

Prognostic Significance of Nuclear Receptor Coactivator-3 Overexpression in Primary Cutaneous Melanoma

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A B S T R A C T

Purpose

To assess the prognostic significance of nuclear receptor coactivator-3 (NCOA3) overexpression in primary cutaneous melanoma.

Patients and Methods

NCOA3 expression was assessed using immunohistochemical analysis of a melanoma tissue microarray (TMA) containing primary melanomas from 343 patients with defined histology and follow-up. The impact of the presence or absence of various prognostic factors on relapse-free survival (RFS) and disease-specific survival (DSS) of melanoma patients was assessed using Cox regression and Kaplan-Meier analysis. The impact of presence or absence of various factors on sentinel lymph node (SLN) metastasis was assessed using logistic regression analysis.

Results

Increasing degree of NCOA3 expression was significantly predictive of SLN metastasis ($P = .013$) and the mean number of SLN metastases ($P = .031$). Kaplan-Meier analysis demonstrated a significant association between NCOA3 overexpression and reduced RFS ($P = .021$) and DSS ($P = .030$). Logistic regression analysis revealed increasing degree of NCOA3 expression to be an independent predictor of SLN status ($P = .017$). Multivariate Cox regression analysis showed the independent impact of NCOA3 expression on RFS ($P = .0095$) and DSS ($P = .021$). NCOA3 was the most powerful factor predicting DSS, outperforming tumor thickness and ulceration.

Conclusion

These results identify NCOA3 as a novel, independent marker of melanoma outcome, with a significant impact on SLN metastasis, RFS, and DSS.

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INTRODUCTION

The incidence of melanoma is rising more rapidly than any other cancer,¹ such that it currently represents the fifth most common malignancy in the United States.² Melanoma frequently exhibits unpredictable clinical behavior. While the vertical thickness of the primary tumor is one of the most important prognostic factors determining survival, many patients with thick melanomas are free of metastasis, while a small subset of patients with thin tumors die of their disease.³ Additional biomarkers are therefore required to improve prognostic algorithms for newly diagnosed melanoma patients. Despite intense investigation, no molecular factors are routinely used in the prognostic evaluation of melanoma patients.⁴

Recent cDNA microarray analyses of melanomas demonstrated the differential expression of several genes in metastatic versus primary mel-

anomas.⁵ These analyses identified the nuclear receptor coactivator-3 (NCOA3) gene to be overexpressed in melanoma metastases when compared with unrelated primary melanomas. NCOA3 (also known as AIB1 or SRC-3) is a member of the steroid receptor coactivator-1 family. The NCOA3 gene maps at a region at 20q12 that is frequently amplified in human breast cancers,⁶ and has been shown to correlate with poor disease outcome in breast cancer.⁷ Increased copy number of 20q has been found in melanoma samples and cell lines.^{8,9} We thus hypothesized that primary melanomas expressing high levels of NCOA3 would exhibit an increased risk of metastasis and correspondingly reduced survival, yielding a molecular prognostic marker for melanoma. In this study, we analyzed the role of NCOA3 expression in predicting the outcome associated with melanoma in a tissue microarray (TMA) containing a large cohort of primary melanoma specimens.

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PATIENTS AND METHODS

Characterization and Construction of Melanoma TMA

We constructed a TMA of 343 primary melanomas with at least 2 years of follow-up, documented relapse, or having undergone SLN biopsy. The demographic breakdown of the cohort is listed in Table 1. Of the 343 patients, 259 had undergone SLN biopsy, thus providing information regarding SLN status. The criteria for undergoing SLN biopsy include the following: melanoma 1.0 mm or more in thickness, or presence of any of the following histologic factors in melanomas under 1.0 mm thick: Clark level IV or V, ulceration, vascular involvement, microsatellites, extensive regression (covering greater than 50% of the diameter of the tumor), or inadequate biopsy (partial biopsy showing melanoma transected at the base). The mean follow-up of this cohort was 49 months, with a median follow-up of 45 months. This molecular prognostic factor analysis was approved by the Committee on Human Research. The TMAs were constructed as previously described¹⁰ by taking 1.0 mm in diameter tissue cores from the paraffin block.^{10,11}

Immunohistochemistry

Slides were deparaffinized and rehydrated in xylene, then microwaved in 10 mmol/L citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and the slides sequentially incubated with Avidin and Biotin blocking reagents. The primary antibody, mouse monoclonal anti-NCOA3 IgG (Abcam #ab14139; Abcam, Cambridge, MA) was then added at a 1:10 dilution and incubated for 60 minutes at room temperature. Biotinylated horse antimouse IgG antibody (Vector Laboratories, Burlingame, CA) was used as a secondary antibody for amplification, followed by incubation with ABC-HRP (Vector Laboratories) for 30 minutes, and diaminobenzidine (DAB)/hydrogen peroxide solution (Sigma; St Louis, MO).

