

Nuclear Odontogenic Ameloblast-Associated Protein (ODAM) Correlates with Melanoma Sentinel Lymph Node Metastasis

Sagar S. Gandhi^{1,2}, Daniel P. Kestler^{2,3}, Charles T. Bruker⁴, James M. McLaughlin^{1,2}, Robert E. Heidel², Sabina Siddiqui⁵, James S. Foster^{2,3}, Keith D. Gray^{1,2}, John Bell^{1,2}, Alan Solomon^{2,3}, James Lewis^{1,2}

¹Department of Surgery, University of Tennessee Medical Center-Knoxville, Knoxville, USA; ²Graduate School of Medicine, University of Tennessee Medical Center-Knoxville, Knoxville, USA; ³Department of Medicine, University of Tennessee Medical Center-Knoxville, Knoxville, USA; ⁴Department of Pathology, Boca Raton Regional Hospital, Boca Raton, USA; ⁵Department of Surgery, University of Michigan School of Medicine, Ann Arbor, USA.
Email: dkestler@utmck.edu

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ABSTRACT

We have examined primary tumor sections from melanoma patients by immunohistochemistry (IHC) for the presence of the odontogenic ameloblast-associated protein (ODAM). Within these patient tissues we have observed a correlation of nuclear ODA staining in the primary tumors with sentinel lymph node (SLN) metastasis. Surgically, SLN invasion in melanoma is considered an important indicator of more aggressive, invasive melanoma and to date there are limited biomarkers which strongly correlate with metastatic disease. The observation that ODA staining in melanoma associates with SLN invasion may have important prognostic implications which could assist in the management of melanoma. Notably, ODA expression may correlate with pathway-signaling we have previously reported to be affected by ectopic ODA expression in cultured melanoma and breast cancer cell lines.

Keywords: Melanoma; Sentinel Lymph Node Metastases; ODA; Immunohistochemistry

1. Introduction

Melanoma metastasis is predicted by factors that reflect biologic behavior such as primary tumor Breslow thickness, mitotic rate, and ulceration [1,2]. Sentinel lymph node (SLN) status in melanoma remains the single most important predictor of overall survival [3-5]. In addition, records from the AJCC Melanoma Staging Database demonstrate that as Breslow thickness increases, a significant decline in both 5- and 10-year survival rates is observed, and recent data demonstrate a significant correlation between survival and the primary tumor mitotic rate. Notably, survival rates of patients with an ulcerated melanoma and similar Breslow thickness are significantly worse compared to non-ulcerated matched primary tumors [2]. Many potential biomarkers for melanoma have been reported, but their clinical significance largely remains undetermined [6]. Molecular factors influencing primary melanoma growth and metastasis reflect dysregulation of normal cellular signaling pathways, and these factors continue to be intensively investigated,

both with respect to potential therapeutic advances and for utility as prognostic indicators [7].

ODA is a protein initially identified as the amyloid-forming component in a rare dental neoplasm, calcifying epithelial odontogenic tumor or Pindborg tumor. The protein has been detected in a broad range of epithelial tissues and in multiple human cancers including those of the breast, esophagus, gastric tissues, and bronchial epithelium [8,9]. The potential role of this protein as a marker of disease status and survival in breast cancer has been reported as increased nuclear ODA staining of primary breast tumors across disease staging [10]. A portion of patients (up to ~13% in late stage) who were ODA-positive exhibited improved survival compared to stage-matched ODA-negative breast cancer patients while the remaining bulk (~87%) of late stage ODA-positive tumor patients did not exhibit a survival benefit, suggesting at least two patients' outcome groups associated with ODA-expressing breast tumors [10]. Based on previous observations that lymph node-positive breast

cancer patients are often positive for nuclear ODA M staining, together with the propensity of melanomas to metastasize into regional lymph nodes, we examined ODA M expression in primary tumors and lymph node biopsies of patients with SLN-positive (Stage III) and SLN-negative (Stage I-II) melanoma. This allowed us to test whether nuclear ODA M staining in primary melanoma could predict sentinel node positivity. We report our findings as follows.

