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microRNA profiling for early detection of nonmelanoma skin cancer

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Summary

Background. microRNAs (miRNAs) are single-stranded, noncoding RNA molecules. Given the vast regulatory potential of miRNAs and their often tissue-specific and disease-specific expression patterns, miRNAs are being assessed as possible biomarkers to aid diagnosis and prediction of different types and stages of cancers, including skin cancer. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common forms of nonmelanoma skin cancer (NMSC). BCC originates from the basal layer of the epidermis, while SCC arises from epidermal keratinocytes or from the dermal appendages. Although NMSCs are currently the most common types of malignancies, both BCC and SCC have a better than 95% cure rate if detected early.

Aim. To identify plasma miRNAs suitable for early detection of NMSC.

Methods. Expression profiles of 741 miRNAs were evaluated using high-throughput real-time quantitative PCR from plasma samples in 42 patients with NMSC and 282 healthy controls (HCs).

Results. Our results demonstrated that in patients with NMSC, compared with HCs, expression levels of miR-30e-3p, miR-145-5p, miR-186-5p and miR-875-5p were significantly ($P < 0.05$) upregulated, while those of miR-19a-3p, miR-25-3p, miR-30a-5p, miR-451 and miR-576-3p were significantly downregulated.

Conclusion. Our study suggests that the miRNAs with significant changes in expression (miR-19a-3p, miR-25-3p, miR-30a-5p, miR-145-5p and miR-186-5p) could serve as novel noninvasive biomarkers for detection of NMSC.

Introduction

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common forms of nonmelanoma skin cancer (NMSC). The head and neck, and especially the nose, are the most common sites for BCC development. BCC arises from the pluripotential primordial cells in the epidermis basal layer, and less often from the outer root sheath of the hair follicle or from the sebaceous gland or other cutaneous appendages. The most common sites affected by SCC are the scalp, ears, lower lip, neck, forearms, legs and dorsa of the hands. SCC presents clinically as growing indurated erythematous papules, nodules or plaques, followed by ulceration and crusting. Although NMSC is the most common types of malignancy, both BCC and SCC currently have a > 95% cure rate with early detection.[1,2]

Recent studies have indicated difference in microRNA (miRNA) expression profiles between normal and neoplastic tissues, and this may lead to the identification of new diagnostic and/or prognostic

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markers. miRNAs are evolutionarily conserved types of single-stranded RNAs, approximately 21–23 nucleotides in length. They are noncoding RNA molecules that regulate gene expression by binding to the 3′ untranslated region (UTR) of the target mRNA, causing its destabilization and degradation.[3,4] miRNAs play important roles in a wide variety of biological processes, including tumorigenesis, cell differentiation, metabolism, apoptosis, signal transduction and organ development.[5] The components of miRNA synthesis, including the microprocessor complex consisting of Drosha, Dicer, DiGeorge syndrome chromosomal region 8 (DGCR8) and the RNA-induced silencing complex (RISC) have recently been investigated regarding their expression in both BCC and SCC, and their expression levels have been shown to be significantly higher in patients with BCC or SCC compared with healthy controls (HCs).[6,7] miRNAs can be detected in serum and plasma, and are used as a biomarker in non-invasive diagnosis of diseases. Therefore, this study aimed to determine circulating miRNAs for early detection of NMSC in plasma, and to our knowledge is the first plasma miRNA profiling study of NMSC in the English literature.

Methods

The study was approved by the Medical Ethical Review Committee, Academic Hospital of the Mersin University, and informed consent was obtained from all participants in accordance with the Helsinki Declaration.

Participants

Participants were recruited from the Dermatology Department of Mersin University. In total, 324 subjects were enrolled in the present study: 42 newly diagnosed patients with NMSC (median age 58 years) and 282 HCs (median age 32 years). The patients had been diagnosed with NMSC by clinical and histopathological examination. The HCs were selected from healthy people with no history of cancer, chronic degenerative neurological disease, diabetes, hypertension, atopy, autoimmune diseases or allergies in general. All medical records were reviewed to extract clinical and demographic data.

