Identifying High-Risk Tumors within AJCC Stage IB–III Melanomas Using a Seven-Marker Immunohistochemical Signature

Robin Reschke †, Philipp Gussek † and Mirjana Ziemer *

Department of Dermatology, Allergology and Venereology, University Medical Center, 04103 Leipzig, Germany; robin.reschke@medizin.uni-leipzig.de (R.R.); philipp.gussek@medizin.uni-leipzig.de (P.G.)
* Correspondence: mirjana.ziemer@medizin.uni-leipzig.de
† These authors contributed equally.

Simple Summary: Immunotherapy and targeted therapy are widely accepted for stage III and IV melanoma patients. Clinical investigation of adjuvant therapy in stage II melanoma has already started. Therefore, methods for relapse prediction in lower stage melanoma patients apart from sentinel node biopsies are much needed to guide (neo)adjuvant therapies. Gene scores such as the “DecisionDx-Melanoma” and the “MelaGenix” score can help assist therapy decisions. However, a seven-marker immunohistochemical signature could add valuable feasibility to the biomarker toolbox.

Abstract: Background: We aim to validate a seven-marker immunohistochemical signature, consisting of Bax, Bcl-X, PTEN, COX-2, (loss of) β-Catenin, (loss of) MTAP and (presence of) CD20, in an independent patient cohort and test clinical feasibility. Methods: We performed staining of the mentioned antibodies in tissue of 88 primary melanomas and calculated a risk score for each patient. Data were correlated with clinical parameters and outcome (recurrence-free, distant metastasis-free and melanoma-specific survival). Results: The seven-marker signature was able to identify high-risk patients within stages IB-III melanoma patients that have a significantly higher risk of disease recurrence, metastasis, and death. In particular, the high sensitivity of relapse prediction (>94%) in sentinel negative patients (stages IB–IIC) was striking (negative predictive value of 100% for melanoma-specific survival and distant metastasis-free survival, and 97.5% for relapse-free survival). For stage III patients (positive nodal status), the negative predictive value was 100% with the seven-marker signature. Conclusions: The seven-marker signature can help to further select high-risk patients in stages IIB-C but also in earlier stages IB-IIA and be a useful tool for therapy decisions in the adjuvant and future neo-adjuvant settings. Stage III patients with measurable lymph node disease classified as high-risk with the seven-marker signature are potential candidates for neoadjuvant immunotherapy.

Keywords: biomarker; relapse; immunotherapy; targeted therapy; melanoma

1. Introduction

The incidence of cutaneous melanoma is rising worldwide [1]. The risk of disease recurrence increases with tumor thickness. For patients with a Breslow thickness of 1 mm (or 0.8 mm and other risk factors such as ulceration, increased mitotic rate or younger age) a sentinel node biopsy for risk stratification and decision of adjuvant therapy should be performed [2,3]. Adjuvant treatment with novel therapies has been shown to significantly prolong the relapse-free survival (RFS) and overall survival (OS) in stage III melanoma [4–9]. There are ongoing and planned clinical trials addressing the benefits of novel adjuvant therapy for stage II melanoma patients [10]. The 5-year melanoma-specific survival (MSS) in patients with stages IIB/IIC is, surprisingly, worse than in patients with stages IIIA/IIIB melanoma (87%/82% vs. 93%/83%) [11]. Therefore, it is argued that stages...
IIB and IIC should also be considered for adjuvant therapy [12]. Staging according to American Joint Committee on Cancer 8th edition (AJCCv8) might not be sufficient to classify risk subgroups. For stage I and II melanoma, 5-year MSS rates of 97% and 94% were reported [11]. Nonetheless, a significant number of patients die from thin melanomas (<1 mm, IB). This suggests that for a high-risk subgroup in stages I–IIA, adjuvant therapy would also be reasonable [13]. Moreover, a worse outcome for patients with recurrent melanoma after negative sentinel lymph node biopsy was documented compared to sentinel-positive patients [14,15]. Therefore, further prognostic biomarkers must be investigated and validated to reliably identify high-risk patients, especially within lower AJCCv8 melanoma stages such as IB–IIA [16]. We tested and validated the immunohistochemical seven-marker signature consisting of five risk markers (Bax, Bcl-X, COX-2, PTEN, presence of CD20 positive B-lymphocytes) and two protective markers (loss of MTAP and β-Catenin) for clinical (routine) use in our patient cohort. The signature was found to be an independent negative predictor for OS and RFS and could separate patients into low-risk and high-risk groups [17].

2. Materials and Methods

2.1. Patient Cohort

Consecutive patients diagnosed with stages IB-III cutaneous melanoma and resected at the university hospital Leipzig between August 2006 and January 2015 were included retrospectively in this study. From a study collective of 299 patients, 88 patients with available formalin-fixed paraffin-embedded (FFPE) primary melanomas were investigated (Figure 1). The clinicopathological characteristics of those patients are given in Table 1.

![Flow chart depicting the workflow from initial sample collection until generation of final study cohort, staining and statistical evaluation of the 7-marker signature.](image-url)
Table 1. Patient and clinicopathological characteristics of the low-risk and high-risk group (all, stage IB–IIIC).

