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ORIGINAL ARTICLE

High expression of MLANA in the plasma of patients with head and neck squamous cell carcinoma as a predictor of tumor progression

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Background: There is a paucity of plasma-based biomarkers that predict outcome in patients with head and neck squamous cell carcinoma (HNSCC) treated with chemoradiation therapy (CRT). Here, we evaluate the prognostic potential of plasma Melanoma-Antigen Recognized by T-cells 1 (MLANA) in this setting.
Methods: *MLANA* expression in HNSCC lines were evaluated by reverse transcription polymerase chain reaction, whereas plasma levels were quantified using ELISA in 48 patients with locally advanced HNSCC undergoing a phase 2 trial with CRT.
Results: *MLANA* is expressed at variable levels in a panel of HNSCC lines. In plasma, levels were elevated in patients with tumor relapse compared to those without ($P < .004$); 73.9% of the patients expressing high plasma MLANA levels progressed with recurrent disease ($P = .020$). Multivariate analysis showed that plasma MLANA levels and tumor resectability were independent prognostic factors for progression free survival.
Conclusion: Plasma MLANA expression appears to be an effective noninvasive biomarker for outcomes in patients treated with CRT, and could potentially guide therapeutic decisions in this context.

KEYWORDS
biomarker, HNSCC, MLANA, plasma, tumor progression

1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) affects 740 000 new patients each year, with approximately 300 000 deaths worldwide.[1,2] Despite significant advances in therapy, only 30%-50% of patients with locally advanced disease survive more than 5 years, and this has not changed over the past 30 years.[3] While current staging systems have an important role to play in predicting outcomes, they do not necessarily affect treatment decisions as they do not take into account response to the different treatment modalities.[4] This is particularly true in the context of locally advanced HNSCC affecting the oropharynx, larynx, and hypopharynx, in which concurrent chemoradiation therapy (CRT), with or without induction chemotherapy (IC), has emerged as the new paradigm of treatment.[5] While the objective of this

strategy is to preserve critical functions (airway, speech, swallowing, body image, and so forth), a proportion of patients fail treatment and require salvage surgery. In the latter cohort, ineffective pretreatment results in unwanted delay of curative therapy. In addition, surgery is made challenging in a heavily pretreated patient and a radiated field.[6] It is generally believed that to personalize treatment decisions in these patients, noninvasive biomarkers that stratify patients according to treatment response would help to optimize appropriate patient-specific therapeutic interventions, quality of life, and outcomes.

Previous attempts in identifying suitable biomarkers to predict treatment response have been based on tumor-specific markers.[7–9] The Melanoma-Antigen recognized by T-cells 1 (MART-1 or MLANA) is a major melanocyte-differentiation antigen that is well-known to be produced by melanoma cells.[10–12] This protein has been described as a marker for the diagnosis of the sinonasal and other mucosal melanomas.[13] While the function of this protein is yet unknown, several studies have demonstrated that MLANA expression in the peripheral blood of advanced melanoma patients correlates with advance disease stage, disease progression, and poorer outcomes.[14,15]

In a screen to identify putative prognostic markers in various compartments of patient plasma, we identified MLANA as a candidate protein correlated with response to CRT. In this study, we demonstrated that *MLANA* is expressed in HNSCC tumor cells and proceeded to evaluated MLANA expression levels in the plasma of patients with locally advanced HNSCC treated as part of a phase 2 trial with IC followed by concomitant CRT.[5] The main objectives were to correlate pretreatment plasma levels of MLANA with specific response to IC, CRT, and disease-specific outcomes following these treatment modalities.

