New insights in melanoma biomarkers: long-noncoding RNAs

Ricardo Moreno-Traspas¹, Igor Vujic^{1,2}, Martina Sanlorenzo^{1,3} & Susana Ortiz-Urda^{*1}

Practice points

- Melanoma is one of the most serious and malignant cancers worldwide, but still there are not efficient serum biomarkers to conduct an early detection, an accurate diagnosis and prognosis, an efficient monitoring of the disease, or a reliable prediction of survival and recurrence.
- Current melanoma biomarkers, including LDH, s100B, MIA, CRP and VEGF, suffer from multiple weaknesses, such as specificity concerns and inability for the detection of early melanoma stages, which make them useless as powerful diagnostic and prognostic tools.
- Despite research lines exploring innovative melanoma biomarkers have obtained promising results in the latest years, they are still far from defining reliable circulating markers with a direct application in clinics, and further research is still needed.
- The world of long-noncoding RNAs (lncRNAs) is becoming more and more attractive for researchers due to the versatility of these molecules, together with their tissue-specific expression and involvement in numerous key cellular processes.
- The secretion of specific sets of lncRNAs by certain tumors into the bloodstream has already been documented. Furthermore, they have been proposed as accurate diagnostic and prognostic tools for these types of cancer. Surprisingly, this is still an unexplored field in melanoma.
- Certain lncRNAs are upregulated in human melanoma tumors, such as BANC1, SPRY4-IT1 and HOTAIR among others. In this review we propose to further investigate their secretion and presence in the serum of patients and, ultimately, assess their potential as melanoma circulating markers.
- Melanoma lncRNAs display interesting characteristics which could be exploited in the field of serum biomarkers, involving high potential informative power about important tumor features, and high specificity and early expression of some of the candidates. Moreover, the technical approach for the detection of melanoma lncRNAs in blood samples from patients would be fast and cheap.

Melanoma is one of the leading cancers worldwide, distinguished for its malignancy and low survival rates. Although the poor outcome could improve with an early diagnosis and a good monitoring of the disease, current melanoma biomarkers display several limitations which make them useless. Interestingly, long-noncoding RNAs are secreted into the bloodstream inside exosomes by a wide range of malignant cells, and several of them have been validated as promising circulating molecular signatures of other tumors, but not melanoma. In this review we propose to explore the booming field of long-noncoding RNAs in order to find potential candidates to be tested as novel melanoma biomarkers, with the ultimate goal of improving melanoma detection, diagnosis and prognosis.

First submitted: 25 February 2016; Accepted for publication: 17 May 2016; Published online: 16 August 2016

¹Department of Dermatology, University of California San Francisco, Mt. Zion Cancer Research Center, 2340 Sutter Street N461, San Francisco, CA 94115, USA

²The Rudolfstiftung Hospital, Academic Teaching Hospital, Department of Dermatology, Medical University Vienna, Juchgasse 25, 1030 Vienna, Austria

³Department of Medical Sciences, Section of Dermatology, University of Turin, Italy

*Author for correspondence: susana.ortiz@ucsf.edu

KEYWORDS

- biomarkers • cancer
- lncRNAs • melanoma

Background

Melanoma is the deadliest form of skin cancer and one of the most aggressive and lethal neoplasms. High mortality rates are mainly due to its fast development and highly metastatic potential, especially to vital organs such as lung, liver and brain [1]. Moreover, the incidence of cutaneous melanoma is one of the highest in the USA, and the number of new diagnosed cases steadily rises every year [2]. However, in recent years patient survival has significantly improved thanks to new US FDA-approved drugs, such as vemurafenib/dabrafenib (BRAF inhibitor) [3–6], trametinib (MEK inhibitor) [6,7], ipilimumab (CTLA-4 antibody) [6,8], pembrolizumab [6,9] and nivolumab (PD-1 antibodies) [6,10]. Unfortunately, most tumors become drug resistant shortly after starting the treatment and undergo lethal progression [3,11–13], only with exception of combined immunotherapy with pembrolizumab and nivolumab. Hence we are still far from being able to efficiently combat melanoma and avoid the fatal outcome of the disease.

Importantly, melanoma survival improves considerably with an early diagnosis, as well as with an adequate treatment and a good monitoring of the disease [14]. At this point melanoma biomarkers have their part as diagnostic, prognostic and predictive tools. Nevertheless, current circulating markers for melanoma have several limitations and are neither efficient enough to detect early stages of the malignancy, nor reliable for prognosis and prediction of treatment response, survival or recurrence. It is of utmost importance to identify novel biomarkers and develop precise and innovative biochemical tests to improve patient's quality of life, survival and cure.

This review aims to give a brief state of the art update about melanoma biomarkers used nowadays and their limitations, as well as set the basis for future research lines in a new and unexplored field with high potential and powerful possibilities: the world of long-noncoding RNAs (lncRNAs). We point out lncRNAs display a wide range of attractive features and potential advantages over traditional melanoma markers, which support further investigation on them: their presence and possible detection in patient serums; their expression along different stages of the malignancy, including early stages; the high specificity of some of them; and their high versatility and involvement in several cellular processes which make them more likely to correlate with the state of the tumor than other molecules such as miRNAs.

State of the field

Biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention” according to the US NIH's Biomarkers Definition Working Group and the Biomarkers Consortium [15]. Nowadays, biomarkers are widely used in basic and clinical research as well as in clinical practice due to their high informative potential and numerous applications, involving disease detection and diagnosis, staging and classification, prognosis and prediction of clinical response to an intervention or treatment [15].

