Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN

ONCOLOGIA, EMATOLOGIA E PATOLOGIA

Ciclo 35

Settore Concorsuale: 06/D3 - MALATTIE DEL SANGUE, ONCOLOGIA E REUMATOLOGIA

Settore Scientifico Disciplinare: MED/06 - ONCOLOGIA MEDICA

EXPLORATORY ANALYSIS OF THE ROLE OF CIRCULATING MICRORNA IN METASTATIC MELANOMA PATIENTS

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Esame finale anno 2023

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ABSTRACT

MicroRNAs act as oncogene or tumor suppressor gene regulators and are actively released from tumor cells in the circulation. Specific microRNAs can be isolated and quantified in the blood, usually in serum or plasma fractions, where they are uncommonly stable. Cell-free microRNAs serve many, and possibly yet unexplored, functional roles and microRNA levels reflect underlying conditions and have been associated with skin cancer presence, stage and evolution. However, the clinical potential of circulating miRNAs in metastatic melanoma remains largely undefined.

From May 2020 to September 2022, we conducted a spontaneous, monocentric, exploratory study on human tissues in vitro, which aimed to evaluate the prognostic and predictive role of circulating miRNAs in metastatic melanoma patients.

At the Medical Oncology Unit of Policlinico Sant'Orsola-Malpighi of Bologna, peripheral venous blood samples from patients with metastatic melanoma treated with checkpoint inhibitors (CPI) were collected before the start of CPI (baseline, T0) and longitudinally, approximately every 3 months (T1, T2, etc). Circulating miRNA quantification was performed by droplet digital PCR (Biorad) using an EvaGreen and LNA primer-based assays. QuantaSoft Program (Biorad) calculated the absolute quantifications of each miRNA, indicated as copies/µL.

After analysis of the literature, we chose to analyze miR-155-5p, miR-320a and miR-424-5p level. All miRNAs except miR-424-5p show a significantly higher level in plasma of patients who are alive after 1 year of follow-up. High/low levels of baseline miR-155-5p, miR-320a and miR-424-5p are significantly associated with overall survival and progression-free survival.

Furthermore, a preliminary analysis on the group of patients who received first-line with anti-PD-1 (N=7), baseline miR-155-5p shows higher levels in responder vs. non responder patients (p 0.06). These data, though promising, are preliminary and need to be further investigated in a larger cohort of patients.

INTRODUCTION

Malignant melanoma is a type of skin cancer that comprises less than 5% of all cutaneous malignancies, although it accounts for the majority of skin cancer-related deaths.

The incidence and mortality rates of melanoma differ widely across the globe. Worldwide, 324635 cases of all newly diagnosed primary malignant cancers are cases of cutaneous melanoma, and annually about 57043 cancer deaths are due to cutaneous melanoma. The incidence of cutaneous melanoma has increased since the early 1970s in predominantly fair skinned populations [1]. Age-cohort period analyses of melanoma incidence in Australia, New Zealand, Norway, Sweden, the UK, and the white population of the USA from 1982 to 2011 revealed that the incidence increased about 3% annually, and will further increase at least until 2022 in Norway, Sweden, the UK, and the USA [2]. In 2020, 14 900 newly diagnosed melanoma cases were expected in Italy, where melanoma is the second most common malignancy in under-50-years men, the third most common in under-50-years women and accounts for 1% of cancer deaths in both sexes [3].

The prognosis of melanoma is excellent for patients who present with localized disease and primary tumors 1 mm or less in thickness, with 10-year melanoma-specific-survival of 95%. For patients with localized melanomas more than 1.0 mm in thickness (stage II), 5-year survival rates range from 82% to 94%, depending on tumor thickness, presence of ulceration, and mitotic rate. When regional nodes are involved (stage III), 5-year survival rates range from 32% to 93%, depending primarily on the nodal tumor burden [4]. It is uncommon for patients with melanoma to present initially with metastatic disease (stage IV). Most patients develop distant metastases after an interval from their original management for localized or regional metastatic disease. Often, metastases become evident within 2 to 3 years of diagnosis, but delayed metastasis is also common, and for melanoma, regional and distant metastases have occurred after disease-free intervals measured in decades [5] . In general, the interval to detection of distant metastases is shorter for patients who initially present with high-stage disease and is longest for patients who present with clinically localized thin melanomas.

Historically, survival outcomes for patients with metastatic melanoma (stage IV) have been poor. Few treatment options were available and median overall survival was 6-10 months and the 5-year survival rate approximately 10% [6]. However, over the past 10 years, increased biological understanding and access to innovative therapeutic substances have markedly changed the prognosis of metastatic melanoma (MM) patients and have transformed advanced melanoma into a new oncological model for treating solid cancers.

Targeted therapy

The mitogen activated protein kinase (MAPK) pathway, which features the sequence RAS, RAF, MEK, and ERK, is one of the major signaling networks involved in melanoma tumorigenesis [7]. A major driver of this pathway is BRAF, which can initiate a cascade of events including phosphorylation and activation of MEK. BRAF mutations are found in 50% of cutaneous melanomas, with most (70–95%) consisting of a V600E substitution, while a smaller proportion (5–30%) are V600K substitutions [8].