2. Materials and Methods

2.1. Melanoma Patient Tumor Tissue

Patients diagnosed with melanoma were identified retrospectively through our institutional tumor registry from years 2000-2006. Only cases with available primary tumor and SLN tissue were evaluated. Histological features of the primary tumors were recorded for both the SLN-negative and positive samples, along with overall survival (OS) and recurrence data obtained from our patient database. Archived formalin-fixed paraffin embedded tumor tissues were cut and immunostained with murine monoclonal anti-ODA M antibody 8B4, as previously reported [10] and detailed below.

2.2. IHC Analysis

Two micrometer thick formalin-fixed paraffin embedded melanoma sections were mounted on charged slides (Fisherbrand Superfrost/Plus, Thermo-Fisher), dried overnight at room temperature, and deparaffinized to water. Sections were immersed in antigen-retrieval solution (Biogenex Citra Plus, BioGenex, San Ramon, CA, USA) and subjected to standard blocking procedures. Anti-ODA M antibody was applied at 1:7500 in diluent (Dako, #S-3022), incubated overnight (5°C), and visualized using the ImmPRESS polymerized enzyme-linked reporter system followed by the ImmPACT diaminobenzidine detection kit (Vector Laboratories, Burlingame, CA, USA). External positive controls were utilized for slide interpretation with each batch of patient tumor-slides while, benign structures present in each study section served as internal controls.

Tumor tissue obtained from each block was stained by hematoxylin-eosin and reviewed in conjunction with immunostained slides. The presence or absence of ODA M immunostaining was determined in the neoplastic cells of each case and staining was assessed and reported, for the nucleus only, as negative or positive. Nuclear positivity was defined by the presence of distinct smooth homogeneous staining of at least 50% of tumor cell nuclei, and negativity was defined as a near complete lack of nuclear immunostaining in essentially all tumor cells of interest (less than 1% of nuclei) as in previous studies [10]. This

was based on the observed distribution of nuclear ODA M staining where positive tumors exhibited staining in essentially all (greater than 90%) cell nuclei while staining was present in less than 1% of cell nuclei in tumors designated as ODA M-negative. Thus, tumors with a percent reactivity between 1% and 90% were rare, and 50% reactivity was chosen as a cutoff to provide a study design that minimized ambiguity. In practice, no tumors exhibited reactivity near the 50%-positive cutoff. Staining for ODA M was graded in blinded fashion by a single peer reviewed pathologist (CTB).

2.3. Statistics

Descriptive statistics were conducted for the Breslow thickness, age, gender, and months to follow-up. An independent samples t-test was used to compare Breslow values on SLN-positive and SLN-negative groups. In the event of a violation of a statistical assumption, a non-parametric Mann-Whitney U test was employed. Unadjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated to compare ODA M staining and SLN status to various discrete variables including recurrence, cancer status, and cancer death. Logistic regression analysis was employed to yield multivariate adjusted odds ratios when predicting for SLN positivity. Statistical significance was assumed at a $p < 0.05$ level and all analyses were conducted using SPSS Version 19 software (SPSS, Chicago, IL).

3. Results

3.1. Patient Sample Populations

Our institutional tumor registry contained 270 cases of primary melanoma treated from 2000-2006. Complete data and adequate tissue samples were available for 44 of these patients (21 SLN-positive patients and 23 SLN-negative patients). Inadequate tissue samples, incomplete medical records, non-sentinel lymph node biopsies and patients who underwent lymphadenectomies were excluded. All patients had invasive melanoma. **Table 1** depicts patient demographics and primary tumor characteristics for each cohort. Demographics were not significantly different but was close to significance in regards to Breslow thickness ($p = 0.09$). Ulceration was not significantly different between cohorts, 13 versus 10 in the SLN-positive and SLN-negative cohorts, respectively ($p = 0.61$).

3.2. ODA M Immunostaining

Immunostaining for ODA M showed that both the nucleus and cytoplasm of benign melanocytes stain strongly positive for ODA M, while in melanoma, staining of the

Table 1. Melanoma patient characteristics^a.

