMING GROWTH FACTOR β AND INTERFERON γ IN METASTATIC LYMPH NODES IN MALIGNANT MELANOMA

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We analyzed the prognostic significance of indoleamine-2,3-dioxygenase (IDO) and type 1 receptors for transforming growth factor beta (TGF- β R1) and interferon gamma (IFN- γ R1) in resected nodal metastases of 48 malignant melanoma patients. In 32 cases the corresponding skin tumors were available. We used immunohistochemical (IHC) staining which was assessed by pathologists and by a computer-aided algorithm that yielded quantitative results, both absolute and relative. We correlated the results with the patient outcome. We identified absolute computer-assessed IDO levels as positively correlated with increased risk of death in a multivariate model (HR = 1.02; 95% CI: 1.002-1.04; $p = 0.03$). In univariate analysis, patients with IDO levels below the median had a better overall survival time (30.3 vs. 17.5 months; $p = 0.03$). TGF- β R1 and IFN- γ R1 expression was modestly correlated ($R = 0.34$; $p < 0.05$) and TGF- β R1 expression was lower in lymph nodes than in matched primary skin tumors ($Z = 2.87$; $p = 0.004$). The pathologists' and computer-aided IHC assessment demonstrated high correlation levels ($R = 0.61$, $R = 0.74$ and $R = 0.88$ for IDO, TGF- β R1 and IFN- γ R1, respectively). Indoleamine-2,3-dioxygenase is prognostic for the patient outcome in melanoma with nodal involvement and should be investigated prospectively for its predictive significance. IHC assessment by computer-aided methods is recommended as it gives IHC more objectivity and reproducibility.

Key words: melanoma, indoleamine-pyrrole 2,3,-dioxygenase, transforming growth factor β , interferon γ , immunohistochemistry.

Introduction

Malignant melanoma of the skin is one of the most aggressive and clinically challenging human tumors. Surgery is the crucial modality to control the disease, and the primary skin tumors themselves are indeed almost always operable. However, melanoma features

a very high risk of recurrence and dissemination. It is significant even for tumors with no regional lymph node involvement (25-60%) [1] and becomes more excessive for patients in whom nodal metastasis were present (37-89%) [2]. The most important factor of risk of nodal metastasis is the tumor clinical stage,

which determines the clinical protocol for the diagnosis and treatment of melanoma patients [3].

Another distinguishing feature of melanoma is its close relationship with the human immune system. Complex biological pathways related to lymphocyte inhibition such as cytotoxic lymphocyte receptor 4 (CTLA4) or programmed death 1 (PD-1) are drawing attention from the whole world of oncology as their outcome in melanoma patients seems to be a spectacular improvement in its management [4, 5]. However, less sophisticated mechanisms responsible for immunosuppression exist and these have not been fully studied in melanoma. Indoleamine-2,3-dioxygenase (IDO) is the enzyme responsible for the first, rate-limiting step of tryptophan decay. Its activity in tissue causes a localized state of immunotolerance as a result of the hypersensitivity of the lymphocytes to either lack of tryptophan or accumulation of the intermediates of its catabolic pathway [6, 7]. Physiologically, it is crucial for pregnancy as it diminishes the T-cell response of the pregnant female against the antigen-distinct fetus [8]. Various human tumors are known to overexpress IDO, most likely in order to achieve the same goal – to avoid the T-lymphocyte-mediated response of the host [9]. Due to the immune-related characteristics of melanoma, IDO can be particularly expected to play a key role in this tumor type. Indoleamine-2,3-dioxygenase is up-regulated at the nuclear level by other biological signaling particles. Two of them draw attention because of their known role in tumors. Transforming growth factor beta (TGF- β) is a multifunctional intracellular signal transducer. It regulates the cell cycle, differentiation and apoptosis, as well as numerous other processes [10]. Alterations of its normal functioning, which are often found in tumors, cause increased growth and aggressiveness and enhance metastasis formation [11]. Interferon γ (IFN- γ) is a pro-inflammatory cytokine. Similarly to TGF- β , it initiates transcription of numerous specific particles, yet in this case they mediate the response to antigens by, among other things, enhancing antigen presentation, acting as a chemotactic agent to immune cells and directly interfering with some antigen types [12]. Anti-tumor activity of interferon gamma has been reported, but the trial results have been ambiguous – with some even suggesting that IFN- γ is responsible for the progression of tumors [13]. As lymphatic spread of melanoma is crucial for the patient prognosis, the present study has been started to collectively analyze IDO, TGF- β and IFN- γ in patients with melanoma-affected lymph nodes with a focus on the patient prognosis.