RNA isolation, reverse transcription and quantitative PCR

Peripheral blood samples were collected into tubes containing EDTA as anticoagulant. They were spun in a centrifuge at 2700 ***g*** for 15 min, and then the plasma was separated and stored at −80 °C until analysis. RNA was isolated (High Pure miRNA Isolation Kit; Roche, Mannheim, Germany) in accordance with the manufacturer's instructions and then stored at −80 °C until required. cDNAs were obtained from the isolated plasma miRNAs (TaqMan MicroRNA Reverse Transcription Kit; cat. no. 4366596; Applied Biosystems, Foster City, CA, USA) and pre-amplification was performed using TaqMan PreAmp Master Mix 29 (PN 4391128 and the Megaplex Human Primer Pools Set v3.0 (PN 4444750) (both Applied Biosystems). Reverse transcription and preamplification were performed using a real-time PCR system LightCycler 480; Roche), and 741 different miRNAs were analysed with a high-throughput real-time quantitative PCR (RT-qPCR) device (Fluidigm; Biomark, South San Francisco, CA, USA) using integrated fluidic circuits (96.96 Dynamic Array IFCs; Fluidgm). The $^{\Delta\Delta}$Ct method was used to calculate the expression levels of the target miRNAs.

Normalization and relative quantification of plasma microRNA expression

Analysis of circulating miRNA is difficult because there are no verified housekeeping genes in serum/plasma that can be used for normalization. To overcome this problem of normalization for miRNA expression in plasma, we used the global mean normalization method for normalization of plasma miRNA expression. Relative expression of miRNAs was calculated by the comparative ΔCT (ΔΔCT) method, and fold change (FC) was calculated by the equation $2^{-\Delta\Delta Ct}$.

Statistical analysis

All statistical analyses were performed with qbase+ software (v2.0; Biogazelle, Zwijnaarde, Belgium). miRNA expression data were normalised according to the global mean normalisation strategy as described above. Global mean normalisation of miRNA qRT-PCR data was performed with qbase+ software Patients with NMSC and HCs were compared with the Mann–Whitney *U*-test. To determine the difference in expression between the NMSC and HC groups, fold changes were calculated. The normalized signal values were transformed from $\log_2$ to the linear scale. $P < 0.05$ was considered statistically significant.

Results

Participant data

Table 1 presents the characteristics of the enrolled participants.

microRNAs

Plasma samples from were examined for the expression of 741 miRNAs using high-throughput RT-qPCR. In this study, 301 miRNAs were expressed in both groups, and of these, 9 were found to be aberrantly expressed: 5 (miR-30a-5p, miR-576-3p, miR-25-3p, miR-19a-3p and miR-451a) were significantly ($P < 0.05$) downregulated and 4 (miR-186-5p, miR-875-5p, miR-30e-3p and miR-145-5p) were significantly upregulated in patients with NMSC compared with HCs ($P < 0.05$) (Table 2).

Discussion

Since the discovery of miRNAs, many studies have identified cancer-specific miRNA signatures in various cancers. Additionally, miRNAs have been used to classify tumour origin and to predict prognosis. There have also been several studies analysing miRNAs specific for several cancer types, including skin, breast, ovarian and gastric cancers.[6] However, almost all the information about skin cancer-related miRNAs in previous studies has been restricted to cancer tissue-derived or cell line-derived miRNAs. Tissue-derived miRNAs have limitations for clinical use because of the invasiveness of specimen collection, and they are of limited value as a screening tool. Therefore, in this study, we investigated the potential role of circulating miRNAs to serve as an early detection marker of NMSC, by comparing plasma miRNA profiles of patients with NMSC with those of HCs. We found nine miRNAs with statistically significant differences between the two groups. Below, we discuss these miRNAs in more detail.

Table 1 Demographic characteristics and medical history of the patients with non-melanoma skin cancer and the healthy control groups.*

	NMSC (n = 42)	HC (n = 282)
Sex		
Female	21 (50)	98 (34.8)
Male	21 (50)	184 (65.2)
Mean age, years	58	32
Smoking		
Yes	6 (14.3)	65 (23)
No	36 (85.7)	217 (77)
HTN		
Yes	13 (31)	2 (0.7)
No	29 (69)	280 (99.3)
DM		
Yes	7 (16.7)	3 (1.06)
No	35 (83.3)	279 (98.94)
Family history of NMSC		
Yes	7 (16.7)	0
No	35 (83.3)	282 (100)

DM, diabetes mellitus; HC, healthy control; HTN, hypertension; NMSC, nonmelanoma skin cancer. *All data are n (%).