Vemurafenib, an inhibitor of mutant BRAF, was approved by the Food and Drug Administration (FDA) in 2011 for the treatment of melanoma patients harboring the BRAF V600E mutation based on improved overall survival (OS) versus dacarbazine (DTIC) in the BRIM-3 phase III study [9]. The melanoma armamentarium expanded again with the FDA approval of dabrafenib and trametinib. Dabrafenib was approved for patients with unresectable or metastatic melanoma harboring the BRAF V600E mutation based on results from a phase III trial showing improved median progression-free survival (mPFS) versus DTIC (5.1 months for dabrafenib vs. 2.7 months for DTIC; HR, 0.30)[10]. The MEK inhibitor trametinib was approved for melanoma patients harboring a BRAF V600E or V600K mutation based on results from a phase III trial showing improved median PFS versus DTIC or paclitaxel (4.8 months for trametinib vs. 1.5 months for DTIC or paclitaxel; HR, 0.45) [11]. In 2014, the FDA approved the use of dabrafenib in combination with trametinib for patients with BRAF V600E- or V600K-mutated melanoma (making dabrafenib/trametinib the first FDA-approved targeted combination therapy) on the base of improved overall response rate and median duration of response versus BRAF inhibitor monotherapy demonstrated in a phase I/II trial [12] and then confirmed in the phase III studies Combi-D and Combi-V [13,14]. Overall, BRAF inhibitors have shown objective response rates (ORR) of approximately 50%, which increase to 70% when combined with MEK inhibitors. Additionally, mPFS increases from 7-9 months with single-agent BRAF inhibitors to 11-14.9 months with BRAF and MEK inhibitors combination. Furthermore, a recently published pooled analysis showed that one third of patients who received first line treatment with dabrafenib and trametinib is alive and 19 % were progression free at 5 years [15]. In the COLUMBUS trial, treatment with the combination of the BRAF inhibitor encorafenib 450 mg and the MEK inhibitor binimetinib 45 mg improved progression-free survival and overall response compared with encorafenib 300 mg or vemurafenib, with better tolerability in patients with locally advanced unresectable or metastatic BRAF V600–mutant melanoma, untreated or progressed after first-line

immunotherapy. The median PFS and OS with encorafenib plus binimetinib were 14.9 months and 33.6 months [16,17].

To sum up, three BRAF-MEK combinations are now approved: vemurafenib and cobimetinib, dabrafenib and trametinib and encorafenib and binimetinib. Essentially, the efficacy data for these treatment combinations are highly comparable, whereas their pharmacokinetics and toxicity profiles differ in some regards.

Immunotherapy

The rationale for using immunotherapy to treat advanced melanoma was based on two observations that suggest involvement of the immune system in the natural history of melanoma. First, a small proportion of patients experience spontaneous tumor regression in primary, but not metastatic, tumors in the absence of systemic intervention, suggesting that melanoma may be an immunologically modulated malignancy [18]. Second, high dose interleukin-2 (HD IL-2), a cytokine that induces T-cell activation and proliferation, demonstrated promising antitumor activity in murine models [19].

Therefore, initial attempts to improve outcomes in patients with advanced melanoma focused on the use of HD IL-2, that was evaluated in a series of phase II melanoma trials [20–23]. Although HD IL-2 may provide durable responses of over 10 years in some patients, its use is limited by severe toxicity that can affect multiple organ systems (e.g., cardiovascular, respiratory, nervous, renal, digestive, and skin) [24]. Despite these limitations, the experience with HD IL-2 provides proof-of-concept that modulation of the immune system might offer durable clinical benefit in melanoma.

Improvements in understanding of tumor immunology have led to the development of targeted immunotherapies aimed at specific immune-checkpoints, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed death-1 (PD-1), and programmed death ligand-1 (PD-L1). CTLA-4 and PD-1 are inhibitory receptors with non-overlapping roles in modulating the adaptive immune response. CTLA-4 acts primarily early in the immune response to regulate T-cell proliferation and migration to the tumor, whereas PD-1 and its ligand PD-L1 regulate T-cell activation and proliferation at the tumor site [25].

Ipilimumab, which targets CTLA-4, was approved by the US Food and Drug Administration (FDA) and the European Medicines Agency in 2011 for the treatment of unresectable or metastatic melanoma. A survival benefit with ipilimumab was demonstrated in two randomized, controlled phase III trials (MDX010-20 and CA184-024) [26,27]. In study MDX010-20, previously treated

melanoma patients received ipilimumab 3 mg/kg plus the melanoma peptide vaccine gp100, ipilimumab 3 mg/kg alone, or gp100 alone [26]. The median OS for these treatment groups was 10.0, 10.1, and 6.4 months, respectively. The hazard ratio (HR) for death compared with gp100 alone was 0.68 ($p < 0.001$) for the ipilimumab plus gp100 group and 0.66 ($p = 0.003$) for the ipilimumab-alone group. In the study CA184-024, previously untreated patients received ipilimumab 10 mg/kg plus DTIC or DTIC plus placebo. The median OS for these treatment groups was 11.2 and 9.1 months, respectively (HR, 0.72; $p < 0.001$) [27].

A meta-analysis of pooled OS data from ipilimumab trials, which included data from 1861 melanoma patients, reported a 3-year OS rate of 22%; furthermore, a plateau in the pooled Kaplan–Meier curve began at approximately 3 years after initiation of therapy, and extended through follow-up of as long as 10 years [28]. More recently, it was demonstrated that higher-doses of ipilimumab (10 mg/kg) showed an advantage in terms of OS (15.7 vs.11.5 months), with more frequent immune-mediated toxicity [29]. Ipilimumab can be associated with different kinds of side effects, due to the immune system activation by CTLA-4 blockade. Collectively, the spectrum of side effects is described as immune-related adverse events (irAEs). irAEs most commonly affected the skin (rash/vitiligo/pruritis), the liver (hepatitis/rise in liver enzymes), the bowel (colitis), and the endocrine system (hypophysitis, thyroiditis, adrenal insufficiency). More rarely, uveitis, conjunctivitis, neuropathy, myopathy, and nephritis have been known to occur.

The success of ipilimumab was closely followed by the development of additional immune-checkpoint inhibitors (CPI). The anti-PD-1 agents, nivolumab and pembrolizumab, have demonstrated improved survival and less toxicity compared with ipilimumab.

In a phase I trial, nivolumab had a 48% objective response rate (ORR) and 32% OS rate at 4 years [30]. In the phase III Checkmate 066 trial, nivolumab demonstrated superior OS in previously untreated melanoma patients without BRAF mutation [31,32]. In the Checkmate 067 trial, nivolumab demonstrated substantially improved ORR, progression-free survival (PFS), and OS compared with ipilimumab as first line treatment [33].