| Variable                        | Category                  | Overall Patients (n = 88) | Low-Risk Patients (n = 40) | High-Risk Patients (n = 48) | p-Value |
|---------------------------------|---------------------------|--------------------------|----------------------------|-----------------------------|---------|
| Follow-up time (months)         | Mean (SD)                 | 94.9 (28.5)              | 73.1 (33)                  | 0.0013                      |
|                                 | Median (min, max)         | 93.5 (60–150)            | 71 (3–136)                 |                             |
| Gender                          | Male                      | 48 (54.6%)               | 21 (23.9%)                 | 27 (30.7%)                  | 0.8304  |
|                                 | Female                    | 40 (45.5%)               | 19 (21.6%)                 | 21 (23.9%)                  |         |
| Age (years)                     | Mean (SD)                 | 63.4 (17.6)              | 64.1 (16.2)                | 0.8556                      |
|                                 | Median (min, max)         | 69.5 (17–90)             | 66 (27–93)                 |                             |
| Tumor thickness                 | Mean (SD)                 | 2.6 (1.7)                | 3.9 (3)                    | 0.012                       |
|                                 | Median (min, max)         | 2.1 (1.1–8)              | 3 (1–14)                   |                             |
| Ulceration                      | Yes                       | 41 (46.6%)               | 14 (15.9%)                 | 27 (30.7%)                  | 0.0557  |
|                                 | No                        | 47 (53.4%)               | 26 (29.5%)                 | 21 (23.9%)                  |         |
| SLN status                      | Positive 1                | 19 (21.6%)               | 4 (4.5%)                   | 15 (17%)                    | n.c.    |
|                                 | Negative 0                | 69 (78.4%)               | 36 (40.9%)                 | 33 (37.5%)                  |         |
|                                 | -cN0                      | 6 (6.8%)                 | 3 (3.4%)                   | 3 (3.4%)                    |         |
|                                 | -pN0                      | 63 (71.6%)               | 33 (37.5%)                 | 30 (34.1%)                  |         |
| pTNM (AJCC8)                    | IB                        | 24 (27.3%)               | 16 (18.2%)                 | 8 (9.1%)                    | n.c.    |
|                                 | IIA                       | 16 (18.2%)               | 5 (5.7%)                   | 11 (12.5%)                  |         |
|                                 | IIB                       | 20 (22.7%)               | 13 (14.8%)                 | 7 (8%)                      |         |
|                                 | IIC                       | 9 (10.2%)                | 2 (2.3%)                   | 7 (8%)                      |         |
|                                 | IIIA                      | 1 (1.1%)                 | 1 (1.1%)                   | 0 (0%)                      |         |
|                                 | IIIB                      | 5 (5.7%)                 | 1 (1.1%)                   | 4 (4.5%)                    |         |
|                                 | IICC                      | 13 (14.8%)               | 2 (2.3%)                   | 11 (12.5%)                  |         |

*p-value: Fisher’s exact test for categorical variables; t-test for continuous variables. In case of less than 5 patients in the subgroups of the risk group by categorical variable, Fisher’s exact test could not be calculated (n.c.). Significant p-values are bold.

2.2. Immunohistochemical Expression Analysis

In this analysis, whole S-100 positive melanoma FFPE tissue sections were used. The 7-marker signature (immunoprint®) determination and scoring was previously described in detail [17]. The analysis was conducted in cooperation with Synvie GmbH, Munich, Germany. The stained slides were evaluated by an external dermatohistopathologist (AG) and two experienced Synvie lab scientists (SS and PN) in a blinded manner. In case of discordant scoring results, a consensus score was assigned. Immunoreactivity was evaluated using an algorithm resulting in a stepwise scoring system (0 to 3+; 0 (negative): 0% positive cells, 3+: greater than 50% positive cells) [17].

2.3. Calculation of the 7-Marker-Signature Risk Score

The 7-marker signature risk score was calculated as previously described by a linear combination of the marker coefficient and the corresponding IHC measurements normalized by the number of markers measured [17]. Based on this risk score, patients were assigned to a high-risk group and a low-risk group using the originally described cut-off of 0.135. Risk score values < 0.135 are considered low-risk; values > 0.135 represent high-risk status (Supplementary Figures S1–S5).
2.4. Statistical Analysis

Statistical analyses were conducted by Staburo GmbH, Munich, which matched the Synvie lab risk score results with the survival information provided from the Clinical Cancer Registry of Leipzig. Using R statistical software version 4.0.4. *p*-values lower than 0.05 were considered to indicate statistical significance. Appropriate statistical tests (e.g., Fisher’s exact test and *t*-test) were used to analyze differences between high-risk and low-risk study groups. Hazard ratio (HR) with 95% confidence interval (CI) were calculated using Cox regression analysis. For the MSS calculation, only melanoma-related deaths were considered. RFS events included all types of events including local (lymph node, satellite, and in-transit) metastasis, distant metastasis (separately documented for distant metastasis-free survival (DMFS) and MSS events. Kaplan–Meier analyses were performed in order to estimate survival probabilities of the high-and low-risk group. The data-cut-off for survival was June 2019. Patients who died after the 5-year follow-up of unknown causes were censored at date of death for MSS, DMFS and RFS. Subsequently, log-rank tests were used to compare survival rates. In order to analyze further correlations between variables, univariate and multivariable cox regression were used. Univariate analyses were conducted for the following variables: tumor thickness, ulceration, nodal status, gender, and age. In case of significant values in univariate analyses, a multivariable analysis was performed for risk models including previously found risk factors with the strongest impact on melanoma outcome (tumor thickness, nodal status, ulceration) with and without the 7-marker signature.

2.5. REMARK

The results of our retrospective study were reported according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines [18].

3. Results

By using the signature’s cut-off, from the 88 patients, 40 patients were characterized as low-risk and 48 as high-risk (Figure 2, Supplementary Table S1).

![7-Marker Signature IHC Expression Profiles from 88 patients](image)

**Figure 2.** 7-marker signature immunohistochemistry (IHC) expression profile. The figure shows the IHC expression profile of all tumor specimens from the cohort ordered by their predicted risk score. Each column represents an individual patient consisting of the expression values of the 7-marker signature (5 risk markers and 2 protective markers). The corresponding risk score is plotted for the 40 low-risk patients (green) and the 48 high-risk patients (red). The horizontal line (at 0.135) corresponds to the signature’s cut-off value that separates the groups. The IHC expression values are scaled between 0 (light blue) and 3 (dark blue) according to the used stepwise scoring system (the expression value of 4—as originally described—did not occur in this particular cohort).

