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Dynamic MicroRNA-Expression in Plasma of Melanoma Patients Correlates With Progression, PD-L1 Status and Overall Survival

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Abstract

Melanoma treatment has improved significantly with the development of immune checkpoint inhibition (ICI), which has greatly enhanced the survival rates of patients with metastatic melanoma. However, a significant number of patients do not respond well to ICI treatment and experience progression. This highlights the critical need for practical means to track melanoma patients' response to ICI. To address this issue, the patterns of circulating miRNAs were studied in liquid biopsies of melanoma patients. These miRNAs have the potential to provide essential information regarding the cancer stage, progression, and the presence of PD-L1 in tumor tissue. A sophisticated flow cytometric test was used to measure up to 63 different miRNAs at once. The study identified a combination of nine miRNAs that are capable of distinguishing between different stages of melanoma, particularly stage IV. Additionally, five miRNAs were pinpointed which are downregulated in patients who do not respond to ICI treatment. Furthermore, two miRNAs were found that correlate to the level of PD-L1 in tumor tissue, and low levels of miR-150-5p were linked to poorer overall survival. These findings suggest that circulating miRNAs could serve as valuable markers to predict the effectiveness of ICI, provide insights into the cancer's stage and PD-L1 status, and ultimately help physicians make better treatment decisions in the future. However, further research is needed to confirm these findings and establish their clinical usefulness.

Introduction

In recent years immune checkpoint inhibitors (ICI) have increased clinical benefit in some malignant solid tumors [1,2]. Especially, malignant melanomas of the skin, because of their high immunogenicity, have been proven to be the tumor entity with the most promising outcome of ICI. Inhibitors of the programmed cell death protein 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) checkpoint and the T -lymphocyte-associated protein 4 (CTLA-4) checkpoint led to tremendous improvement of survival of patients with metastatic melanoma and even resulted in durable remission [3,4]. Nevertheless, a considerable portion of patients show primary resistance to immune checkpoint inhibition. 60% - 70% non-responders to PD-1 monotherapy (nivolumab, pembrolizumab) and 40% - 50% for the combined therapy with anti-CTLA-4 (ipilimumab) have been reported [5,6]. The pathways involved in ICI response or non-response, as well as the diagnostic and predictive markers for deciding which and when a particular therapy should be used for individualized (personalized) therapy are not yet very well understood.

While reported predictive markers in liquid biopsies from melanoma patients seem to be promising candidates for predicting the outcome of ICI therapy and have been correlated with therapy outcomes [7-15], they may lack the sensitivity and/or specificity needed to accurately depict specific tumor- or metastasis-associated mechanisms. Furthermore, they may not be sufficient to monitor tumor development over the course of therapy and predict therapy response, particularly in therapy-naïve patients before the initiation of ICI treatment.

Therefore, biomarkers, which can discriminate between certain risk groups (stages) of melanoma, and melanoma progression and predict the response to therapy, are urgently needed [16]. The use of circulating microRNAs (miRNAs) in liquid biopsies might fulfill these tasks.

MiRNAs are 19-24 nt long, noncoding RNA molecules, which post-transcriptionally regulate mRNA translation by binding to the 3`UTR of messenger RNAs, thereby reducing mRNA and thus protein expression [17,18]. MiRNA-mediated control of gene expression is involved in stem cell organization and cell differentiation, as well as cancer development, progression, and metastasis [19-21]. Therefore, miRNAs or miRNA networks can be used as tumor biomarkers [22-25]. Indeed, the potential of miRNAs to function as a biomarker is increasingly acknowledged either by detection in cancer tissues or in body fluids like blood, plasma, urine, and CSF as circulating cell-free miRNAs in liquid biopsies [14,26]. Most notably, there is increasing evidence for the involvement of miRNAs in tumor immune escape and the possibility to monitor these pathways in liquid biopsies [14,27,28].

While circulating miRNAs and other liquid biopsy parameters show promising potential as biomarkers linked to melanoma stage, progression, progression-free survival (PFS), and overall survival (OS), there is a pressing need for reproducible, sensitive, non-invasive, and clinically user-friendly biomarkers to inform clinical decision-making in both pre- and post-melanoma therapy scenarios [29]. Apart from sensitivity and specificity, an ideal biomarker should possess the unique quality of being generated by an assay adaptable to routine clinical practice, providing results within days rather than weeks. This aligns with the intention to implement biomarker-driven clinical practices [30].

Although miRNAs and other biomarkers play a role in pathways related to tumor formation and immune processes, an in-depth understanding of these pathways isn't essential for biomarker development. The initial phase involves identifying biomolecules or distinctive features (classifiers) that differentiate patients and enable discrimination in areas like disease stages, treatment responses, or prognoses. Turning a classifier into a biomarker requires independent validation and clinical relevance.

While comprehending pathways can aid in designing personalized therapies, it's not a mandatory step in biomarker discovery.

We present a multi-miRNA discovery method, utilizing the Abcam FirePlex flow cytometry assay, for monitoring melanoma stages and progression in liquid biopsies (plasma). The goal is to establish a miRNA classifier indicative of PD-L1 expression levels, facilitating early therapeutic decision-making. In our initial discovery cohort (n=153 patients), we successfully differentiate between melanoma stages (< IV vs. IV) and progressive vs. non-progressive melanoma through distinct miRNA patterns in plasma. Additionally, we identify specific miRNAs in liquid biopsies indicative of negative, low, or high PD-L1 expression in melanoma tissue, correlating with the OS of melanoma patients. However, independent validation is essential to establish these findings as potential biomarkers.

Materials and Methods

Study design and liquid biopsy collection

This was a monocentric prospective observational study that included plasma samples from an existing biobank ("LiquiMel"). For the biobank, citrate blood samples from routinely drawn blood were collected with the patient's informed consent, and centrifuged for 10 min at 1800 x g. 2 ml Plasma was aliquoted and frozen at -80°C until miRNA multiplexing. Blood values and other clinically relevant parameters were known from patient records. Patients` plasma samples were included in different cohorts of melanoma stages, with and without metastasis, before or during therapy. Cohort 1: Melanoma patients in stages I - III with no or only locoregional metastatic sites (lymph nodes) (without any form of therapy) compared to treatment-naïve stage IV melanoma patients who actively metastasize (Table S2). Cohort 2: Treatment-naïve stage IV melanoma patients who actively metastasize at therapy-start (baseline) compared to an independent group of stage IV melanoma patients with the first occurrence of progressive disease after the start of immune therapy (progress) (Table S4). Cohort 3: Treatment-naïve, actively metastasizing stage IV melanoma patients with negative, compared to low and high PD-L1 immunostaining of melanoma tissue (Table S6). Because the time point of sampling is important in this study, the baseline samples were taken directly on the day of the first therapy before infusion. Progress samples were collected on average within 16 days before or after the staging in which the progressive disease was first diagnosed. "Progressive disease" is defined as at least a 20 % growth in the size of the metastasis or as the appearance of a new metastasis, which means increasing tumor mass [31].