In the phase I trial KEYNOTE-001 of pembrolizumab, there was a 5-year OS rate of 34% in patients with previously treated and treatment-naive advanced melanoma and 41% in untreated patients only [34]. In the phase II trial (KEYNOTE-002), pembrolizumab at two different dosages (2 and 10 mg/kg every 3 weeks) were compared to investigator choice chemotherapy in patients pretreated with ipilimumab, and both doses improved PFS and ORR [35].

In a phase III trial comparing pembrolizumab with ipilimumab (KEYNOTE-006 trial), after a median follow-up of 57,7 months, mOS was 32,7 months in the combined pembrolizumab groups and 15,9 months in the ipilimumab group. Additionally, treatment-related grade 3–5 adverse

events were less frequent with pembrolizumab (13.3% and 10.1%) than with ipilimumab (19.9%) [36].

Ipilimumab in combination with nivolumab has been studied in melanoma clinical trials. In the CheckMate-067 phase III trial, nivolumab alone or nivolumab plus ipilimumab were compared with ipilimumab alone in 945 previously untreated patients with metastatic melanoma [33]. The rates of objective response were 57.6% in the nivolumab-plus-ipilimumab group, 43.7% in the nivolumab group, and 19.0% in the ipilimumab group. The mPFS was 11.5 months in the nivolumab plus-ipilimumab group, 6.9 months in the nivolumab group, and 2.9 months in the ipilimumab group. Treatment-related grade 3/4 adverse events were observed in 59% of patients in the nivolumab plus ipilimumab group, in 22% of the nivolumab group, and 28% of the ipilimumab group. At the last follow-up, 6.5-year OS rate was 49% in the combination group and 42% in the nivolumab group, as compared with 23% in the ipilimumab group. The median overall survival was 72.1 months for patients treated with nivolumab plus ipilimumab, 36.9 months in the nivolumab group, and 19.9 months in the ipilimumab group [37].

Despite progress in treatment of advanced disease, the majority of patients treated with targeted therapy develop resistance while on therapy and approximately 60% and 20−30% of melanoma patients show primary and secondary resistance to PD-1 checkpoint inhibition, respectively [38].

Primary resistance to CPI occurs when there is a failure to induce an effective antitumor immune response at any of the three stage of the cancer immune cycle [antigen presentation and T-cell activation, T-cell trafficking and tumor infiltration and T-cell killing activity within the tumor microenvironment (TME)]. Primary resistance mechanisms are poor immunogenicity, impaired dendritic cells maturation (IL-6, IL-10 from TME, lipid accumulation, IL-35), downregulation of chemokines required for T-cell recruitment, upregulation of endothelin B receptor, overexpression of VEGF, induction of indoleamine 2,3-dioxygenase (IDO), upregulation of regulatory T cells and expression of innate anti-PD1 resistance signature (IPRES) transcriptional signature. Acquired resistance occurs in patients who relapse after exhibiting initial response to immunotherapy. Mechanisms that give rise to secondary resistance are the loss of beta-2 microglobulin, loss-of-function mutations in JAK/STAT pathway, upregulation of PD-L1, upregulation of immune checkpoint markers [e.g. lymphocyte activation gene 3 (LAG3), T-cell immunoglobulin and mucin domain 3 (TIM-3)] [39,40].

Biomarkers in melanoma

As in other types of cancer, the search for tumor markers in melanoma is being intensively investigated to provide better tools for less invasive disease management. Historically, the lack of effective therapies against advanced melanoma has limited the utility of these markers. Fortunately, encouraging results obtained with new therapeutic strategies has stimulated renewed interest in this field.

For many years, lactate dehydrogenase (LDH) has been the only serum biomarker used in metastatic melanoma. LDH is an ubiquitous enzyme having a tetrameric structure. Subunits that can be of two types: M (muscle type) or H (heart type) encoded by two genes LDH-A and LDH-B, respectively. LDH catalyzes the conversion of pyruvate to lactate. This conversion is essential in hypoxic and anaerobic conditions when adenosine triphosphate (ATP) production by oxidative phosphorylation is disrupted. In malignancy, the growth of tumor cells consumes oxygen supply and hypoxia is quite common. Throughout the decades, several studies have shown that LDH is a marker of metastases especially in liver [41,42] and is an important independent prognostic factor as patients with increased LDH had reduced survival [43–45].

Predictive biomarkers for immunotherapy can be classified into three major groups: tumor-intrinsic biomarkers, which are expressed by tumor cells (e.g., PD-1/PDL-1, tumor mutational burden); tumor microenvironment biomarkers (e.g., tumor-infiltrating lymphocyte); and systemic biomarkers (e.g., circulating factors, microbiota).

Tumor mutational burden (TMB) is an index that summarizes the mutational load of a tumor. Since a high number of mutations could translate into a high number of neoantigens that the immune system can recognize, it has been hypothesized that TMB could act as a proxy for CPI effectiveness. As shown by Cristescu et al. [46], melanomas with high TMB have a response rate to pembrolizumab of 42% versus only 9% of melanomas with low TMB. However even though high TMB alone can identify a subset of tumors particularly sensitive to CPI, it should be highlighted that cancers with low TMB could also derive benefits from immunotherapies, albeit in a small percentage of patients (5%) [47]. Theoretically, even in a context of low mutational burden, strong immunogenic neoantigens could be generated, albeit with a lower probability compared to a high TMB context.

Programmed cell death 1 receptor (PD-1) is a checkpoint molecule present on T-cells, B-cells, and natural killer cells, which can interact with its ligands: PD-L1, expressed on tumor cells. PD-L1 is, indeed, the most commonly recognized biomarker to predict immunotherapy response in patients with different solid tumors, including cutaneous melanoma [48]. For pembrolizumab,

nivolumab, and combined regimens, there is a significantly higher benefit in patients with positive PD-L1 melanomas. Notwithstanding, PD-1/PD-L1 inhibitors appear to also have activity in subsets of patients who do not meet IHC positivity to PD-L1 [49]. This is mainly because PD-L1 expression might represent only a component of T cell–related biology that is relevant to a favorable tumor immune microenvironment.