Material and methods

We analyzed 48 melanoma patients who underwent a successful regional lymph node dissection between 2005 and 2009 in the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology,

Gliwice Branch. Requirements for inclusion in the study were that the patients had metastases from melanoma confirmed in histological examination and the formalin-fixed, paraffin-embedded (FFPE) nodal specimen was available to the researchers. The study was approved by the Bioethics Committee of the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch. Due to the retrospective type of the study the Committee waived the requirement of a written individual consent for the use of the archival FFPE tissue samples and the anonymized patient data in this study. The clinical characteristics of the patients and their disease are summarized in Table I. For each patient, overall survival (OS) and distant-metastasis-(or inoperable local/locoregional recurrence)-free survival (MFS) were calculated to objectively evaluate the prognosis.

Immunohistochemical staining

The FFPE tissue samples from the skin metastatic lymph nodes ($n = 48$) and, when available, matched primary skin tumors ($n = 32$) were cut into $4.5 \mu\text{m}$ slices and placed on charged glass slides (cat. no. K8020; Dako, Glostrup, Denmark). The slides were fixed by heating for 60 minutes at 60°C and depara-

Table I. Clinical and pathological characteristics of the patients

PARAMETER	NO. OF CASES (%)
Age	
Median	56.9
Range	21.4-85.5
Sex	
Male	31 (64.6)
Female	17 (35.4)
Initial nodal metastasis	
Yes	30 (62.5)
No	18 (37.5)
Number of metastatic lymph nodes	
1	15 (31.2)
2-3	20 (41.7)
4-9	8 (16.7)
10+	5 (10.4)
Metastasis dimension	
Micrometastasis ($\leq 2 \text{ mm}$)	5 (10.4)
Macrometastasis ($> 2 \text{ mm}$)	43 (89.6)
Extracapsular extension	
Yes	14 (29.2)
No	34 (70.8)

raffinized directly thereafter. Due to the high concentration of antibodies used, staining against IDO had to be performed manually. After deparaffinization, antigen unmasking using a Dako PT-Link kit (cat. no. PT10126) for 20 minutes followed; slides were then washed for 5 minutes in Dako TRS low pH buffer (cat. no. K8005). Endogenous peroxidase was blocked with 3% H₂O₂ solution (5-minute incubation followed by 5-minute wash as in the previous step). Slides were subsequently incubated with a specific monoclonal mouse antibody against IDO (4D2 clone, cat. no. MCA 5433Z; AbD Serotec, Kilington, UK) in 1 : 50 dilution for 60 minutes at room temperature and then washed. Next, Dako's EnVision Flex+ visualization kit was applied according to the recommended protocol: 15-minute incubation with linker → 5-minute wash in buffer → 20-minute incubation with horseradish peroxidase solution → 5-minute wash in buffer → incubation with Permanent HRP Green chromogen solution (cat. no. ZUC-070-100; Zytomed, Berlin, Germany) → wash in distilled water for 1 minute. The next step involved counterstaining of the nuclei with hematoxylin for 2 minutes and a 30 second wash in tap water. The final steps included de-hydration of slides by brief heating in an oven and a 30-second wash in xylene and their automated covering by Tissue-Tek Film Coverslipper (Sakura Finetek, Tokyo, Japan). In addition to the staining of the tumor specimen as described above, three reference controls were made. The positive control involved staining of normal human spleen as described. The negative control was made the same way as the positive one with the exception of incubation with the primary anti-IDO antibody, which was replaced by a universal mouse control (cat. no. IR750; Dako). Finally, a unicolor positive control was made using the same protocol as for the positive one, but omitting the counterstaining with hematoxylin.