Ethic committee approval

We got the ethical approval for this study from the IRB Ethical Review Board of Ham-burg and the Medical Association of Lower Saxony for analysis of human materials and all patients gave their written informed consent for this study.

miRNA profiling

The transcription of miRNAs was measured via flow cytometric quantification of barcode-labeled fluorescent miRNA hydrogel micro-particle ("FirePlex Particle Technology ", Abcam) according to the manufacturer's protocol as previously described [32]. Briefly, 25 µl of protease-digested plasma was added to customized fireplex particles (~35 µL) and incubated under shaking (1125 rpm) at 37 °C for 60 min. After binding of miRNAs, the particles, which contain a complementary sequence, were rinsed twice with rinse buffer A followed by a labeling reaction (RT, 60 min, 1125 rpm). During labelling each miRNA is ligated to two linkers. After washing of miRNA-linker-bound particles with rinse buffers B

and A, the miRNA-linkers were eluted with H_2O from the particles at 55 °C. The miRNA linkers were amplified by PCR and the PCR products were transferred back to the initial particles (shaking at 1125 rpm, 37 °C, 60 min). A fluorescent reporter was added (RT, 15 min, 1125 rpm) that binds to the miRNA linker complex. Fluorescence of the particles was then measured by flow cytometry (with Guava easycyte 8HT, Millipore). The raw data obtained from flow cytometry were then processed with the "FirePlex Analysis Workbench software" (Abcam). For the normalization, the geometric mean of the five overall most stably expressed miRNAs was determined (regardless of stage, therapy, or outcome) (Table S1).

PD-L1 staining in tumor tissue

PD-L1 staining in melanoma tumor tissue and determination of the tumor proportion score (TPS) was done as part of the ongoing diagnosis. The immunohistochemical staining was carried out using monoclonal rabbit anti-PD-L1 (clone 28-8) according to the manufacturer's protocol with PD-L1 IHC 28-8 pharmDx Kit (Dako). TPS was scored by a pathologist according to the manufacturer's interpretation manual [33].

Statistical analysis

Statistical analysis was performed using R 4.1.1 (https://www.r-project.org/). miRNA expression was log2-transformed after setting negative values to 0 and adding 1 to increase data symmetry and approximate a normal distribution. miRNAs were considered differentially expressed if significantly altered and the calculated transcriptional fold change was ≥ 1.5-fold (log2-FC: ≥ 0.585). For each hypothesis, we conducted a two-way analysis of variance (ANOVA) including the different miRNAs and the respective grouping variable for each cohort with an interaction term. ANOVA was performed with heteroscedasticity-corrected covariance matrices (hccm) to adjust for unequal variances. Pair-wise differences in each subgroup were then compared by the Games-Howell post-hoc test. Results were considered statistically significant if $p < 0.05$.

Statistical differences for baseline demographics were tested with the chi^2 test for categorical data and ANOVA (> 2 groups) or Welch's t-test (2 groups) for numerical data.

Pathway analysis was performed with the web tool miRTargetLink 2.0 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) using a unidirectional search with a set of miRNAs and performing enrichment analysis on this miRNA set in the database of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Survival analyses were conducted with the R packages survival and survminer [34,35]. OS is presented in Kaplan-Meier plots; hazard ratios were calculated with multi-variable Cox regression as a post-hoc analysis.

Results

Baseline demographics

For this study, we included a total of 153 patients in different stages of cutaneous malignant melanoma with a median age of 64 [range: 17.0, 96.0] years. For 101 (66.1%) of these patients the v-Raf murine sarcoma viral oncogene homolog B (BRAF) status was known. 59 patients (38.6%) had a wildtype BRAF and 42 patients (27.5%) had a BRAF V600 mutation. Ten patients (6.5%) were negative in PD-L1 immunostaining of the tumor tissue, 20 were positive (13.1%) and 40 (26.1%) had elevated serum LDH concentrations.

Table 1. Demographics and patient characteristics of the analyzed melanoma patients.

	Overall (N=153)
Age	
Mean (SD)	62.4 (15.0)
Median [Min, Max]	64.0 [17.0, 96.0]
Sex	
Male	83 (54.2%)
Female	70 (45.8%)
BRAF	
Wildtype	59 (38.6%)
Mutated	42 (27.5%)
Unknown	52 (34.0%)
PD-L1 staining in tumor tissue	
negative	10 (6.5%)
low	9 (5.9%)
high	11 (7.2%)
Missing	123 (80.4%)
LDH (ULN = 225)	
Elevated	40 (26.1%)
Normal	70 (45.8%)
Missing	43 (28.1%)
Stage	
I	17 (11.1%)
II	30 (19.6%)
III	57 (37.3%)
IV	49 (32.0%)
Number of metastatic sites	
0	104 (68.0%)
1	17 (11.1%)
2	19 (12.4%)
≥ 3	13 (8.5%)

104 patients were in stages I to III and had no distant metastases. In total 17 patients were in stage I (11.1%), 30 in stage II (19.6%), and 57 in stage III (37.3%). All these patients were therapy naïve at blood sampling. 49 patients were in stage IV with active distant metastases and one or more metastatic sites (Table 1).

Differences in miRNA expression between stage I-III and stage IV melanoma patients

To answer the question of whether there is a difference in miRNA expression pattern between patients without active distant metastases in stages I to III and actively metastasizing patients in stage IV, we

compared the expression of 63 miRNAs (Table S1) based on hypothesis-driven literature search, meaning the miRNAs were selected based on their reported association with cancer development as oncomirs or tumor suppressors. Our selection did not exclusively focus on miRNAs specific to malignant melanoma but rather encompassed those involved in general carcinogenesis. In cohort one (Table S2) blood plasma of 104 patients in lower stages (< IV) and 35 actively metastasizing patients in stage IV were examined. The correlation with anatomical sites of metastases is not discussed in this report. To exclude possible influences of any kind of melanoma therapy we only included therapy naïve patients that did not get immunotherapy or targeted therapy at blood sampling. The group < IV consisted of 17 patients (12.2%) in stage I, 30 patients (21.6%) in stage II, and 57 patients (41.0%) in stage III. The overall median age of patients in this cohort is 66.0 [17.0, 96.0] years. The gender distribution in stages < IV was n = 51 female (49.04%) and n = 53 male (50.96%), but in stage IV there was a higher male proportion with 62.9 % (n = 22) in contrast to females with 37.1% (n = 13). In stage IV 12 patients had one distant metastatic site (34.3%), 13 patients had two (37.1%) and 10 patients had three or more distant metastatic sites (28.6%).