Evaluation of Immunohistochemical Staining

The regions of most intense staining were scored for each tissue array core. Expression of NCOA3 protein was graded using the following scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, intense staining. The arrays were scored by a pathologist blinded to the identity of the cases (R.W.S.), and each score was replicated by a separate, independent scoring trial. A consensus score was determined for the few instances of discrepant scoring across replicated trials. Specificity controls for NCOA3 staining included breast tumor, melanoma cell lines (LOX and FEM), and melanoma tissue sections. Positive controls included breast tumor sections, as well as LOX and FEM cells, while

negative controls included tonsil tissue and the breast carcinoma cell line T47D. The negative control used for immunohistochemistry included the use of phosphate buffered saline instead of primary antibody, with all other experimental conditions kept constant.

Statistical Analyses

Statistical methods used to assess the significance of various prognostic factors on melanoma outcome were previously described.^{10,12,13} For both RFS and DSS, the definition of high NCOA3 scores (defined as a score of 2 or 3) was originally selected on the basis of the best cut offs for predictive value of DSS on Kaplan-Meier analysis, and the same cut offs were uniformly and consistently used in all subsequent univariate and multivariate analyses of RFS and DSS. The association between high NCOA3 expression and RFS or DSS was assessed using the Fisher's exact test and both univariate and multivariate Cox regression. For SLN status, the best cutoffs were determined to be a score of 0 versus 1 or 2 versus 3 on the basis of the results of univariate logistic regression analysis, and the same cutoffs were uniformly and consistently used for all subsequent analyses examining SLN status. The association between increasing NCOA3 expression and SLN metastasis was assessed using χ^2 analysis and both univariate and multivariate logistic regression. The association between increasing NCOA3 expression and mean SLN count was assessed using the analysis of variance (ANOVA) and the directional Le test. With the exception of this directional analysis, all *P* values reported are two sided. In addition to the prognostic factors analyzed by the American Joint Committee on Cancer (AJCC), the following factors were included in the data set: mitotic rate, tumor vascularity, presence or absence of microsatellites, vascular involvement, and regression. The coding for clinical or pathologic attributes was performed as previously described.¹⁰

RESULTS

Given our cDNA microarray results suggesting differential expression of the NCOA3 gene in metastatic melanomas when compared with unrelated primary tumors, we aimed to examine the prognostic impact of NCOA3 expression at the protein level in a TMA containing 343 primary melanoma cores. NCOA3 expression was analyzed using a commercially available monoclonal antibody targeting human NCOA3, and scored by an observer blinded to patient outcomes.

NCOA3 expression was absent in 20 (5.8%; Fig 1A) and intense (score of 3) in 111 (32.4%; Fig 1B) of the 343 cores examined. Expression of NCOA3 did not significantly correlate with histologic subtype of melanoma (data not shown), with the possible exception of desmoplastic melanomas, in which only one primary tumor exhibited intense staining. In addition, NCOA3 expression did not correlate with several known histologic prognostic factors for melanoma, such as tumor thickness, ulceration, Clark level, mitotic rate, vascular involvement, microsatellites, or tumor vascularity (data not shown).

First, we analyzed the association between NCOA3 expression and melanoma outcome by univariate analysis. High NCOA3 expression (defined as a score of 2 or 3) significantly increased the risk of melanoma relapse (52.2% v 35.9%; *P* = .010, Fisher's exact test) and reduced the RFS of melanoma patients in this cohort when analyzed by Kaplan-Meier analysis (*P* = .021, log-rank test; Fig 2A). High NCOA3 expression was associated with increased risk of death due to melanoma (31.9% v 18.5%; *P* = .021, Fisher's exact test) and reduced DSS by Kaplan-Meier analysis (*P* = .030, log-rank test; Fig 2B).

