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REVIEW

Blood Biomarkers of Uveal Melanoma: Current Perspectives

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Manuel F Bande Rodríguez[1,2]
Beatriz Fernandez Marta[1]
Nerea Lago Baameiro[3]
Maria Santiago-Varela[1,2]
Paula Silva-Rodríguez[2,4]
María Jose Blanco-Teijeiro[1,2]
Maria Pardo Perez[3]
Antonio Piñeiro Ces[1,2]

[1]Unidad de Retina Quirúrgica y Tumores Intraoculares del Adulto (URQTIA), Servicio de Oftalmología Xerencia de Xestión Integrada de Santiago de Compostela, SERGAS, Santiago de Compostela, Spain; [2]Tumores Intraoculares en el Adulto, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain; [3]Grupo Obesidómica, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain; [4]Fundación Pública Galega de Medicina Xenómica, Clinical University Hospital, SERGAS, Santiago de Compostela 15705, Spain

Abstract: The detection of metastases in patients with a diagnosis of uveal melanoma (UM) is a controversial issue. While only 1% of the patients have detectable metastases at the time of diagnosis, up to 30% of them will develop liver metastases within 5 years of treatment. UM spreads hematogenously, therefore, blood biomarkers may be helpful for prognosis and monitoring the disease progression. Despite the great progress achieved thanks to the genetic analysis of UM biopsies, this is an invasive technique and is limited by the heterogeneity of the tumor. The present review considers the current understanding in the field regarding biomarkers for the diagnosis and prognosis of UM and its metastasis, primarily to the liver. General covered topics include non-conventional markers such as proteins previously identified in cutaneous melanoma and UM cell lines, circulating tumor cells, microRNAs (miRNA), and circulating DNA, and how each may be critical in the development of novel blood biomarkers for UM.

Keywords: uveal melanoma, biomarker, circulating tumor cells, microRNAs, circulating DNA, exosome

Introduction

The detection of metastases in patients with a diagnosis of uveal melanoma (UM) is a controversial issue. Only 1% of the patients show metastases at diagnosis;[1] however, up to 30% of them will develop liver metastases within 5 years of treatment. The most accepted hypothesis explaining this phenomenon is based on the theory of early dissemination and micrometastasis, which are not detected by current screening methods, and may be in a quiescent status until some sort of factor, still unknown, promotes its progression.

Recent studies highlight the importance of cytogenetic characteristics in the prognosis of UM. Thus, chromosome 3 loss is associated with a reduction in the probability of 5-year survival from approximately 100% to 50%.[2,3] In turn, chromosome 8 gain and 1 loss correlate significantly with poorer survival.[2,4] Similarly, in recent years, gene expression profiles (GEP) have been used to categorize UMs according to their messenger RNA (mRNA) expression profile. GEPs are used to classify UMs for disease-specific mortality risk with class 1A being very low risk (2% risk at 5 years), class 1B being low risk (21% risk at 5 years), and class 2 being high risk (72% at 5 years).[2] Unfortunately, these tests require an invasive technique to obtain the tumor samples from either enucleation or intraoperative biopsy by fine needle aspiration (FNA).[5]

In oncology, blood biological markers are used to facilitate diagnosis, establish a prognosis, and predict the therapeutic response of a neoplasm in a non-invasive

Correspondence: Manuel F Bande Rodríguez
Unidad de Retina Quirúrgica y Tumores Intraoculares del Adulto (URQTIA), Servicio de Oftalmología Xerencia de Xestión Integrada de Santiago de Compostela, SERGAS, Santiago de Compostela, Spain
Tel +34981951756
Fax +34981956189
Email verman017@hotmail.com

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way. One of the greatest challenges pursued by modern medicine is to predict the risk of suffering a pathological event in a healthy person or a specific patient. Therefore, there is growing interest in the identification of diagnostic and prognostic biomarkers at circulating level. The ideal biomarker and its implementation should be specific, sensitive, predictive, rapid, cost-effective, stable in vivo and in vitro, non-invasive, and of sufficient preclinical and clinical relevance to modify decisions regarding the pathological process in which it is applied.[6]

Currently, imaging methods are often used to clinically detect and monitor cancer metastasis. Because liver metastases are the most common for metastatic UM, abdominal ultrasound and a liver biochemical function test are considered adequate.[7,8] Hepatic ultrasound is a non-invasive, accessible, and relatively inexpensive method of metastasis screening. This method is used routinely in the initial evaluation of UM patients by European specialists (79%); however, this is not used by North American specialists (3%), they rely primarily upon liver function tests and chest x-rays.[7] The sensitivity of ultrasound for the detection of UM metastasis ranges from 40% to 89% and its specificity is close to 96%.[9] Although computed tomography/positron emission tomography (CT/PET) imaging would seem to be the most sensitive, given its very high cost and low availability, its use is impractical. On the other hand, MRI is also superior to CT for detecting UM associated retinal detachments and extra-scleral extensions.[10] Thus, there is no evidence that CT surpasses ultrasound for the early diagnosis of liver metastases. The most established guideline is to perform six-monthly systemic follow-ups (liver ultrasound + liver function test) during the first 5 years and annually thereafter. Systemic monitoring is recommended to be lifelong.[11]