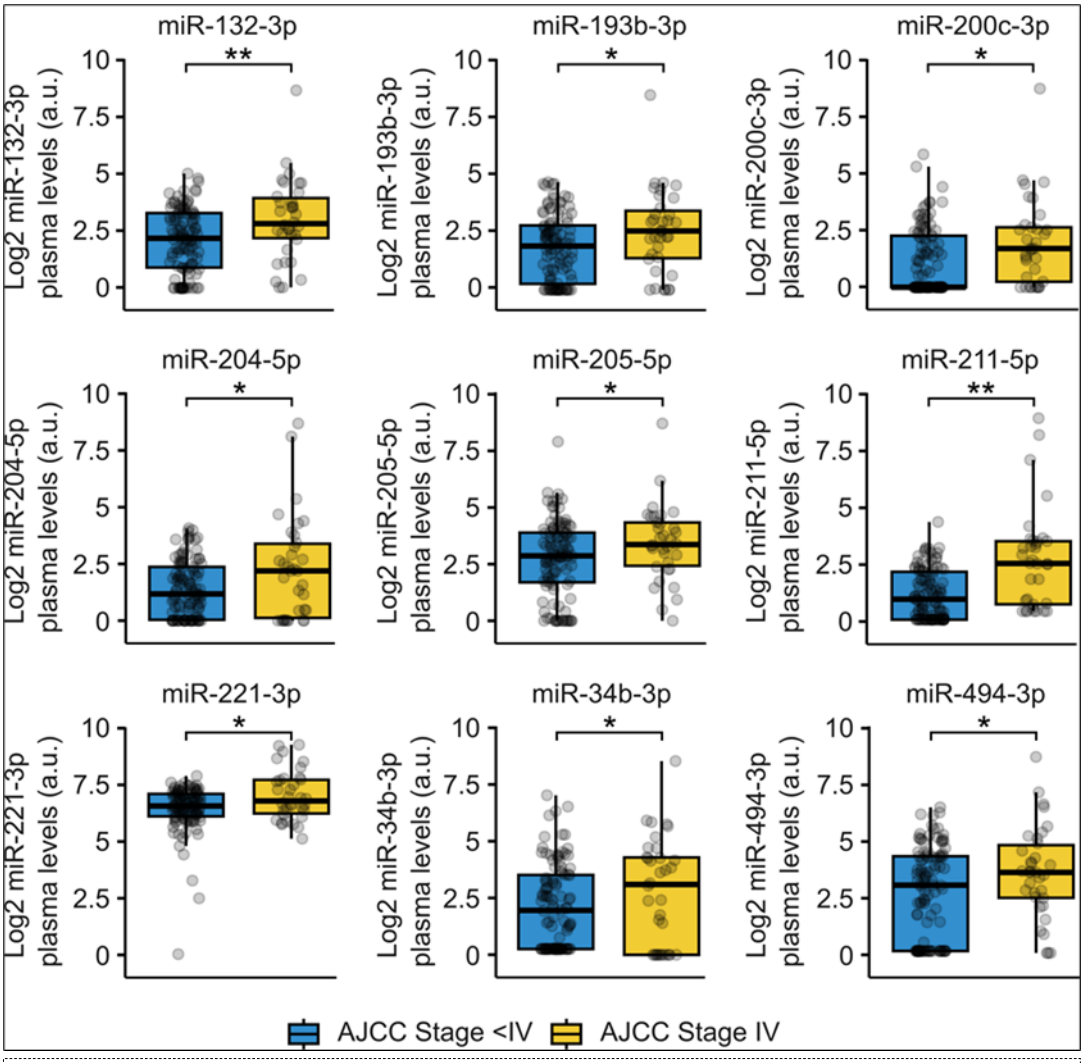

Figure 1. Display of the transition from stage I to III melanoma (< IV) to metastatic melanoma Stage IV (IV) by a 9-miRNA signature. Data from patients in cohort 1 was used for analysis (Table S2). Statistical differences were tested with the Games-Howell-post hoc test. *: $p < 0.05$, **: $p < 0.01$.

Two-way ANOVA (analysis of variance) was conducted to examine the effects of clinical stage and type of miRNA on miRNA expression. There was no significant interaction between the type of miRNA and certain clinical stages (<IV compared to IV), however a significant main effect for clinical stage (< IV vs. IV) was present (ANOVA, $F_{1, 8590} = 53.8$, p = 2.38e-13). A post-hoc Games-Howell test showed that on the individual miRNA level, a set of nine miRNAs (miR-132-3p, miR-193b-3p, miR-200c-3p, miR-204-5p, miR-205-5p, miR-211-5p, miR-221-3p, miR-34b-3p, miR-494-3p) was significantly different between groups < IV and IV (Figure 1). All of these miRNAs were downregulated in stages < IV and were upregulated in stage IV, which may be indicative of an increase in tumor mass by metastases and a correspondingly higher amount of secreted miRNAs into patients' blood. This would correlate with patients' data for tumor burden, measured during staging by radiologists (progressive disease is defined as at least a 20 percent growth in the size of the metastasis or appearance of a new metastasis [31]).

Overrepresentation analysis on this set of nine miRNAs showed several enriched pathways involved in carcinogenesis, including microRNAs in cancer (p-value = 1,55e-15), proteoglycans in cancer (p-value = 2.02e-12), or pathways in cancer (p-value = 2.59e-11) (Table S3). Moreover, receiver operating characteristic (ROC) analyses showed that expression levels of this set of miRNAs are useful better classifiers for discriminating stage IV patients from patients with lower stages than of the single miRNAs alone (AUC = 0.739, p = 1.485513e-05) (Figure 2, Figure S1).

Therapy naïve stage IV melanoma patients show different miRNA expression patterns than melanoma patients at disease progress under therapy

Data from cohort 2 were used (Table S4) to compare miRNA expression between therapy naïve patients with stage IV melanoma (baseline, n = 35) and stage IV melanoma patients at first disease progression after the start of immune therapy (progress, n = 14).

The median age was higher in the baseline subgroup with 67.0 [20.0, 96.0] years compared to 57.5 [35.0, 76.0] years in the progress subgroup. The proportion of patients with BRAF mutated melanoma

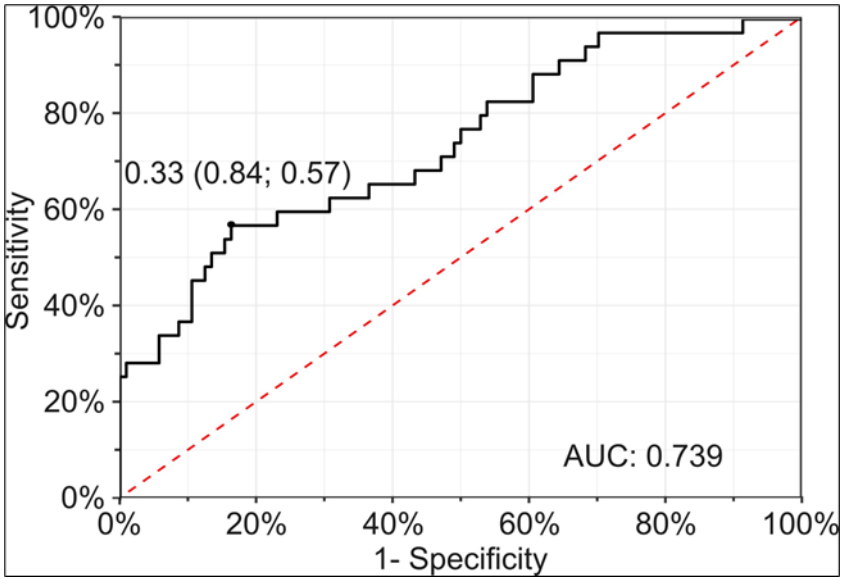

Figure 2. ROC analysis of the nine miRNA classifier to distinguish stage IV melanoma patients from melanoma patients with lower stages (p = 1.485513e-05). Cutoff-value is the Youden-Index. Specificity and Sensitivity at cutoff are shown in parentheses. The red line indicates a random classifier. AUC, area under the curve. Data from Cohort 1.