A tumor marker is any substance related with the presence or progress of cancer. Tumor markers can be either unique products of the malignant cells, molecules expressed in greater amounts by the tumor than by normal cells, or substances released by other cells of the body in response to the tumor. The most common patient's specimen used in cancer clinics to detect specific tumor biomarkers is blood serum due to two main reasons: the fact that many distinctive molecules associated with cancer are secreted into the bloodstream, and the ease and rapidity of the sample collection from the patient plus the low cost of the subsequent analysis.

• Current biomarkers in melanoma

The field of circulating markers in melanoma is limited to a few proteins: LDH, s100B, MIA, CRP and VEGF (Table 1). The most relevant melanoma biomarker to date is LDH, which stands out as the only statistically significant marker for disease progression [16], reason why it has been incorporated in the AJCC (American Joint Committee on Cancer) staging system [17] for late melanoma (stage IV M1c disease). However, all melanoma biomarkers including LDH bear numerous problems and limitations, mainly regarding to specificity concerns and their unsuitability for early detection of melanoma stages 0/I (Table 1).

• In the search for novel biomarkers

Nowadays, there is a wide range of innovative research lines in the field trying to discover new tools and approaches to improve clinical diagnosis, prognosis and prediction of response to therapy in melanoma. Lower serum concentrations of vitamin D and increased levels of IL-8 have been recently found to be significantly

Table 1. Traditional melanoma biomarkers, indicating their pros and cons.

Melanoma biomarker	Description	Pros	Cons	Ref.
LDH	Serum lactate dehydrogenase	Correlation with melanoma metastasis in the liver and decreased survival. Predictive potential for recurrence and response to therapy	Specificity concerns, no utility for detection, diagnosis and staging of the disease	[16–21]
S100B	–	Potential for prognosis, staging and monitoring response to therapy	Specificity concerns, limitation for early detection	[22–31]
MIA	Melanoma-inhibiting activity	Correlation with advanced melanoma, metastasis and poor prognosis	Lower specificity than LDH and S100B, no utility for early detection and staging of the disease	[29,32–35]
CRP	C-reactive protein	Potential for the diagnosis of stage IV melanoma and prognosis	Low specificity, no utility for early detection and staging of the disease	[36,37]
VEGF	Vascular endothelial growth factor	Correlation with late melanoma stages and prognostic potential	No correlation with survival rates, low specificity, sensitivity and positive predictive value, no utility for early detection	[38–40]

helpful as melanoma predictors in combination with classical biomarkers [41]. Circulating tumor cells are currently being investigated to determine their suitability for predicting melanoma metastasis and clinical staging [42]. The potential of analyzing cell-free tumor DNA containing hallmark mutations of melanoma in patient's blood has been proposed for monitoring tumor burden and response to therapy [43,44]. Moreover, recent studies have reported the possibility to detect serum epigenetic biomarkers, such as specific DNA methylation patterns of melanoma, in this cell-free tumor DNA [45,46]. Importantly, promising results are being achieved in the field of miRNome: highly specific serum miRNA signatures have been identified as melanoma hallmarks of late stages and recurrence, and their long-term beneficial impact in melanoma diagnosis, prognosis and monitoring of the disease has been proposed [47–49].

However, all these studies are preliminary approaches, the obtained results are not significant enough and there are several methodological and clinical issues that should be resolved before the introduction of any of these candidate molecules as routine tested biomarkers in melanoma. Therefore, there is still no general consensus about their use in the clinical setting, and further research and novel research lines are strongly needed.

LncRNAs as cancer biomarkers

Large-scale transcriptome analyses have shown that the genomes of complex organisms encode a great amount of RNAs which do not have any protein-coding potential; indeed, around 50% of cellular transcripts are different types of

noncoding RNAs (ncRNAs) [50]. Among them, lncRNAs, which have a length ranging from 200 bp to over 10 kb, have been gaining special interest in the latest years since they are expected to constitute a complex system of regulatory factors that act on different levels of gene expression, mainly displaying transcriptional, post-transcriptional and epigenetic operating mechanisms. Their development-dependent and tissue-specific expression patterns [51] together with their great versatility, make them very attractive biomolecules for researchers. Recent studies have proved the involvement of lncRNAs in multiple key cellular processes such as X-chromosome silencing, pluripotency, cell fate determination, cell-cycle regulation, chromatin remodeling and DNA damage response [52,53]. Importantly, lncRNAs display aberrant expression patterns and are substantial contributors to cancer development [54]. Indeed, they participate in the tumorigenic progression and the acquisition of numerous malignant features, such as protection against apoptosis, evasion of growth suppression and enhanced proliferation, deregulation of cellular energetics, invasion and metastasis [55,56].

Interestingly, lncRNAs can be packaged within exosomes [57,58], cell-derived vesicles with a diameter between approximately 30–100 nm which are secreted by a wide range of mammalian cell types. In the field of cancer, numerous studies *in vitro*, *in vivo* and clinical assays indicate that exosomal release rate is increased in cancer cells in contrast with healthy cells, and their content is also different [59,60]. It is possible to successfully isolate these membranous bodies from diverse biological samples, like blood plasma, serum and urine by a simple and fast

procedure. This fact opens the window for using the molecules contained inside the exosomes, which constitute a unique footprint of the tumor, as specific cancer biomarkers.