Almost half of our patients had stages IB–IIA melanomas (*n* = 40). Of these, 8/24 stage IB (33%) and 11/16 stage IIA (9%) were classified as high-risk. In stage IIB, 7/20 melanomas (35%), and in stage IIC, 7/9 (78%) melanomas qualified as high-risk. The only stage IIIA melanoma was stratified as low-risk. Four of five stage IIB melanomas presented as high-risk (80%). Eleven of 13 melanomas of stage IIIIC patients were labeled...
high-risk (85%). Twenty-one of 47 melanomas without ulceration (45%) qualified as high-risk, whereas 27 of 41 melanomas with ulceration (66%) were stratified as high-risk ($p$-value 0.0557). An equal distribution of low- and high-risk ($n = 36$ vs. $n = 33$) was found amongst patients with stages IB–IIC negative nodal status. However, of patients with positive nodal status ($n = 19$), almost four times more melanomas were classified as high-risk ($n = 15$ vs. $n = 4$ low-risk) (Supplementary Figures S6–S8). Results showed that higher values of the seven-marker signature score were significantly associated with shorter MSS in a univariate Cox analysis (HR = 2.049; 95% CI = 1.212 to 3.463; $p$ = 0.0074). Furthermore, tumor thickness and positive nodal status were significant risk factors of MSS ($p = 0.0011$ and $p = 0.0027$), but ulceration was not. In a multivariable Cox regression model including these prognostic factors/variables (and ulceration) for MSS (Table 2), only tumor thickness remained significant with a $p < 0.05$ (HR = 1.208; 95% CI 1.045 to 1.398 $p = 0.0108$). The $p$-value for the seven-marker signature resulted in $p = 0.0600$ and failed significance by a narrow margin. In a multivariable analysis for DMFS, the seven-marker signature remained significant beside tumor thickness (HR = 1.808; 95% CI = 1.159 to 2.819; $p < 0.0090$) (Supplementary Table S2). Both the numerical and the dichotomous risk score was utilized for Cox regression analyses with the endpoint RFS. Univariate Cox regression showed that seven-marker signature, tumor thickness and nodal status were associated with recurrence (all HR $\geq 1.25$; all $p < 0.0005$). A multivariable Cox regression using the dichotomous risk score (Table 2) demonstrated that besides nodal status and tumor thickness, the seven-marker signature was a significant independent risk factor for relapse (HR = 30.604 95% CI 4.112 to 228.757; $p = 0.0008$). Receiver operating characteristic (ROC) curves for the risk score and the reference model could be calculated for the endpoints MSS and RFS (Figure 3).

**Figure 3.** ROC (receiver operating characteristics) curves for the two prognostic models (reference vs. seven-marker signature model) with the endpoints MSS (A) and RFS (B). The plots illustrate varying prognostic abilities, i.e., sensitivity and specificity, for the seven-marker signature and conventional prognostic factors, such as tumor thickness, ulceration, and nodal status. As a measure of quality comparing the different models, area under the curve (AUC) can be calculated. A (endpoint MSS): with an AUC of 70.9% the model including the seven-marker signature shows superiority over the reference model (AUC = 62.8%). B (endpoint RFS): the model including the seven-marker signature showed a higher AUC (76.4% vs. 64.7%).
### Table 2. Univariate and multivariable Cox regression analysis.

| Patients/Cohort | Variables/Prognostic Factors | Univariate | Multivariable |
|-----------------|-----------------------------|------------|--------------|
| Entire cohort   |                             |            |              |
| (n = 88)        |                             |            |              |
| (Events: MSS: 16 | 2.05 (1.21–3.46) | 0.007      | 1.73 (0.98–3.05) | 0.060 |
| Nodal status    | 4.51 (1.68–12.07) | 0.003      | 2.66 (0.91–7.77) | 0.074 |
| Age at diagnosis| 1.00 (0.97–1.03) | 0.880      | —            | —    |
| Gender          | 1.01 (0.38–2.71) | 0.988      | —            | —    |
| RFS: 32)        |                             |            |              |
| (n = 88)        |                             |            |              |
| (Events: RFS: 32) | 38.68 (5.27–284.15) | <0.001     | 30.60 (4.11–227.76) | <0.001 |
| Tumor thickness (mm) | 1.25 (1.13–1.38) | <0.001     | 1.14 (1.02–1.27) | 0.022 |
| Ulceration      | 2.03 (1.00–4.12) | 0.051      | 1.11 (0.51–2.42) | 0.792 |
| Nodal status    | 3.79 (1.86–7.71) | <0.001     | 2.15 (1.01–4.56) | 0.046 |
| Age at diagnosis| 1.00 (0.98–1.03) | 0.737      | —            | —    |
| Gender          | 0.60 (0.29–1.24) | 0.167      | —            | —    |

HR—Hazard Ratio, significant p-values are bold; 7 mrs—seven-marker signature risk score: high-risk or low-risk; nodal status—N ≥ 1a; gender—male or female. * Due to the absence of MSS events in the low-risk group, the numerical risk score variable (continuous variable) was used for Cox regression analysis (instead of the dichotomous risk score variable). Since the small scale of the metric risk score leads to very high Hazard ratio values, the score was transformed linearly (multiplied by 10). The interpretation of HR for risk score now does not refer to the increase in the score by 1, but by 0.1. For more detailed results of Cox regression analysis, see also Supplementary Table S2.