2 | MATERIALS AND METHODS

2.1 | Cell line culture

The HNSCC cell lines FaDu (ATCC HTB-43), HN13 (CVCL_5519), SCC4 (ATCC CRL-1624), SCC14 (CVCL_7810), SCC25 (ATCC CRL-1628), NCC-HN19, NCC-HN64, NCC-HN90, and NCC-HN120 were maintained in RPMI-1640, MEM, or DMEM (ThermoFisher, Waltham, Massachusetts) supplemented with 10% fetal bovine serum (ThermoFisher) and 0.01 μg/mL of penicillin-streptomycin (ThermoFisher). The cells were grown in an incubator at 37°C with 5% CO_2. The NCC-HN series were all patient-derived lines that have been previously characterized.[16]

2.2 | RNA extraction, cDNA synthesis, and reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction was performed using TRIzol reagent (ThermoFisher) according to the manufacturer's instructions. After extraction, 25 μg of total RNA were treated with RQ1 RNase-Free DNase (Promega, Madison, Wisconsin) to eliminate the presence of genomic DNA and 2 μg of total RNA were subjected to cDNA synthesis using the SuperScript III First-Strand Synthesis System (ThermoFisher). The cDNA obtained was diluted 10-fold before its use. The *MLANA* mRNA expression was determined by RT-PCR through a *Veriti 96-Well Thermal Cycler* (ThermoFisher). The primer sequences are given in Supporting Information Table S1.[17] The expression of beta-actin (*ACTB*) was used to normalize the RT-PCR data.

2.3 | Patients

This study involved plasma samples from 48 patients with locally advanced HNSCC who underwent organ preservation protocol as part of a phase 2 clinical trial to test the effect of IC followed by CRT, between 2009 and 2010 at the Department of Head and Neck Surgery, Barretos Cancer Hospital (Barretos, SP, Brazil).[5] The study was approved by the institution ethics committee (number: 231/2009), and pretreatment plasma samples were obtained from the hospital tissue bank. Inclusion criteria for this study were histologically confirmed locally advanced stage III or IV a-b (M0) squamous cell carcinoma of the larynx, oral cavity, oropharynx, or hypopharynx, with no prior treatment or cancers, and informed consent to undergo the treatment as outlined. All patients were required to have measurable disease by Response Evaluation Criteria in Solid Tumors (version 1.1), an Eastern Cooperative Oncology Group-Performance Status ≤ 2, age of at least ≥ 18 years, and adequate liver, renal, and bone marrow function. Patients with oral cavity cancer were included only if they had unresectable disease. Exclusion criteria included a history of another malignancy, previously received chemotherapy, radiotherapy, or surgery (except diagnostic biopsy) for the primary tumor or lymph nodes, presence of a serious concomitant illness, and a psychiatric illness that would preclude the delivery of the treatment.

2.4 | MLANA sandwich ELISA-like assay

Quantification of MLANA in patient plasma was conducted by a sandwich ELISA protocol in triplicate for each patient, following the manufacturer's instructions (LSBio, Seattle, Washington, EUA #LS-F12003). Briefly, 50 μL of plasma was incubated with the capture antibody and biotinylated detection antibody. After washing off excess unbound detection antibody, Avidin-Horseradish Peroxidase conjugate was added. After 3 washes, tetramethylbenzidine solution was added and incubated for 45 minutes. The reaction was stopped by an acidic "stop" solution provided by the manufacturer. Absorbance was determined at $\lambda = 450$ nm with a Tecan microplate reader (Tecan Trading AG, Switzerland).

Concentrations were determined by comparison with a standard curve generated by the standards provided in the kit.

2.5 | Statistical analyses

The Mann-Whitney, Chi-square, or Fisher exact tests were used to evaluate the associations among MLANA expression, treatment response, disease progression, and clinical variables, as appropriate. A previous definition of tumor response to treatment and disease progression was used in this study.[18] Briefly, complete response was defined as disappearance of all detectable lesions, and disease progression as the appearance of a new lesion or increase of any lesion classified as measurable at initial examination (including tumor recurrence). A multivariate logistic regression was performed to identify independent variables associated with tumor progression. The Kaplan-Meier method was used to estimate progression-free survival (PFS) of patients, and the log-rank test was used to examine the differences between groups. The PFS was defined as the date of assignment until tumor progression or death resulting from any cause. If the patient had no evidence of the aforementioned events, survival was censored at the time of the last documented evaluation of efficacy/contact. Cox proportional hazard regression analysis was performed in a step-forward fashion to estimate the hazard ratios (HRs); the multivariate Cox regression model included clinical and molecular variables with P value <.20 in the univariate analysis to build a final model. The final model was further adjusted for clinically relevant variables. A P value <.05 was necessary to determine statistically significant differences. All statistical analyses were performed using SPSS statistics 23.0 (IBM, New York New York).