The presence of tumor related lncRNAs in extracellular circulating exosomes has been demonstrated by several studies in hepatocellular [61], gastric [62] and prostate cancers [63], among others. Interestingly, several circulating lncRNAs have been proved to have a great potential as markers for certain tumors: DD3^{PCA3}, MALAT1 and PCAT-1 in prostate cancer (urine location) [64]; MALAT1 in lung cancer [65]; HOTAIR in colorectal [66] and cervical cancer [67]; H19 and LINC00152 in gastric cancer [62,68]; and POU3F3 in esophageal squamous cell carcinoma [69]. It is especially interesting the case of DD3^{PCA3} as a prostate cancer biomarker, since it displays better specificity and positive and negative predictive values than PSA, the traditional protein biomarker of this type of cancer [64]. This example illustrates how lncRNAs might be better circulating tools for diagnosis, prognosis and monitoring of the disease than current protein biomarkers.

LncRNAs as melanoma biomarkers

As it has been shown with several examples in the previous section, there are lncRNAs that are currently being used as biomarkers of certain tumors. However, still no published studies have investigated this possibility in melanoma. The only exception is a very preliminary and limited study about a novel serum lncRNA, SNHG5, that might be used as a novel tumor marker of malignant melanoma in the long-term [70]. This study shows the significant increased serum levels of SNHG5 in malignant melanoma patients in comparison with squamous cell carcinoma patients and normal subjects [70]. However, SNHG5 did not increase according to the melanoma stage, so SNHG5 level seems to reflect tumor-bearing status of the patient but not the progression of the disease [70]. It is also important to note that these results are limited by the low number of analyzed patient samples, so further research using a higher number of samples and including other cancer types is still needed before concluding the real utility of this lncRNA as a diagnostic tool for melanoma.

Interestingly, there is a pretty wide range of lncRNAs highly expressed by melanoma cells, which display leading roles in the tumorigenic progression from normal melanocytes to

malignant melanoma cells. We aim to propose these lncRNAs (Table 2) as possible candidates in the field of melanoma circulating markers. It is especially interesting that some of these lncRNAs are differentially expressed along the different stages of the tumorigenic progression. This fact suggests how the hypothetical presence of these molecules in the bloodstream could be used not only to determine whether a patient suffers melanoma, but also as an informative tool about the stage of the disease which might help to provide a more personalized and efficient treatment therapy.

• BANCER

BANCER is a 693 bp lncRNA identified first in BRAF-mutant human melanomas [71]. Approximately 50% of melanomas harbor activating BRAF mutations, 90% of those produce active mutant BRAF^{V600E} protein [81] which induces the upregulation of a wide range of proteins and transcripts involved in cell survival, proliferation and invasion. Flockhart *et al.* reported BANCER as one of these activated genes downstream BRAF [71]. BANCER is recurrently overexpressed in both primary and metastatic melanoma cell lines and tissues, and its levels increase with tumor stages, which evidences its potential oncogenic role throughout the spectrum of disease progression [71,72]. Interestingly, high levels of BANCER correlate with a low survival rate in human patients, suggesting the utility of BANCER as a predictor of poor clinical outcome [72]. Nevertheless, the secretion of BANCER inside membrane vesicles and its presence in the serum has not been investigated yet.

• SPRY4-IT1

SPRY4-IT1 is derived from an intron of the *SPRY4* gene and plays an important role in melanoma pathogenesis as a tumor suppressor by inhibiting the MAPK pathway [73]. SPRY4-IT1 was first identified upregulated in melanoma cell lines when compared with normal melanocytes and keratinocytes using a lncRNA microarray [73]. More interestingly, increased levels of SPRY4-IT1 were found in 30 different patient samples classified as primary *in situ*, regional metastatic, distant metastatic and nodal metastatic melanoma [73], what suggests its potential utility for melanoma staging and early detection. SPRY4-IT1 is actually present in the plasma of esophageal squamous cell carcinoma patients [69].

Table 2. Upregulated long-noncoding RNAs in melanoma, indicating their relevance for the disease and their potential as prospective biomarkers.

Melanoma lncRNA	Description	Relevance to melanoma	Candidate biomarker for	Ref.
BANCR	BRAF-activated noncoding RNA	Overexpressed in primary and especially in metastatic melanoma, involved in cell motility and proliferation, predictor of survival	Primary, malignant, invasive and metastatic melanoma, tumorigenic progression, survival	[71,72]
SPRY4-IT1	–	Overexpressed in melanoma tumors ranging from primary to highly metastatic, involved in cell growth, invasion, migration and apoptosis protection	Primary, malignant, invasive and metastatic melanoma	[73]
HOTAIR	HOX transcript antisense RNA	Overexpressed in metastatic melanoma, involved in cell motility and invasion	Invasive and metastatic melanoma	[74,75]
Llme23	–	Overexpressed in melanoma cell lines, oncogenic role	Malignant melanoma	[76]
CASC15	Cancer susceptibility candidate 15	Overexpressed in metastatic melanoma, involved in malignant progression, predictor of disease recurrence	Invasive and metastatic melanoma, tumorigenic progression, survival and recurrence	[77]
MALAT-1	Metastasis-associated lung adenocarcinoma transcript 1	Overexpressed in malignant melanoma and especially in metastatic melanoma, involved in cell motility	Malignant, invasive and metastatic melanoma	[78]
UCA1	Urothelial carcinoma-associated 1	Overexpressed in malignant melanoma, involved in cell motility	Malignant and invasive melanoma	[78]
ANRIL	Antisense noncoding RNA in INK4 locus	Expressed in several melanoma cell lines, polymorphism associated with melanoma risk	Primary and malignant melanoma, melanoma risk	[79,80]

• HOTAIR

HOTAIR is a 2.2 kilobase lncRNA residing in the human *HOXC* locus. It has been reported to be involved in chromatin reprogramming to promote cancer metastasis by repressing the expression of metastatic suppressor genes genome-wide in a PRC2 dependent manner [74]. In the field of melanoma, HOTAIR is upregulated in metastatic tissue in comparison with primary melanoma, and involved in promoting cell motility and invasiveness [75]. There is strong evidence that HOTAIR is secreted outside the cell inside exosomes in high concentrations [58,82] and its use as a serum biomarker for other cancer types has been suggested [66,67].