Due to unsuitability of FFPE archival material for direct cytokine detection, staining against type 1 receptors of TGF- β and IFN- γ (TGF- β R1 and IFN- γ R1, respectively) was used. In this case, the effective antibody concentrations allowed for the use of the Ventana BenchMark Ultra (Ventana, Tucson, USA) automated staining device. The preparation of the slides (cutting, placing on slides, heating in oven) was identical to the protocol for IDO. Next, slides were placed in the staining device and deparaffinized with EZ Prep Kit (Ventana Tucson, USA cat. no. 950-102) according to the kit instructions. Antigen unmasking with Ultra CC1 kit (cat. no. 950-224; Ventana) for 64 minutes at 95°C followed. Next, a 32-minute incubation at 36°C with polyclonal primary rabbit antibodies was performed: anti-TGF- β R1 (cat. no. 102784; Genetex, Irvine, USA) in 1 : 250 dilution and anti-IFN- γ R1 (cat. no. 103098; Genetex) in 1 : 100 dilution. Slides were subsequently incubated

with the UltraView Red visualization system (cat. no. 5269814001; Ventana) according to the kit protocol and finally automatically dehydrated and covered. Controls were made in a corresponding way to the ones described for IDO; the control for TGF- β R1 consisted of cells from the A431 line and for IFN- γ R1 normal tonsil tissue was used. The negative controls involved the same procedure as for positive ones, with the primary antibodies replaced by a universal rabbit immunoglobulin mix (cat. no. IR600; Dako).

Immunohistochemical assessment and quantification

The level of expression of IDO, TGF- β R1 and IFN- γ R1 as detected by IHC was assessed by two experienced pathologists with no insight into the clinical data. Whenever the assessment of a slide varied between the two, a final mark was given based on a collegial discussion, with the computer-aided score (as described below) revealed to them to aid the final decision. The assessment involved assigning a semi-quantitative score of IHC staining intensity in a commonly used 3-step scale (+: weak staining, ++: intermediate staining, +++: strong staining). An example of staining of boundary intensities is shown in Fig. 1. The highest intensity observed in the tumor cells within the given slide was entered into the database. None of the tumor samples in the whole set was completely devoid of the staining reaction in the melanoma cells; therefore the "0" score was not used, though it was initially considered.

In addition, slides were digitized using the Panoramic 250 microscopic scanner (3D Histech, Budapest, Hungary) to allow for an objective, quantitative assessment of the staining intensity. This was performed using Image J graphics processing software (National Institute of Health, Bethesda, USA) and a custom variant of the color deconvolution algorithm developed by the authors. Using the unicolor controls for each chromogen used for staining, it is capable of extracting the counterstaining chromogen signal from the slide image, thus leaving only the color of the chromogen of interest in the processed image. A given area of this image (which should be cytoplasm of the tumor cells in 40 \times magnification, as shown in Fig. 2B) can be set to measure the median value of the chromogen intensity (ranging from 0 to 255, with 255 representing a complete lack of color which also means the lowest possible IHC staining intensity; shown in Fig. 2C). In our study we set 3 to 5 such single areas on a given tumor sample approximately 350 μ m in size and calculated the mean value. In order to obtain the proper (positive) correlation between these values and the semiquantitative assessment, they were inverted by being subtracted from 255. The resulting value can be used directly or normalized to the maximal observed one in the

whole series – which returns a percentage score. We recorded both of these values for each case.

Statistical analysis

Database generation and all statistical analysis were performed by Statistica 11 with a supplementary medical statistical kit (StatSoft, Tulsa, USA). For optimal choice of statistical methods all variables were checked for the normality of distribution using the Shapiro-Wilk test. As none of them featured a normal distribution, only non-parametric tests were used. Comparison of two groups was done by the Mann-Whitney U-test; in the case of more than three, the non-parametric Kruskal-Wallis ANOVA was used. Wilcoxon's test was used for comparison of differences across matched pairs of variables. Correlations were assessed by Spearman's R coefficient. Survival was analyzed by the multivariate Cox model (with forward regression) and the univariate Kaplan-Meier model (log-rank test). To objectively correlate semiquantitative and objective scales to each other, receiver operating characteristic (ROC) curves were used. Values within 95% confidence intervals (CI) ($p < 0.05$) were considered statistically significant.