Newer genomic technologies can be used to evaluate complexities of tumor and host immune cell interactions within the tumor microenvironment, going beyond the measurement of single analytes such as PD-L1. Ayers et al examined the T cell-inflamed gene expression profiling (GPE) in the tumor microenvironment, using RNA isolated from formalin-fixed paraffin-embedded (FFPE) tumor tissue samples and found that IFN-γ–related gene expression signatures predict response to PD-1 checkpoint blockade in melanoma [50]. Similarly, Gide et al. performed transcriptomic and immune profiling on 158 melanoma samples and identified two gene clusters associated with better outcomes with anti-PD-1 monotherapy. In particular, IFN-related genes (such as TBX21, STAT1, IRF1, TNF, and IFNG) and tumor-infiltrating T-cell genes (CCL5, CXCL13, and IL-2) were highly expressed, suggesting activated T-cells enriched tumoral microenvironment in responders with IFN-secretive phenotype [51].

Melanoma cells release a myriad of compounds into circulation either by active secretion or as result of cell death. Others are endogenously produced in response to the disease process. As can be expected, the concentration of these substances, i.e., biomarkers, can change during the course of the illness in response to progression or therapeutic intervention. These markers include nucleic acid, protein, metabolites, and microvesicles. In addition, during disease progression, some cells can detach from the primary tumor and be incorporated into the circulatory compartment and as such serve as biomarkers themselves. Among the required characteristics, an ideal tumor marker should have high sensitivity and specificity, ideally 100%, and should be easily quantified in accessible samples. Blood is a very accessible specimen that can be obtained repeatedly providing a more dynamic picture of the disease process versus a tissue biopsy that encompasses a single point in time. Circulating biomarkers offer information related to the diagnosis, staging, prognosis, and monitoring of the disease process [52].

Circulating microRNAs

MicroRNAs (miRNAs) are endogenous single-stranded nucleotides, 18–25 in length, that bind to 3′-untranslated regions of a target gene, which in turn might regulate multiple cellular processes through modulation of RNA translation. To date, miRNAs are by far the most studied non-coding

RNAs (ncRNAs) in cancer and are certainly the most well-studied RNA source as liquid biopsies. Due to their high abundance and inherent stability in a variety of bodily fluids (e.g. blood, urine, stool and saliva), miRNAs have been regarded as one of the most promising non-invasive biomarker sources [53–55].

The miRNA biogenesis begins with transcribing gene into large primary transcript (pri-miRNA), which is 5′ capped and 3′ polyadenylated in structure. The transcription is typically mediated by RNA polymerase II, although some pre-miRNAs are generated by RNA polymerase III [56,57]. The pri-miRNAs are then cleaved by a microprocessor complex, composed of RNA-binding protein DGCR8 and type III RNase Drosha, into a ~ 85-nucleotide stem–loop structure called precursor miRNA (pre-miRNA). Following transportation by Ran/GTP/Exportin 5 complex from nucleus to cytoplasm, the pre-miRNAs are processed by another RNase III enzyme Dicer to a ~20–22-nucleotide miRNA/miRNA duplex. After the duplex is unwound, the mature miRNA is incorporated into a protein complex termed miRNA-induced silencing complex (miRISC) and guides RISC to target mRNA [58]. MiRNAs can target hundreds of mRNAs and regulate different biological processes, including immune cell differentiation [59].

Several studies have demonstrated the release of extracellular RNAs in blood and biological fluids including urine, saliva, seminal, ascites, and cerebrospinal fluid [60–63]. In addition, the expression profile of circulating miRNAs is different if considering biological fluids' origin and different pathophysiological conditions, thus indicating that extracellular miRNAs may be selectively released from cells and not only passively released from necrotic or injured cells. Studies suggest two major ways for miRNA release into the extracellular microenvironment: miRNAs can be packaged and transported in extracellular vesicles (EVs) or associated with RNA binding proteins or lipoproteins (Figure 1) [64].

Currently, majority of non-coding RNA(ncRNA)-based liquid biopsy biomarker candidates are investigated for diagnostic and screening purposes [65]. Since miRNAs are frequently overexpressed in cancers, they are plausibly suitable for monitoring of cancer progression and recurrence as well. Accordingly, several studies have demonstrated the utility of circulating ncRNAs, including miRNAs, for cancer monitoring [66]. Furthermore, considering that tissue-based profiling of various ncRNA types in multiple cancers have clarified the functional roles of miRNAs in cancers, there is an increasing research interest to understand the molecular profiles of these novel ncRNAs in the circulation for potential blood-based cancer biomarker discovery [65].

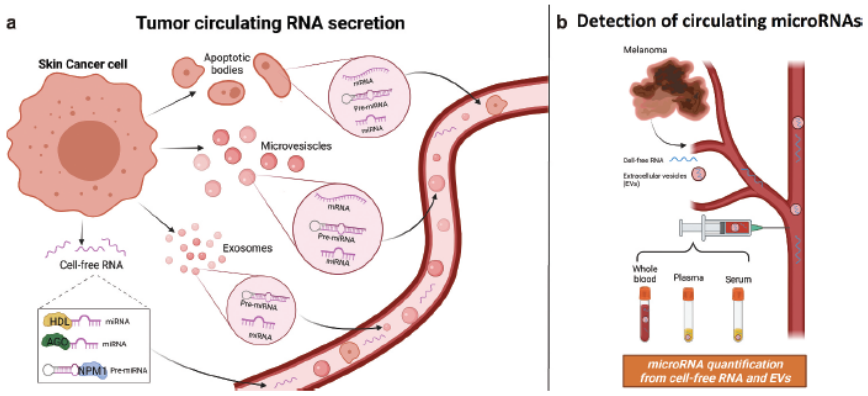

Figure 1. **RNA release from tumor cells. A)** microRNAs are released from the cancer cell into the extracellular microenvironment packaged and transported in extracellular vesicles, including exosomes, microvesicles and apoptotic bodies; or associated with RNA-binding proteins, such as Argonaute (AGO) and nucleophosmin1 (NPM1), or with lipoproteins, including high-density lipoprotein (HDL). **B)** Melanoma cells release RNAs in the extracellular microenvironment and in the bloodstream. RNA molecules are either complexed with RNA-binding proteins or lipoproteins or packaged in extracellular vesicles (EVs). microRNAs can be detected in whole blood, plasma, and serum samples. Circulating miRNA levels are different in melanoma *vs.* normal *vs.* other tumors and can be used as diagnostic biomarkers.