Conventional Markers: Hepatic Serology

As mentioned above, the liver is involved in most cases of UM metastasis. Currently, liver function tests (LFT), liver ultrasounds, chest radiography, and in some cases, CT is used to follow-up patients treated for UM. According to the Collaborative Ocular Melanoma Study Group (COMS),[12] the LFT should include alanine aminotransferase (ALT), which was formerly known as glutamic-pyruvic transaminase (GPT); aspartate aminotransferase (AST), which was formerly known as glutamic-oxalacetic transaminase (GOT); alkaline phosphatase (FAL); gamma glutamyl transpeptidase (GGP); lactic dehydrogenase (LDH); and bilirubin. The LFT results are considered abnormal if AST > 2× the major normal reference limit (LNR), ALT > 2 on LNR, FAL > 1.5 on LNR, and bilirubin ≥ 2.0 mg/100 mL.

Different studies have shown that serum levels of liver enzymes increase in the presence of metastasis;[8,13] however, most of these studies find low sensitivity. For instance, some studies give maximum sensitivity of LFT to ALT (sensitivity, 21%) and alkaline phosphatase (sensitivity, 25%).[13,14] Others report the highest sensitivity being for LDH at 67%.[9] Although the sensitivities of each of the enzymes individually are low, Eskelin et al[15] observed that using a panel of enzymes, including ALT, AST, FAL, LDH, and bilirubin, that at least one of those is altered in 70% of the patients who develop metastases. Kaiserman et al[16] obtained similar results in a patient series. They found that 50% of the patients with liver metastases showed an increase in at least one of the enzymes 6 months prior to image diagnosis.

According to COMS,[12] the sensitivity, specificity, positive, and negative predictive value associated with at least one abnormal LFT prior to the first diagnosis of metastasis were 14.7%, 92.3%, 45.7%, and 71.0%, respectively. The values increased to 24.1%, 98.2%, 75.3%, and 85.4% if the time to obtain the results of the analyses was limited to 90 days prior to the diagnosis of metastasis. Table 1 shows the diagnostic properties of each liver enzyme in more detail. Despite limitations in this field of research, determination of circulating levels of these enzymes is used in specialized eye oncology centers.

Non-Conventional Markers: Non-Hepatic Serology

The low sensitivity and specificity of liver enzyme levels and the low positive predictive value greatly encourage the search for new serological markers capable to detect disease progression. This challenge has led researchers to initially focus on serum markers, previously described in cutaneous melanoma, to test its possible application for UM (Table 2). This is the case for the protein S100β, that was one of the first biomarkers tested in UM, due to its utility for monitoring cutaneous melanoma.[17] Thus, S100β is a powerful prognostic marker in stages III and IV skin melanomas.[18] The S100β belongs to a family of low molecular weight proteins present in vertebrates and are characterized by two calcium-binding sites with helix-loop-helix structures (EF-hand).[19] S100β was first isolated from neurological tissues and as such, it is

Table 1 Diagnostic Properties of Liver Enzymes in Uveal Melanoma Metastatic Disease (UM)

ENZYME	Eskelin et al[9]					
	Sensitivity (%)		Specificity (%)		Likelihood Ratio (+)	
FAL	27		93		5.0	
AST	43		95		5.8	
ALT	38		90		3.8	
LDH	67		96		14.7	
	COMS Report 23[9]–90 days earlier					
ENZYME	Sensitivity (%)		Specificity (%)		Positive predictive values (%)	
	Large	Medium	Large	Medium	Large	Medium
FAL	18.9	14.4	99.3	99.3	92.3	72.7
AST	10.0	7.1	98.9	99.6	79.2	66.7
ALT	12.8	7.3	98.6	99.4	80.0	61.5
Bilirubin	4.2	0	99.1	99.6	66.7	0
> 1 altered (LFT)	26.7	19.6	97.8	98.5	83.6	61.1

Note: Data from Eskelin et al.[9]
Abbreviations: ALT, alanine-aminotransferase; aspartate-aminotransferase (AST), FAL, alkaline phosphatase; LDH, lactic dehydrogenase; LFT, liver function tests.