Results

No correlations were found between the clinical variables and any expression scores of the analyzed particles. An internal analysis of protein expression correlations revealed a modest, yet significant association between TGF- β R1 and IFN- γ R1 levels ($R = 0.34$; $p < 0.05$). In 32 patients, for whom the primary tumor specimens were available for the assessment of cytokine expression, a comparison was made between matched primary and nodal metastatic tumors. Significant differences were found in the TGF- β R1 expression profiles ($Z = 2.87$; $p = 0.004$). A review of the subjective IHC scores of TGF- β R1 suggests that its expression is diminished in lymph node metastases (+/+ +/+ +/+ +: $n = 15/10/7$) compared to the corresponding primary skin tumors ($n = 4/9/19$).

A multivariate analysis of all clinical and IHC expression variables identified IDO expression level, as measured by absolute chromogen signal intensity, as significantly, positively affecting the risk of death (HR = 1.02; 95% CI: 1.002-1.04; $p = 0.03$). The only clinical variable influencing the risk of death was the number of metastatic lymph nodes (HR = 1.06; 95% CI: 1.01-1.11; $p = 0.009$). It was also the only

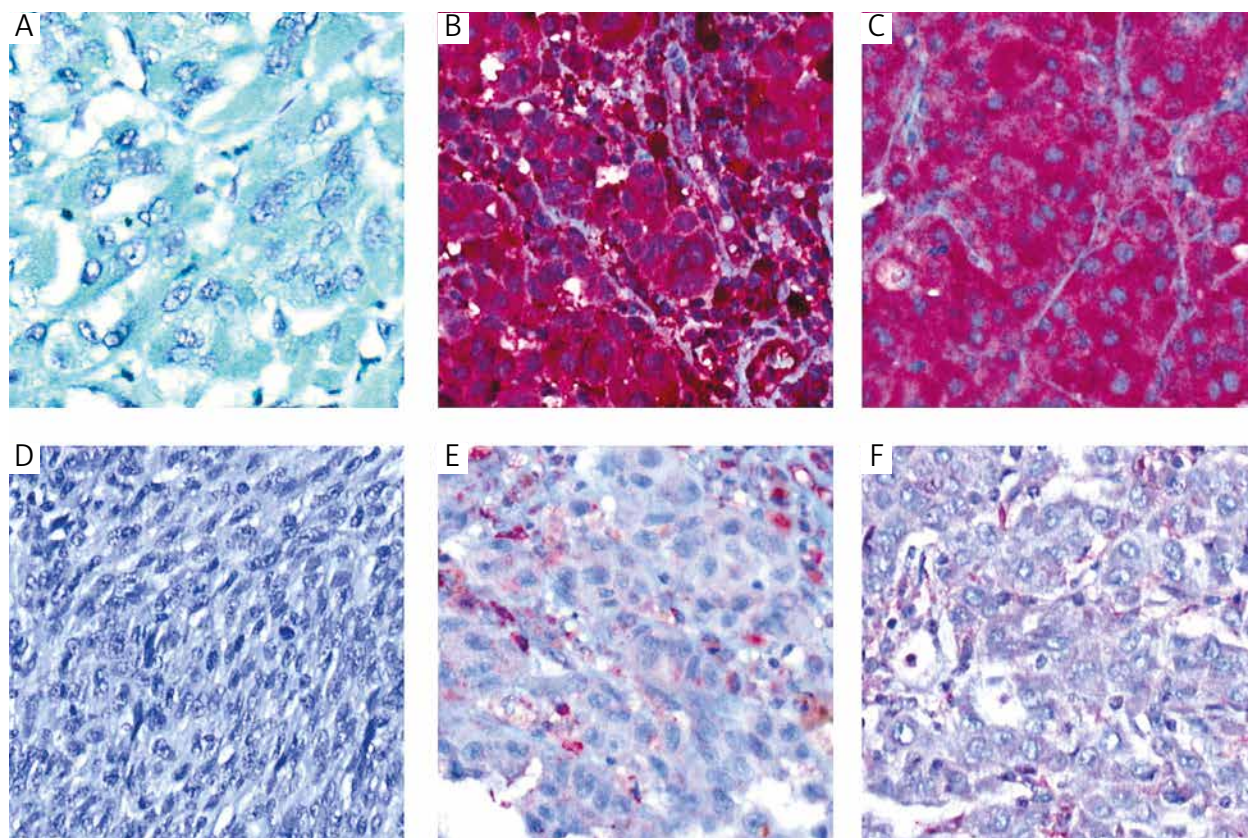


Fig. 1. Examples of melanoma cells of strong and weak IHC staining intensity: A) Strong (+++) anti-IDO staining (green chromogen). B) Weak (+) anti-IDO staining. C) Strong (+++) anti-TGF- β R1 staining (red chromogen). D) Weak (+) anti-TGF- β R1 staining. E) Strong (+++) anti-IFN- γ R1 staining (red chromogen). F) Weak (+) anti-IFN- γ R1 staining. All images captured under $40\times$ magnification by Panoramic 250 Scanner (see methods); all scale bars equal $50\ \mu\text{m}$