Selection of miRNAs

MiR-155 is highly expressed in activated B cells, T cells and other immune cells and plays key roles in modulating humoral and innate cell-mediated immune responses. Ji and colleagues demonstrated that miR-155 enhances the anti-tumor response by epigenetically restricting CD8+ T cell differentiation and functional exhaustion [67]. Moreover, it was showed that in a melanoma mouse model, miR-155 expression in CD8+ T cells reflects the strength of in situ antigen stimulation, independent of the inflammatory environment. Anti–PD-1 treatment leads to both increased miR-155 expression and tumor control by specific CD8+T cells. In addition, low miR-155 target gene signature in tumors is associated with prolonged overall survival in melanoma patients, raising the hypothesis that high miR-155 expression in CD8+ tumor-infiltrating T cells may be a surrogate marker of the relative potency of in situ antigen-specific CD8+ T-cell responses [68].

MiR-320a exhibits abnormal expression levels in multiple malignancies and is involved in the formation, progression, and metastasis of cancer. In a recent study conducted in patients with advanced non-small cell lung cancer (NSCLC), plasma exosomal microRNAs were investigated. It was found that a high level of miR-320 family could be correlated with an unfavorable response to anti-PD-1 treatment and could be potential predictive factor of anti-PD-1 therapy [69].

In a study performed on human ovarian cancer cell lines OVCAR-3 and tumour ovarian tissues, it has been demonstrated that miR-424 overexpression reduced PD-L1 and CD80 expression through direct binding to the 3'-UTRs of these genes. Furthermore, low miR-424 and high PD-L1 expression were significantly correlated and strongly associated with chemoresistant phenotypes

in ovarian cancer and restoration of miR-424 expression enhanced the sensitivity of cancer cells to drug treatment and was accompanied by T-cell activation by blocking the PD-L1 immune checkpoint in both in vitro and in vivo models [70]. In a more recent study the serum miRNA profiling was performed in NSCLC patients treated with anti-PD1 drugs, showing that some miRNAs including miR-424-5p were significantly increased in responders [71].

OBJECTIVES

In the present study we aimed to evaluate the prognostic and predictive role of circulating miRNAs in metastatic melanoma patients treated with CPI. Specifically, the miRNAs we chose to analyze are: miR-155-5p, miR-320a and miR-424-5p. The choice of these miRNAs was performed after a careful analysis of the literature.

METHODS

The study protocol was approved by the Ethics Committee of Bologna University Hospital. All participants provided written informed consent for the use of their samples for research purposes. Patients with unresectable locally advanced or metastatic cutaneous melanoma were enrolled in the Medical Oncology Unit of Policlinico Sant'Orsola-Malpighi of Bologna, from May 2020 to May 2022.

In the present study, peripheral venous blood samples from patients with metastatic melanoma treated with CPI were collected before the start of CPI treatment (baseline, T0) and longitudinally, approximately every 3 months (T1, T2, etc). Fresh blood was collected in EDTA tubes (BD Vacutainer). For plasma separation EDTA tubes were centrifuged at 1900xg for 10 minutes at 4°C, while for serum plain tubes were centrifuged at 1100xg at room temperature. Plasma and serum has been stored at -80°C until use. Total RNA was extracted from 200 µL of plasma samples using miRNeasy kit (Qiagen) after adding cel-miR-39-3p as spike-in molecule. The conversion of RNA to cDNA was performed using 2 µL of RNA template and the miRCURY LNA RT kit (Qiagen). Circulating miRNA quantification was performed by droplet digital PCR (Biorad) using an EvaGreen and LNA primer-based assays. QuantaSoft Program (Biorad) calculated the absolute quantifications of each miRNA, indicated as copies/µL.

Tumor burden was quantified by adding the sum of the longest dimensions of all measurable baseline target lesions according to RECIST v1.1 criteria.

OS was defined as the time elapsed between the first blood draw (T0) and death from any cause. PFS was defined as the time elapsed between the start of immunotherapy treatment and the progression of disease. The best performing cut-off level for classifying patients into low and high miRNA level groups was performed using the web application Cutoff Finder. We analyzed differences across the groups using Mann-Whitney and Unpaired t-test according to D'Agostino-Pearson Test for normality. Statistical analyses were performed using Microsoft Excel, Statistical Package for the Social Sciences (SPSS) program version 28.0 (IBM, Armonk, NY, USA) and R studio.

RESULTS

Patients characteristics and treatments

Fourteen patients affected by metastatic cutaneous melanoma were included in this analysis. According to American Joint Commission on Cancer (AJCC) 8th edition, 4 patients were M1a (29%), 3 patients M1c (21%) and 7 patients M1d (50%). Median age at the time of study enrollment was 65 years (range 39-86 years). Five patients harbored a BRAF mutation (36%). Half of the patients (N=7) had elevated basal lactate dehydrogenase (LDH) level. All the patients had at least 3 metastases: 3-5 metastases in 8 patients (57%), >5 in 6 patients (43%). Median baseline tumor size was 105 mm (range 48-299 mm), 6 patients had baseline tumor burden <100 mm (43%) and 8 patients >100 mm (57%). Patients' characteristics are summarized in Table 1.