The model for MSS extended by the seven-marker signature proved to be superior compared to the reference model in terms of area under the curve (AUC) (70.9% vs. 62.8%) (Figure 3A). ROC curves with the endpoint RFS (using the dichotomous risk score) also showed higher values for the seven-marker signature model in AUC (76.4% vs. 64.7%) (Figure 3B). For survival estimation/comparison of patients with low- and high-risk seven-marker signature score, Kaplan–Meier (KM) curves were calculated for the endpoints MSS, DMFS, and RFS. As depicted in the respective KM-plots (Figure 4), a high-risk seven-marker signature score led to a significantly lower survival probability in all endpoints—MSS, DMFS, and RFS (at five years, 84%, 63% and 63% for high-risk, and at ten years, 77.2%, 63.2% and 44.2% vs. 100% for low-risk; p < 0.0001). In the low-risk group (40 patients), only one locoregional relapse occurred. In sum, in the high-risk group all events but one were predicted correctly by the seven-marker signature. In this cohort, the seven-marker signature had a sensitivity and negative predictive value (NPV) of 100% for MSS and DMFS, and 97% and 97.5%, for RFS, respectively. Of 48 patients with a high-risk seven-marker signature score, 16 had an MSS, 25 a DMFS, and 32 an RFS event. This results in a specificity and a positive predictive value (PPV) for MSS of 55.6% and 33.3%, for DMFS of 63.5% and 52.1%, and for RFS of 70.9% and 66.7%. Thus, approximately two-thirds of the patients with a high-risk seven-marker signature, but only one patient with a low-risk seven-marker signature, had an event. To evaluate patients with low(er) risk for metastasis and disease recurrence according to AJCCv8 staging, we performed separate KM analyses of 40 IB–IIA patients (Figure 5, Supplementary Table S3). Approximately half of the patients (n = 19) showed a high-risk result. For all endpoints, MSS, DMFS and RFS, there was a statistically significant difference in survival of patients with low- and high-risk signature (p = 0.029; p = 0.0023; p = 0.00035). Of the 19 high-risk patients, four had an MSS, seven a DMFS and nine patients an RFS event. All events were correctly indicated by a high-risk score of the seven-marker signature at the same time that no patient with a low-risk score had an event (sensitivity and NPV = 100%) (Table 3). To evaluate the benefit of the seven-marker signature for patients stratified by nodal status, we conducted KM analyses by patient subgroups with negative and positive nodal status. The KM analysis of the nodal status negative patients (Figure 6, Supplementary Table S4) yielded significant differences for survival in the high- vs. the low-risk group for the endpoints MSS, DMFS and RFS (p = 0.0029; p < 0.0001; p < 0.0001). Of 69 patients with negative nodal status—N ≥ 1a; gender—male or female.
status, 33 were stratified as high-risk and 36 as low-risk according to the seven-marker signature. Among the high-risk patients, MSS events occurred in eight patients, DMFS events in 14 patients and RFS events in 18 patients. In the low-risk group, one patient had an RFS event (sensitivity/NPV: 94.7%/97.2%), but none had a DMFS or MSS event (sensitivity/NPV: 100%/100%). The highest specificity (70%) of the seven-marker signature was reached for the endpoint RFS among patients with negative nodal status. In contrast, KM-plots for patients with positive nodal status (Figure 7, Supplementary Table S5) showed significantly different survival probabilities for DMFS and RFS \( (p = 0.02; p = 0.01) \), but not for MSS \( (p = 0.11) \). Again, the seven-marker signature could predict all events (MSS, DMFS and RFS). Of 15 high-risk patients with positive nodal status (stage IIIA-IIIC), eight had an MSS, eleven a DMFS and thirteen an RFS event. In the four low-risk patients, no melanoma-specific event occurred. Considering all KM analyses of all subgroups with the endpoint RFS among patients with positive nodal status, the highest test quality values were achieved with 66.7% and 86.7% for specificity and PPV, and 100% for both sensitivity and NPV.

Table 3. The event table shows numbers of patients at risk and the distribution of events in the low-risk and high-risk groups with the resulting predictive power of stratification by the seven-marker signature, indicated by sensitivity, specificity, PPV and NPV.

| Patients/Cohort | MSS |     |     |     | DMFS |     |     |     | RFS |     |     |
|----------------|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|
|                | High Risk | Low Risk | Total | High Risk | Low Risk | Total | High Risk | Low Risk | Total | High Risk | Low Risk | Total |
| Entire cohort  | 32  | 40  | 72   | 23  | 40  | 63   | 16  | 39  | 55   | 16  | 39  | 55   |
| (n = 88)       |     |     |       |     |     |       |     |     |       |     |     |       |
|                | Event | 16  | 0    | 16   | 25  | 0    | 25  | 32  | 1    | 33  |     |     |
|                | Total | 48  | 40   | 88   | 48  | 40   | 88  | 48  | 40   | 88  |     |     |
| Sensitivity    | 100% | PPV | 33.3% | 100% | PPV | 52.1% | 97% | PPV | 66.7% |     |     |     |
| Specificity    | 55.6% | NPV | 100% | 63.5% | NPV | 100% | 70.9% | NPV | 97.5% |     |     |     |
| Increase in risk | 183.3% | 183.3% |       |     |     |       |     |     |       |     |     |     |
| Stage IB–IIC   | 25  | 36  | 61   | 19  | 36  | 55   | 15  | 35  | 50   |     |     |     |
| (n = 69)       |     |     |       |     |     |       |     |     |       |     |     |     |
|                | Event | 8   | 0    | 8    | 14  | 0    | 14  | 18  | 1    | 19  |     |     |
|                | Total | 33  | 36   | 69   | 33  | 36   | 69  | 33  | 36   | 69  |     |     |
| Sensitivity    | 100% | PPV | 24.2% | 100% | PPV | 42.4% | 94.7% | PPV | 54.6% |     |     |     |
| Specificity    | 59%  | NPV | 100% | 65.5% | NPV | 100% | 70%  | NPV | 97.2% |     |     |     |
| Increase in risk | 209.1% | 209.1% |       |     |     |       |     |     |       |     |     |     |
| Stage IB–IIA   | 15  | 21  | 36   | 12  | 21  | 33   | 10  | 21  | 31   |     |     |     |
| (n = 40)       |     |     |       |     |     |       |     |     |       |     |     |     |
|                | Event | 4   | 0    | 4    | 7   | 0    | 7   | 9   | 0    | 9   |     |     |
|                | Total | 19  | 21   | 40   | 19  | 21   | 40  | 19  | 21   | 40  |     |     |
| Sensitivity    | 100% | PPV | 21.1% | 100% | PPV | 36.8% | 100% | PPV | 47.4% |     |     |     |
| Specificity    | 58.3% | NPV | 100% | 63.6% | NPV | 100% | 67.7% | NPV | 100% |     |     |     |
| Increase in risk | 210.5% | 210.5% |       |     |     |       |     |     |       |     |     |     |
| Stage III      | 7   | 4   | 11   | 4    | 4    | 8    | 2    | 4    | 6    |     |     |     |
| (n = 19)       |     |     |       |     |     |       |     |     |       |     |     |     |
|                | Event | 8   | 0    | 8    | 11   | 0    | 11  | 13   | 0    | 13  |     |     |
|                | Total | 15  | 4    | 19   | 15  | 4    | 19  | 15  | 4    | 19  |     |     |
| Sensitivity    | 100% | PPV | 53.3% | 100% | PPV | 73.3% | 100% | PPV | 86.7% |     |     |     |
| Specificity    | 36.4% | NPV | 100% | 50%  | NPV | 100% | 66.7% | NPV | 100% |     |     |     |
| Increase in risk | 126.7% | 130.1% |       |     |     |       |     |     |       |     |     |     |