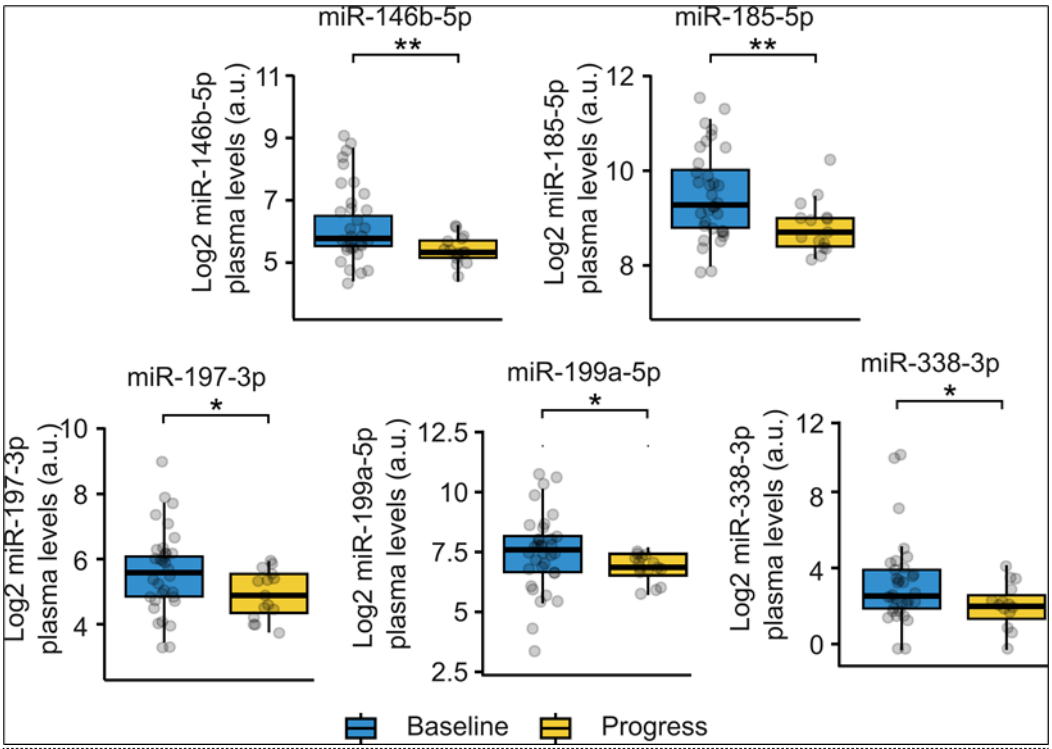

Figure 3. Differentially expressed miRNAs between therapy naïve stage IV melanoma patients (baseline) and stage IV melanoma patients at progress during therapy (progress). Data from patients in cohort 2 (Table S4) were used for analysis. Statistical differences were tested with the Games-Howell-post hoc test. *: $p < 0.05$.

was higher in the progress subgroup (64.3% vs. 28.6%), however, LDH decreased during therapy with 62.9% of patients showing elevated LDH at baseline and 35.7% of patients at progress. Other covariates showed similar distribution patterns between the two groups (Table S4).

A two-way ANOVA was conducted to examine the effects of baseline-/progress-patients and type of miRNA on miRNA expression. No interaction effect could be observed, but a significant main effect for the two groups of melanoma patients (baseline vs. progress) was present (ANOVA, $F_{1,2952} = 61.993$, $p = 4.80e\text{-}15$). A post-hoc Games-Howell test showed that on the individual miRNA level, five miRNAs – namely miR-146b-5p, miR-185-5p, miR-197-3p, miR-199a-5p, and miR-338-3p – were downregulated in patients at progress (Figure 3). A subsequent pathway analysis yields significantly enriched pathways for this 5-miRNA set including pathways in cancer (p-value = 3.12335e-10), proteoglycans in cancer (p-value = 3.12335e-10), or microRNAs in cancer (p-value = 2.68618e-09) (Table S5).

It might, however, be interesting to note that there is no overlap in the miRNA expression patterns between cohort 1 and 2 (compare Fig.1 and Fig.3), although the first cohort discriminates between patients in stage < IV and IV, where stage IV group already represents metastasized melanoma patients which underwent some further progression compared to the stage < IV group patients. Also, the trend in miRNA expression is different between the two cohorts (in cohort 1 we detected an upregulation of the miRNAs involved (Figure 1), whereas in cohort 2 we found a downregulation of miRNAs with further progression). Apparently, in stage IV melanoma patients (cohort 2) further progression after the start of therapy might occur and is “controlled” by another set of miRNAs, with possibly different targets.

Differences in miRNA expression based on PD-L1 levels in melanoma

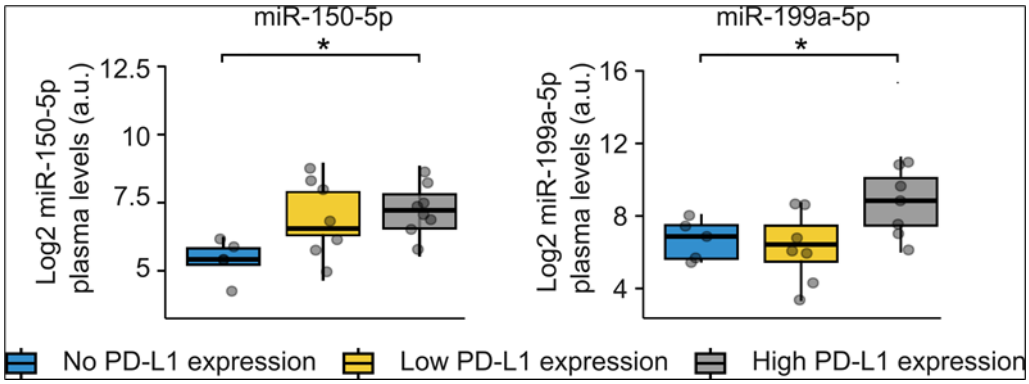

Figure 4. Analysis of miRNA expression depending on PD-L1 expression. Statistical differences were tested with the Games-Howell-post hoc test. *: $p < 0.05$. Data from cohort 3 (Table S 6).

For 20 therapy naïve baseline patients, the tumor proportion score (TPS) of PD-L1 positive tumor cells was known by histological staining. These patients (cohort 3) were grouped by their TPS into negative (<1%), low (1-20%), and high (>20%) PD-L1 expression in tumor tissue. The median age of this cohort was 63.5 [20.0, 96.0] years with eight (40.0%) female and 12 (60.0%) male patients. Six patients (30.0%) showed a BRAF mutation, and 16 patients (80.0%) had elevated LDH levels (Table S6).

Two-way ANOVA revealed a significant difference in miRNA expression between the TPS groups (ANOVA, $F_{2,1071} = 18.578$, $p = 1.17e\text{-}08$). Two miRNAs (miR-150-5p and miR-199a-5p) were found to be able to distinguish between the PD-L1 negative, low, and high patients (Figure 4). In these two miRNAs, there was a significant upregulation from negative to highly positive PD-L1 tumors.