Table 1 Patients' characteristics

Characteristic	**No. of patients**	**Percentage %**
Age		
Median (yr) - range	65 (39-86)	
Gender		
Female	5	36
Male	9	64
BRAF mutation status		
wild type	9	64
mutated	5	36
Elevated baseline LDH level	7	50
Baseline no. of metastases		
<3	0	
3-5	8	57
>5	6	43
M stage (AJCC 8)		
M1a	4	29
M1b	0	
M1c	3	21
M1d	7	50
Baseline Tumor Size		
Median (mm) - range	105 (48- 299)	
<100 mm	6	43
>100 mm	8	57

Seven patients received first line treatment with anti-PD1 (4 patients nivolumab and 3 patients pembrolizumab), one patient received the combination of nivolumab and ipilimumab after relapsing on adjuvant treatment with nivolumab, three patients received the combination of nivolumab and ipilimumab for progressive disease on BRAF/MEK inhibition and three patients

received first line treatment with nivolumab and ipilimumab. Median duration of anti-PD1-based therapy was 8 months (95% CI: 3,5 -12,5). Four patients received subsequent systemic treatments (two patients received ipilimumab and two patients were rechallenged with BRAF/MEK inhibition) (Figure 2). Three patients received concomitant radiotherapy and one patient elettrochemotherapy.

The database lock occurred on September 23, 2022. The median follow up time for the alive patients was 15.4 months. Objective response rate was 36%, median PFS 3.5 months and median OS 7.3 months. Four patients developed treatment-related toxicity.

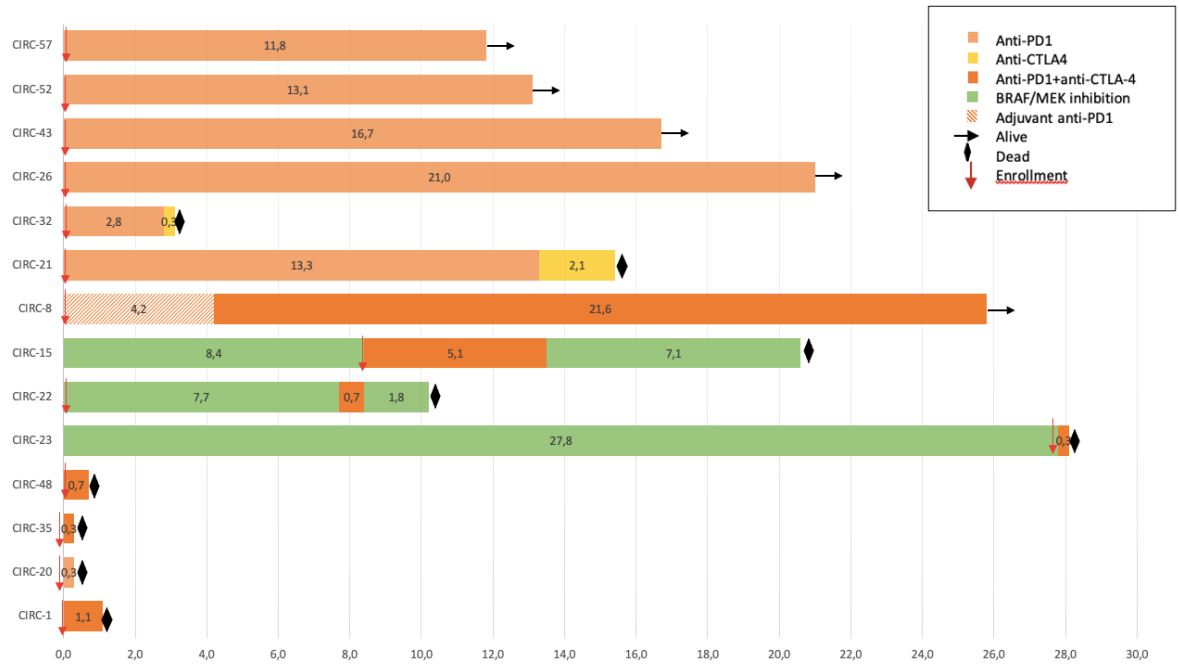

Figure 2 The swimmer plot shows the treatment history for all the patients. Each bar represents one patient.

MiRNAs analysis

MiR-155-5p, miR-320a and miR-424-5p expression from metastatic melanoma patients' serum and plasma samples were analysed by ddPCR in our laboratory. The miRNA level was higher in plasma than serum samples, therefore we chose to analyse their expression in plasma samples.

We analyzed miR-155-5p, miR-320a and miR-424-5p circulating levels before starting treatment with CPI (T0). As shown in Figure 3, all miRNAs except miR-424-5p show a significantly higher level in plasma of patients who are alive after 1 year of follow-up.

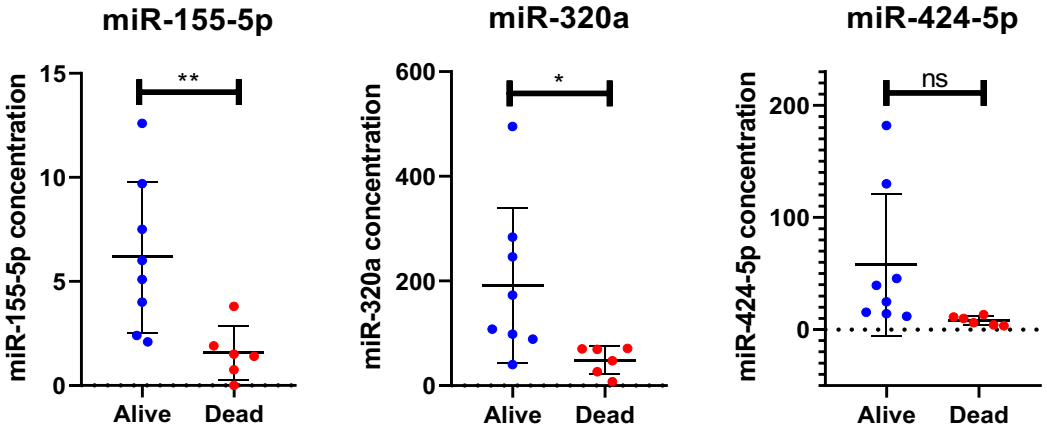

Figure 3 Baseline circulating levels of miR-155-5p, miR-320a, buy not miR-424-5p, are higher in patients who are still alive after 1 year from the start of CPI

Furthermore, high/low levels of baseline miR-155-5p, miR-320a and miR-424-5p are significantly associated with overall survival and progression-free survival (Figure 4). We found no correlation between basal miRNA level and the development of drug toxicity.