• Llme23

The 1600 bp lncRNA Llme23 displays a pro-oncogenic role via the interaction and subsequent block of the tumor suppressor protein PSF [76]. Interestingly, Llme23 is exclusively overexpressed in human melanoma cell lines [76]; therefore, defining the suitability of Llme23 as a melanoma biomarker would provide clinicians with a highly specific tool for melanoma screening and detection. Further research is needed to determine Llme23 secretion and presence in patient's plasma.

• CASC15

CASC15 is a long intergenic noncoding RNA (lincRNA) localized in chromosome 6 in a frequently altered genomic segment in metastatic melanoma tissues, and is actually involved in melanoma progression, metastasis and recurrence [77]. CASC15 transcript levels increase steadily during melanoma progression to advance stages of distant metastasis, being actively upregulated in metastatic melanoma cell lines [77]. Furthermore, CASC15 levels were independent predictors of disease recurrence and survival in a study with a cohort of 141 patients with stage III lymph node metastasis [77]. Future research in the field of melanoma biomarkers should study the secretion and possibility to detect this lncRNA in serum samples from melanoma patients.

• MALAT-1

MALAT-1 is an approximately 8000 bp lncRNA, also known as NEAT2. Its expression, function and molecular mechanism of action have been extensively studied in the cancer field, reporting MALAT-1 upregulation in several tumors and a wide range of oncogenic and metastatic promoting functions [83]. In the melanoma field, MALAT-1 data are still very limited. Thanks to Tian *et al.* it is known that melanoma tumors

highly express MALAT-1 compared with adjacent normal tissues [78]. Moreover, melanoma with lymph node metastasis displays elevated MALAT-1 levels [78]. Interestingly, it has been reported the presence of MALAT-1 in both plasma and urine samples from patients suffering prostate cancer, and the utility of this lncRNA as a diagnosing biomarker [84,85].

• UCA-1

In the same study carried out by Tian *et al.*, it was also reported the markedly increased levels of the 1.4 kb lncRNA UCA-1 in melanoma tumors compared with adjacent normal tissue, especially at advanced stages (stages III/IV) [78], where it seems to have a potential function in invasion and metastasis [78]. UCA-1 packaging in membranous vesicles and release to the blood circulation has not been studied yet.

• ANRIL

ANRIL is an antisense 3834 bp lncRNA codified by a gene located within the *INK4/ARF* locus. ANRIL consists of 19 exons and suffers alternative splicing, leading to several short, long and circular isoforms, some of them differentially expressed in some melanoma cell lines [79]. Importantly, GWAS studies identified a SNP (*rs1011970*) associated with melanoma risk [80]. However, ANRIL putative oncogenic role and involvement in melanoma pathogenesis is still unknown, and further investigation is needed in order to determine its presence in serum exosomes and its potential utility as a biomarker.

Discussion

One of the main problems in the field of melanoma circulating markers is the lack of early blood indicators. Thus, biopsies are performed when there is a potential cutaneous melanoma (stage 0/I) and it is necessary to confirm the malignancy of the suspicious skin alteration. The main limitation here is that the potential tumorigenic cutaneous lesion has to be detected either by the patient or by an oncologist. Nevertheless, in some cases this primary detection is not early enough for stage 0/I melanomas due to on the one hand the lack of knowledge of the patient to detect a potentially malignant skin alteration which is further hindered by the absence of symptoms in some early melanomas, and on the other hand the fact that some melanoma tumors are highly malignant and invasive, and start invading deeper skin layers when their size

is still not very big (less than 1 mm). Another important limitation is that a biopsy requires the tumor to have a certain size and weight, so the collection and analysis of the sample is possible.

Secreted lncRNAs could be the key to replace biopsies. Since the expression of several lncRNAs in early melanoma stages has already been described, they could be used as biomarkers analyzed in regular blood tests to detect stage 0/I melanoma in patients with still any symptoms, avoiding this way the human eye detection limitation. Moreover, lncRNAs expressed by malignant melanoma cells might provide accurate information about the size and the malignancy of the tumor, as well as its progression stage, what hypothetically makes them not only a good diagnostic but also prognostic tool.

The possibility to detect early melanoma cases avoiding biopsies would have several advantages. On the one hand, the use of a noninvasive and simple technique, performed daily for regular blood tests in health centers and hospitals all around the world. On the other hand, both the process of taking the sample and the subsequent analysis are faster in comparison to biopsies. Furthermore, blood tests involve less sanitary professionals, equipment and, thus, less budget. Hence the replacement of biopsies for the collection and analysis of patient blood samples would greatly facilitate early detection of cutaneous melanoma in the whole population. For instance, it would be easier to keep a close surveillance of risk groups, such as pilots and cabin crew [86] or individuals with a high risk of developing melanoma due to their genetic background.

lncRNAs display numerous features which make them good candidates to be investigated as clinical tools for early detection and also diagnosis and prognosis of melanoma patients. First of all, the fact that lncRNAs are secreted into the bloodstream makes melanoma lncRNAs very likely to be present in the serum of patients, as it has already been reported for other types of cancer. Although naked lncRNAs are less stable in the blood compared with proteins or miRNAs, their secretion inside exosomes dismisses this problem. Second, their differential expression during melanoma progression suggests their potential utility for clinical staging of the disease. Third, as it has been previously described, some lncRNAs are expressed in early stages of the malignancy, so they could be used for early melanoma detection (one of the main needs in the field) in the case of being proved as valid biomarkers for melanoma.