Correlation with overall survival

Examining these preliminary results, we speculated whether the miRNA expression pattern could be correlated with patients' survival characteristics in our basic cohort (Table 1). Therefore, we

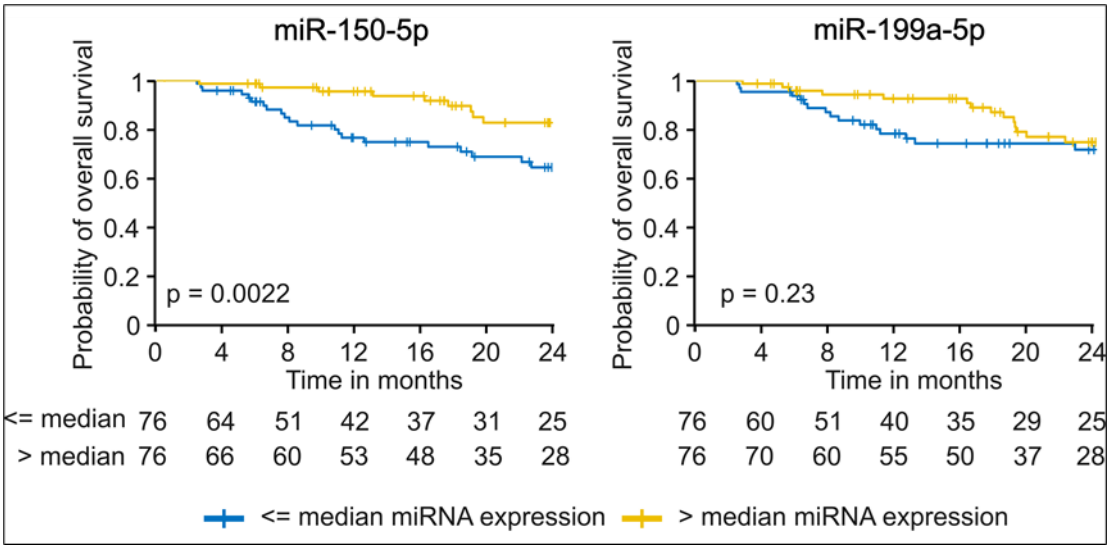

Figure 5. Kaplan-Meier curves for overall survival grouped by high (> median) or low (≤ median) miRNA expression for miR-150-5p and miR-199a-5p. For n=152 patients' miRNA expression data was available. OS was calculated as the time from the first blood draw to death of any cause. Patients without events were censored with the last contact date. <= median, > median, number at risk for each given time point grouped by lower or higher than the median miRNA expression, respectively.

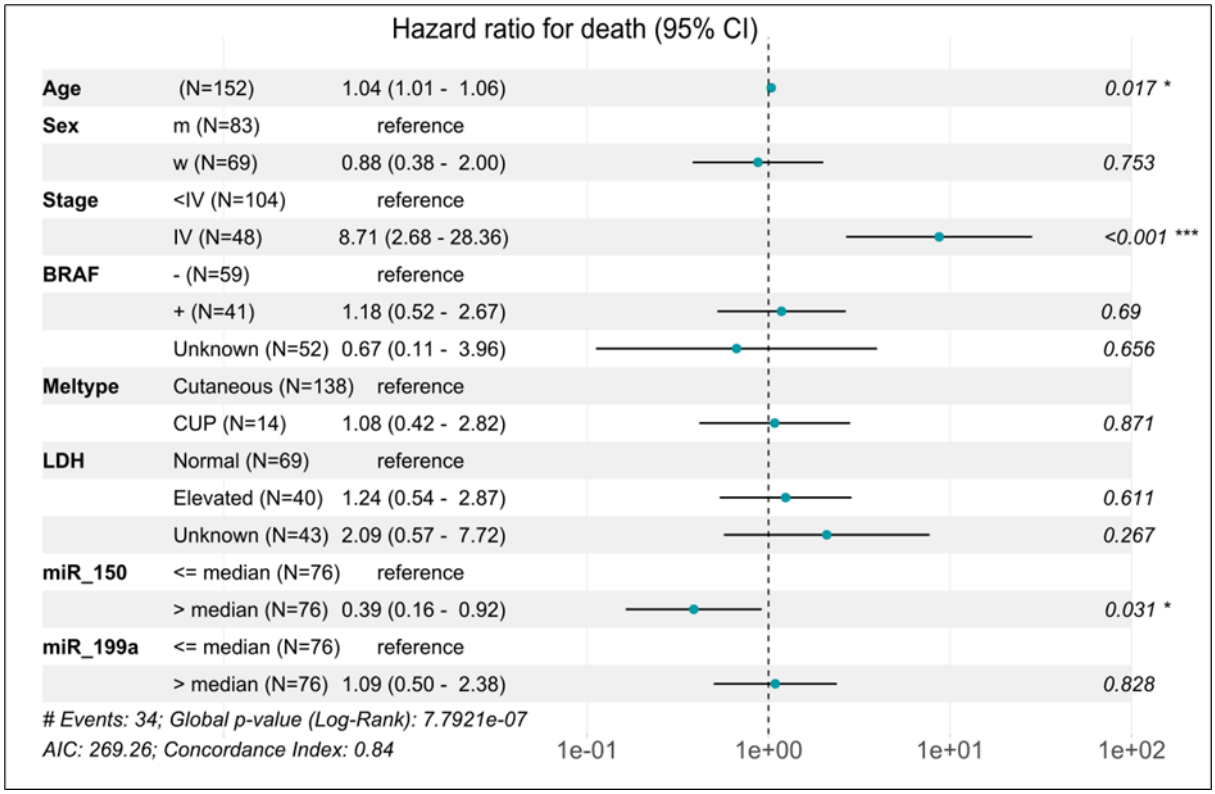

Figure 6. Multivariable Cox regression for OS. After adjustment for confounding covariates (age, sex, AJCC stage (8th edition), BRAF status, melanoma subtype, LDH, miR-150-5p expression, miR-199a-5p expression) high miR-150-5p expression ($p = 0.031$) was associated with better survival whereas higher AJCC stage ($p < 0.001$) and age ($p = 0.017$) were associated with worse survival in melanoma patients.

investigated the OS of n=152 patients, grouped by “low” or “high”. In this setting, “low” corresponds to the expression of miRNA-150-5p and miRNA-199a-5p lower than the median of the corresponding miRNA for all members of the cohort (n=152), whereas “high” corresponds to higher expression than the median, respectively (Figure 5). miRNA-199a-5p did not show significant differences between the groups.

However, miR-150-5p exhibited significant differences in favor of high expression in a Kaplan-Meier analysis ($p = 0.0022$). To adjust for baseline differences in patients with lower and higher miR-150-5p expression than the median (Table S8), respectively, we performed a successive multivariable Cox regression (Figure 6). The previously observed differences remained significant with a more favorable OS for patients with higher miR-150-5p expression (HR (95%): 0.39 (0.16-0.92, $p = 0.031$). Furthermore, as expected, a higher stage and age were associated with a worse OS (HR (95%): 8.71 (2,68-28.36, $p < 0,001$; HR (95%): 1.04 (1.01-1.06, $p = 0.017$, respectively), whereas other covariates like sex, BRAF mutation status, LDH or melanoma type were not significantly altered.