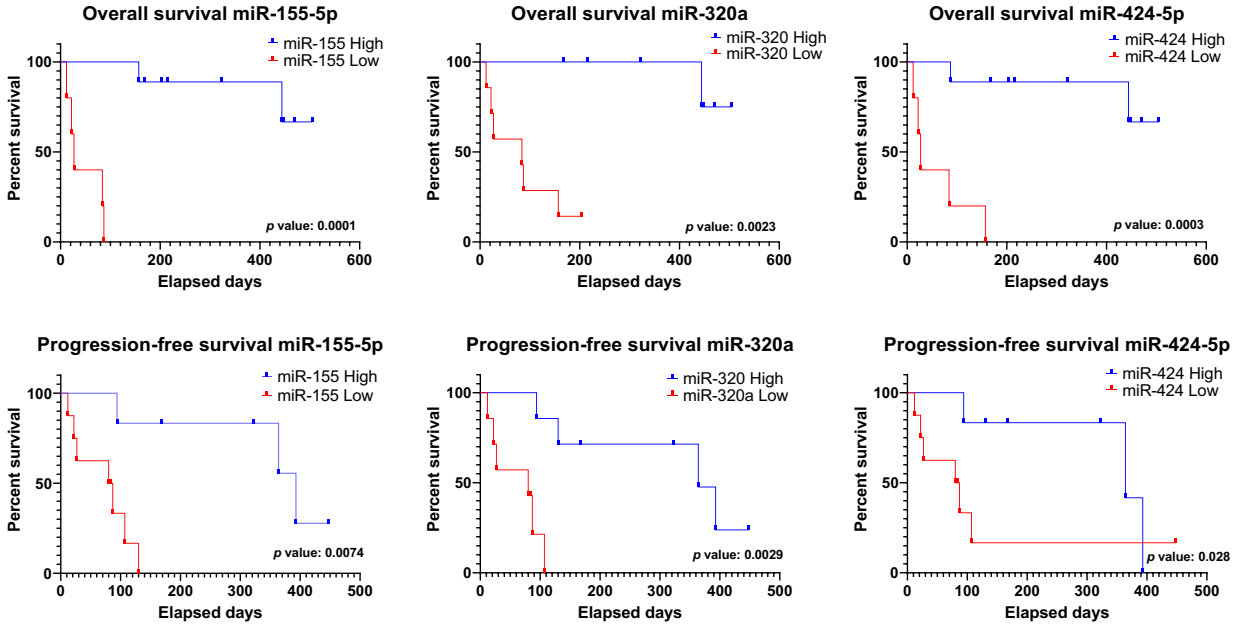

Figure 4 Gehan-Breslow-Wilcoxon analysis for overall survival and progression-free survival analysis in patients with high or low circulating miRNAs levels.

Longitudinal analysis of circulating miRNAs concentrations suggests a good concordance among the three selected miRNAs and an hypothetical role in describing the clinical course in terms of objective response to CPI treatment (Figure 5).

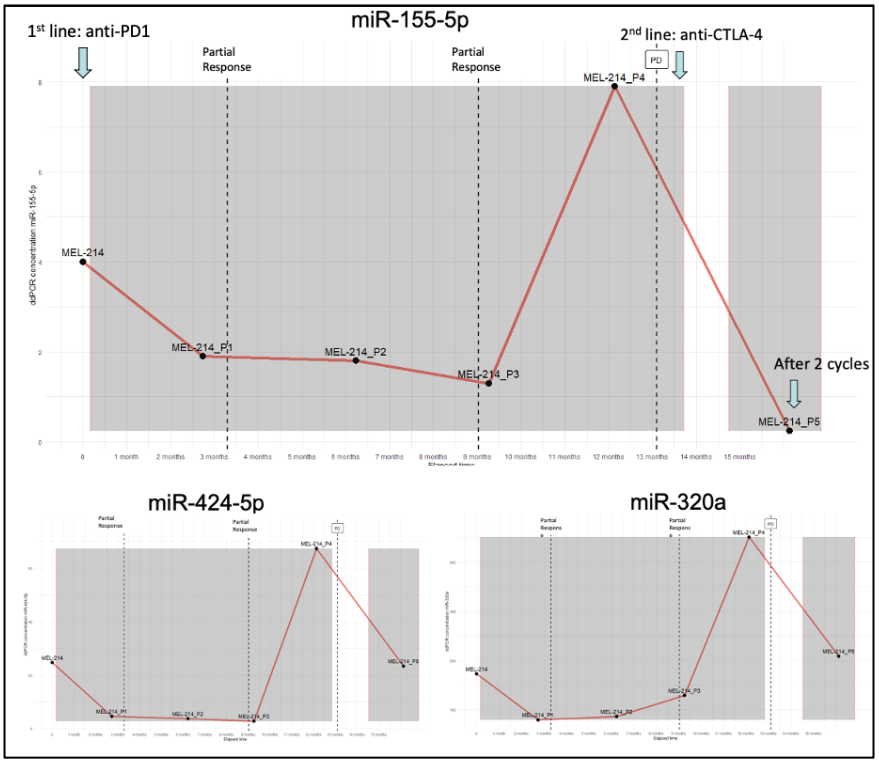

Figure 5 Longitudinal analysis of miR155, miR-424-5p and miR-320a in a patient with initial partial response to anti-PD1, progressive disease at 12 months and subsequent second line with anti-CTLA-4 for metastatic NRAS Q61R mutant melanoma

Finally, we analyzed circulating miRNA levels specifically in the group of patients who received first-line anti-PD-1 treatments, to observe if the miRNA levels were significantly associated with objective response according to RECIST criteria. This is a preliminary analysis on a pilot group of 7 patients. As can be observed in Figure 6, baseline miR-155-5p shows higher levels in responder vs. non responder patients (p 0.06).