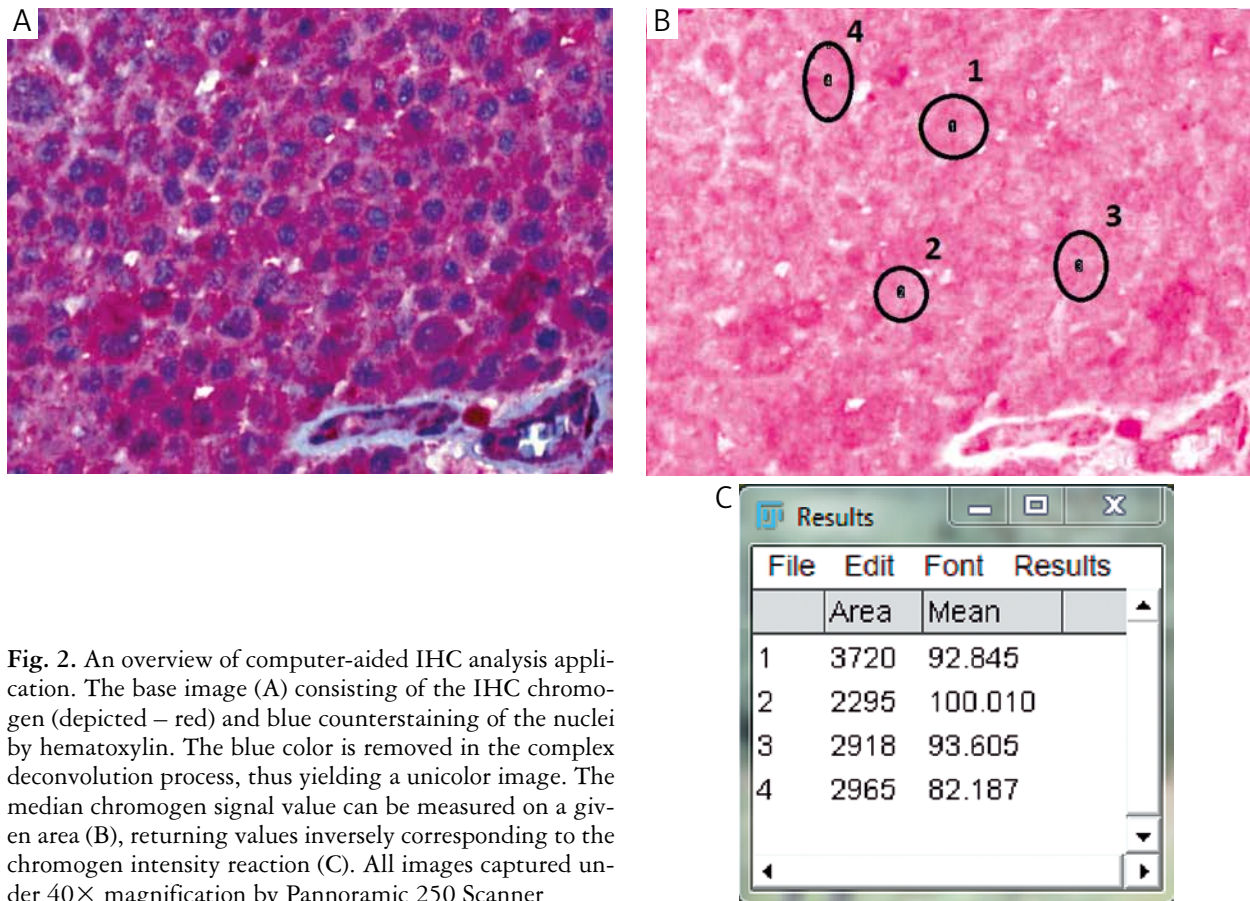


Fig. 2. An overview of computer-aided IHC analysis application. The base image (A) consisting of the IHC chromogen (depicted – red) and blue counterstaining of the nuclei by hematoxylin. The blue color is removed in the complex deconvolution process, thus yielding a unicolor image. The median chromogen signal value can be measured on a given area (B), returning values inversely corresponding to the chromogen intensity reaction (C). All images captured under 40 \times magnification by Panoramic 250 Scanner