In addition to risk of relapse and death, increasing NCOA3 expression correlated significantly with positive SLN status, a measure of micrometastasis to the regional nodal basin, by logistic regression analysis (*P* = .013). Patients with an NCOA3 staining

Table 1. Summary of Patient Demographics

Demographic	No.	%
Age, years		
Median	53	
Sex		
Male	227	66.2
Anatomical location		
Head and neck	60	17.5
Trunk	137	39.9
Extremity	146	42.6
Histologic tumor type		
Superficial spreading melanoma	159	46.4
Nodular melanoma	121	35.3
Acral melanoma	19	5.5
Lentigo maligna melanoma	11	3.2
Desmoplastic melanoma	9	2.6
Melanoma not otherwise classified	24	7.0
Tumor thickness, mm		
T1 (\leq 1.0)	22	6.4
T2 (1.01-2.0)	110	32.1
T3 (2.01-4.0)	97	28.3
T4 ($>$ 4.0)	114	33.2

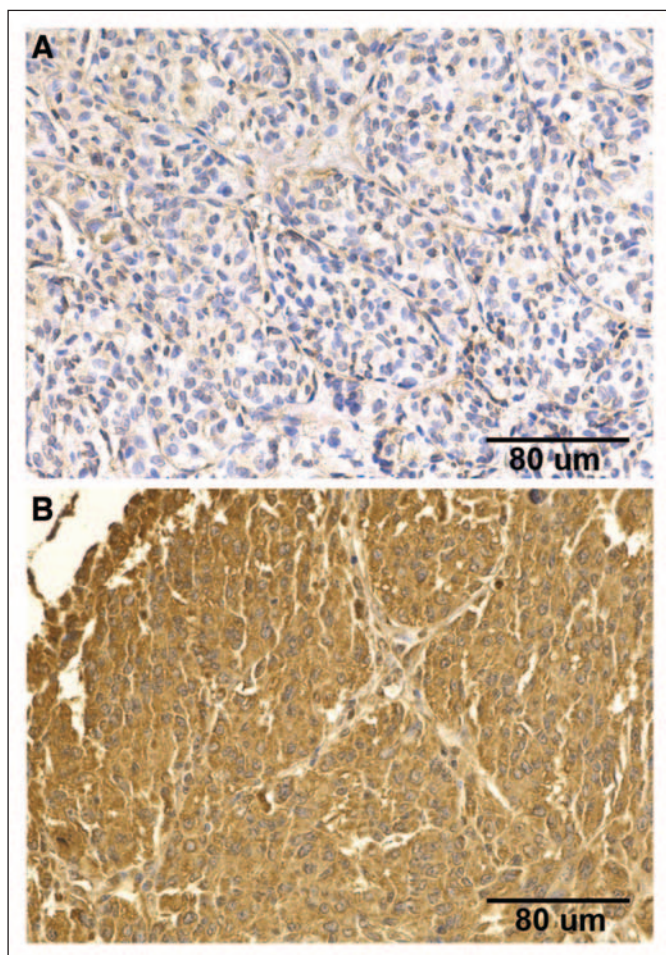


Fig 1. Photomicrographs of primary melanoma demonstrating (A) absent and (B) intense nuclear receptor coactivator-3 immunostaining.

score of 0 had a 7.1% prevalence of SLN positivity, which increased to 27.4% in patients with a score of 1 or 2, and 38.3% in patients with a score of 3 ($P = .036$, χ^2 test). Intriguingly, level of NCOA3 expression also correlated with SLN tumor burden as measured by mean number of SLNs involved. Thus, in patients with an NCOA3 staining score of 0, the mean number of nodes involved was 0.07. This increased to 0.47 nodes in patients with a score of 1 or 2, and 0.57 nodes in patients with a score of 3 (ANOVA $P = .0004$; Le directional text $P = .030$).

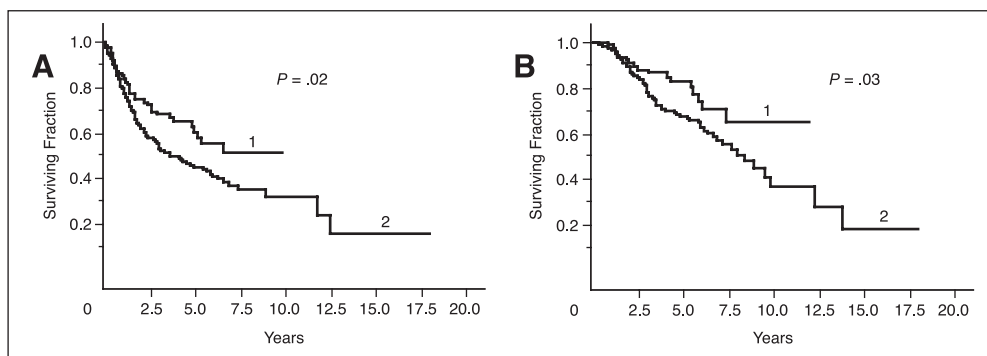


Fig 2. Kaplan-Meier analysis of (A) relapse-free survival and (B) disease-specific survival according to nuclear receptor coactivator-3 expression level.