These findings indicate that overexpression of only one miRNA (miR-150-5p) might be predictive not only for high PD-L1 expression in melanoma patients but also for OS of melanoma patients.

Discussion

Melanoma of the skin is the deadliest form of skin cancer with increasing incidences. Melanoma has been recognized for a long time as a therapy-resistant cancer with no (or little) success of therapies in the past. However, ICI has revolutionized therapy outcomes in the last decade, which has been

demonstrated by an increase in OS, PFS, and other surrogate survival parameters in many studies up to now. However, positive response to ICI seems to be restricted to only a subgroup of melanoma patients, and many patients do not respond and/or develop resistance to therapy. The reasons for this are still not understood very well. Thus, there is an urgent need for diagnostic, progression, and predictive biomarkers. It is still necessary to have early diagnostic tools to differentiate between healthy and melanoma-suffering individuals, and, in case of a melanoma diagnosis, to predict the progression of disease. In times of modern, individualized terms of precision medicine (targeted therapies as well as ICI) it is, furthermore, important to find predictive biomarkers, to characterize patients in their ability to respond to a certain therapy (ideally before the start of therapy) and how these patients might respond (or non-respond) during therapy.

A variety of markers has been described in the past including gene expression, ctDNA (circulating tumor DNA), circulating tumor cells, epigenetic markers like DNA methylation and histone modifications, BRAF (and other gene) mutational status, as well as protein expression (e.g. PD-L1-expression in tissue and liquid biopsies [15,29]. However, novel circulating biomarkers are still lacking, and, to date, the only ("validated") marker that has been incorporated in clinical routine use seems to be LDH, which appears to be an independent, significant predictor of survival (when detected at high serum levels), although only in advanced melanoma stages [29].

Search for new, reliable biomarkers for diagnostics, progression, and prediction is, therefore, still ongoing and urgently needs to be investigated and to be validated for clinical use.

It turned out in the last years that circulating miRNAs (in liquid biopsies, e.g. blood (-plasma)) might be good candidates for the future to be used as biomarkers for disease progression, therapy resistance, and therapy response [16,36].

In this paper, we used a clinically applicable, fast, and reliable assay (Abcam FirePlex) to measure miRNA expression patterns in the plasma of melanoma patients in different stages of disease and therapy. We have been able to show, that

- A plasma circulating miRNA expression pattern can discriminate between stage < IV and stage IV melanoma patients.

Several candidates for this kind of discrimination have been discussed in the literature [37-40]. However, their use in a clinical setting (fast, reliable, validated) still awaits application. Using the Abcam FirePlex assay we have been able to show here, that on liquid biopsy (melanoma plasma) level, miRNAs are good candidates to discriminate between stages of melanoma progression (< IV vs. IV). The identified nine miRNAs (miR-132-3p, -193b-3p, -200c-3p, -204-5p, -205-5p, -211-5p, -221-3p, -34b-3p and -494-3p) are all known to be involved in melanoma development and progression and might belong to a group of outstanding, pathway determining biomarker candidates for the future of precision therapy in the field of malignant melanoma [41-46].

- Therapy naïve stage IV melanoma patients show a different miRNA expression pattern than melanoma patients at further disease progress under therapy.

This might be an important finding, indicating that further progression in stage IV melanoma patients is controlled by another miRNA pattern and their associated targets (genes) compared to simple differentiation between low stages (< IV) vs. advanced stages (IV) (see cohort1, Fig 1). There is no overlap between the significant miRNA expression patterns that discriminate between stage < IV and IV patients and those who suffered further progress in stage IV (results from cohorts 1 and 2, see Figs.

1 and These results might be indicative of the participation of different miRNA pathways involved in melanoma development and should be considered, after further validation, in next-generation therapy regimes. Further pathway analyses of gene targets associated with the miRNA changes have to show, specifically, which regulatory pathways are involved and how their knowledge can be used for better clinical prognosis and prediction.

- Expression of liquid biopsy miR-150-5p and miR-199a-5p can discriminate between PD-L1 negative and high expressing patients.

In a small cohort (n=20, cohort 3) we have been able to show, that two miRNAs (miR-150-5p and miR-199a-5p) can discriminate between negative expression (< 1%), low expression (1 – 20 %), and high expression (> 20%) of PD-L1 in liquid biopsies (plasma) of melanoma patients according to the TPS for immune-histologically detection of PD-L1 in melanoma tissue (Figure 4). There was a significant upregulation of both miRNAs from negative to high expression. This finding might indicate that plasma detection of this duo of miRNAs might reveal PD-L1 expression, which is seen as an important parameter in predicting therapy response for ICI in melanoma patients. The advantage of this liquid biopsy-based detection would be the use of a non-invasive assay, which might facilitate its clinical application.

- These miRNAs have recently been shown to be involved in an indirect regulation of PD-L1 expression. MiR-150 negatively regulates STAT1 and EIF4B, whereas miR-199a negatively regulates mTOR expression [47]. A subsequent KEGG enrichment analysis for these two miRNAs, furthermore, indicates the involvement of different cancer-associated pathways like regulation of TWIST1 (p-value = 8.73 e-05) or immune response (p = 1.39e-04; Table S7). TWIST1 knockdown in breast cancer cells was reported to downregulate their metastatic abilities [48]. In Melanoma TWIST1 is upregulated [49]. Its overexpression triggers EMT [50] and is likely to affect progression. Hence, the less the expression of these miRNAs, the worse it could be for the patient's overall survival.

- Expression of miR-150-5p is correlated with overall survival.

Besides being indicative of the level of PD-L1 expression in melanoma patients, the expression of miR-150-5p was also correlated to OS in a cohort of n=152 patients. Especially, its overexpression was correlated with better OS, according to Kaplan-Meier analysis (p = 0.0022) (Figure 5). This could be confirmed by a successive multivariable Cox regression, yielding HR of 0.39 (95% CI:0.16 – 0.92) for high expression of miRNA-150-5p compared to low expression of this miRNA (Figure 6).

Conclusions

In summary, this study suggests, particularly within discovery cohorts, that the assessment of liquid biopsy-derived miRNA expression levels could serve as promising candidates for diagnostic, prognostic, and predictive biomarkers. These biomarkers could offer a rapid, reliable, and easily applicable approach for staging melanoma patients, especially in the context of therapy. It's important to note that this investigation does not explicitly delve into a detailed analysis of the targets of the detected miRNAs or the associated pathways/networks. Preliminary insights have been provided for a selected set of miRNAs. The primary focus, as a proof of principle, was to demonstrate that circulating plasma miRNAs may evolve into valuable biomarkers for melanoma development and therapy in a clinically applicable setting. However, further validation is essential through ongoing and newly designed, larger validation cohorts.