parameter that had an impact on distant metastasis risk (HR = 1.07; 95% CI: 1.02-1.13; $p = 0.009$). To verify the significance of the prognostic value of IDO expression level, the patients were divided by the median IDO channel value of 47.39 and thus compared by the Kaplan-Meier univariate model measuring OS. The result of this comparison is shown in Fig. 3.

Correlations were also measured between the scores acquired using subjective (semiquantitative pathologist's assessment) and objective (computer-yielded absolute and relative quantitative values) methods of IHC assessment to validate the algorithms used. The resulting coefficients were significant for all particles analyzed ($p < 0.05$): strong correlations between IDO and TGF- β R1 scores ($R = 0.61$ and $R = 0.74$, respectively) and very strong for IFN- γ R1 ($R = 0.88$) were recorded.

Discussion

Indoleamine-2,3-dioxygenase, tumor growth factor β and interferon γ in melanoma

The discovery of the immune checkpoints in recent years has led to a substantial improvement in the management of patients with advanced or metastatic melanoma. The interest in IDO and its regulators in

melanoma is therefore justified. It has been studied by a few other research groups, and most of these focused on involved or sentinel lymph nodes. Chevillet *et al.* investigated 120 melanomas of the skin and 85 matched lymph nodes (both involved and not). They observed better OS ($p = 0.04$) in patients who demonstrated elevated expression of IDO in both the primary tumors and lymph nodes, independently. Worth noticing is the fact that the better prognosis of patients with lower nodal IDO expression was independent of whether the nodes were metastatic or not [14]. Speckaert *et al.* analyzed 116 sentinel lymph nodes from melanoma patients using IHC and flow cytometry. They observed a significant, positive correlation between IDO expression and the fraction of CTLA4-expressing cells. Enhanced IDO expression was independently associated with worse OS ($p = 0.01$) and disease-free survival ($p = 0.015$) of the patients. Again, the observed correlations were significant in non-metastatic lymph nodes as well [15]. Lee *et al.* observed elevated IDO expression in 69% of melanomas of the skin and a significantly more frequent presence of IDO-positive lymph nodes in melanoma compared to a control group of lymph nodes from breast cancer patients [16]. These findings are in full compliance with our observations of a better OS in patients demonstrating low IDO nod-

al expression and support the conclusion that IDO is a negative prognostic factor in melanoma.

A modest, yet significant correlation was observed between TGF- β 1 and IFN- γ 1. While many *in vitro* experimental studies have well demonstrated how IDO is induced by TGF- β and IFN- γ [17, 18], correlations between these particles in tumor specimens are not widely described. Lee et al. found significant correlations between genes encoding both TGF- β and IFN- γ with the *Indo* gene encoding IDO ($R = 0.95$ and $R = 0.78$, respectively; $p < 0.0001$) [19]. Why this has not been fully demonstrated in our study can be explained by the limitation of methods used. Due to the aged material we only assessed the TGF- β and IFN- γ receptors. While their expression level may indirectly represent the exposure and susceptibility of cells to cytokine stimulation [20], methods assessing the cytokines directly (flow cytometry, blots and enzymatic assays) do obviously better demonstrate the role of the cytokine in biological processes and are thus more recommended [21]. This especially concerns TGF- β , whose role in tumors is not only due to the hyperactivity of this pathway, but primarily is a result of its alterations not present in normal cells – which needs investigation using highly complex methods [22]. However, the above described methods all require fresh tissue material, which would need a prospective study type.