	SLN-NEGATIVE	SLN-POSITIVE	ODAM-NEGATIVE	ODAM-POSITIVE
AGE (Median)	68 (44 - 85)	54 (34 - 75)	68 (34 - 85)	56 (39 - 80)
GENDER (M:F)	10:13	12:9	8:15	14:7
LOCATION				
Extremities	14	8	13	9
Trunk	4	9	7	6
Head	5	4	3	6
MEDIAN BRESLOW (Range)	2 mm (0.35 - 7)	3.38 mm (0.32 - 16)	2.48 mm (0.35 - 10)	2.75 mm (0.32 - 16)
ULCERATION PRESENT	10	13	13	10

^aCohort Demographics are separated by sentinel lymph node-negative (Stage I/II), sentinel lymph node-positive (Stage III), ODA-negative, and ODA-positive melanoma.

cytoplasm is consistently diminished and nuclear staining is variable. In ODA-negative melanoma there is no nuclear staining of the primary tumor (**Figure 1**), while ODA-positive melanoma exhibits readily demonstrable staining in the cell nucleus (**Figure 2**). Primary tumors in the SLN-positive cohort were significantly more likely to exhibit nuclear localization of ODA as recorded in **Table 2**. Sixteen of 21 specimens (76%) in the SLN-positive patients stained for ODA compared to 5 of 23 (22%) in the SLN-negative patients (Odds Ratio (OR) = 11.52, 95% CI 2.81, 47.23). SLN staining for ODA corresponded with the primary tissue staining pattern in all specimens. No SLN stained positive for ODA unless the primary tumor was ODA-positive as well. Our study had 13 thin melanomas (≤ 1 mm), 5 of which (38%) were ultimately Stage III. Notably, of these five, four stained positive for ODA. Also, 4 of 13 thin melanomas were ulcerated; 1 of these was SLN-negative, 3 were SLN-positive, and ODA status correlated with SLN status in all samples from ulcerative tumors.

3.3. Recurrence and Survival Analyses

Median follow-up for the SLN-positive and negative cohorts was 37 months (range of 7 - 68) and 47 months (range of 4 - 64), respectively. Four patients were lost to follow-up, all of which were in the SLN-negative group (half were ODA-positive). Logistic regression analysis, given in **Table 3**, found that when controlled for Breslow thickness and ulceration, participants that stained ODA-positive were 35 times more likely to be SLN-positive (OR = 35, 95% CI 4.05 - 302.26). Breslow thickness was close to showing a significant association (OR 1.48, 95% CI 0.96 - 2.27, $p = 0.077$).

As shown in **Table 4**, disease recurrence developed in 10 of 18 (55.5%) node positive patients and 2 of 22 (9.1%) node negative patients (OR = 12.5, 95% CI 2.23,

70.19). Three patients in the SLN-positive group were found to have metastatic disease during staging after initial wide local excision and SLN biopsy and were thus considered never disease free. Subset analysis of ODA-positive patients irrespective of nodal status demonstrated that 10 of 20 (50%) ODA-positive patients had recurrence versus 2 of 20 (10%) in the ODA-negative group (OR = 9, 95% CI 1.64, 49.45). Overall survival (OS) was 10 of 21 (48%) in the SLN-positive group and 18 of 23 (79%) in the SLN-negative group (3.96, 95% CI 1.07, 14.67). OS in the ODA-positive group was 11 of 21 (52%) versus 17 of 23 (74%) for ODA-negative primary tumors (OR = 0.39, 95% CI 0.11, 1.38) as displayed in **Table 5**.

4. Discussion

Our study revealed that SLN-positive primary tumors (Stage III) were significantly more likely to exhibit nuclear ODA upon IHC staining, and thus suggests that nuclear ODA localization is associated with more invasive tumors. Furthermore, 4 of 5 SLN-positive thin melanomas (< 1 mm) were found to be nuclear ODA-positive. Since an estimated 5% - 8% of thin melanomas metastasize to lymph nodes, SLN biopsies are not routinely performed in these cases [5]. We propose that preoperative staining of melanoma, and particularly thin melanoma, for ODA may guide operative management and patient treatment.