3 | RESULTS

3.1 | MLANA expression in HNSCC cell lines

Given that our prior screens identified MLANA expression as a potential marker of response to CRT, we sought to evaluate if *MLANA* is expressed in HNSCC cells. RT-PCR was performed to determine the presence of *MLANA* transcripts in 9 different HNSCC lines. This analysis showed that *MLANA* is expressed at variable levels in 8 of the 9 HNSCC cell lines examined, compared to the melanoma cell line (SK-MEL-25), which acted as the positive control (Figure 1).

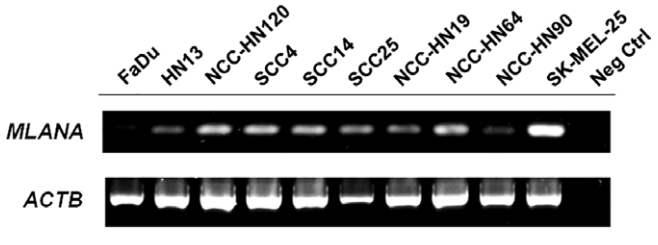

FIGURE 1 Electrophoresis image showing expression pattern of Melanoma-Antigen recognized by T-cells 1 (*MLANA*) in a panel of head and neck squamous-cell carcinoma (HNSCC) cell lines as indicated based on reverse transcription polymerase chain reaction (RT-PCR). The melanoma cell line SK-MEL-25 was used as *MLANA* expression positive control; blank as *MLANA* negative control (Neg Ctrl); RT-PCR of beta-actin (ACTB) is used as a loading control

TABLE 1 Clinical and pathologic characteristics of patients included in the study

Variables	Category	No. of patients	%
Median age, 56 y (range, 37-76 y)			
Sex	Male	46	95.8
	Female	2	4.2
Tobacco use	Never	2	4.2
	Former	12	25.0
	Current	34	70.8
ECOG-PS	0	2	4.2
	1	42	87.5
	2	4	8.3
Tumor grade	G1	6	12.5
	G2	37	77.1
	G3	5	10.4
Primary site	Oral cavity	3	6.3
	Oropharynx	27	56.3
	Hypopharynx	6	12.5
	Larynx	12	25.0
HPV status[a]	Positive (p16+)	2[b]	4.2
	Negative (p16−)	41	85.4
	Unavailable	5	10.4
Resectability status	Resectable	31	64.6
	Unresectable	17	35.4
T classification	T2	4	8.3
	T3	28	58.3
	T4	16	33.3
N classification	N0	10	20.8
	N1	5	10.4
	N2	25	52.1
	N3	8	16.7
Stage	III	14	29.2
	IV	34	70.8
Disease progression	No progression	20	41.7
	Progression	28	58.3
CRT response	Complete response	30	62.5
	Incomplete response	16	33.3
	Missing cases	2	4.2

Abbreviations: CRT, chemoradiation therapy; HPV, human papillomavirus.
[a] HPV status was determined retrospectively using archival tumor specimens.
[b] Both patients were seen with oropharyngeal tumors, were currentl smokers, men, and stage IV disease.

3.2 | Patient characteristics

The clinical and histological features of the 48 patients with locally advanced HNSCC enrolled in this study are

presented in Table 1, and details of the treatment protocol have been previously published.[5] The median follow-up for this cohort was 4 years. The patients were predominantly men ($n = 46$; 95.8%), with age ranging from 37 to 76 years (median: 56 years). Tobacco use (current and former) was reported by 95.8% ($n = 46$) of patients, while only 4.2% of the cases were human papillomavirus (HPV)-associated cancers ($n = 2$). Primary tumor sites included oral cavity ($n = 3$; 6.3%), oropharynx ($n = 27$; 56.3%), hypopharynx ($n = 6$; 12.5%), and larynx ($n = 12$; 25.0%). Majority of tumors were locally advanced (T3/T4, 91.6%), and 62.6% ($n = 30$) of the patients demonstrated complete response to the treatment protocol. During the follow-up period, 58.3% ($n = 28$) of patients developed tumor progression or recurrence.