Figure 1. Schematic protocol for analyzing the presence of circulating melanoma long-noncoding RNAs in the blood sample of a patient. The protocol starts with collecting a sample of peripheral venous blood from the patient (regular procedure for venepuncture) into EDTA-K2 tubes to avoid coagulation, and its storage at 4°C until the subsequent processing. To obtain plasma, the whole blood is centrifuged at 160 g at RT to get rid of erythrocytes and blood cells. Plasma is stored at -80°C until exosome extraction. Plasma exosome vesicles can be precipitated either by ultracentrifugation, a precipitation reagent or a commercial kit. Exosomes are then lysed and the RNA is purified, either by using TRIzol or a commercial kit. Finally, the analysis is performed by synthesizing the cDNA and quantifying the levels of the lncRNAs of interest by qRT-PCR with specific primers. lncRNA: Long-noncoding RNA; RT: Room temperature.

Fourth, most of current protein biomarkers suffer from specificity issues, whereas some lncRNAs are highly melanoma-specific. Fifth, when compared to miRNAs, lncRNAs stand out for being much more versatile and display a higher number of cellular functions, what may suggest a better correlation with the state and progression of the malignancy. Finally, it might be possible that these lncRNAs are present in higher levels in plasma, or secreted more often or even earlier than other biomarkers.

Despite all the advantages lncRNAs might offer as biomarkers, there are some concerns that should be taken into account in the process of validating candidate lncRNAs as serum melanoma markers. As it has been mentioned above, it is important not only that the lncRNA at issue is secreted into the bloodstream, but also that it does it inside exosomes so it is protected against the degradative action of RNase enzymes present

in the plasma. Moreover, it has to be released in a sufficient quantity to be detected by regular analysis techniques. Also it is relevant to point out the fact that several of the lncRNAs expressed by melanoma cells are also expressed and secreted by other tumors. This is the case, for instance, of MALAT-1 in lung cancer [65] or HOTAIR in colorectal [66] and cervical cancer [67]. Therefore, the optimal solution to avoid tumor specificity issues would be the establishment of a set of a certain number of lncRNAs secreted by melanoma cells, so the detection of this molecular signature in a patient's blood could work as a highly specific indicator of cutaneous melanoma.

Overall, lncRNAs constitute a promising field of study with a great potential of success. However, we would like to stress that all the points presented in this review are mainly theoretical and speculative; further research is needed in order to confirm the suitability of lncRNAs

as melanoma diagnostic and prognostic tools, as well as validate their potential utility as serologic biomarkers with a clinical application.

Conclusion

Melanoma is one of the leading cancers worldwide, but still there are not efficient serum biomarkers to conduct an early detection and an efficient monitoring of the disease. Therefore, it is of utmost importance to discover novel circulating markers in order to implement diagnosis, prognosis and treatment of this malignancy. In this issue lncRNAs stand out as good candidates due to their potential informative power about important tumor features, the theoretical possibility to use them as early-stage melanoma markers and the advantages and amenities of their detection method in comparison to biopsies. The documented secretion of several lncRNAs by other tumors into the bloodstream and their use as current cancer biomarkers support this hypothesis, leaving the door open for future and promising research lines on the challenge of finding efficient, reliable and specific biomarkers to detect early melanoma cases and accurately monitor the evolution of the disease.

Future perspective

It is of utmost importance to discover novel circulating markers for melanoma in order to implement early detection, diagnosis and prognosis, as well as to accurately monitor the evolution of the tumor and predict response to therapy and recurrence. We propose lncRNAs as good candidates to fulfill at least some of these needs, basing on the one hand on their likely secretion and medium/long-term presence in the blood circulation, and on the other hand on their specificity, differential expression along the different stages of the malignancy, involvement on the

acquisition of multiple malignant features and upregulation of some of them in early tumors.

We would like to propose and briefly describe a methodological approach for detecting circulating melanoma lncRNAs in patient's blood samples, in order to illustrate how simple, fast and inexpensive would it be in contrast with biopsies. The basic procedure would involve two important steps: first the isolation of the plasma exosomes, and second the RNA extraction for the subsequent analysis by qRT-PCR (Figure 1). Once the presence of a melanoma lncRNA in patient's blood is confirmed, the next step will be to determine the relevance and validity of the candidate lncRNA as a diagnostic and prognostic tool, as well as to test and optimize several remarkable parameters such as the precision, accuracy, sensitivity and specificity of the analytical method, and the sensitivity and specificity of the clinical test.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Acknowledgements

The authors are grateful to T Dattels for his generous support and to the Society for Dermatology and Venerology KA Rudolfstiftung.

Financial & competing interests disclosure

This study was supported by National Cancer Institute of the NIH under award number K08CA155035 and the Melanoma Research Alliance Young Investigator Award. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