Endpoints: melanoma-specific survival (MSS), distant metastasis-free survival (DMFS), relapse-free survival (RFS); positive predictive value (PPV), negative predictive value (NPV); High-risk, Low-risk.
Figure 4. Kaplan–Meier analysis of the entire cohort (n = 88 patients; AJCCv8 stages IB—IIIC) stratified with the seven-marker signature into high risk or low-risk. Estimates of event-free survival for the endpoints MSS, RFS and DMFS at 5- and 10-year follow-up are displayed for: high-risk (in red), low-risk (in green), and all patients (black). Corresponding tables with the number of patients at risk are given below.
Figure 5. Kaplan–Meier analysis of the sub-cohort with AJCCv8 stages IB—IIA stratified into high-risk or low risk-by the seven-marker signature. Survival estimates for the endpoints MSS, RFS, and DMFS at 5- and 10-year follow-up are displayed for: high-risk (in red), low-risk (in green), and all patients (black).
Figure 7. Kaplan–Meier survival estimates of patients with positive nodal status stratified into high-risk and low-risk by seven-marker signature. Endpoints were MSS, RFS, and DMFS at 5- and 10-year follow-up. High-risk (in red), low-risk (in green), and all patients (black). Corresponding tables with the number of patients at risk are given below.

Figure 6. Kaplan–Meier survival estimates of patients with negative nodal status stratified into high-risk and low-risk by the seven-marker signature. Endpoints were MSS, RFS, and DMFS at 5- and 10-year follow-up. High-risk (in red), low-risk (in green), and all patients (black). Corresponding tables with the number of patients at risk are given below.
Figure 6. Kaplan–Meier survival estimates of patients with negative nodal status stratified into high-risk and low-risk by the seven-marker signature. Endpoints were MSS, RFS, and DMFS at 5- and 10-year follow-up. High-risk (in red), low-risk (in green), and all patients (black). Corresponding tables with the number of patients at risk are given below.

Figure 7. Kaplan–Meier survival estimates of patients with positive nodal status stratified into high-risk and low-risk by seven-marker signature. Endpoints were MSS, RFS, DMFS at 5- and 10-year follow-up. High-risk (in red), low-risk (in green) according to the seven-marker signature, and all patients (black). Corresponding tables with the number of patients at risk are given below.
4. Discussion

The seven-marker signature was initially developed and validated in German melanoma cohorts of Regensburg and Hamburg to be an independent prognostic tool for shorter OS and RFS [17]. This study aimed to investigate (and validate) the seven-marker signature as a prognostic risk tool in an independent melanoma cohort from Leipzig. The seven-marker signature was examined in 88 stage I–III melanoma patients with complete clinicopathological and a minimum of 5-year follow-up data. Multivariable Cox regression analysis was performed to evaluate whether the seven-marker signature contributes prognostic information independent of tumor thickness, ulceration, lymph node status, age and gender. In the multivariable model, the seven-marker signature remained consistently significant for DMFS and RFS and thus was a statistically independent prognostic factor. The seven-marker signature indicated all MSS and DMFS events and all but one RFS event. The sensitivity for MSS, DMFS and RFS was 100%, 100% and 97.0%, respectively. A low-risk signature result could almost exclude subsequent recurrence and metastasis (NPV of 100% for MSS and DMFS, and 97.5% for RFS). Compared with larger cohorts in the literature, in terms of AJCCv8 stages, the cohort studied here has fewer stage IB patients, a relatively high proportion of stage II patients, and a comparable proportion of stage III patients [19–21]. The KM analyses of stages IB–IIA underline the predictive potential of the seven-marker signature for disease outcome. For AJCCv8 stages IB and IIA, high values for 5-year MSS of approximately 88–94% and RFS of 76–90% are reported in the literature [11,12,22]. In our study, the 5-year-survival differed significantly in low-risk and high-risk classified patients. For all endpoints (MSS, DMFS and RFS), 5-year survival was 100% in low-risk patients. MSS, DMFS and RFS were significantly lower in high-risk patients (at 5 years, 84%, 63% and 63%, and at 10 years, 77.2%, 63.2% and 44.2%). Accordingly, our results show even better discrimination of low-risk and high-risk patients compared with the seven-marker signature of the subgroup analysis (patients with <2 mm tumor thickness) in the discovery cohort, in which a 5-year OS and RFS of 91% and 92% for low risk and 79% and 57% for high-risk were found [17]. The majority of patients with thin melanoma (stages IA, IB, and IIA) have a 10-year MSS of 98%, 94%, and 88%, respectively. Despite the relatively lower intrinsic mortality risk, the overall number of deaths from thin melanomas is similar or even higher than from thicker melanomas or melanomas diagnosed at higher stages (>stage IIA) due to their high incidence [13,23]. This highlights the importance of identifying high-risk patients especially in lower stages for follow-up care optimization and reasonable adjuvant therapy [24]. Thus, a study focusing on stage IB melanomas showed that computer-assisted multidimensional primary tumor measurements may be useful in predicting patients at highest risk of recurrence [25]. Another study proved the sentinel tumor burden of >0.5 mm to be an independent risk parameter for MSS [26]. Our results also show that risk assessment with the seven-marker signature could be expanded to patients with negative nodal status, including stage IB–IIC patients. Prospectively, once classified as high-risk by the seven-marker signature, indication for adjuvant therapy could also be discussed for stages I and II. Patients also could benefit from a risk-adapted follow-up regimen and imaging. Together with our results in patients with positive nodal status (stages IIIA–IIIC), this suggests that the seven-marker signature allows classification of patients at “high-risk” for disease recurrence independently of nodal status with a sensitivity of 94.4% to 100%, and therewith, may add valuable diagnostic information in addition to sentinel node biopsy. In patients with low-risk melanomas, disease recurrence can almost be excluded (NPVs of 97.2% (nodal negative) to 100% (nodal positive)). Currently, 9–21% of patients with negative sentinel node will experience disease recurrence [15,27]. In our study, 26% of patients with negative nodal status had a disease recurrence, of which all but one could be identified by a high-risk seven-marker signature. We observed a high sensitivity of relapse prediction (>94%) in stages IB–IIC. The sensitivity of a 31-gene and 8-gene score (such as the “DecisionDx-Melanoma” and “MelaGenix” scores) has been reported to be only 64–79% and 32–76% for stages I–II melanoma [19,28–32].
At the same time, the specificity of the seven-marker signature was 70%, similar to the reported specificity of the 13- and 8-gene scores with 70–86% and 43–77%.