3.3 | Plasma MLANA expression correlates with disease progression

Plasma MLANA levels in this cohort ranged between 1.11 and 931.02 pg/mL, with mean and median concentrations of 228.40 and 165.9 pg/mL, respectively. Significant higher plasma levels of MLANA were detected in patients with disease progression, compared to those who did not ($P < .038$; Figure 2A). However, there were no associations between plasma MLANA and CRT response ($P = .831$; Figure 2B).

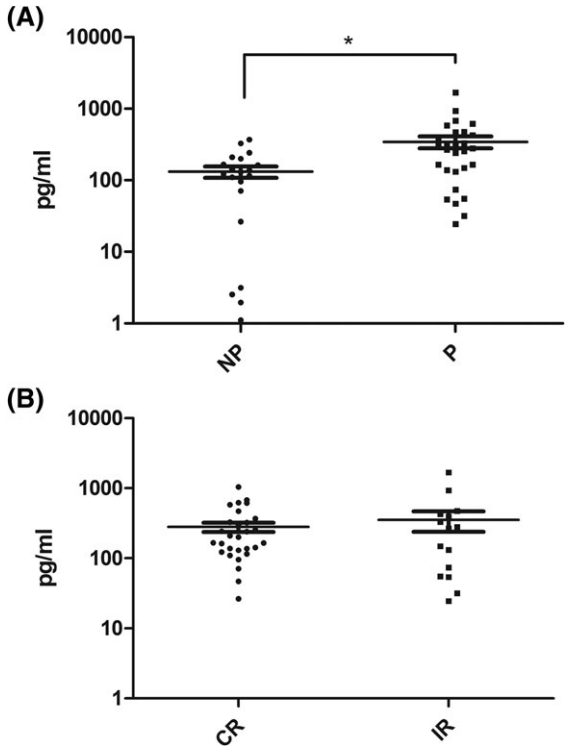

FIGURE 2 Plasma Melanoma-Antigen recognized by T-cells 1 (MLANA) levels in patients with locally advanced head and neck squamous-cell carcinoma (HNSCC). A, Comparison between patients presenting with disease progression and patients with no progression (Mann-Whitney test; $*P < .038$). B, Comparison between patients with complete response to chemoradiation therapy (CRT) and patients with incomplete response (Mann-Whitney test; $P = .831$)

Using the median as cutoff,[19,20] plasma levels of MLANA was scored as low and high, and analyzed for associations with clinical characteristics, primary tumor site, grade, stage, HPV status, and response to CRT. There were no associations seen between MLANA levels and clinico-pathologic variables tested (Table 2).

We subsequently performed Kaplan-Meier analysis to evaluate the association between plasma MLANA levels and PFS. Based on the cutoffs defined above, 72% of patients with high plasma MLANA levels developed disease progression at 4 years, compared to 44% in patients with low MLANA levels ($P = .020$; Figure 3).

Furthermore, a multivariate Cox regression model adjusted for age, tumor resectability, and plasma MLANA levels showed that MLANA expression (HR = 2.40; 95% CI 1.11-5.18; $P = .026$) and tumor resectability (HR = 2.66; 95% CI 1.26-5.63; $P = .010$) were independent prognostic factors for PFS (Table 3).

4 | DISCUSSION

Over the last 3 decades, management and prognosis of patients with HNSCC have improved through multimodality treatment protocols incorporating surgery, radiotherapy, and chemotherapy. Nevertheless, the 5-year survival rates remain unchanged at 50%.[3] The potential of noninvasive biomarkers that can not only prognosticate but guide treatment decisions is an urgent and unmet clinical need.