Table 2 Cutaneous Melanoma Biomarkers

Study	Marker	No Metastasis		N Number (Metastases)	N Number Control
		N Number (Not)	N number (Treated)		
Missotten et al[24]	S–100β	44 0.04 μg/L	–	20 0.06 μg/L	58 0.03 μg/L
Schaller et al[30]	MIA	131 6.6 ng/mL	–	8 26.28 ng/mL	NO
Reiniger et al[31]	MIA	125 7.27 ng/mL	160 7.43 ng/mL	20 13.03 ng/mL	NO
Haritoglou et al[39]	MIA	18 5.64 ng/mL		14 13.11 ng/mL	
	OPN	18 47.39 ng/mL		14 152.01 ng/mL	
Kadkol et al[37]	OPN	–	37 7.15 ng/mL	15 17.62 ng/mL	30 6.71 ng/mL
Suesskind et al[40]	GDF–15	170 1.5 ng/mL		18 10.53 ng/mL	18 1.09 ng/mL
Missotten et al[25]	MIA	104 5.63 ng/L	–	30 8.13 ng/L	50 5.18 ng/l
	S–100β	104 0.07 μg/L		30 0.23 μg/L	50 0.06 μg/L
Barak et al[38]	MIA S–100β OPN	–	38	18	44
Barak et al[41]	MIA S–100β OPN CK18	–	43	32	53

a sensitive indicator of brain-tissue damage and neurological dysfunction.[20] It is present in the retina where it participates in the signaling pathways between photoreceptors and Müller cells.[21] Additionally, high levels of S100β are detected in vitreous and aqueous humor.[22] Cochran et al were the first to demonstrate the presence of S100β in histological slices of choroidal melanoma. However, subsequent studies have shown that the tissue expression of S100β is less relevant in other tissues compared to that in cutaneous melanoma.[23] In addition, serum concentration of S100β lacks a predictive value and does not correlate with other prognostic factors in UM.[24,25]

Melanoma inhibitory activity (MIA) protein was discovered in cutaneous melanoma cell cultures and identified for its ability to inhibit the incorporation of thymidine by melanoma cell lines.[26] Subsequently, MIA has been found to be specifically expressed in cutaneous melanoma cells, chondrocytes, and in an advanced breast cancer subgroup.[27] MIA specifically inhibits the binding of melanoma cells to fibronectin and laminin, and therefore is suspected to regulate the detachment of melanoma cells from the extracellular matrix, which is an important step in the metastatic cascade.[28] Moreover, it has been shown that there is a good correlation between the stage of the skin tumor and the concentration of MIA protein in the blood.[29] Shaller and collaborators[30] noted that MIA is expressed in primary and metastatic UM lesions, and reported a statistically significant elevation of serum MIA levels in patients who develop metastases. On the contrary, Reiniger and other authors demonstrated, in a cohort of 305 UM patients, that serum MIA concentration does not reach enough value as a predictive marker, and therefore it is not useful to estimate the risk of developing metastatic disease.[31] Under the above, it is important to note that although progression of UM cannot be excluded in those patients with normal serum MIA levels, abnormal levels of MIA may indicate a probable presence of metastatic disease.[31]

Another protein of interest is osteopontin (OPN), which is a phosphoprotein capable of binding to calcium, with high affinity for hydroxyapatite playing an important role in bone mineralization and dystrophic calcification.[32] This protein has been described in the context of various physiological functions, including chemotaxis, cell migration, cell adhesion, angiogenesis, apoptosis, extracellular cell-matrix interactions, immune regulation, and tumor metastasis.[33] Thus, it was shown that osteopontin actively promotes a tumorigenic phenotype and contributes to metastasis.[34] Increased osteopontin expression is associated with aggressive behavior and metastasis in breast, colon, prostate, lung, liver, and ovarian cancers. Moreover, a soluble form of this protein is secreted into the blood showing a positive correlation with advanced or metastatic stage cancers.[35] The discovery that cutaneous melanoma cells with invasive behavior express more osteopontin at tissue level[36] led to the study of this marker in the context of UM. Various studies have shown that osteopontin is diffusely expressed in tissue sections of UM liver metastases and that serum levels of osteopontin correlate with melanoma metastases in the liver with significant specificity and sensitivity.[37,38]

Growth Differentiation Factor 15 (GDF-15/MIC-1) is a divergent member of the TGF-ß superfamily. Members of this family are important regulators of cellular physiological processes, including cell survival, proliferation, differentiation, migration, and apoptosis.[42] GDF-15 is highly expressed in many melanoma cell lines compared to that in normal human melanocytes, and is also overexpressed in biopsy specimens of metastatic cutaneous melanoma.[43] It is therefore hypothesized to have melanoma promoting properties. Patients with clinically detectable metastases have significantly higher serum GDF-15 levels compared to those without clinically detectable metastases, as well as to healthy individuals.[40] Interestingly, paralleling skin melanoma, concentrations of GDF-15 are higher in patients with clinically detectable UM metastases compared to those UM patients that are in remission or those without clinically detected UM metastases.[40]

Liver Biomarkers

Metastasis is a highly selective and complex process that includes the proliferation and growth of tumor cells in the parenchyma of target organs to complete the metastatic process requiring the formation of new blood vessels (angiogenesis).[44] Thus, it is assumed that the interactions between tumor cells and the microenvironments of the target organ are the primary determinants in the outcome of the metastatic process.[45] The fact that the liver is the main target organ in UM metastasis raises the suspicion that growth factors synthesized in the liver, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin growth factor type 1 (IGF-1), and their receptors, may play a role in the acceptance and survival of the metastatic UM cells.[46] Hendrix and other authors found that hepatocyte growth factor receptor (HGFR/c-Met) expression correlates with the invasive capacity of UM cell lines.[47] On the other hand, a close and positive correlation has been described among the immunoreactivity of

the EGF receptor (EGFR) in UM tissue, the progression to metastasis, and a decrease in survival.[48]

