ORIGINAL ARTICLE

Investigation of microRNA-10b values for the discrimination of metastasis due to melanoma

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Background: Melanoma is one of the most invasive cutaneous cancers with characteristics such as rapid progression and distant metastasis. The early diagnosis and staging of melanoma can help better manage the patients. The current study is aimed to assess the values of microRNA-10b (miRNA-10b) in the discrimination of metastatic melanomas. **Materials and Methods:** The current cross-sectional study has been conducted on forty patients diagnosed with melanoma since 2011. Cell culture of melanoma cell lines derived from the cancerous tissue, including WM115, BLM, K1735, WM793, and A375M, was cultured. In order to assess miRNA-10b levels, the real-time polymerase chain reaction was utilized. The absence (n = 20)/presence (n = 20) of metastasis was diagnosed with chest computed tomography or chest X-ray. The values of miRNA-10b for the discrimination of metastasis incidence were assessed. **Results:** The demographic characteristics, including age and gender of the metastatic and nonmetastatic patients, were similar ($P > 0.05$). The specimen cultures were positive for miRNA-10b in 14 (35%) of the metastatic cases versus 4 (20%) of the nonmetastatic ones ($P = 0.004$). The quantitative analysis of miR-2b revealed significantly higher levels in metastatic cases (−1.59 ± 1.13 in metastatic vs. −0.16 ± 0.67 in nonmetastatic cases; $P = 0.001$). The measured area under the curve for the value of miRNA-10b was 0.923 ($P < 0.001$; 95% confidence interval: 0.811–1) with sensitivity and specificity of 100% and 94.4%. **Conclusion:** Based on this study, metastatic melanoma was associated with elevated levels of miRNA-10b. This marker had the sensitivity and specificity of 100% and 94.4% for the discrimination of metastatic melanoma from nonmetastatic ones.

Key words: Melanoma, microRNAs, real-time polymerase chain reaction

How to cite this article: Mokhtari M, Rouhanizadeh N, Hajialiasgar S. Investigation of microRNA-10b values for the discrimination of metastasis due to melanoma. J Res Med Sci 2021;26:108.

INTRODUCTION

Melanoma, a melanocyte-derived cancer, is responsible for only 1% of all skin-related malignancies, while it is one of the most invasive cancers.[1] Unfortunately, despite the remarkable progress in the treatment of melanoma, the overall 5-year survival of melanoma patients in advanced stages has not altered dramatically, yet. Besides, the incidence of melanoma has increased within a few decades. Therefore, the determination of molecular mechanisms responsible for cell lines changes to melanoma, and its development is necessary.[2,3]

Finding relevant biomarkers associated with melanoma is a significant problem for scientists because, due to the typical formalin fixation of the tissues and their paraffin embedding, macromolecules are poorly preserved. Nevertheless, due to the reasonably well preservation of microRNAs (miRNAs), these molecules have deviated from the attractions toward themselves.[4]

miRNAs are small noncoding RNAs consisting of 20–22 nucleotides regulating posttranscriptional gene expression; therefore, they play a crucial role in regulating biological processes.[5] Novel studies in the literature have represented the aberrant expression of miRNAs in human malignancies. miRNAs may have both roles of oncogenesis or tumor suppression in carcinogenesis.[6,7]

miRNAs can be appropriately extracted from formalin-fixed paraffin-embedded tissues,

Access this article online

Quick Response Code:

Website: www.jmsjournal.net

DOI: 10.4103/jrms.JRMS_573_20

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Submitted: 18-May-2020; **Revised:** 22-Jun-2020; **Accepted:** 15-Jul-2021; **Published:** 29-Nov-2021

represent considerable genetic alterations, and exhibit a melanoma-specific signature.[8,9] Furthermore, miRNAs have been demonstrated as prognostic markers of melanoma.[10,11] Therefore, a panel of six miRNAs was associated with postrecurrence survival[12] and four miRNAs with brain metastasis. Besides, investigations regarding new panels are in progress.[13]