- Marcovall J, Ferreres JR, Martín C *et al.* Patterns of visceral metastasis in cutaneous melanoma: a descriptive study. *Actas Dermosifiliogr.* 104(7), 593–597 (2013).
- Little EG, Eide MJ. Update of the current state of melanoma incidence. *Dermatol. Clin.* 30(3), 355–361 (2012).
- Alcalá AM, Flaherty KT. BRAF inhibitors for the treatment of metastatic melanoma: clinical trials and mechanisms of resistance. *Clin. Cancer Res.* 18(1), 33–39 (2012).
- Ascierto PA, Minor D, Ribas A, Lebbe C. Phase II trial (BRAK-2) of the BRAF inhibitor dabrafenib (GSK2118436) in patients with metastatic melanoma. *J. Clin. Oncol.* 31(26), 3205–3211 (2013).
- McArthur GA, Chapman PB, Robert C *et al.* Safety and efficacy of vemurafenib in BRAF(V600E) and BRAF(V600K) mutation-positive melanoma (BRIM-3): extended follow-up of a Phase 3, randomised, open-label study. *Lancet Oncol.* 15(3), 323–332 (2014).
- Marzuka A, Huang L, Theodosakis N, Bosenberg M. Melanoma treatments: advances and mechanisms. *J. Cell. Physiol.* 230(11), 2626–2633 (2015).
- Thota R, Johnson DB, Sosman JA. Trametinib in the treatment of melanoma. *Expert Opin. Biol. Ther.* 15(5), 735–747 (2015).
- Hodi FS, O'Day SJ, McDermott DF *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* 363(8), 711–723 (2010).

- 9 Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J. Clin. Oncol.* 33(17), 1974–1982 (2015).
- 10 Wolchok JD, Kluger H, Callahan MK *et al.* Nivolumab plus ipilimumab in advanced melanoma. *N. Engl. J. Med.* 369(2), 122–133 (2013).
- 11 Kugel CH, Aplin AE. Adaptive resistance to RAF inhibitors in melanoma. *Pigment Cell Melanoma Res.* 27(6), 1032–1038 (2014).
- 12 Roesch A. Tumor heterogeneity and plasticity as elusive drivers for resistance to MAPK pathway inhibition in melanoma. *Oncogene* 34(23), 2951–2957 (2015).
- 13 Wellbrock C. MAPK pathway inhibition in melanoma: resistance three ways. *Biochem. Soc. Trans.* 42(4), 727–732 (2014).
- 14 Bataille V. Early detection of melanoma improves survival. *Practitioner* 253(1722), 29–32 (2009).
- 15 Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 69(3), 89–95 (2001).
- 16 Deichmann M, Benner A, Bock M *et al.* S100-Beta, melanoma-inhibiting activity, and lactate dehydrogenase discriminate progressive from nonprogressive American Joint Committee on Cancer stage IV melanoma. *J. Clin. Oncol.* 17(6), 1891–1896 (1999).
- 17 Balch CM, Buzaid AC, Soong SJ *et al.* Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J. Clin. Oncol.* 19(16), 3635–3648 (2001).
- 18 Einhorn LH, Burgess MA, Vallejos C *et al.* Prognostic correlations and response to treatment in advanced malignant melanoma. *Cancer Res.* 34(8), 1995–2004 (1974).
- 19 Garg R, McPherson TA, Lentle B, Jackson F. Usefulness of an elevated serum lactate dehydrogenase value as a marker of hepatic metastases in malignant melanoma. *Can. Medical Assoc. J.* 120(9), 1114–1116 (1979).
- 20 Campora E, Repetto L, Giuntini P *et al.* LDH in the follow-up of stage I malignant melanoma. *Eur. J. Cancer Clin. Oncol.* 24(2), 277–278 (1988).
- 21 Agarwala SS, Keilholz U, Gilles E *et al.* LDH correlation with survival in advanced melanoma from two large, randomised trials (Oblimersen GM301 and EORTC 18951). *Eur. J. Cancer* 45(10), 1807–1814 (2009).
- 22 Guo HB, Stoffel-Wagner B, Bierwirth T, Mezger J, Klingmüller D. Clinical significance of serum S100 in metastatic malignant melanoma. *Eur. J. Cancer* 31A(11), 1898–1902 (1995).
- 23 Von Schoultz E, Hansson LO, Djureen E *et al.* Prognostic value of serum analyses of S-100 beta protein in malignant melanoma. *Melanoma Res.* 6(2), 133–137 (1996).
- 24 Henze G, Dummer R, Joller-Jemelka HI, Böni R, Burg G. Serum S100: a marker for disease monitoring in metastatic melanoma. *Dermatology* 194(3), 208–212 (1997).
- 25 Abraha HD, Fuller LC, Du Vivier AW, Higgins EM, Sherwood RA. Serum S100 protein: a potentially useful prognostic marker in cutaneous melanoma. *Br. J. Dermatol.* 137(3), 381–385 (1997).