Among the growth factors synthesized by the liver, special attention is paid to IGF-1 since it is the only growth factor that has been studied in the serum of patients with UM.[49] IGF-1 is a soluble protein produced by the liver that binds to a transmembrane receptor called IGF-1R. This receptor is expressed throughout the body, but primarily in cartilage, bone, liver, kidneys, lung, and the central nervous system.[50] IGF-1R plays an important role in the cell cycle, anaerobic respiration, pediatric growth, and aging. IGF-1R is also important in the development of metastatic tumors, cell proliferation, and the prevention of apoptosis.[51] Unlike other markers, serum IGF-1 levels tend to decrease in patients who develop UM metastasis.[52]

In recent years, studies have been published that describe a possible relationship between Vascular Endothelial Growth Factor (VEGF) levels in serum of patients with UM and the risk of developing metastasis. Boyd et al and later Missotten et al showed that VEGF concentrations increase in eye aqueous humor in association with UM.[53,54] While determining the amount of VEGF in the serum of patients with UM, it has been observed that the levels are not elevated at the time of enucleation and that only those patients with metastases have a high concentration of VEGF in their serum.[55] A recent study by Barak and collaborators presented similar confirming results. Therefore, although no correlation has been found between VEGF expression by the primary tumor and histologic parameters, VEGF levels are significantly higher in patients with metastatic disease when they value themselves interpersonally before and after metastasis.[56] An increase in VEGF on serial measurements could indicate the development of UM metastases.

Other Biomarkers

There are other biological factors expressed by most malignant tumors, including UM, that can be measured in serum. These include factors associated with tumor angiogenesis (basic FGF or IL-8),[57] adhesion (ICAM and VCAM),[58] and invasion (matrix metalloproteinases 1 and 9).[59]

Patients with eosinophilia show a trend towards longer survival in various types of cancer, including melanoma. Recently, elevated serum concentrations of cationic eosinophil protein (ECP) were indicated to increase survival of patients with UM.[60]

It is worth mentioning the carcinoembryonic antigen (CEACAM-1), which was first described in UM by Michelson and other authors.[61] These researchers demonstrated an increase of CEA in the sera of patients with UM metastasis. However, the inconvenience with this marker is that it may also be increased in other types of tumors (eg, ovarian and colon cancer).[62]

Finally, we mention the recent work of Song and collaborators who evaluated different serum biomarkers using a multiple immunoassay based on magnetic microspheres. They were selected after a literature search and with an in silica analysis of gene and protein databases reported to be useful for the detection of UM. To OPN, MIA, CEACAM-1, MIC-1 were added proteins related to the progression of cutaneous melanoma (periostin/POSTN)[63] and others whose increase was associated with poor prognosis in other types of cancer (Heat shock protein 27/HSP27 and Spondin 1/SPON1).[64,65] Multivariate analysis identified HSP27 and OPN as the best combination of markers to discriminate UM patients from healthy controls. The combination of MIA and MIC-1 was also useful in differentiating between patients with metastatic and disease-free UM, although this was not statistically significant due to the small sample size.[66]

Proteins Identified from UM Cells Cultured in vitro

A comprehensive study of the proteome of the primary UM cell culture UM-A was the first of its kind in the literature and represents an important first step towards the use of proteomic technology in the study of UM tumor cells.[67] A total of 683 proteins derived from 393 different genes were identified. Of these 683 proteins, 18% were related to carcinogenic processes by their association with cell proliferation, invasion, metastasis, oncogenesis, and drug resistance.

The expression of some of these new identified proteins was validated by immunodetection in UM-A cells and other UM cell lines established by other groups, including UW-1, OCM-1, SP6.5, and 92.1. These proteins include MUC18 (MelCAM/CD146) and high mobility group protein (HMG-1). The expression of MUC18 has been associated with increased metastatic potential in other neoplasms, such as prostate and bladder cancer.[68] Our work shows for the first time the relationship between MUC18 and in vitro invasion of UM. On the other hand, HMG-1 intervenes in the transcriptional regulation of genes that play a key role in different biological processes of neoplastic progression and metastasis.[69] Subsequently, the analysis of secreted proteins (secretomes) of the mentioned UM cell lines was also characterized, finding proteins relevant to the development of cancer, such as cathepsin D, PMEL/ME20M, DJ-1/PARK7,

and syntenin 1. Interestingly, DJ-1, ME20M, and cathepsin D were detected in the sera of patients with UM at higher levels compared to those of healthy individuals.[70,71] The reference studies performed by our group and those of others using UM cell lines are listed in Box 1.