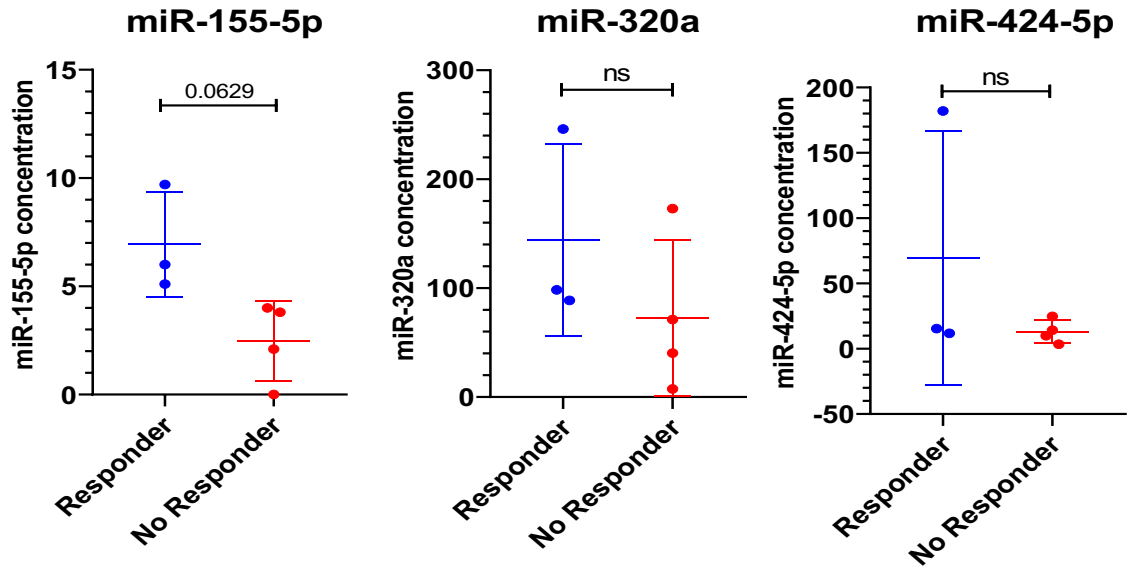

Figure 6 Analysis of T0 miR-155-5p, miR-320a and miR-424-5p expression in responder and non-responder patients treated with anti-PD-1 therapy

DISCUSSION

Although immunotherapy has revolutionized the treatment of metastatic melanoma, approximately 60% of melanoma patients show primary resistance to PD-1 blockade, and 20−30% of initial responders will develop secondary (acquired) resistance to CPI. Therefore, it is key to find biomarkers that can predict the response or resistance to immunotherapy.

The aim of our study was to explore the role of circulating miRNAs in metastatic melanoma treated with CPI; in particular we analyzed miR-155-5p and miR-424-5p on the basis of data showing their involvement in the T-cell immune response [67,70] and miR320a because of its high specificity for melanoma [72].

We found that lower baseline levels of miR-155-5p, miR-320a and miR-424-5p are associated with a poor prognosis and higher baseline miR-155-5p level can predict response to anti-PD1.

The short mPFS and mOS are explained by the fact that the population included in the study is mostly with poor prognosis [all patients with ≥3 metastases, 71% M1c or M1d, 50% with elevated LDH, 57% with high tumor burden (>100mm)]. Furthermore, half of the patients had brain involvement and of these, 3 patients received the combination of nivolumab and ipilimumab when progressing to BRAF/MEK inhibition, that showed to be significantly less effective than in treatment naïve patients [73].

It is noteworthy that the correlation between the baseline level of miR-155-5p and the response to anti-PD1 agent, if confirmed, could have important clinical implications suggesting that treatment intensification is necessary for patients with low baseline level of miR155-5p (e.g. association of anti-PD1 with anti-CTLA-4 or anti-LAG3 or with loco-regional treatments, such as radiotherapy, whenever feasible).

While many studies have focused on identifying circulating miRNA biomarkers that can distinguish between melanoma patients and healthy control individuals, relatively few studies have investigated the prognostic role of circulating miRNAs and fewer studies focused on the identification of circulating miRNAs as predictors of response to therapies.

Huber and colleagues identified a set of microRNAs (miR-146a-5p, miR-155-5p, miR-125b-5p, miR-100-5p, let-7e, miR-125a-5p, miR-146b-5p, and miR-99b-5p) that are responsible for the conversion of monocytes into myeloid suppressor cells (the accumulation of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment can hinder immunotherapy efficacy). They measured the expression levels in the plasma of melanoma patients with advanced disease (stage IIIC unresectable and stage IV) and in a group of healthy donors and they found that baseline levels clustered with the clinical efficacy of CTLA-4 or PD-1 blockade [74].

As such, the clinical potential of circulating miRNAs in metastatic melanoma patients remains largely undefined and our study aims to shed some light on it.

One of the strengths of our study stays in its prospective nature with longitudinal sampling during treatment. The concentration of circulating biomarkers can change during the course of illness in response to progression of disease or therapeutic intervention. The analysis of blood samples over a period of time will allow a dynamic assessment of the disease, which is very difficult to achieve with tissue biopsies.

Another aspect to be considered is that circulating miRNAs originate from multiple sources including primary cancer cells, tumor metastases, immune cells, and cells of the tumor microenvironment, therefore they have the disadvantage of a reduced tumor specificity; however, they reflect the complex tumor–host interaction more than what is occurring in the tumor cells and they and mirror the systemic and comprehensive effects of disease or its evolution over time.

Even though promising, our results are preliminary and need to be further investigated in a larger and more homogeneous cohort of patients. Therefore, we aim to enroll a larger cohort of untreated metastatic melanoma patients candidate to receive anti-PD1 therapy.

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