Next, we examined the impact of NCOA3 expression on melanoma outcome by multivariate analysis. Multivariate Cox regression analysis was performed, including NCOA3 status and six clinical and histologic prognostic factors evaluated by the AJCC staging committee for melanoma, including tumor thickness and ulceration, the factors currently used in the AJCC staging classification for cutaneous melanoma.^{3,14} This analysis revealed NCOA3 status to be an independent predictor of both RFS (Table 2) and DSS (Table 3). In the Cox regression analysis of DSS, NCOA3 emerged as the most powerful factor determining survival, surpassing both tumor thickness and ulceration. Subsequently, multivariate logistic regression analysis demonstrated NCOA3 expression to be an independent predictor of SLN status when the six other prognostic factors were included in the model (Table 4).

Finally, we aimed to assess the predictive value of various prognostic factors when all 12 factors included in the data set (and available for analysis) were entered in the multivariate models. In addition to the seven factors mentioned previously, this analysis included mitotic rate, degree of tumor vascularity, as well as presence or absence of vascular involvement, microsatellites, and regression. NCOA3 overexpression remained significantly predictive of DSS and SLN status (data not shown), when all 12 factors were included in the multivariate models.

DISCUSSION

Gene expression profiling has shown tremendous promise to revolutionize current approaches to cancer classification and prognosis. However, significant challenges have prevented the validation of gene expression signatures into clinically useful biomarkers. Previous gene expression profiling analyses of primary and metastatic melanomas have identified a plethora of candidate melanoma progression genes and markers.^{5,15,16} In one recent analysis, expression of several of these candidate markers was shown to correlate with reduced overall survival.¹⁶ However, few studies have demonstrated the independent prognostic impact of a molecular factor when complete clinical and histologic information is included in multivariate models.

NCOA3 was identified in our gene expression profiling study as overexpressed in melanoma metastases when compared with primary tumors,⁵ suggesting its possible role as a marker of melanoma prognosis. No prior analysis of NCOA3 protein expression in melanoma has been reported to date. In this study, we show that NCOA3 overexpression in primary cutaneous melanoma correlates significantly

Table 2. Cox Regression Analysis of Impact of Clinical, Histologic, and Molecular Factors on RFS of Melanoma Cohort

Prognostic Factor	Risk Ratio	χ^2	<i>P</i> (two-tailed)
Clark level	2.22	16.77	< .00005
Ulceration	1.94	13.86	.0002
NCOA3 level (2 or 3 v 0 or 1)	1.69	6.72	.0095
Tumor thickness	1.27	5.28	.022
Site	1.39	3.61	.057
Age	1.03	0.22	.64
Sex	1.04	0.04	.85

Abbreviations: RFS, relapse-free survival; NCOA3, nuclear receptor coactivator-3.

with melanoma relapse and disease-specific death. NCOA3 expression was significantly predictive of RFS and DSS of this cohort when the six prognostic factors analyzed by the AJCC staging committee were included in the multivariate models. Importantly, NCOA3 expression outperformed tumor thickness in each of these analyses, and emerged as the most powerful factor predicting DSS. Since the publication of the revised AJCC staging classification for melanoma,¹⁴ few molecular prognostic factors have been shown to be of independent prognostic significance when the six factors analyzed by the AJCC staging committee have been included in multivariate models, highlighting the potential significance of the findings correlating NCOA3 expression and outcome associated with melanoma.

In addition, NCOA3 status continued to provide independent prognostic information regarding DSS and SLN status when all 12 available prognostic factors were included in the model. This analysis included factors such as mitotic rate and vascular involvement, which have been shown in various analyses to be more powerful than ulceration.^{12,17} Taken together, these results suggest the utility of NCOA3 as a novel, independent molecular marker of melanoma outcome, with a significant impact on important outcome measures for melanoma. While these results need to be confirmed by other investigators in a multicenter setting, our data strongly suggest the inclusion of NCOA3 for further exploration as a molecular marker for melanoma.