The pro-oncogenic characteristic of DJ-1 was first described regarding its ability to transform NIH-3T3 cells when expressed alone or in combination with other oncogenes, such as c-Myc or H-Ras.[77] DJ-1 appears to regulate several cellular signaling pathways that are related to survival. Thus, DJ-1 modulates the PI3K/Akt pathway by inactivating PTEN.[78] Apart from regulating this pathway, DJ-1 also interacts with other genes such as Von Hippel Lindau (VHL), which is a tumor suppressor gene involved in the ubiquitination and degradation of the factor inducible by hypoxia (HIF-1a) under normoxia.[79] On the other hand, several studies have shown a link between p53 and DJ-1 of which some suggest an anti-apoptotic function of DJ-1 through the repression of the transcriptional activity of p53. DJ-1 may interfere with the binding of p53 to promoter DNA in order to suppress the transcription of the proapoptotic factor Bax, thereby inhibiting caspase activation.[80]

Our group has demonstrated that DJ-1 presents significantly higher serum concentrations in patients with choroidal nevi that has manifested some risk factors for malignancy.[81] The risk factors include symptoms at the time of diagnosis of UM, acoustic shadow on ultrasound, nevus height > 1.5 mm, and a basal diameter > 8 mm.[82] The mean serum level of this group of patients was 89.56 ng/mL compared to 37.39 ng/mL for the nevus group without associated risk factors and 32.98 ng/mL for the control group.[83] This indicates that DJ-1 could be a potential marker of malignant transformation of choroid nevi. On the other hand, Chen et al have related DJ-1 to the presence of UM metastases. In their study, they observed a sensitivity and specificity of DJ-1 circulating levels with metastasis detection of 74.1% and 94.3%, respectively.[84]

Box 1 Summary of Proteomics Studies in Tumors and Uveal Melanoma (UM) Cell Lines

Pardo et al[67]	Global UM cell line proteome (UM-A)
Pardo et al[71]	Differential proteome of UM cell lines (less and more aggressive)
Pardo et al[70]	Global secretome proteome of 5 UM cell lines
Zuidervaart et al[72]	Differential cell line proteome derived from a primary tumor and metastasis
Wang et al[73]	Differential proteome of irradiated UM cell lines.
Yan et al[74]	Differential proteome of irradiated UM cell lines.
Angi et al[75]	Comparative analysis of high and low risk tumors of metastasis.
Surman et al[76]	Proteome derived from ectosomes secreted uveal melanoma cells

In contrast to DJ-1, PMEL/ME20M is a melanocyte-specific protein. ME20M plays a central role in the biogenesis of melanosomes.[85] It participates in the ripening of stage I and stage II melanosomes. The release of a soluble form of this protein ME20-S/M-alpha may protect tumor cells from antibody-mediated immunity.[86] We found significantly higher concentrations of ME20-S in patients in the UM untreated group and in patients with liver metastases than in the choroid nevus or control group. Figure 1.[87] In addition, it is important to note that patients with UM treated without metastasis had protein concentrations similar to those in the control group.[87] Regarding the analysis of the effects of height and base on levels of ME20-S in patients with untreated choroidal nevus or UM, positive associations were found between serum levels of ME20-S and height of melanocytic lesion.[87] Thus, we conclude, that PMEL(ME20-S) might be a promising serum marker for UM and useful for monitoring metastatic disease.

In summary, osteopontin (OPN), calcium-binding protein B S100 (S-100B), cytokine-1 inhibitor of macrophages (MIC-1), and Parkinsonian protein (DJ-1/PARK7) appear to have higher serum concentration in metastatic UM patients. However, these proteins are not specific to melanoma and may undergo wide interindividual variability as in the case of VEGF. Although the combination of serum markers (eg, OPN, MIA, and S100B) provides a more sensitive method for detecting metastases than LFT liver metastases, these studies are of few patients and their use has not yet been adopted in universal clinical practice. We advocate large multicenter studies with specific melanoma biomarkers (MIA and ME20-S) and sequential measurement of levels of these proteins in patient sera.

Circulating Tumor Cells

Circulating tumor cells (CTCs) were first described in 1869 by Thomas Ashworth,[88] describing them as "tumor-like cells that can be seen in blood." However, it was not until 1990 that research regarding CTCs and their association with oncology in the clinic began.[89] To date, the confirmation and enumeration of CTCs have been accomplished using flow cytometry techniques, direct cytometry