**Strengths and Limitations**

Compared to the previously published study, where tissue micro array spots were analyzed, we used entire tissue sections for staining with the biomarkers, which allows for a broader and more representative understanding of the melanoma histology. The seven-marker signature reliably identified a subgroup of patients with an increased risk of relapse with PPVs for RFS ranging between 47.4% (stage IB–IIA) and 64.6% (all stages IB-III). The highest PPV of 86.7% was observed in patients with positive nodal status (Table 3). Lower PPVs were observed for the endpoint MSS (e.g., 33.3% for the whole cohort, 21.1% for stage IB/IIA) and may be partially explained by the short length of the follow-up (minimum 5 years). Patients may still experience late metastasis with subsequent death after the last follow-up of this study. The high NPV (97.5% to 100%) observed in the different subcohorts could support rule-out decisions if therapy and follow-up management is concerned. A limitation of the study is the relative high number of dropouts due to no available tissue.

5. Conclusions

The results of this study need to be further validated in larger cohorts, and ideally in prospectively collected cohorts (a prospective study with more samples). A prospective (biomarker) study would avoid a follow-up-bias in favor of patients with metastatic events. A prognostic marker/signature should reliably identify patients at high-risk of recurrence/metastasis and, thus, patients who could benefit from adjuvant therapies or adapted follow-up. Therefore, high sensitivity is of primary importance. In addition, melanoma-specific deaths were predicted consistently as high-risk by the seven-marker signature. Prospectively, upcoming neoadjuvant treatment regimens could be offered more individually to high-risk patients in the future. One could imagine that sentinel lymph nodes labeled with a magnetic seed detector would be removed after neoadjuvant treatment, a concept that has already proven to be successful for patients with measurable index nodes [33]. Thus, the seven-marker signature could serve as a stratification tool to guide decision making, particularly for stage I and II melanoma patients, and help in creating a more personalized medicine as well as avoiding invasiveness of therapy, frequency of follow-up, hospitalization and treatment costs in patients stratified as low-risk [34–37]. The seven-marker signature proved to be a valid prognostic tool to reliably classify patients with increased recurrence and metastatic risk, independent of AJCCv8 staging.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/cancers13122902/s1, Figure S1: 7-marker-signature high-risk, Figure S2: 7-marker-signature low-risk, Figure S3: 7-marker-signature high-risk, Figure S4: 7-marker-signature low-risk, Figure S5: 7-marker-signature low-risk, Figure S6: Patient and clinicopathological characteristics (AJCCv8), Figure S7: Patient and clinicopathological characteristics (AJCCv8), Figure S8: Patient and clinicopathological characteristics (AJCCv8), Table S1: Patient and clinicopathological characteristics (AJCCv8), Table S2: Univariate and multivariable Cox regression analysis of prognostic factors for MSS, DMFS and RFS, Table S3: Patient and clinicopathological characteristics of the low-risk and high-risk group (stage IB–IIA), Table S4: Patient and clinicopathological characteristics of the low-risk and high-risk group (patients with nodal status pN0), Table S5: Patient and clinicopathological characteristics of the low-risk and high-risk group (stage IIIA–IIIC).

**Author Contributions:** Conceptualization: R.R., P.G. and M.Z.; data curation R.R., P.G. and M.Z.; writing—original draft preparation, R.R. and P.G.; writing—review and editing M.Z., R.R. and P.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** The analysis was conducted in cooperation with Synvie GmbH, Munich, Germany, based on a research contract for financial support of the stainings. Synvie had part in the design of the study and in the analyses and interpretation of data. We acknowledge financial support from Leipzig
University Library for Open Access Publishing. Leipzig University Library had no role in the design of the study; in the collection, analyses, or interpretation of data.

**Institutional Review Board Statement:** This study was approved by the University Ethics Committee Leipzig, Germany (Ref.nr. 169/19-ek). The study was reported according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available within the article.

**Conflicts of Interest:** Mirjana Ziemer received lecture fees from Bristol-Myers Squibb, Roche Pharma AG, MSD Sharp & Dohme GmbH, Pfizer Pharma GmbH, Sanofi-Aventis Deutschland GmbH and Novartis Pharma GmbH, received financial support for congress participation from Bristol-Myers Squibb, Novartis Pharma and Amgen GmbH and served as a member of expert panels on cutaneous adverse reactions for Pfizer INC. Robin Reschke and Philipp Gussek declare no conflict of interest.

**Abbreviations**

AJCCv8 American Joint Committee on Cancer 8th Edition Cancer Staging  
Bax Bcl-2-associated X protein  
CI confidence interval  
COX-2 Cyclooxygenase-2  
DMFS distant metastasis-free survival  
FFPE formalin-fixed paraffin-embedded  
HR hazard ratio  
IHC Immunohistochemistry  
MTAP S-methyl-5′-thioadenosine phosphorylase  
NPV negative predictive value  
PPV positive predictive value  
OS overall survival  
PTEN Phosphatase and Tensin Homolog  
REMARK Reporting Recommendations for Tumor Marker Prognostic Studies  
RFS relapse-free survival