Table 2 The microRNAs found to be upregulated or downregulated in the nonmelanoma skin cancer group compared with the healthy control group.

miRNA	Fold change	P
Upregulated		
miR-186–5p	8	0.01
miR-875–5p	11.5	0.01
miR-30e-3p	23	0.02
miR-145–5p	26	0.02
Downregulated		
miR-30a-5p	−12.3	< 0.01
miR-576–3p	−9	0.04
miR-25–3p	−5.7	0.03
miR-19a-3p	−5.2	0.01
miR-451a	−3.4	< 0.01

miRNA, microRNA.

The expression level of miR-30a-5p was found to be decreased by 12-fold in the NMSC group compared with the HC group in our study. Sand *et al.*[8] found that this miRNA was downregulated in tumour biopsies from patients with cutaneous SCC. They suggested that differentially expressed miRNAs may play a role in the molecular pathogenesis of cutaneous SCC, and might be candidates for further validation and functional analysis. Kimura *et al.*[9] showed that miR-30a-5p was upregulated in both head and neck SCC and oesophageal SCC cell lines compared with normal squamous epithelial cell lines.

miR-25 is a member of the miR-106b-25 cluster, which exerts potential proliferative, anti-apoptotic and cell cycle-promoting effects in a variety of cancer types. Xu *et al.*[10] showed that miR-25 was upregulated in oesophageal SCC tumour tissues, and demonstrated that the relative expression of miR-25 between tumour and adjacent noncancer tissues correlated with lymph-node metastasis and TNM (tumour, node, metastasis) stage in patients with oesophageal SCC. It was shown that miR-25 can promote cell migration and invasion by inhibiting E-cadherin expression in

oesophageal SCC.[10] Wu *et al.*[11] reported strong expression of miR-25 in oesophageal SCC tissues and sera, and that increased miR-25 level in serum was significantly associated with lymph node metastasis. Xu *et al.*[12] showed that miR-25-3p expression in a tongue SCC cell line and tissue specimens was significantly lower than that in normal tongue epithelial cells, and suggested that miR-25-3p functions as an oncogene, inhibiting the proliferation of tongue SCC cells, regulating cell cycle-related expression of proteins (such as p21, p27, cyclinD1, AKT and FOXO1), and playing an important role in the occurrence and development of SCC of the tongue. By contrast, in the current study, we found that miR-25-3p was downregulated 5.7-fold in the NMSC compared with the HC group.

In the current study, we found that miR-19a-3p was downregulated 5.2-fold in the NMSC group compared with the HC group. It has been reported that miR-19a (miR-19a-3p) belongs to the miR-17-92 cluster, also known as Oncomir-1, which consists of six members (hsa-miR-17, hsa-miR-18a, hsa-miR-19a, hsa-miR-19b-1, hsa-miR-20a and hsa-miR-92) that are responsible for enhanced cell proliferation and suppression of apoptosis.[13] The miR-17-92 cluster collaborates with the sonic hedgehog pathway in both medulloblastoma and BCC.[14]

Zhu *et al.*[15] showed that miR-451 was upregulated in multidrug resistant (MDR) cancer cell lines compared with their parental lines, and reported that miR-451 is involved in activating the expression of P-glycoprotein, the product of the *MDR1* gene, which confers cancer cell resistance against a wide range of chemotherapy drugs. Those authors suggested that miR-451 may function as an activator of MDR1/P-glycoprotein expression, which indicates that such an approach to targeting this miRNA may offer novel therapeutic opportunities for the treatment of MDR cancers. By contrast, in this study, we found that expression levels of miR-451 were decreased by 3.4-fold in patients with NMSC who were newly diagnosed and had not yet received any treatment.

In our study, miR-186-5p expression level was increased eightfold in the NMSC compared with the HC group. Ries *et al.*[16] previously reported miR-186 (miR-186-5p) to be downregulated twofold in their study, and they suggested that the aberrant expression levels of miR-186, miR-494 and miR-3651 in whole blood samples of patients with oral SCC might provide the possibility to establish a minimally invasive screening method for this cancer. Zhou *et al.*[17] found that the 3'-UTR P2X7 sequence was a putative target site for miR-186. The receptor P2X7 is a membrane-bound, ligand-operated channel that regulates cell growth through mediation of apoptosis. miR-186 expression level was found to be higher in cancer than in normal cells, and treatment with miR-186 inhibitors increased P2X7 mRNA.[17]

We also found that miR-875-5p level was upregulated (by 11.5-fold) in the NMSC compared with the HC group, and also found that miR-875 was expressed only in the plasma of patients with NMSC. Based on these findings, we suggest that miR-875 might serve as a novel noninvasive biomarker for NMSC.