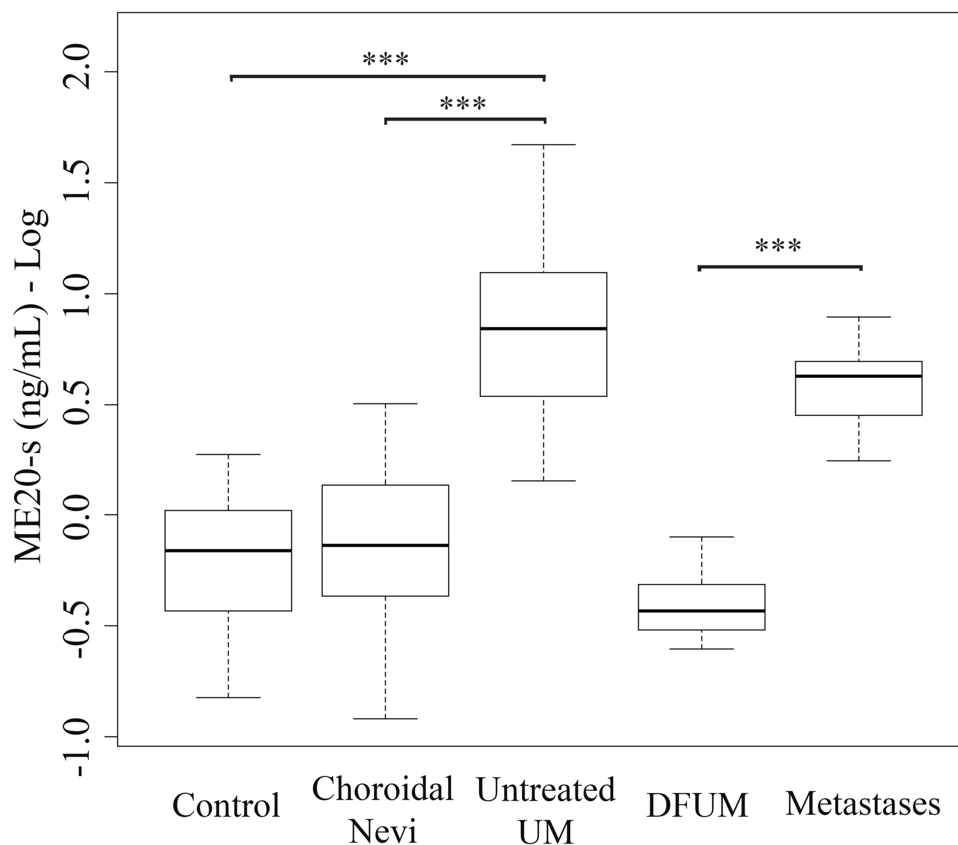

Figure 1 Logarithmic scale representation of serum ME20-S levels in five groups of patients (Control, Choroidal Nevi, Untreated UM, 10-year disease-free (DFUM), hepatic metastatic UM). Significance level: ***p<0.001. Adapted from Bande MF, Santiago M, Mera P, et al. ME20-S as a potential biomarker for the evaluation of uveal melanoma. Invest Ophthalmol Vis Sci. 2015;56(12):7007–7011. Copyright 2015 The Association for Research in Vision and Ophthalmology, Inc.[87]

analysis, fluorescent in situ hybridization, and polymerase chain reaction (PCR) analysis of RNA and DNA of tumor cells. The identification of CTCs in the blood is becoming an important clinical tool, even though CTCs are rarely seen in cancer patients; however, it is estimated that there is one CTC for every mononuclear cell.[90]

In the specific case of UM, the presence of CTCs in the blood of patients was described as early as 1969 using standard microscopic techniques and staining with orange acridine nuclear dye.[91] Over the years, techniques used for patients with cutaneous melanoma have been applied in patients with UM.[92] The most frequently used method for the detection of CTCs in patients with UM has been PCR. Tyrosinase mRNA was able to be detected in blood samples from three out of six patients diagnosed with UM.[93] Two of the patients who were positive for tyrosinase mRNA had metastatic disease with the third initially being non-metastatic, but who did develop metastases nine months later. Based on these results, the investigators studied a larger cohort of 36 patients with UM.[94] Contrary to expectations, CTCs were not detected using tyrosinase PCR in any of the 51 blood samples they collected and analyzed. Blood samples were also taken before and after primary treatment in 41 patients with UM with no metastatic disease being detectable at the time of diagnosis.[95] Treatment was based on clinical indications and included enucleation, brachytherapy, or transpupillary thermotherapy. Patients were followed up for the development of metastases or for a minimum period of 5 years. At the start of the study, 16 of the 41 patients tested positive for CTCs. A total of 11 of these 16 patients (69%) were negative for CTCs after treatment of the primary tumor. Although a positive correlation was found between the presence of CTCs and the probability of metastasis at 5 years, there was no correlation between the positivity of CTCs and clinical risk factors, such as tumor size, histology, or treatment method.

On the other hand, Callejo et al published an important finding using multi-marker PCR that allows the simultaneous detection of several molecular markers.[90] They studied 30 patients with UM, 5 at the time of initial diagnosis and the other 25 at various times after diagnosis and initial

treatment, ranging from 1 to 17 years. PCR was used to detect tyrosinase and MART-1 mRNA expression. They collected an average of four blood samples per patient; one at the time of the initial visit and subsequent samples at 3-month intervals. They detected CTCs in 29 of the 30 patients in more than 119 visits. However, no correlation was found between the presence of CTCs and tumor size, type of treatment, or time since treatment. The authors attributed their differing results compared to those of previous studies to their use of a more sensitive approach involving the use of more blood samples (four versus one or two), the collection of large amounts of blood (30 mL versus 5–15 mL), a larger number of PCRs (20 versus one), and to their inclusion of more than one marker. Despite this argument, mRNA analysis is known to be susceptible to false positives due to "illegitimate" transcription or contamination.[96]