Intriguingly, the metastases examined in the gene expression profiling study that overexpressed NCOA3 were primarily lymph node metastases, suggesting the potential importance of NCOA3 expression to melanoma lymph node metastasis. The results presented herein corroborate this hypothesis by demonstrating that increased NCOA3 expression was an independent predictor of SLN status,

Table 3. Cox Regression Analysis of Impact of Clinical, Histologic, and Molecular Factors on DSS of Melanoma Cohort

Prognostic Factor	Risk Ratio	χ^2	<i>P</i> (two tailed)
NCOA3 level (2 or 3 v 0 or 1)	1.91	5.29	.021
Clark level	1.75	5.00	.025
Ulceration	1.65	4.89	.027
Tumor thickness	1.34	4.69	.030
Age	1.09	1.65	.20
Site	1.41	2.27	.13
Sex	1.00	0.0002	.99

Abbreviations: DSS, disease-specific survival; NCOA3, nuclear receptor coactivator-3.

Table 4. Logistic Regression Analysis of Impact of Clinical, Histologic, and Molecular Factors on SLN Metastasis

Prognostic Factor	χ^2	<i>P</i> (two-tailed)
Decreasing age	12.92	.0003
Tumor thickness	8.10	.0044
NCOA3 expression (3 v 1 or 2 v 0)	5.70	.017
Clark level	5.14	.023
Sex	1.43	.23
Ulceration	0.79	.37
Site	0.09	.76

Abbreviations: SLN, sentinel lymph node; NCOA3, nuclear receptor coactivator-3.

which has emerged both as a standard technique to evaluate regional lymph node metastasis and as an important predictor of melanoma outcome.¹⁸⁻²⁰ Not only did high NCOA3 expression correlate with a significantly increased risk of SLN metastasis, increasing NCOA3 expression was shown to significantly impact SLN tumor burden, further strengthening this association between NCOA3 status and regional lymph node metastasis. To our knowledge, these studies are the first to identify a molecular marker expressed in melanoma cells that predicts SLN metastasis and tumor burden, suggesting its potential utility in identifying candidates to undergo this procedure.

Consistent with the strong correlation between NCOA3 expression and SLN status was the observation that high NCOA3 expression was rarely seen in the small subset of desmoplastic melanomas included in this analysis. Spindle cell melanomas in general, and desmoplastic melanomas in particular, have been shown to metastasize infrequently to SLNs,^{21,22} suggesting that the molecular program for lymph node metastasis may be lacking in this tumor subtype. Therefore, which patients with desmoplastic melanomas should undergo SLN biopsy and whether they ought to is controversial. Given its correlation with SLN metastasis, it would be intriguing to examine NCOA3 expression in a larger cohort of desmoplastic melanomas undergoing SLN biopsy for its possible utility in this problematic patient population.

Given the predominance of patients undergoing SLN biopsy in this data set, it is possible that some of the results reported herein may have been skewed by selection bias. Controversy still exists as to the selection of patients undergoing SLN biopsy in the setting of thin (< 1.0 mm) and thick (> 4.0 mm) tumors, and particular histologic subtypes, such as desmoplastic melanoma. Thus, the selection criteria used to recommend SLN biopsy may have influenced the reported outcome data. As a result, the significance of NCOA3 overexpression may be most relevant to patients undergoing SLN biopsy. Our results await confirmation in cohorts that do not undergo SLN biopsy.

Finally, these results suggest the potentially broad-based significance of NCOA3 as a prognostic marker in cancer given its apparent importance in hormone-sensitive and hormone-independent malignancies. In the setting of breast cancer, NCOA3 overexpression has been linked to tamoxifen resistance.²³ Given the limited therapeutic utility of tamoxifen in melanoma,²⁴ further studies will be required to elucidate the mechanism(s) by which NCOA3 promotes tumor progression in melanoma. Intriguingly, NCOA3 has been shown to be present in a complex containing inhibitor of kappa B kinase, and to enhance nuclear factor kappa B-mediated gene expression.²⁵ Given the known importance of nuclear factor

kappa B to melanoma progression,^{10,26,27} these observations suggest a potential pathway by which NCOA3 may contribute to melanoma progression and metastasis.

In conclusion, our results show a significant correlation between NCOA3 overexpression and increased risk of relapse and

reduced survival associated with melanoma. In addition, they reveal NCOA3 expression to be independently predictive of several measures of melanoma outcome, including DSS, RFS, and SLN status, suggesting NCOA3 as a novel prognostic marker for primary cutaneous melanoma.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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