ODA is expressed in a broad range of normal epithelial tissues and malignancies [8,9]. During dental development the protein is secreted from ameloblasts and associated with the junctional epithelium at the incisor enamel interface [11,12]. Late in dental development ODA localizes to the ameloblast nucleus and mediates direct activation of MMP-20/enamelysin gene expression [13]. Thus, while its roles have not been fully delineated,

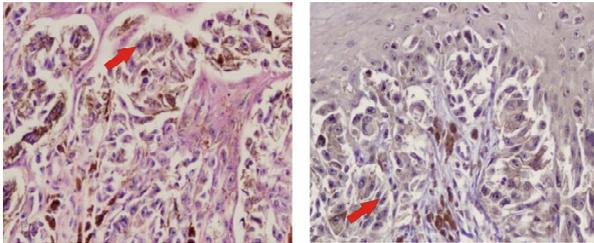


Figure 1. ODA-negative primary melanoma (stage I). Left: hematoxylin-eosin stain. Right: anti-ODAM immunostain. Arrows indicate tumor cell nuclei. Note brown staining of phagocytic cells with lack of nuclear staining in tumor cells. Original magnifications 200 \times .

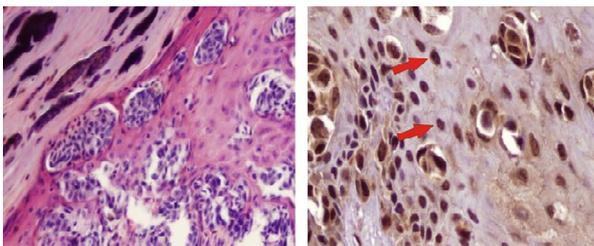


Figure 2. ODA-positive primary melanoma (stage III). Left: hematoxylin-eosin stain (original magnification 100 \times). Right: anti-ODAM immunostain (original magnification 200 \times). The arrows indicate two of numerous ODA-positive tumor cell nuclei.

ODAM exhibits potential cell signaling functions both in the nucleus and through interactions with extracellular matrix components, suggesting classification of ODA as a matricellular protein [12-14]. Proteins of this subset include the tenascin, osteopontin, thrombospondin, SPARC (osteonectin), SPARCL1 (Hevin), and CCN proteins. This class of proteins has been suggested to contribute to melanoma progression by supporting cellular release from keratinocyte control [15].

The high degree of cellular organization of normal differentiated tissues is often lost in cancer. The detection of ODA nuclear localization in melanoma is consistent with the observation of other proteins, such as activating transcription factor 2 (ATF2), showing increased localization to the nucleus in metastatic melanoma [16]. Nuclear re-localization of forkhead box 03 (FOXO3a), β -catenin, and a number of other proteins is proving to be a determining factor in tumor cell growth and invasiveness associated with a broad range of malignancies including melanoma [17-19].

As of yet, no singular molecular biomarkers have proven clinical utility for predicting the progression of melanoma to metastasis [20-22]. Previous studies have discussed biomarkers such as S100B and lactate dehydrogenase as prognostic indicators for melanomas [6,20-24]. These biomarkers are elevated in advanced disease, and their presence indicates poor prognosis and diminished

survival. However, they are not routinely used in clinical practice for early stage disease. Similarly, markers for lymphatic vessel density have been utilized as predictors of SLN metastasis, but this requires analysis of multiple antigens by IHC and a high degree of variability has been reported [25]. Recent reports have also described two potential biomarkers for melanoma identified by monoclonal antibodies KBAb2 and PNL2 and observed in over 85% of cases but the associated antigens have not, to date, been identified [26].

A previous retrospective study of breast cancer tumor sections at our institution demonstrated a statistically significant correlation between the presence of nuclear ODA and tumor stage [10]. Our current study also demonstrates that ODA expression correlates with melanoma recurrence, survival, and that Breslow thickness approached significance when associated with ODA staining.