Supplementary Material

Table S1: Hypothesis-(literature research) driven list of miRNAs analyzed in this study using the Abcam FirePlex Assay; Table S2: Demographics and patient characteristics for melanoma patients in cohort 1; Table S3: Top 20 KEGG pathways enriched in the 9-miRNA signature examined in Fig.1 for discriminating lower stage melanoma patients and actively metastasizing stage IV melanoma patients; Figure S1 : ROC analysis of the single nine miRNAs to distinguish stage IV melanoma patients from melanoma patients with lower stages. Table S4: Demographics and patient characteristics for melanoma patients in cohort 2 (actively metastasizing stage IV melanoma patients at therapy-start (baseline) and stage IV melanoma patients with first occurrence of progressive disease after start of immune therapy (progress); Table S5: Top 20 pathways enriched in the five-miRNA signature examined in Fig. 3 for discriminating therapy naïve stage IV melanoma patients (baseline) and stage IV melanoma patients at progress during therapy (progress); Table S6: Demographics and patient characteristics in cohort 3 (for treatment-naïve, actively metastasizing stage IV melanoma patients with negative, com-pared to low and high PD-L1 immunostaining of melanoma tissue); Table S7: Top 20 pathways enriched in the two-miRNA signature examined in Fig. 4 for discriminating miRNA expression depending on PD-L1 expression in tumor tissue; Table S8: Baseline differences in patients with lower or higher miR-150-5p expression than the median, respectively.

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Abbreviations

ANOVA - analysis of variance; AUC - Area Under the Curve**;** BRAF-v-Raf murine sarcoma viral oncogene homolog B; ctDNA- circulating tumor DNA; CTLA-4 - T-lymphocyte-associated protein 4; EIF4B - eukaryotic translation initiation factor 4B; EMT - epithelial-mesenchymal transition; Hccm- heteroscedasticity-corrected covariance matrices; HR - Hazard Ratio; ICI - immune checkpoint inhibition; IHC - immunohistochemical; KEGG - Kyoto Encyclopedia of Genes and Genomes; LDH- L - Lactate dehydrogenase; miRNA - microRNA; mTOR - mammalian or mechanistic target of rapamycin; OS - overall survival; PD-1 - programmed cell death protein; PD- L1 - programmed cell death-ligand 1; PFS - progression-free survival; STAT1 - signal transducer and activator of transcription 1;TPS- tumor proportion score; TWIST1 - Twist Family BHLH Transcription Factor 1

References

1. Swart M, Verbrugge I, Beltman J B. (2016) Combination Approaches with Immune-Checkpoint Blockade in Cancer Therapy. *Front Oncol*. *6*, 233, doi:10.3389/fonc.2016.00233.
2. Khair D O, Bax H J, Mele S, et al. (2019) Combining Immune Checkpoint Inhibitors: Established and Emerging Targets and Strategies to Improve Outcomes in Melanoma. *Front Immunol*. *10*, 453, doi:10.3389/fimmu.2019.00453.
3. Ugurel S, Rohmel J, Ascierto P A, et al. (2017) Survival of patients with advanced metastatic melanoma: the impact of novel therapies-update 2017. *Eur J Cancer*. *83*, 247-257, doi:10.1016/j.ejca.2017.06.028.
4. Schadendorf D, van Akkooi A C J, Berking C, et al. (2018) Melanoma. *Lancet*. *392*, 971-984, doi:10.1016/S0140-6736(18)31559-9.
5. Larkin J, Chiarion-Sileni V, Gonzalez R, et al. (2015) Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med*. *373*, 23-34, doi:10.1056/NEJMoa1504030.
6. Robert C, Schachter J, Long G V, et al. (2015) Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med*. *372*, 2521-2532, doi:10.1056/NEJMoa1503093.
7. Lim S Y, Lee J H, Diefenbach R J, et al. (2018) Liquid biomarkers in melanoma: detection and discovery. *Mol Cancer*. *17*, 8, doi:10.1186/s12943-018-0757-5.
8. Diem S, Kasenda B, Spain L, et al. (2016) Serum lactate dehydrogenase as an early marker for outcome in patients treated with anti-PD-1 therapy in metastatic melanoma. *Br J Cancer*. *114*, 256-261, doi:10.1038/bjc.2015.467.

9. Weide B, Martens A, Hassel J C, et al. (2016) Baseline Biomarkers for Outcome of Melanoma Patients Treated with Pembrolizumab. *Clin Cancer Res*. *22*, 5487-5496, doi:10.1158/1078-0432.CCR-16-0127.
10. Martens A, Wistuba-Hamprecht K, Yuan J, et al. (2016) Increases in Absolute Lymphocytes and Circulating CD4+ and CD8+ T Cells Are Associated with Positive Clinical Outcome of Melanoma Patients Treated with Ipilimumab. *Clin Cancer Res*. *22*, 4848-4858, doi:10.1158/1078-0432. CCR-16-0249.
11. Martens A, Wistuba-Hamprecht K, Geukes Foppen M, et al. (2016) Baseline Peripheral Blood Biomarkers Associated with Clinical Outcome of Advanced Melanoma Patients Treated with Ipilimumab. *Clin Cancer Res*. *22*, 2908-2918, doi:10.1158/1078-0432.CCR-15-2412.
12. Ferrucci P F, Gandini S, Cocorocchio E, et al. (2017) Baseline relative eosinophil count as a predictive biomarker for ipilimumab treatment in advanced melanoma. *Oncotarget*. *8*, 79809-79815, doi:10.18632/oncotarget.19748.
13. Fassler M, Diem S, Mangana J, et al. (2019) Antibodies as biomarker candidates for response and survival to checkpoint inhibitors in melanoma patients. *Journal for immunotherapy of cancer*. *7*, 50, doi:10.1186/s40425-019-0523-2.
14. Huang S K, Hoon D S. (2016) Liquid biopsy utility for the surveillance of cutaneous malignant melanoma patients. *Mol Oncol*. *10*, 450-463, doi:10.1016/j.molonc.2015.12.008.
15. Ugurel S, Schadendorf D, Horny K, et al. (2020) Elevated baseline serum PD-1 or PD-L1 predicts poor outcome of PD-1 inhibition therapy in metastatic melanoma. *Annals of oncology : official journal of the European Society for Medical Oncology*. *31*, 144-152, doi:10.1016/ j.annonc.2019.09.005.
16. Pagni F, Guerini-Rocco E, Schultheis A M, et al. (2019) Targeting Immune-Related Biological Processes in Solid Tumors: We do Need Biomarkers. *Int J Mol Sci*. *20*, doi:10.3390/ijms20215452.
17. Lujambio A, Lowe S W. (2012) The microcosmos of cancer. *Nature*. *482*, 347-355, doi:10.1038/ nature10888.
18. Oliveto S, Mancino M, Manfrini N, et al. (2017) Role of microRNAs in translation regulation and cancer. *World J Biol Chem*. *8*, 45-56, doi:10.4331/wjbc.v8.i1.45.
19. Stagg J, Loi S, Divisekera U, et al. (2011) Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A*. *108*, 7142-7147, doi:10.1073/pnas.1016569108.
20. Liu H T, Gao P. (2016) The roles of microRNAs related with progression and metastasis in human cancers. *Tumour Biol*. doi:10.1007/s13277-016-5436-9.
21. Adams B D, Kasinski A L, Slack F J. (2014) Aberrant regulation and function of microRNAs in cancer. *Curr Biol*. *24*, R762-776, doi:10.1016/j.cub.2014.06.043.
22. Zhang X L, Xu L L, Wang F. (2017) Hsa_circ_0020397 regulates colorectal cancer cell viability, apoptosis and invasion by promoting the expression of the miR-138 targets TERT and PD-L1. *Cell Biol Int*. *41*, 1056-1064, doi:10.1002/cbin.10826.
23. Nouraee N, Calin G A. (2013) MicroRNAs as cancer biomarkers. *Microrna*. *2*, 102-117.
24. Lan H, Lu H, Wang X, et al. (2015) MicroRNAs as potential biomarkers in cancer: opportunities and challenges. *Biomed Res Int*. *2015*, 125094, doi:10.1155/2015/125094.