Several groups have demonstrated different approaches to the identification and validation of prognostic factors in HNSCC, largely based on analyses of the primary tumor tissue itself. These include using single gene expression such as mRNA-based signatures, miRNA expression, single protein biomarkers, or complex multiprotein signatures.[21–25] Few studies have focused on plasma-based biomarkers as prognostic factors in HNSCC. Le et al[26] found that serum Osteopontin levels correlated with tumor hypoxia, suggesting that this protein could be used to identify patients at high risk for tumor recurrence. Similarly, Gehrmann et al[27] showed that sHsp70 levels in the blood of HNSCC patients might be useful for tumors detection, and also for the monitoring of the therapeutic response to radiation therapy. In other studies, plasma levels of LCN2/matrix metallopeptidase 9 complex, MMP2, TIMP1, TIMP2, and TIMP3 were significantly correlated to the large tumor size, lymph node involvement, tumor differentiation and prediction of the tumor stage, and T status in patients affected with OSCC.[28–30] Moreover, circulating miRNAs in the plasma of HNSCC patients such as miR-142-3p, miR-186-5p, miR-195-5p, miR-374b-5p, and miR-574-3p also represent promising plasma markers for overall prognosis and posttreatment surveillance.[31] Despite their identification, few have been tested in the context of a specific treatment regime in a trial setting and/or compared to accepted clinical predictors for

TABLE 2 Correlation between plasma MLANA levels with clinical and pathologic characteristics. All *P* values were based on 2-tailed *t* tests

Variable	Categories	No. of cases	MLANA		*P* (2-tailed *t* tests)
			Low *n* (%)	High *n* (%)	
Age (y)	≤60	27	17 (51.5)	16 (48.5)	1.000
	>60	11	8 (53.5)	7 (46.7)	
Sex	Male	46	23 (50.0)	23 (50.0)	.490
	Female	2	2 (100.0)	0 (0.0)	
Tobacco use	Never	6	4 (66.7)	2 (33.3)	.183
	Former	37	19 (51.4)	18 (48.6)	
	Current	5	2 (40.0)	3 (60.0)	
Primary site	Oral cavity	3	1 (33.3)	2 (66.7)	.900
	Oropharynx	27	15 (55.6)	12 (44.4)	
	Hypopharynx	6	3 (50.0)	3 (50.0)	
	Larynx	12	6 (50.0)	6 (50.0)	
HPV status	Positive (p16+)	2	1 (50.0)	1 (50.0)	1.000
	Negative (p16−)	41	21 (51.2)	20 (48.8)	
Resectability status	Resectable	31	17 (54.8)	14 (45.2)	.764
	Unresectable	17	8 (47.1)	9 (52.9)	
Stage	III	12	7 (58.3)	5 (41.7)	.117
	IV	26	16 (61.5)	10 (38.5)	
CRT	Complete response	30	16 (53.3)	14 (46.7)	1.000
Response	Incomplete response	16	8 (50.0)	8 (50.0)	

Abbreviations: CRT, chemoradiation therapy; MLANA, Melanoma-Antigen recognized by T-cells 1.

outcomes: TNM classification, HPV status, and other high-risk factors (perineural invasion, extranodal extension, and so forth).[32,33]

Undoubtedly, there is tremendous potential in the use of serum/plasma-based markers to prognosticate and guide therapy. It is noninvasive, hence obviates the need to obtain tumor tissues through biopsies, or extract information from poorly archived tissue. It avoids the need for expertise in tissue processing, complex immunohistochemical, and gene expression analyses. Furthermore, blood-based markers can be expanded as dynamic investigations and performed over a time course, with a potential use in posttreatment tumor surveillance. We identified plasma MLANA as a potential prognostic factor for CRT response based on prior screens in various plasma compartments, and sought to validate these findings in a uniformly treated patient cohort using a readily available ELISA kit. The data shown above demonstrated that in this specific setting of patients with advanced HNSCC treated with CRT, high MLANA levels are associated with disease progression on univariate and multivariate analyses. While this is a relatively small patient cohort of 48 patients, the uniformity of this cohort with regards to treatment protocol and follow-up evaluation contributes to the accuracy of these results, and hence supports the need to conduct such studies under a controlled context, such as the confines of a clinical trial.