The family of mammalian miR-10 consists of miR-10a and miRNA-10b has identical seed sequences and plays notable roles in various regulatory pathways.[14] miRNA-10b deregulation has been detected in numerous types of malignancies, including hepatocellular carcinoma, breast cancer, colorectal cancer, and also melanoma.[4,15,16] Although this marker's upregulation is associated with poor prognosis in melanoma, the clinical significance of miRNA-10b with melanoma metastasis is unclear.[17] Therefore, in the current study, we have aimed to compare the levels of miRNA-10b among metastatic melanoma patients versus nonmetastatic ones.

METHODS

Study population

The current cross-sectional study has been conducted on forty patients with the first-time presentation and diagnosis of melanoma (none of the samples were taken from those with recurrence) referred to Al-Zahra Hospital affiliated to Isfahan University of Medical Sciences since 2011.

The Ethics Committee of Isfahan University of Medical Sciences approved the study protocol (IR.MUI.MED.REC.1398.108). After that, the included patients were reassured about their personal information confidentiality, and written consent was obtained.

Those patients with documented diagnosis of melanoma whose medical records were available were included. Besides, the patients were selected among those whose melanoma had a depth of over 1.5 mm based on the Breslow thickness as accounted high risk for metastasis and Clark level 2.[18]

Inadequate or improper tissue specimens and more than 20% defects in the medical records were considered exclusion criteria.

The study population was selected through convenience sampling with 1:1 block. Therefore, data of twenty patients with distant metastasis as the case group and twenty other ones without metastasis as the controls were recruited.

The medical records of the patients were assessed to find the traces of metastasis based on reports, including history, physical examination, complete blood count, serum biochemistry analysis, chest X-ray and computed tomography (CT) imaging, cranial CT or magnetic resonance imaging, whole-body scan, and abdominal CT or ultrasonography.

Procedure

Cell culture of melanoma taken from the melanoma-involved skin cell lines, including WM115, BLM, K1735, WM793, and A375M, was cultured in Dulbecco's modified Eagle's medium containing streptomycin/penicillin and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2.

Real-time polymerase chain reaction

In order to assess miRNA-10b, the specimen were recruited, dewaxed, and microdissected. Therefore, manual microdissection was performed. Sections were dewaxed and digested in 500 µl of lysis buffer (0.05 M Tris, pH 7.65, 0.1% SDS, and 100 µg/ml proteinase K overnight at 37°C). Lysates were stored at −20°C. Total RNA was purified from tissue digests by Tri-reagent (Sigma-Aldrich, Gillingham, UK) separation based on the protocols for tissue or plasma represented by the manufacturer. By the separation, the total RNA was precipitated with 1.25 times the volume of absolute ethanol using the QIAGEN RNeasy kit (QIAGEN, Valencia, CA, USA) solation kit considering the manufacturer's protocols. The RNA was extracted in 25 µl of RNase-free buffer and stored at −20°C.

In order to prepare the miRNAs, cDNA was primarily synthesized from the total RNA. This process was done by the real-time (RT) reaction and then preamplified to improve sensitivity and detectable miRNAs using TaqMan Megaplex Preamplification Primers (Human Pool A v2.0) and TaqMan Preamplification Master Mix in a 25-µl reaction. Preamplified cDNA was diluted with 0.1xTE (pH 8.0) to 100 µl.