Computer-aided IHC assessment

IHC is a widely accessible and affordable method of protein assessment. It may be applied to archived tissue specimens, which is a significant advantage over methods requiring fresh material. Its major drawback in research, however, is relatively poor reproducibility of the results across different researchers, arising from the subjectivity of immunostaining intensity assessment, especially in the case of cytoplasmic protein location. Therefore quantification of the chromogen in stained tissues is desired to make the results more objective [23]. This approach is not popular, yet there are studies that aimed to validate the semiquantitative pathologist's assessment this way. Rizzardi *et al.* used a color deconvolution algorithm similar to ours when analyzing the staining against S100 antigen in ovarian cancer cells. They obtained a significant ($p < 0.0001$) correlation coefficient of 0.78 [24]. De Matos *et al.* also managed to get a significant and strong correlation ($p = 0.0001$; $R = 0.71$) for their validation of the pathologist's assessment of anti-galectin-3 IHC staining in thyroid cancer [25]. This approach is still in development, and the results of the studies cannot be yet compared across different platforms. However, such observations – in line with ours – do show that the IHC method has a not yet fully utilized potential for objective research use and such validation should become more common. This

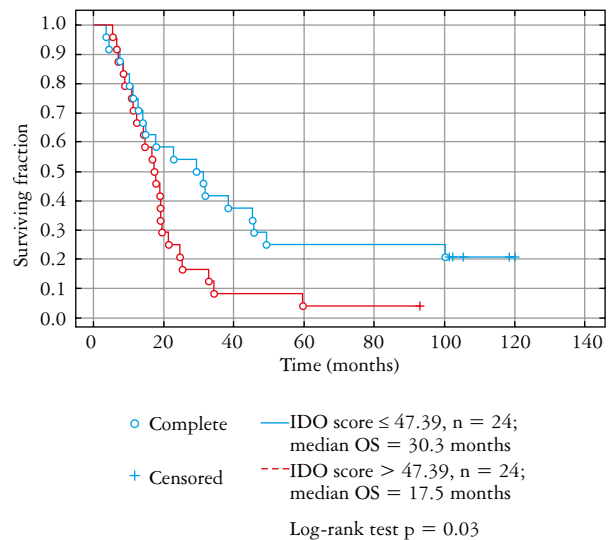


Fig. 3. Kaplan-Meier OS curves for the whole group of patients as divided by median absolute intensity of anti-IDO staining reaction

especially concerns rare IHC antibodies and protocols, which do not have appropriate reference controls widely established.

Potential application of the results

Indoleamine-2,3-dioxygenase due to its unspecific immunosuppressive properties can theoretically affect the currently used immunotherapeutic modalities, especially those which involve blocking the lymphocyte-inhibiting signal cascades – such as the previously mentioned anti-CTLA4 and anti-PD-1 therapeutic antibodies [26]. Moreover, IDO itself is promisingly considered a therapeutic target whose inhibition may lead to restoration of an adequate immune response to the tumor. Three inhibitors are undergoing phase I and II clinical trials, namely 1-methyl-D-tryptophan [27] and two noncompetitive inhibitors: INCB024360 [28] and NLG919 [29]. This study, while it did confirm IDO's prognostic role in lymph node-metastatic malignant melanoma, can by no means prove its predictive role in immunotherapy. We are not in possession of any data related to patient immunotherapy at any stage of the treatment protocol – it was not a standard of care at the time the patients from our study were treated. However, the biology of IDO and our study results should be a strong encouragement for the organizers of immunotherapy clinical trials on melanoma to include IDO and its regulators in the array of biomarkers that are prospectively analyzed for their predictive and prognostic significance. Confirmation of such a value of IDO may further improve the immunotherapy of melanoma patients by introducing a new treatment modality to help fight their disease.

Conclusions

IDO expression has a prognostic role in malignant melanoma with lymph node involvement. It is a negative predictor of overall patient survival and is significantly associated with increased risk of death. This finding is in line with the results of other studies and requires validation in prospective, controlled observations that may identify its predictive significance as well. TGF- β 1 is down-regulated in lymph node metastasis and positively correlated with IFN- γ 1 expression. Whether this phenomenon or the two cytokines in general have a clinical impact on melanoma in the affected patients should be further investigated by more complex methods that assess the cytokines directly. Computer-aided IHC analysis is a valuable tool that should be further developed, as it may improve the research usability of IHC staining. Through the means of automated, quantitative analysis, this method becomes more objective and repeatable across different researchers, while preserving its simplicity and affordability as compared to other protein assessment methods.

The authors declare no conflict of interest.

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