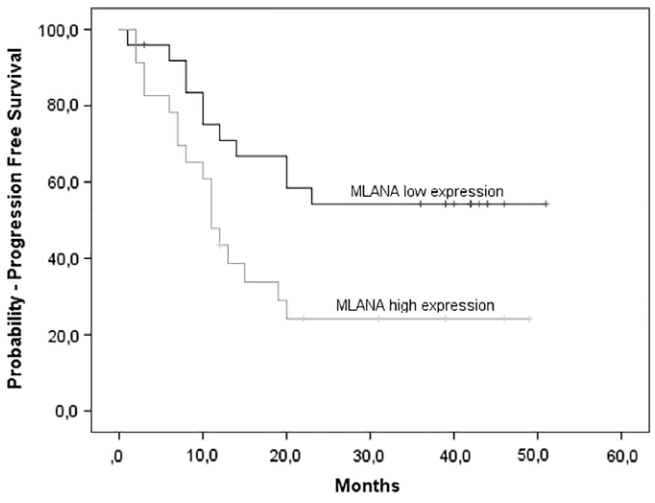

FIGURE 3 Kaplan-Meier plots showing correlation between high versus low plasma Melanoma-Antigen recognized by T-cells 1 (MLANA) levels and progression-free survival (PFS) ($P = .020$)

MLANA was first identified by 2 independent groups Coulie et al[34] and Kawakami et al,[11,12] as an antigenic target of T lymphocytes on melanomas. Notwithstanding, the expression of this antigen has been detected in melanocytes, and nonmelanocytic cells damaged by inflammatory processes, such as keratinocytes, normal skin cells adjacent to the actinic keratosis, retinal pigment epithelium, and tumors such as melanomas, clear cell sarcomas, and adrenocortical carcinomas[12,35–37] Our data also demonstrate that *MLANA* is expressed in HNSCC cells, which we believe have not been shown before. The MLANA protein has been used to develop peptide vaccines against melanoma cells, while plasma protein levels have been evaluated for its role in

TABLE 3 Multivariate analysis (Cox regression model) for factors predicting progression-free survival (PFS)

Characteristic	Adjusted HR (95% CI)	*P* value
MLANA expression		**.026**
Low expression	1 (ref.)	
High expression	2.40 (1.11-5.18)	
Tumor resectability		**.010**
Resectable	1 (ref.)	
Unresectable	2.66 (1.26-5.63)	

Abbreviations: CI, confidence interval; HR, hazard ratio; MLANA, Melanoma-Antigen recognized by T-cells 1.

predicting melanoma recurrence and prognosis.[11–15,32,38] Interestingly, it has been shown that MLANA expression is a useful diagnostic blood marker for sinonasal melanomas, which is one of the most aggressive melanomas arising in the head and neck region.[13] Previous studies have also reported that increase plasma MLANA levels in melanoma patients correlated with stage and tumor recurrence.[14,15] Functional characterization of MLANA suggests that it may be related to melanin expression, coat color phenotype, and aberrant melanosomes in knockout mice.[39] Moreover, increased melanin expression has been shown to decrease the response to chemotherapy or radiotherapy in metastatic melanoma cells, and correlates with disease progression.[40] In fact, it was even suggested that the inhibition of melanogenesis might improve radiotherapy response, although extending these findings to CRT response in HNSCC is purely speculative.[40–42]

There are a number of pitfalls in this study including a small patient cohort, availability of plasma samples from only a single time point, and potential technical issues associated with evaluating plasma MLANA levels in frozen plasma samples. Furthermore, as we did not have any a priori knowledge of the distribution of MLANA levels across patient samples, we used the median plasma level as a cutoff to conduct our analyses in this cohort. We anticipate that this will need further refinements based on validation studies in larger cohorts. Nevertheless, this remains the first study (to our knowledge) reporting a significant association between plasma MLANA expression and prognosis of HNSCC patients treated with CRT. Expanding these findings in larger patient cohorts would be critical to validate these results.

In conclusion, our results indicate that high plasma levels of MLANA is an independent predictor of disease progression in patients with locally advanced HNSCC treated with IC followed by CRT. These findings suggest that the evaluation of plasma MLANA expression could prognosticate patients undergoing these treatment modalities and better stratify those likely to develop tumor recurrences. It may also be used in future studies to identify patients a priori who may fail organ-preservation protocols, directing them to surgical resection at an earlier stage.

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CONFLICT OF INTEREST

The authors disclosed no potential conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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