We wanted to assess the gatekeepers for testing on clinical tissue samples. Therefore, a semi-quantitative scoring system to assess miRNA expression was used. *In situ* hybridization for miRNA-10b was based on a described method[19] using 6-µm thick formalin-fixed, paraffin-embedded sections. These were dewaxed, rehydrated, and digested with proteinase K at 7.5 µg/ml in 50 mM Tris-HCl pH 7.6 at 37°C for 30 min, then prehybridized in Exiqon hybridization buffer (Exiqon, Vedbaek, Denmark) at 56°C for 15 min and hybridized for 2 h with 80 nM double-DIG-labeled miRNA-10b probe with 30% locked nucleic acid substitutions complimentary to full-length miRNA-10b CA + CAA + ATT + CGG + TTC + TAC + AGG + GTA (base after + is locked) (Exiqon), given stringent washes with 5 × SSC, 1 × SSC, and 0.2 × SSC buffers at 55°C for 5 min per wash, blocked with DIG-blocking reagent (Roche, Mannheim, Germany) in maleic acid

buffer containing 2% sheep serum at 25°C for 15 min, and detected with alkaline phosphatase-conjugated anti-digoxigenin (1:600 in blocking reagent, Roche) for 15 min and enzymatic development using 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyl phosphate substrate (Roche) at 25°C for 180 min. A nuclear fast red counterstain (Vector Laboratories, Burlingame, CA, USA) was used at 25°C for 2 min. The slides were mounted with Eukitt mounting medium (VWR, Herlev, Denmark). Negative controls were carried out using a scrambled probe (80 nM) or the scrambled probe double-DIG-labeled scramble probe (Exiqon, Cat #99004-15) and by the omission of the probe in the hybridization protocol. A double-DIG-labeled probe for U6 snRNA (cacgaatttgcgtgtcatcctt, Exiqon) (0.1 nM) was used as a positive control to test for RNA retention.[4]

Eventually, the positivity of miRNA-10b was represented qualitatively, while the delta Ct method was used to represent miRNA-10b specimen levels quantitatively. Therefore, the delta Ct method with a miRNA-10b reference standard (Horizon Diagnostics) was used in each experiment and adjusted according to the estimated tumor cells.[17]

Statistical analysis

The obtained data were entered into the Statistical Package for Social Sciences (version 22, IBM Corporation, Armonk, NY, USA). Descriptive statistics were reported as mean (standard deviation) for continuous variables with normal distribution or median (interquartile range) for those with nonnormal distribution and frequency (percentage) of patients for categorical variables. The independent sample *t*-test (Mann–Whitney for data without normal distribution) was used to measure the mean score difference between metastatic and nonmetastatic cancers. Furthermore, the miRNA-10b performance was assessed using the area under the curve (AUC) of the receiver operating characteristics and measures of diagnostic accuracy including sensitivity, specificity, and accuracy value. The optimal cutoff point of the model was determined using Youden index, calculated as (sensitivity + specificity − 1). The level of statistical significance was considered at $P = 0.05$.

RESULTS

In the current study, forty patients, including twenty ones with metastatic melanoma and twenty ones without metastasis, were assessed. The two assessed groups were similar in terms of age ($P = 0.59$) and gender distribution ($P = 0.34$), demonstrated in Table 1.

Table 2 represents the comparison of quantitative and qualitative assessments of miRNA-10b among the two studied groups. Both qualitative and quantitative measurements revealed a significant difference between metastatic and nonmetastatic melanoma patients which is represented in detail in Table 2 ($P < 0.05$).

Table 1: Demographic information of the study population

Variables	Metastatic melanoma	Nonmetastatic melanoma	*P*
Age	44.50±13.54	44.50±14.78	0.59*
Gender (%)			
Male	9 (22.5)	12 (30)	0.34**
Female	11 (27.5)	8 (20)	

*Independent *t*-test, **Chi-square

Table 2: Quantitative and qualitative assessments of microRNA-10b in the study population

Variables	Metastatic melanoma	Nonmetastatic melanoma	*P*
Quantitative miRNA-10b levels	−1.59±1.13	−0.16±0.67	0.001*
Qualitative miRNA-10b assessments (%)			
Positive	14 (70)	4 (20)	0.004**
Negative	6 (30)	16 (80)	

*Mann-Whitney, **Chi-square. miRNA=MicroRNA

The receiver operating characteristic was depicted to assess the values of miRNA-10b for the prediction of metastasis incidence among melanoma patients. Based on Figure 1, the measured AUC was 0.923 ($P < 0.001$; 95% confidence interval: 0.811–1) with sensitivity and specificity of 100% and 94.4%.