To overcome these limitations, immunomagnetic classification particles with anti-melanoma antibodies, which recognized human high molecular weight-melanoma-associated antigen (HMW-MAA), have been used. Ulmer and collaborators were the first to suggest this approach for patients with UM.[97] These investigators examined the blood of 52 patients with non-metastatic UM and identified CTCs in 19% of the samples (1–5 cells/50 mL), with a positive relationship established to clinical prognostic factors (ciliary body invasion, advanced local tumor stage, and anterior tumor localization). Likewise, Suesskind other authors found 14% of the peripheral blood samples from 81 patients with UM to be positive for CTCs but found no correlation with clinical parameters.[98] In a study carried out by Mazzini et al, the malignant cells were isolated by filtration in a cohort of 31 UM patients. By stratifying patients into groups based on the number of CTCs (less than or greater than 10 CTCs per 10 mL of blood) and the presence of CTC groups, a significant difference was found in LBD, tumor height, disease-free survival, and overall survival ($p < 0.05$).[99]

A similar study by our group assessed the level of peripheral blood CTC in patients with nevus and non-metastatic choroidal melanoma. No CTC was found in patients with choroidal nevus, while at least one CTC/7.5mL was found in 50% of the patients with UM. The highest number of CTCs was recorded in the patient with the largest choroidal melanoma, which presented extrascleral extension and epithelial pathology. However, no significant relationship was found between the positivity of CTC and the clinical-pathological parameters of the lesions (basal diameter of the tumor and height of the tumor).[83]

Our results above were corroborated by another recent article, conducted by Bidard and collaborators. In this work, the CTC count and DNA levels of tumor cells were associated with the presence of miliary liver metastases and with the volume of metastases. CTC was correlated and both have poor prognostic significance.[100]

In a study conducted by Terai et al, it was found that CTCs are detectable more frequently and in greater numbers in arterial blood samples compared to that of venous samples in UM.[101] Patients with UM liver metastases and extrahepatic metastases show a higher number of arterial CTCs compared to patients with liver metastases alone. There is no significant association between the number of arterial CTCs and the tumor load in the liver in patients with liver metastases.

Taken together, the data obtained to date from the different studies using CTCs of UM have been inconsistent. This is probably caused by variations in test procedures. Thus, further work is necessary to reconcile these differences.

MicroRNA

MicroRNAs (miRNAs) are a family of small non-coding RNAs that regulate a wide range of biological processes including carcinogenesis. In cancer cells, miRNAs have been found to be heavily dysregulated. The functional characterization of miRNAs, especially their interaction with oncogenes, tumor suppressor genes, and other cancer-related genes may help us to better understand the tumorigenesis process.[102] Several studies have been conducted on UM using miRNA expression tests. Therefore, expression of certain miRNAs (let-7b, miR-199a, miR-199a, miR-143, miR-193b, and miR-652) was associated with chromosome 3 status, gene expression profile classes, and prognosis.[103]

In a study by Nannan Liu and collaborators using UM cell lines with higher and lower invasive potential, it was shown that MiR-9 is involved in the regulation of the NF-kB pathway.[77] MiR-9 expression is reduced in melanoma cells with high invasive capacity. This reduced expression appears to negatively modulate the expression of NF-Kb1, thereby suppressing cell migration and invasive capacity that is regulated by the NF-kB1 signaling pathway. Meanwhile, Chen et al studied the expression of miR-124a in UM cell lines using RT-PCR. They determined that miR-124a expression is down-regulated in UM cells. They found that miR-124a inhibits the functions of CDK4, CDK6, cyclin D2, and EZH2, which results in inhibition of migration and invasion of UM cells.[109]

Achberger and collaborators were the first to study miRNA in the sera of patients with UM. They extracted blood from six patients with UM at the time of diagnosis that did not have clinical or radiographic evidence of metastasis. Plasma and cell levels of miRNA were determined using quantitative PCR (qPCR) assays. Plasma levels of miR-20a, 125b, 146a, 155, 181a, and 223 were all higher in the patients at the time of diagnosis compared with that of controls. Plasma levels of miR-20a, 125b, 146a, 155 and 223 increased and miR-181a levels decreased when metastasis manifested.[104]

The results of the miRNA studies involving UM are summarized in Table 3. However, after completion of all these works, it is clear that only a small number of miRNAs were common among the different studies and several miRNAs even exhibit discordant patterns of expression among them. These discrepancies are possibly due to quality differences in the clinical samples, the different classification and inclusion criteria used in each study, sample processing variations, previous cytotoxic treatments, tumor heterogeneity, and underestimation of hypoxia and infection. It is therefore important to re-evaluate the current strategies used in miRNA profiles and to be cautious with respect to interpreting the existing data.[105]

Circulating DNA

The mechanism of DNA release into the bloodstream is not completely clear. It has been suggested that the presence of high levels of ctDNA in the blood of patients with tumors is caused by apoptosis and the necrosis of tumor cells or by the release of intact cells into the bloodstream and their subsequent lysis.[110]