- 26 Mocellin S, Zavagno G, Nitti D. The prognostic value of serum S100B in patients with cutaneous melanoma: a meta-analysis. *Int. J. Cancer* 123(10), 2370–2376 (2008).
- 27 Bottoni U, Izzo P, Richetta A *et al.* S100 serum level: a tumour marker for metastatic melanoma. *Melanoma Res.* 13(4), 427–429 (2003).
- 28 Hamberg AP, Korse CM, Bonfrer JM, de Gast GC. Serum S100B is suitable for prediction and monitoring of response to chemoimmunotherapy in metastatic malignant melanoma. *Melanoma Res.* 13(1), 45–49 (2003).
- 29 Krähn G, Kaskel P, Sander S *et al.* S100 beta is a more reliable tumor marker in peripheral blood for patients with newly occurred melanoma metastases compared with MIA, albumin and lactate-dehydrogenase. *Anticancer Res.* 21(2B), 1311–1316 (2001).
- 30 Buer J, Probst M, Franzke A *et al.* Elevated serum levels of S100 and survival in metastatic malignant melanoma. *Br. J. Cancer* 75(9), 1373–1376 (1997).
- 31 Molina R, Navarro J, Filella X, Castel T, Ballesta AM. S-100 protein serum levels in patients with benign and malignant diseases: false-positive results related to liver and renal function. *Tumour Biol.* 23(1), 39–44 (2002).
- 32 Bosserhoff AK, Kaufmann M, Kaluza B *et al.* Melanoma-inhibiting activity, a novel serum marker for progression of malignant melanoma. *Cancer Res.* 57(15), 3149–3153 (1997).
- 33 Meral R, Duranyildiz D, Tas F *et al.* Prognostic significance of melanoma inhibiting activity levels in malignant melanoma. *Melanoma Res.* 11(6), 627–632 (2001).
- 34 Bosserhoff AK, Buettner R. Expression, function and clinical relevance of MIA (melanoma inhibitory activity). *Histol. Histopathol.* 17(1), 289–300 (2002).
- 35 Stahlecker J, Gauger A, Bosserhoff A *et al.* MIA as a reliable tumor marker in the serum of patients with malignant melanoma. *Anticancer Res.* 20(6D), 5041–5044 (2000).
- 36 Deichmann M, Benner A, Waldman V *et al.* Interleukin-6 and its surrogate C-reactive protein are useful serum markers for monitoring metastasized malignant melanoma. *J. Exp. Clin. Cancer Res.* 19(3), 301–307 (2000).
- 37 Deichmann M, Kahle B, Moser K, Wacker J, Wüst K. Diagnosing melanoma patients entering American Joint Committee on Cancer stage IV, C-reactive protein in serum is superior to lactate dehydrogenase. *Br. J. Cancer* 91(4), 699–702 (2004).
- 38 Ugurel S, Rappl G, Tilgen W, Reinhold U. Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival. *J. Clin. Oncol.* 19(2), 577–583 (2001).
- 39 Osella-Abate S, Quagliano O, Savoia P *et al.* VEGF-165 serum levels and tyrosinase expression in melanoma patients: correlation with the clinical course. *Melanoma Res.* 12(4), 325–334 (2002).
- 40 Pelletier F, Bermont L, Puzenat E *et al.* Circulating vascular endothelial growth factor in cutaneous malignant melanoma. *Br. J. Dermatol.* 152(4), 685–689 (2005).
- 41 Ene CD, Anghel AE, Neagu M, Nicolae I. 25-OH vitamin D and interleukin-8: emerging biomarkers in cutaneous melanoma development and progression. *Mediators Inflamm.* 2015, 904876 (2015).
- 42 Kiyohara E, Hata K, Lam S, Hoon DS. Circulating tumor cells as prognostic biomarkers in cutaneous melanoma patients. *Methods Mol. Biol.* 1102, 523–522 (2014).
- 43 Tsao SC, Weiss J, Hudson C *et al.* Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for *BRAF* and *NRAS* mutations. *Sci. Rep.* 5, 11198 (2015).
- 44 Gray ES, Rizo H, Reid AL *et al.* Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget* 6(39), 42008–42018 (2015).
- 45 Hatzimichael E, Syed N, Lo Nigro C, Rao B, Crook T. How detection of epigenetic alterations of blood-borne DNA could improve melanoma diagnosis. *Expert Rev. Mol. Diagn.* 14(6), 639–642 (2014).
- 46 Marini A, Mirmohammadsadegh A, Nambiar S *et al.* Epigenetic inactivation of tumor suppressor genes in serum patients with