25. Zeng J, Chen Q W, Yu Z Y, et al. (2016) Regulation of intrinsic apoptosis in cycloheximide-treated macrophages by the Sichuan human strain of Chinese Leishmania isolates. *Acta Trop*. *153*, 101-110, doi:10.1016/j.actatropica.2015.10.010.
26. Anfossi S, Babayan A, Pantel K, et al. (2018) Clinical utility of circulating non-coding RNAs - an update. *Nat Rev Clin Oncol*. doi:10.1038/s41571-018-0035-x.
27. Eichmuller S B, Osen W, Mandelboim O, et al. (2017) Immune Modulatory microRNAs Involved in Tumor Attack and Tumor Immune Escape. *J Natl Cancer Inst*. *109*, doi:10.1093/jnci/djx034.
28. Yi M, Xu L, Jiao Y, et al. (2020) The role of cancer-derived microRNAs in cancer immune escape. *J Hematol Oncol*. *13*, 25, doi:10.1186/s13045-020-00848-8.
29. Tonella L, Pala V, Ponti R, et al. (2021) Prognostic and Predictive Biomarkers in Stage III Melanoma: Current Insights and Clinical Implications. *Int J Mol Sci*. *22*, doi:10.3390/ijms22094561.
30. Ou F S, Michiels S, Shyr Y, et al. (2021) Biomarker Discovery and Validation: Statistical Considerations. *J Thorac Oncol*. *16*, 537-545, doi:10.1016/j.jtho.2021.01.1616.
31. Therasse P, Arbuck S G, Eisenhauer E A, et al. (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst*. *92*, 205-216, doi:10.1093/jnci/92.3.205.
32. Tackett M R, Diwan I. (2017) Using FirePlex() Particle Technology for Multiplex MicroRNA Profiling Without RNA Purification. *Methods Mol Biol*. *1654*, 209-219, doi:10.1007/978-1-4939-7231-9_14.
33. Dako. PD-L1 IHC 28-8 pharmDx Interpretation Manual Melanoma. Available online: https://www.agilent.com/cs/library/usermanuals/public/29120pd-l1-ihc-28-8-interpretation-manual-melanoma.pdf (accessed on
34. Therneau T M G, P. M.(2000)*Modeling Survival Data: Extending the Cox Model*. 1 ed.; Springer, New York, NY: pp. XIV, 350.
35. Kassambara A K, M.;, Biecek, P. survminer: Survival Analysis and Visualization. Available online: https://rpkgs.datanovia.com/survminer/ (accessed on
36. Pardini B, Sabo A A, Birolo G, et al. (2019) Noncoding RNAs in Extracellular Fluids as Cancer Biomarkers: The New Frontier of Liquid Biopsies. *Cancers (Basel)*. *11*, doi:10.3390/cancers11081170.
37. Jayawardana K, Schramm S J, Tembe V, et al. (2016) Identification, Review, and Systematic Cross-Validation of microRNA Prognostic Signatures in Metastatic Melanoma. *J Invest Dermatol*. *136*, 245-254, doi:10.1038/JID.2015.355.
38. Lu T, Chen S, Qu L, et al. (2019) Identification of a five-miRNA signature predicting survival in cutaneous melanoma cancer patients. *PeerJ*. *7*, e7831, doi:10.7717/peerj.7831.
39. Huber V, Vallacchi V, Fleming V, et al. (2018) Tumor-derived microRNAs induce myeloid suppressor cells and predict immunotherapy resistance in melanoma. *J Clin Invest*. *128*, 5505-5516, doi:10.1172/JCI98060.

40. Nakahara S, Fukushima S, Okada E, et al. (2020) MicroRNAs that predict the effectiveness of anti -PD-1 therapies in patients with advanced melanoma. *J Dermatol Sci*. *97*, 77-79, doi:10.1016/ j.jdermsci.2019.11.010.
41. Pinto R, Strippoli S, De Summa S, et al. (2015) MicroRNA expression in BRAF-mutated and wild -type metastatic melanoma and its correlation with response duration to BRAF inhibitors. *Expert Opin Ther Targets*. *19*, 1027-1035, doi:10.1517/14728222.2015.1065818.
42. DiVincenzo M J, Latchana N, Abrams Z, et al. (2020) Tissue microRNA expression profiling in hepatic and pulmonary metastatic melanoma. *Melanoma Res*. *30*, 455-464, doi:10.1097/ CMR.0000000000000692.
43. Diaz-Martinez M, Benito-Jardon L, Alonso L, et al. (2018) miR-204-5p and miR-211-5p Contribute to BRAF Inhibitor Resistance in Melanoma. *Cancer Res*. *78*, 1017-1030, doi:10.1158/0008-5472.CAN-17-1318.
44. Vitiello M, D'Aurizio R, Poliseno L. (2018) Biological role of miR-204 and miR-211 in melanoma. *Oncoscience*. *5*, 248-251, doi:10.18632/oncoscience.443.
45. Yamazaki H, Chijiwa T, Inoue Y, et al. (2012) Overexpression of the miR-34 family suppresses invasive growth of malignant melanoma with the wild-type p53 gene. *Exp Ther Med*. *3*, 793-796, doi:10.3892/etm.2012.497.
46. Bellenghi M, Pontecorvi G, Care A. (2019) On exosome functional role in cancer: miR-494 complex regulation in melanoma cells and corresponding exosomes. *Transl Cancer Res*. *8*, 725-728, doi:10.21037/tcr.2019.04.08.
47. Wang Q, Lin W, Tang X, et al. (2017) The Roles of microRNAs in Regulating the Expression of PD-1/PD-L1 Immune Checkpoint. *Int J Mol Sci*. *18*, doi:10.3390/ijms18122540.
48. Yang J, Mani S A, Donaher J L, et al. (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. *117*, 927-939, doi:10.1016/j.cell.2004.06.006.
49. Ansieau S, Morel A P, Hinkal G, et al. (2010) TWISTing an embryonic transcription factor into an oncoprotein. *Oncogene*. *29*, 3173-3184, doi:10.1038/onc.2010.92.
50. Beck B, Lapouge G, Rorive S, et al. (2015) Different levels of Twist1 regulate skin tumor initiation, stemness, and progression. *Cell Stem Cell*. *16*, 67-79, doi:10.1016/j.stem.2014.12.002.