miR-30e-3p was upregulated by 23-fold in the NMSC compared with the HC group. Previous studies of miR-30e* (miR-30e-3p) found that it has some shared cancer-related targets. Lower expression of this miRNA in elderly patients with advanced ovarian papillary serous carcinoma (OPSC) was associated with a significantly poorer survival rate, strongly suggesting that it could be a critical oncogene and play important roles in the aetiology of advanced OPSC in elderly patients.[18]

Noguchi *et al.*[19] reported that ectopic expression of miR-145, which was downregulated in canine and human melanoma cell lines, inhibited cellular growth, an effect that was partially mediated by reduction of c-Myc protein. Kano *et al.*[20] suggested that miR-145 inhibits the actin-binding protein Fascin homologue 1 in oesophageal SCC. Sand *et al.*[21] suggested that the ability of miR-145 to downregulate the tumour suppressor IRS1 should be further investigated in BCC, based on these preliminary data. miR-145 targets epidermal growth factor receptor and nucleoside diphosphate linked moiety X-type motif 1 in lung adenocarcinoma.[21] It was found to be significantly downregulated (3.4-fold) in BCC compared with non-lesional skin.[21] Another study found that miR-145 was downregulated in tumour biopsies from patients with cutaneous SCC; the authors suggested that differentially expressed miRNAs may play a role in the molecular pathogenesis of cutaneous SCC, and might be candidates for further validation and functional analysis.[8] In the study of Wang *et al.*,[22] miR-145 expression was frequently downregulated in oesophageal SCC specimens and cell lines compared with adjacent normal tissues. The authors suggested that overexpression of miR-145 inhibits tumour growth in part by targeting c-Myc.[22] They also suggested that miR-145 may act as a tumour suppressor in oesophageal SCC, and that its dysregulation may be involved in the initiation and development of human oesophageal SCC.[22] Contrary to these results, we found that miR-145 was upregulated by 26-fold in NMSC versus HC samples.

Finally, miR-576-3p expression was found to be decreased ninefold in the NMSC group in this study. We could not find any publications in the skin cancer literature review about this miRNA, and we therefore cannot compare this study with other studies.

BCC is the most common type of cancer in humans, but there is little information about its molecular pathogenesis. Defects in the Hedgehog and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathways have been found to play a carcinogenic role in the molecular pathogenesis of BCC.[23] The miRNA processing component TRBP [human immunodeficiency virus-1 transactivating response (TAR) RNA-binding protein], which is a cofactor of Dicer, is a target of MAPK/ERK phosphorylation. Phosphorylation of TRBP's p stabilizes the miRNA processing complex, resulting in higher overall miRNA levels with upregulation of progrowth miRNAs and downregulation of antigrowth miRNAs.[24] The miRNA maturing enzymes Drosha and Dicer and the miRNA effector RISC were reported to be dysregulated in epithelial skin tumours, including BCC.[6,8] Dysregulation of expression of Dicer, Drosha and the RISC components has been found in SCC.[7,8] Irregularities in the miRNA synthesis exist in SCC similar to those recently identified in BCC.[21] In recent studies investigating the relationship between skin cancer and miRNA, it was found that expression of cell cycle-related molecules such as c-Myc, p21, p27, cyclinD1, FOXO1 and AKT were related to the cancer process.[12,22]

An associateon between miRNAs and many types of cancer, including NMSC, has been identified, and has been the subject of many studies. However, lack of clarity about his association may be behind the different results between various studies. It is likely that the difference between our results and those in the literature may be related to differences in the material (tissue, plasma, cells) used, and the patient profile of the study groups.

Conclusion

In conclusion, we believe that five miRNAs (miR-30a-5p, miR-19a-3p, miR-145-5p, miR-25-3p, miR-186-5p) are potential markers for NMSC, while the other four miRNAs need to be evaluated and supported with further research.

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What's already known about this topic?

- miRNAs are key players in the stage, progression and metastasis of cancers.
- Anti-tumour or cancer miRNAs can function as oncogenes.
- Although the role of miRNAs in NMSC is just beginning to be understood, recent studies have reported preliminary evidence that miRNAs are involved in NMSC.

What does this study add?

- While there is growing interest in evaluating the miRNAs as biomarkers in a variety of cancers, to our knowledge, no previous study has specifically examined the association between plasma-based miRNAs and early detection of NMSC.
- This is also the first study in the English literature to use plasma miRNA profiling study of NMSC.

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