Circulating DNA is the genetic material that releases all cells – both healthy and tumor cells. This ctDNA reflects at the molecular level the characteristics of the cells of origin. For this reason, its analysis in a blood sample makes it possible to obtain molecular information on the tumor being studied. In addition, ctDNA reflects the mutations present throughout the tumor, not just in a sample of the tumor obtained by biopsy. More than 80% of the UMs present with mutations in the proto-oncogenes GNAQ and GNA11.[5,111] For that reason, PCR techniques for ctDNA of UM patients include screening for GNAQ c.626A>T, GNAQ c.626A>C, and GNA11 c.626A>T.[100]

In a cohort study conducted by Bidard et al,[84] ctDNA was detected in 84% of the patients with metastatic UM. The detection of ctDNA correlates with the presence of liver metastases, tumor size, and CTCs. ctDNA levels are strongly associated with progression-free survival and overall survival.[100] In patients with detectable mutations, ctDNA is detected more frequently than CTCs and therefore appears to have a higher prognostic value. Both CTCs and ctDNA were associated with miliary liver metastases. These small perivascular metastatic foci within the liver are the result of hematogenous spread, which may explain the increased detection of CTCs and the increased release of ctDNA into the bloodstream.[100]

Conclusions and Future Prospects

Genetic biopsy methods for UM have a number of limitations: (1) concern that the biopsy may spread tumor cells leading to a poorer prognosis of survival, (2) concern that

Table 3 Summary of microRNA (miRNA) Studies Involved in Uveal Melanoma (UM) Detection

	Type of Sample	miRNA Overexpressed in Metastatic UM	miRNA Overexpressed in Non-Metastatic UM	miRNA Under-Expressed in Metastatic UM
Worley et al[103]	Histological	let-7b, miR-199a, miR-199a*, miR-143, miR-193b and miR-652		
Liu et al[106]	Cell lines			miR9
Radhakrishnan et al[107]	Histological	miR-196a, miR-549, miR-497, miR-885-5p, miR-585, miR-640, miR-512-5p, miR-556-5p, miR-135b, miR-325, miR-99a, miR-33a	miR-495, miR-18a, miR-586, miR-493, miR-377, miR-376c, miR-369-3p, miR-34c-5p, miR-26a-2, miR-218, miR-19b-1, miR-154, miR-181a, miR-133a, miR-129, miR10a, miR1, Let-7e	
Yang and Wei[108]	Histological	miRNA-20a, miRNA-106a, miRNA-17, miRNA-21 and miRNA-34a		miRNA-145 and miRNA-204
Chen et al[109]	Cell lines			miR-124a
Achberger et al[104]	Serum	miR-20a, 125b, 146a, 155 and 223		miR-181a

the biopsy will worsen the visual prognosis of the eye (14.3% experienced intravitreal hemorrhage),[112] (3) the belief that FNA may lead to misdiagnosis due to sampling failures, and (4) the lack of conviction that cytopathologic analyses of FNA biopsy specimens can effectively distinguish between tumors with low-risk and high-risk for metastasis due to tumor heterogeneity.[113] In addition, it is important to note that both methods (GEP and MLPA) are only prognostic, not diagnostic. For these reasons, it is important to identify other non-invasive biomarkers that will allow the detection and diagnosis of UM at different stages of disease and tumorigenesis.

The use of appropriate blood biomarkers in clinical practice with periodic monitoring of their levels would allow health-care providers to guide the management of patients with UM, to establish therapy protocols, and to directly evaluate the progress or control of the disease. The combination of using these biomarkers, in combination with the tests currently established during routine check-ups, would represent a great advancement in the decision-making process and in the clinical approach for treating these patients.

Moreover, new molecular players such as extracellular vesicles including exosomes have emerged into play lately. Exosomes are vesicles from 30 to 150 nm derived from cells containing various proteins, nucleic acids, and lipids. These vesicles facilitate intercellular communication and cause micro-environmental changes. Interest in exosomes as specific and accurate biomarkers has intensified dramatically in recent years due to their ability to transport a variety of molecular components from the cells of origin.[114] Exosomes released by tumor cells have also been shown to play a very active role in tumorigenesis, metastasis, and response to therapy through the transfer of oncogenes and onco-miRNAs between cancer cells and the tumor stroma.[115] Exosomes in target organs trigger the necessary molecular responses for inflammation and vascularization to host CTCs.

In the future, the quantitation of CTCs and their subsequent genetic analysis may allow for the replacement of primary tumor biopsy with simple blood collection. The ctDNA is found in the blood of patients with advanced cancer and may be used as a source of biological material to determine the somatic genetic characteristics of these tumors. Detection of the gene mutation in ctDNA could also be very useful for prognostic classification of UMs as it is in other types of cancer.[116,117]

The diagnostic approach to UM using serum markers would avoid or delay the use of other more aggressive diagnostic methods such as tumor biopsy. However, some studies show differing results. The unification of the criteria for inclusion and processing of samples promises more reliable results in the future.

Disclosure

The authors report no conflicts of interest in this work.

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