**References**

1. Eggermont, A.M.; Spatz, A.; Robert, C. Cutaneous melanoma. *Lancet* 2014, 383, 816–827. [CrossRef]
2. Swetter, S.M.; Tsao, H.; Bichakjian, C.K.; Curiel-Lewandrowski, C.; Elder, D.E.; Gershenwald, J.E.; Guild, V.; Grant-Kels, J.M.; Halpern, A.C.; Johnson, T.M.; et al. Guidelines of care for the management of primary cutaneous melanoma. *J. Am. Acad Dermatol.* 2019, 80, 208–250. [CrossRef] [PubMed]
3. Azzola, M.F.; Shaw, H.M.; Thompson, J.F.; Soong, S.J.; Scoller, R.A.; Watson, G.F.; Colman, M.H.; Zhang, Y. Tumor mitotic rate is a more powerful prognostic indicator than ulceration in patients with primary cutaneous melanoma: An analysis of 3661 patients from a single center. *Cancer* 2003, 97, 1488–1498. [CrossRef] [PubMed]
4. Long, G.V.; Hauschild, A.; Santinami, M.; Atkinson, V.; Mandalà, M.; Chiarion-Sileni, V.; Larkin, J.; Nyakas, M.; Dutriaux, C.; Haydon, A.; et al. Adjuvant Dabrafenib plus Trametinib in Stage III BRAF-Mutated Melanoma. *N. Engl. J. Med.* 2017, 377, 1789–1801. [CrossRef] [PubMed]
5. Eggermont, A.M.M.; Blank, C.U.; Mandalà, M.; Long, G.V.; Atkinson, V.; Dalle, S.; Haydon, A.; Lichinitser, M.; Khattak, A.; Carlino, M.S.; et al. Adjuvant Pembrolizumab versus Placebo in Resected Stage III Melanoma. *N. Engl. J. Med.* 2018, 378, 1789–1801. [CrossRef] [PubMed]
6. Weber, J.; Mandalà, M.; Del Vecchio, M.; Gogas, H.J.; Arance, A.M.; Cowey, C.L.; Dalle, S.; Schenker, M.; Chiarion-Sileni, V.; Marquez-Rodas, I.; et al. Adjuvant Nivolumab versus Ipilimumab in Resected Stage III or IV Melanoma. *N. Engl. J. Med.* 2017, 377, 1782–1835. [CrossRef]
7. Dummer, R.; Hauschild, A.; Santinami, M.; Atkinson, V.; Mandalà, M.; Kirkwood, J.M.; Chiarion Sileni, V.; Larkin, J.; Nyakas, M.; Dutriaux, C.; et al. Five-Year Analysis of Adjuvant Dabrafenib plus Trametinib in Stage III Melanoma. *N. Engl. J. Med.* 2020, 383, 1139–1148. [CrossRef]
8. Ascierto, P.A.; Del Vecchio, M.; Mandalà, M.; Gogas, H.; Arance, A.M.; Dalle, S.; Cowey, C.L.; Schenker, M.; Grob, J.J.; Chiarion-Sileni, V.; et al. Adjuvant nivolumab versus ipilimumab in resected stage IIIIB-C and stage IV melanoma (CheckMate 238): 4-year results from a multicentre, double-blind, randomised, controlled, phase 3 trial. *Lancet Oncol.* 2020, 21, 1465–1477. [CrossRef]
9. Eggermont, A.M.M.; Blank, C.U.; Mandalà, M.; Long, G.V.; Atkinson, V.G.; Dalle, S.; Haydon, A.M.; Meshcheryakov, A.; Khattak, A.; Carlino, M.S.; et al. Longer Follow-Up Confirms Recurrence-Free Survival Benefit of Adjuvant Pembrolizumab
in High-Risk Stage III Melanoma: Updated Results From the EORTC 1325-MG/KEYNOTE-054 Trial. J. Clin. Oncol. 2020, 38, 3925–3936. [CrossRef] [PubMed]

10. Poklepovic, A.S.; Luke, J.J. Considering adjuvant therapy for stage II melanoma. Cancer 2020, 126, 1166–1174. [CrossRef]

11. Gershenwald, J.E.; Scolyer, R.A.; Hess, K.R.; Sondak, V.K.; Long, G.V.; Ross, M.I.; Lazar, A.J.; Faries, M.B.; Kirkwood, J.M.; McArthur, G.A.; et al. Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J. Clin. 2017, 67, 472–492. [CrossRef]

12. Kanaki, T.; Stang, A.; Gutzmer, R.; Zimmer, L.; Chorti, E.; Sucker, A.; Ugurel, S.; Hadaschik, E.; Gräger, N.S.; Satzger, I.; et al. Impact of American Joint Committee on Cancer 8th edition classification on staging and survival of patients with melanoma. Eur. J. Cancer 2019, 119, 18–29. [CrossRef] [PubMed]

13. Whiteman, D.C.; Baade, P.D.; Olsen, C.M. More people die from thin melanomas (≤1 mm) than from thick melanomas (>4 mm) in Queensland, Australia. J. Investig. Dermatol. 2015, 135, 1190–1193. [CrossRef] [PubMed]

14. Gambichler, T.; Scholl, L.; Bechra, F.G.; Stockfleth, E.; Stücker, M. Worse outcome for patients with recurrent melanoma after negative sentinel lymph biopsy as compared to sentinel-positive patients. Eur. J. Surg. Oncol. 2016, 42, 1420–1426. [CrossRef] [PubMed]

15. Thomas, D.C.; Han, G.; Leong, S.P.; Kashani-Sabet, M.; Vetto, J.; Pockaj, B.; White, R.L.; Faries, M.B.; Schneebaum, S.; Mozzillo, N.; et al. Recurrence of Melanoma After a Negative Sentinel Node Biopsy: Predictors and Impact of Recurrence Site on Survival. Ann. Surg. Oncol. 2019, 26, 2254–2262. [CrossRef]

16. Weiss, S.A.; Hanniford, D.; Hernando, E.; Osman, I. Revisiting determinants of prognosis in cutaneous melanoma. Cancer 2015, 121, 4108–4123. [CrossRef] [PubMed]

17. Meyer, S.; Fuchs, T.J.; Bosserhoff, A.K.; Hofstädter, F.; Pauer, A.; Roth, V.; Buhmann, J.M.; Moll, I.; Anagnostou, N.; Brandner, J.M.; et al. Seven-marker signature and clinical outcome in malignant melanoma: A large-scale tissue-microarray study with two independent patient cohorts. PLOS ONE 2012, 7, e38222. [CrossRef]

18. McShane, L.M.; Altman, D.G.; Sauerbrei, W.; Taube, S.E.; Gion, M.; Clark, G.M. REporting recommendations for tumour MARKer prognostic studies (REMARK). Eur. J. Cancer 2015, 41, 1690–1696. [CrossRef]

19. Zager, J.S.; Gastman, B.R.; Leachman, S.; Gonzalez, R.C.; Fleming, M.D.; Ferris, L.K.; Ho, J.; Miller, A.R.; Cook, R.W.; Covington, K.R.; et al. Performance of a prognostic 31-gene expression profile in an independent cohort of 523 cutaneous melanoma patients. BMC Cancer 2018, 18, 130. [CrossRef] [PubMed]

20. Eggermont, A.M.M.; Bellomo, D.; Arias-Mejias, S.M.; Quattrocchi, E.; Sominidi-Damodaran, S.; Bridges, A.G.; Lehman, J.S.; Hieken, T.J.; Jakub, J.W.; Murphree, D.H.; et al. Identification of stage I/IIA melanoma patients at high risk for disease relapse using a clinicopathologic and gene expression model. Eur. J. Cancer 2020, 140, 11–18. [CrossRef]