DISCUSSION

The current report has been conducted to assess the association of miRNA-10b with distant melanoma metastasis. Our findings favor miRNA-10b use as a marker in pathological specimens for the prediction of distant metastasis among melanoma patients, as both quantitative and qualitative assessments of miRNA-10b revealed remarkable higher levels and positivity among the metastatic cases in comparison to nonmetastatic ones, respectively.

miRNAs are among the most novel markers currently being applied to diagnose and determine the prognosis of human malignancies; thus, numerous studies are in progress to investigate these markers. Although tissue biopsies are still the gold standard approach for diagnosing malignancies, this is not a valuable means for monitoring patients in RT. It has been demonstrated that miRNA deregulation occurs following malignancies, and the increased expression of them may occur due to their secretion by the tumor cells into the bloodstream. As the serum levels of miRNAs are highly stable, consistent, and reproducible, increased levels of particular miRNAs can be an efficacious marker for the disease diagnosis or prediction of prognosis.[20] For instance,

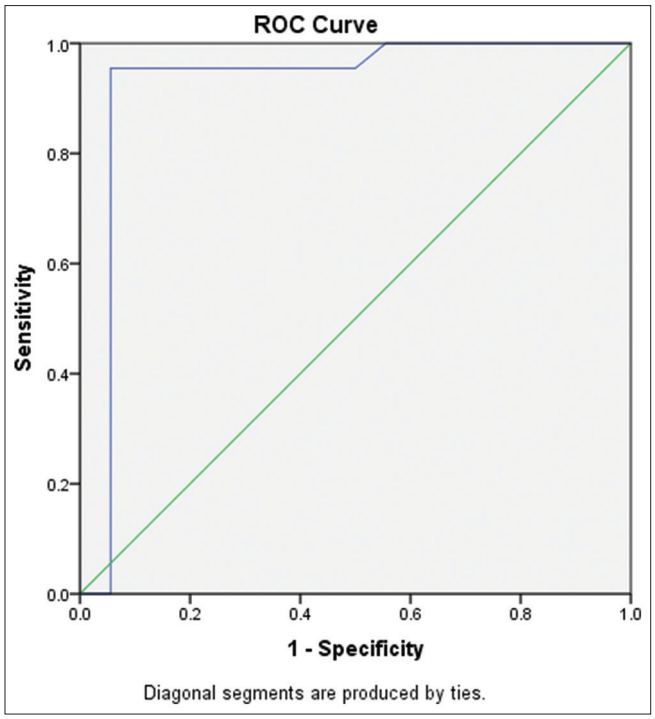

Figure 1: The receiver operating characteristic of microRNA-10b for the prediction of metastasis incidence in melanoma

it is shown that the decrease in the expression of miR-206 in the serum of melanoma patients was associated with poor clinical outcomes. In other words, suppression of miR-206 may lead to the progression of melanoma.[21]