These observations underlie recent research in our laboratory which demonstrated profound growth inhibition *in vitro* and *in vivo* with human breast cancer cells engineered to express ODA. This corresponds with suppression of the PI3K/AKT/mTOR signaling pathway [27,28]. Notably, melanoma exhibits frequent dysregulation of the PI3K/AKT/mTOR and RAS/RAF/MAPK signaling pathways [7,29,30] and we have observed growth suppression *in vitro* and AKT inhibition upon ectopic ODA expression in human melanoma cell lines [28]. This suggests potential impacts on tumor cell behavior, disease progression, and therapeutic outcomes when ODA is expressed in these malignancies. Yet, in the presence of functional, or dysregulated signaling pathway components, these effects may differ. Our studies thus serve to highlight the complex interactions between signaling pathways in melanoma, given that single drug inhibitors can yield contrary effects on tumor behavior dependent upon the cellular context [31-34].

Recognizing the complexity of ODA function with respect to cellular localization, and the participation of multiple signaling pathways in driving melanoma growth, clarification of the role of ODA expression in melanoma biology is necessary both in mechanistic terms, and as support for any possible clinical utility of staining for ODA. The study described herein was of moderate size and retrospective in nature. Thus, the correlation of ODA expression with SLN positivity and overall survival will be better delineated by analyses comprised of greater sample size and longer follow-up intervals, which will allow for increased statistical power in the results. Further, a clear assessment of the association of ODA with melanoma spread will also depend on studies which place ODA expression in the context of evolving subtype classifications for melanoma [35] and the associated molecular hallmarks of these subtypes (e.g.; BRAF V600E, c-KIT, NRAS, cyclin D).

Table 2. Distribution of ODAM staining of primary tumor tissue^a.

	ODAM-POSITIVE (n = 21)	ODAM-NEGATIVE (n = 23)	ODDS RATIO/CI
SLN-POSITIVE	16	5	11.52/95% CI (2.81, 47.23)
SLN-NEGATIVE	5	18	

^aODAM staining of primary tumor tissue for lymph node-positive melanoma (Stage III) and lymph node-negative melanoma (Stage I/II).

Table 3. Logistic regression analysis results for ODAM staining, Breslow thickness values, and ulceration of primary melanomas.

	ODDS RATIO	95% CI	p-value
ODAM	35	(4.05, 302.3)	0.001
BRESLOW	1.48	(0.96, 2.27)	0.07
ULCERATION	2.08	(0.31, 14.16)	0.46

Table 4. Recurrence rates of melanoma patients subdivided by sentinel lymph node and ODAM status.

	RECURRENCE	NO RECURRENCE	NEVER DISEASE FREE	ODDS, RATIO/CI
SLN-POSITIVE	10	8	3	12.5/95% CI (2.23, 70.19)
SLN-NEGATIVE	2	20	1	
ODAM-POSITIVE	10	10	1	9.0/95% CI (1.64, 49.45)
ODAM-NEGATIVE	2	18	3	

Table 5. Overall survival of melanoma patients subdivided by sentinel lymph node and ODAM status.

	ALIVE	MEDIAN FOLLOW-UP (months)	ODDS RATIO/CI
SLN-POSITIVE	10	37	3.96/95% CI (1.07, 14.67)
SLN-NEGATIVE	18	47	
ODAM-POSITIVE	11	38	0.39/95% CI (0.11, 1.38)
ODAM-NEGATIVE	17	46	

5. Conclusion

In summary, our results indicate that nuclear localization of ODAM in primary melanoma specimens corresponds with an increased likelihood of SLN metastasis and as such may have important prognostic implications. Further, this correlation may foster an understanding of the complex genetics, host-interaction and signaling pathways involved with both nodal and distant metastases in melanoma. Future studies will require analysis of additional specimens from melanoma or other malignancies exhibiting ODAM expression, with an aim of further correlating ODAM expression with disease characteristics, and its possible relationship to known regulatory biomarkers.

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