- cutaneous melanoma. *J. Invest. Dermatol.* 126(2), 422–431 (2006).
- 47 Friedman EB, Shang S, de Miera EV *et al.* Serum microRNAs as biomarkers for recurrence in melanoma. *J. Transl. Med.* 10, 155 (2012).
- 48 Alegre E, Sanmamed MF, Rodriguez C *et al.* Study of circulating microRNA-125b levels in serum exosomes in advanced melanoma. *Arch. Pathol. Lab. Med.* 138(6), 828–832 (2014).
- 49 Margue C, Reinsbach S, Philippidou D *et al.* Comparison of healthy miRNomes with melanoma patient miRNomes: are microRNAs suitable serum biomarkers for cancer? *Oncotarget* 6(14), 12110–12127 (2015).
- 50 Claverie JM. Fewer genes, more noncoding RNA. *Science* 309(5740), 1529–1530 (2005).
- 51 Kapranov P, Cheng J, Dike S *et al.* RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* 316(5830), 1484–1488 (2007).
- 52 Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10(3), 155–159 (2009).
- 53 Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell* 136(4), 629–641 (2009).
- 54 Wapinski O, Chang HY. Long noncoding RNAs and human disease. *Trends Cell Biol.* 21(6), 354–361 (2011).
- 55 Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. *Mol. Cancer* 10, 38 (2011).
- 56 Huarte M. The emerging role of lncRNAs in cancer. *Nat. Med.* 21(11), 1253–1261 (2015).
- This review illustrates the importance of long-noncoding RNAs (lncRNAs) in cancer, their high versatility and involvement in multiple malignant processes.
- 57 Kharaziha P, Ceder S, Li Q, Panaretakis T. Tumor cell-derived exosomes: a message in a bottle. *Biochim. Biophys. Acta* 1826(1), 103–111 (2012).
- 58 Gezer U, Özgür E, Cetinkaya M, Isin M, Dalay N. Long non-coding RNAs with low expression levels in cells are enriched in secreted exosomes. *Cell. Biol. Int.* 38(9), 1076–1079 (2014).
- 59 Riches A, Campbell E, Borger E, Powis S. Regulation of exosome release from mammary epithelial and breast cancer cells – a new regulatory pathway. *Eur. J. Cancer* 50(5), 1025–1034 (2014).
- 60 Le MT, Hamar P, Guo C *et al.* miR-200-containing extracellular vesicles promote breast cancer cell metastasis. *J. Clin. Invest.* 124(12), 5109–5128 (2014).
- 61 Takahashi K, Yan IK, Kogure T, Haga H, Patel T. Extracellular vesicle-mediated transfer of long non-coding RNA ROR modulates chemosensitivity in human hepatocellular cancer. *FEBS Open Bio.* 4, 458–467 (2014).
- 62 Li Q, Shao Y, Zhang X *et al.* Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer. *Tumour Biol.* 36(3), 2007–2012 (2015).
- 63 Isin M, Uysaler E, Özgür E *et al.* Exosomal lncRNA-p21 levels may help to distinguish prostate cancer from benign disease. *Front. Genet.* 6, 168 (2015).
- 64 Su YJ, Yu J, Huang YQ, Yang J. Circulating long noncoding RNA as a potential target for prostate cancer. *Int. J. Mol. Sci.* 16(6), 13322–13338 (2015).
- This review describes how certain lncRNAs display better features as circulating biomarkers for prostate cancer than traditional protein markers.
- 65 Guo F, Yu F, Wang J *et al.* Expression of MALAT1 in the peripheral whole blood of patients with lung cancer. *Biomed. Rep.* 3(3), 309–312 (2015).
- 66 Svodoba M, Slysokova J, Schneiderova M *et al.* HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. *Carcinogenesis* 35(7), 1510–1515 (2014).
- 67 Li J, Wang Y, Yu J, Dong R, Qiu H. A high level of circulating HOTAIR is associated with progression and poor prognosis of cervical cancer. *Tumour Biol.* 36(3), 1661–1665 (2015).
- 68 Zhou X, Yin C, Dang Y, Ye F, Zhang G. Identification of the long non-coding RNA H19 in plasma as a novel biomarker for diagnosis of gastric cancer. *Sci. Rep.* 5, 11516 (2015).
- 69 Tong YS, Wang XW, Zhou XL *et al.* Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. *Mol. Cancer* 14, 3 (2015).
- 70 Ichigozaki Y, Fukushima S, Jinnin M *et al.* Serum long non-coding RNA, snoRNA host gene 5 level as a new tumor marker of malignant melanoma. *Exp. Dermatol.* 25(1), 67–69 (2015).
- First study in proving the potential utility of a lncRNA as a serum biomarker of malignant melanoma.
- 71 Flockhart RJ, Webster DE, Qu K *et al.* BRAFV600E remodels the melanocyte transcriptome and induces BANCER to regulate melanoma cell migration. *Genome Res.* 22(6), 1006–1014 (2012).
- 72 Li R, Zhang L, Jia L *et al.* Long non-coding RNA BANCER promotes proliferation in malignant melanoma by regulating MAPK pathway activation. *PLoS ONE* 9(6), e100893 (2014).
- This article described how the levels of BANCER, a characteristic melanoma lncRNA, are directly related with a poor outcome of the disease.
- 73 Khaitan D, Dinger ME, Mazar J *et al.* The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. *Cancer Res.* 71(11), 3852–3862 (2011).
- This study reported the correlation between increased levels of SPRY4-IT1 and the progression of melanoma, suggesting a putative role of this lncRNA in staging and early detection of the disease.
- 74 Gupta RA, Shah N, Wang KC *et al.* Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 464(7291), 1071–1076 (2010).
- 75 Tang L, Zhang W, Su B, Yu B. Long noncoding RNA HOTAIR is associated with motility, invasion, and metastatic potential of metastatic melanoma. *Biomed. Res. Int.* 2013, 251098 (2013).
- 76 Wu CF, Tan GH, Ma CC, Li L. The non-coding RNA Linc23 drives the malignant property of human melanoma cells. *J. Genet. Genomics* 40(4), 179–188 (2013).
- 77 Lessard L, Liu M, Marzese DM *et al.* The CASC15 long intergenic noncoding RNA locus is involved in melanoma progression and phenotype switching. *J. Invest. Dermatol.* 135(10), 2464–2474 (2015).
- The authors of this article showed that the levels of the lncRNA CASC15 were predictors of recurrence and survival in a cohort of 141 melanoma patients with lymph node metastasis.
- 78 Tian Y, Zhang X, Hao Y, Fang Z, He Y. Potential roles of abnormally expressed long noncoding RNA UCA1 and MALAT-1 in metastasis of melanoma. *Melanoma Res.* 24(4), 335–341 (2014).
- 79 Burd CE, Jeck WR, Liu Y *et al.* Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. *PLoS Genet.* 6(12), e1001233 (2010).

- 80 Maccioni L, Rachakonda PS, Bermejo JL *et al.* Variants at the 9p21 locus and melanoma risk. *BMC Cancer* 13, 325 (2013).
- 81 Cancer Genome Atlas Network. Genomic classification of cutaneous melanoma. *Cell* 161(7), 1681–1696 (2015).
- 82 Wang J, Zhou Y, Lu J *et al.* Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. *Med. Oncol.* 31(9), 148 (2014).
- 83 Gutschner T, Hämmerle M, Diederichs S. MALAT1 – a paradigm for long noncoding RNA function in cancer. *J. Mol. Med.* 91(7), 791–801 (2013).
- 84 Ren S, Wang F, Shen J *et al.* Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer. *Eur. J. Cancer* 49(13), 2949–2959 (2013).
- 85 Wang F, Ren S, Chen R *et al.* Development and prospective multicentre evaluation of the long noncoding RNA MALAT-1 as a diagnostic urinary biomarker for prostate cancer. *Oncotarget* 5(22), 11091–11102 (2014).
- 86 Sanlorenzo M, Wehner MR, Linos E *et al.* The risk of melanoma in airline pilots and cabin crew: a meta-analysis. *JAMA Dermatol.* 151(1), 51–58 (2015).