Bai *et al*. conducted a study in order to assess the prognostic values of miRNA-10b for melanoma. They represented this marker as an independent prognostic marker for melanoma patients in overall survival, disease-free survival, lymph node metastasis, and even staging of the disease. They demonstrated that miRNA-10b was considerably increased among the invasive melanoma cell lines and the serum derived from these patients. They eventually concluded that melanoma cell lines would secrete miRNA-10b into the circulation leading to upregulated serum samples. Besides, they considered a potential oncogenic role for melanoma; therefore, the level of miRNA-10b was correlated with the worsening of melanoma clinical outcomes.[17] Consistent with our study, Saldanha *et al*. reported dramatically increased tissue levels of miRNA-10b among those patients with metastatic melanoma; however, they represented poor values for tissue miRNA-10b as a prognostic factor of melanoma.[4] This is while another study showed that the upregulation of miRNA panel consisting of miRNA-10b, 21, 200c, 373, and 520c was associated with tumor activity regulation, the incidence of metastasis, and stemness potential.[22] Jukic *et al*. designed a study to evaluate miRNA expression's relationship with age among melanoma cases. They found higher levels of miRNA-10b among the older patients and represented the notable differences in the value of this biomarker to determine melanoma prognosis in different ages.[23] Despite the differences in the design of these studies, they all together present consistent findings to our study about the usefulness of miRNA-10b for evaluating the prognosis of melanoma. Studying the expression of miRNA-10b in tissue specimen instead of serum is a novel approach used in the current study; however, serum assessments are less invasive, inexpensive, and better accessible.

A significant limitation of this study is its small sample population. In addition, some confounding variables may affect the expression of the miRNA-10b gene such as duration of melanoma, site of metastasis, and site of primary lesion that may have been neglected. Besides, other possible unmeasured confounders interfere with the expression of miRNA-10b may have been missed. Further studies are strongly recommended.

In the current study, we found that miRNA-10b had exceptional values with 100% sensitivity and 94.4% specificity for predicting metastasis in melanoma. To the best of our knowledge, the current study is the second one assessing the values of miRNA-10b in melanoma; however, the studies to find a stand-alone factor for the valuation of prognosis in melanoma are in progress. On the other hand, most of the previous reports insist on a panel of markers, while the markers' cost-beneficence and cost-efficacy should be considered.[24] Bai *et al*. were the first group of scientists to assess the values of miRNA-10b for the detection of melanoma and its stage prediction. They represented considerable sensitivities of 76% and 74% and specificities of 88% and 82% for the discrimination of healthy subjects from malignant ones and detection of progressed melanoma, respectively.[17]

Another issue in this regard is the high levels of miRNA-10b expression in vast numbers of malignancies such as esophageal squamous cell carcinoma,[25] diagnosis of gastric cancer,[26] bone metastasis in breast cancer,[27] and hepatocellular carcinoma discrimination from chronic liver diseases,[28] a fact that shows the requirement for further investigations about the use of this biomarker for the assessment of prognosis or follow-up of melanoma.

The upregulation of miRNA-10b has been demonstrated in different malignancies other than melanoma, as well. For instance, the elevated expression of serum miRNA-10b has been demonstrated among patients with bone metastatic breast cancer. Zhao *et al*. showed increased biomarker levels among patients with metastatic breast cancer, than healthy subjects, and even nonmetastatic breast cancer.[27] Another report showed the values of miRNA-10b not only for the hepatocellular carcinoma diagnosis but also for the

differentiation of this malignancy from other chronic liver diseases.[28] The latter malignancy was gastric cancer that showed to be directly associated with increased levels of a microRNA panel consisting of miRNA-10b-5p, miR-296-5p, miR-195-5p, miR-132-3p, miRNA-10b-5p, miR-20a-3p, and miR-185-5p.[26] Although these studies identify the high value of miRNA-10b as a biomarker for the diagnosis or prognosis determination of malignant conditions, they show that the upregulation of miRNA-10b is not dedicated to melanoma and further investigations for unique biomarkers are required. Perhaps, using a panel instead of mere miRNA-10b is to achieve the most specific markers; however, we found the specificity of 94.4%.

CONCLUSION

Metastatic melanoma was remarkably associated with increased levels of miRNA-10b. Besides, this marker had the sensitivity and specificity of 100% and 94.4% for the discrimination of metastatic melanoma from nonmetastatic ones. Therefore, this biomarker can be applied for the management of patients suspected of metastatic melanoma. Further studies are strongly recommended.

Acknowledgments

We are grateful to Dr. Ali Safaei for his efforts in the preparation of the current report.

Financial support and sponsorship

The study was sponsored by Isfahan University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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