21. Schoffer, O.; Schülein, S.; Arand, G.; Arnholdt, H.; Baaske, D.; Bargou, R.C.; Becker, N.; Beckmann, M.W.; Bodack, Y.; Böhme, B.; et al. Tumour stage distribution and survival of malignant melanoma in Germany 2002–2011. BMC Cancer 2016, 16, 936. [CrossRef] [PubMed]

22. Svedman, F.C.; Pillas, D.; Taylor, A.; Kaur, M.; Hansson, J. Stage-specific survival and recurrence in patients with cutaneous malignant melanoma in Europe—A systematic review of the literature. Clin. Epidemiol. 2016, 8, 109–122. [CrossRef]

23. Criscione, V.D.; Weinstock, M.A. Melanoma thickness trends in the United States, 1988–2006. J. Investig. Dermatol. 2010, 130, 793–797. [CrossRef]

24. Faries, M.B.; Thompson, J.F.; Cochrane, A.J.; Andtbacka, R.H.; Mozzillo, N.; Zager, J.S.; Jahkola, T.; Bowles, T.L.; Testori, A.; Beitsch, P.D.; et al. Completion Dissection or Observation for Sentinel-Node Metastasis in Melanoma. Cancer Med. 2018, 7, 130. [CrossRef] [PubMed]

25. Rosenbaum, B.E.; Schafer, C.N.; Han, S.W.; Osman, I.; Zhong, H.; Brinster, N. Computer-assisted measurement of primary tumor area is prognostic of recurrence-free survival in stage IB melanoma patients. Mod. Pathol. 2017, 30, 1402–1410. [CrossRef]

26. Satzger, I.; Leiter, U.; Gräger, N.; Keim, U.; Garbe, C.; Gutzmer, R. Melanoma-specific survival in patients with positive sentinel lymph nodes: Relevance of sentinel tumor burden. Eur. J. Cancer 2019, 123, 83–91. [CrossRef] [PubMed]

27. Kunte, C.; Geimer, T.; Baumert, J.; Konz, B.; Volkenandt, M.; Flaim, M.; Ruzicka, T.; Berking, C.; Schmid-Wendtner, M.H. Prognostic factors associated with sentinel lymph node positivity and effect of sentinel status on survival: An analysis of 1049 patients with cutaneous melanoma. Melanoma Res. 2010, 20, 330–337. [CrossRef]

28. Keller, J.; Schwartz, T.L.; Lizalek, J.M.; Chang, E.S.; Patel, A.D.; Hurley, M.Y.; Hsueh, E.C. Prospective validation of the prognostic 31-gene expression profiling test in primary cutaneous melanoma. Cancer Med. 2019, 8, 2205–2212. [CrossRef] [PubMed]

29. Greenhaw, B.N.; Zitelli, J.A.; Brodland, D.G. Estimation of Prognosis in Invasive Cutaneous Melanoma: An Independent Study of the Accuracy of a Gene Expression Profile Test. Dermatol. Surg. 2018, 44, 1494–1500. [CrossRef] [PubMed]

30. Hsueh, E.C.; DeBloom, J.R.; Lee, J.; Sussman, J.J.; Covington, K.R.; Middlebrook, B.; Johnson, C.; Cook, R.W.; Slngluff, C.L.; McMasters, K.M. Interim analysis of survival in a prospective, multi-center registry cohort of cutaneous melanoma tested with a prognostic 31-gene expression profile test. J. Hematol. Oncol. 2017, 10, 152. [CrossRef] [PubMed]

31. Marchetti, M.A.; Coit, D.G.; Dusza, S.W.; Yu, A.; McLean, L.; Hu, Y.; Nanda, J.K.; Matsoukas, K.; Mancebo, S.E.; Bartlett, E.K. Performance of Gene Expression Profile Tests for Prognosis in Patients With Localized Cutaneous Melanoma: A Systematic Review and Meta-analysis. JAMA Dermatol. 2020, 156, 953–962. [CrossRef]
32. Amaral, T.M.S.; Hoffmann, M.C.; Sinnberg, T.; Niessner, H.; Sülberg, H.; Eigentler, T.K.; Garbe, C. Clinical validation of a prognostic 11-gene expression profiling score in prospectively collected FFPE tissue of patients with AJCC v8 stage II cutaneous melanoma. *Eur. J. Cancer* 2020, 125, 38–45. [CrossRef]

33. Schermers, B.; Franke, V.; Rozeman, E.A.; van de Wiel, B.A.; Bruining, A.; Wouters, M.W.; van Houdt, W.J.; Ten Haken, B.; Muller, S.H.; Bierman, C.; et al. Surgical removal of the index node marked using magnetic seed localization to assess response to neoadjuvant immunotherapy in patients with stage III melanoma. *Br. J. Surg.* 2019, 106, 519–522. [CrossRef]

34. Hodges, M.; Jones, E.; Jones, T.; Pearlman, N.; Gajdos, C.; Kounalakis, N.; McCarter, M. Analysis of melanoma recurrence following a negative sentinel lymph node biopsy. *Melanoma Manag.* 2015, 2, 285–294. [CrossRef]

35. Moody, J.A.; Botham, S.J.; Dahill, K.E.; Wallace, D.L.; Hardwicke, J.T. Complications following completion lymphadenectomy versus therapeutic lymphadenectomy for melanoma—A systematic review of the literature. *Eur. J. Surg. Oncol.* 2017, 43, 1760–1767. [CrossRef]

36. De Vries, M.; Hoekstra, H.J.; Hoekstra-Weebers, J.E. Quality of life after axillary or groin sentinel lymph node biopsy, with or without completion lymph node dissection, in patients with cutaneous melanoma. *Ann. Surg. Oncol.* 2009, 16, 2840–2847. [CrossRef]

37. Hu, Y.; Briggs, A.; Gennarelli, R.L.; Bartlett, E.K.; Ariyan, C.E.; Coit, D.G.; Brady, M.S. Sentinel Lymph Node Biopsy for T1b Melanoma: Balancing Prognostic Value and Cost. *Ann. Surg. Oncol.* 2020, 27, 5248–5256. [CrossRef]