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Original Article

A panel of miRNAs as prognostic indicators for clinical outcome of skin cutaneous melanoma

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Abstract: Skin cutaneous melanoma (SCM) is the most dangerous type of skin tumor with great potential to develop metastases. Deregulation of microRNAs (miRNAs or miRs) is involved in SCM, which could function as new markers for the diagnosis and prognosis of SCM patients. Therefore, we attempted to identify and develop specific miRNAs as prognostic and predictive markers for SCM patient survival. Expression profiles of miRNA and genes and the corresponding clinical information of 448 SCM samples were downloaded from The Cancer Genome Atlas TCGA dataset, the dataset of gene expression. PCA (Principal Component Analysis) were performed on the differentially expressed miRNAs. The impacts of miRNA expressions and miRNA-interactions on survival were evaluated by Cox proportional hazard regression model. Biological processes and network of putative and validated targets of miRNAs were analyzed by bioinformatics. A total of 96 differentially expressed miRNA were obtained by the analysis of normal samples and skin cancerous melanoma specimen, including 17 up-regulated miRNA and 79 down-regulated miRNA, which accounting for 17.7% and 82.3% respectively. Five interested miRNA, were identified. The up-regulated hsa-mir-202, hsa-mir-206, hsa-mir-3681 hsa-mir-122 and down-regulated hsa-mir-1246 and hsa-mir-129-2 were found to be significantly associated with survival time of SCM patients. Our results demonstrated that miR-202, miR-206, miR-3681, miR-122, miR-1246 and the 5 miRNA-interactions could serve as prognostic and predictive markers for survival of patients with skin cutaneous melanoma, suggesting a potential application in improvement of prognostic tools and treatments.

Keywords: Skin cutaneous melanoma, microRNA, pronostic marker, survival analysis

Introduction

Skin tumor is one of the most malignant tumor and has risen as a worldwide public health problem with its increasing occurring rate and risk [1, 2]. Early diagnosis and treatment of SCM can decrease the death and malformation rate in clinical trials [3]. However, the molecular of the occurrence and development of skin cancer hasn't been made clear, which affected the diagnosis and prognosis of the disease. Therefore, we attempt to identify and develop specific miRNAs as prognostic and predictive markers for SCM patients' survival, which will be beneficial for improving the clinical management of SCM.

MicroRNA (miRNA OR miRs), are non-coding small RNAs that can regulate gene expression through inhibition of translation or degradation of target mRNAs. MiRNAs could act as oncogenes or tumor suppressors in a variety of tumor types. There have been reports of the association between expression of miRNA with the survival of patients [4, 5]. On one hand, we conclude that miRNAs may be able to function as prognostic and predictive indicator of tumors. On the other hand, however, the precise molecular mechanism of these miRNA remains unclear.

The main purpose of this study is to identify specific miRNAs that are closely associated with tumor progression and survivals and can function as prognostic and predictive marker for SCM patients. Another goal is to in this study, we found that hsa-mir-202, hsa-mir-206, hsa-mir-3681 hsa-mir-122, hsa-mir-1246 and

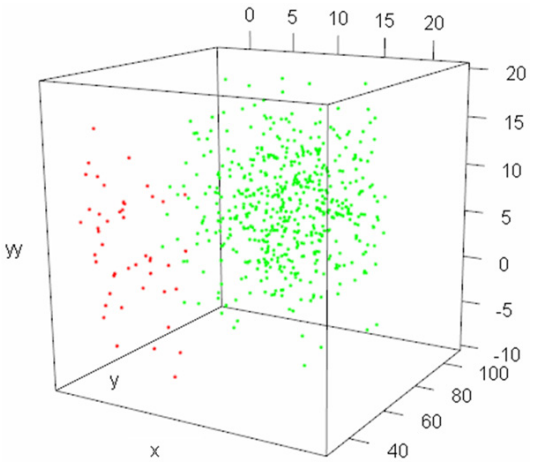


Figure 1. Principal component analysis (PCA) of miRNA expression values. The horizontal axis means the first principal component counts for 50% while the vertical axis stands for the send principal component count for 11%. The green dots in the figure represent cancer samples while the red ones stand for the normal samples. We can see it clearly from the figure that the normal samples assemble in the left while the cancer samples gather in the right, indicating the significant difference of the two kinds of samples.

hsa-mir-129-2 and p53 signal pathway could function as prognostic and predictive markers for survivals of SCM patients. Furthermore, the discovery of the important role of target genes of miRNA in the process of protein phosphorylation could provide evidence for new treatment of SCM.

Materials and methods

Source of miRNA data and data-preprocessing

A total of 448 expression data of miRNAs and genes and the 447 clinical information of patients with skin cutaneous melanoma were downloaded from The Cancer Genome Atlas [6] data portal. The data were deep-sequenced by illumine HiSeq. The normalized data was extracted out and data with no expression value was cut out. The level 3 data were already normalized between each sample and then the normalization in each samples were implemented with the generalized linear model by Limma package in R language, so as to exclude the batch effect.

Differentially expressed miRNA screening

Differentially expressed miRNA between normal tissues and skin cancer tissue samples were identified by SAMR package [7]. logFC >1 and FDR <0.05 were used as the cut-off criteria. PCA (Principal Component Analysis) were used to ensure the accuracy of the screened miRNA, which can clearly differentiate the normal tissues from the SCM samples.

Survival analysis

Clinical information of all patients was summarized to obtain the censoring rate. The survival status of patients was determined using the Kaplan-Meier survival analysis and statistical significances of overall survival and progression-free survival were determined using the Logp-Rank test. Single component Cox model was used to analyze the relation between the differentially expressed miRNA and the survival time of patients. Raw data was ranked by the survival days and then the survival status (1 is death and 2 stands for survival), cumulative survival, standard error, cumulative event and number remaining successively. Afterwards, we calculated the survival rate by these results.

miRNA biomarker screening and Cox regression model confirmation

Data were divided into two groups, the training and the testing. The Cox proportional hazard regression model was used to analyze the expression profile of miRNA to obtain the miRNAs closely associated with the survival [8]. $P<0.01$ was used as the cut-off criterion. 5 miRNA was screened out ($P\leq0.05$). The survival ability of each patient can be predicted by Cox regression model constructed by the five miRNA. Besides, each miRNA has Cox regression correlation coefficient. Each miRNA has a regression relating coefficient and thus a corresponding risk coefficient. Patients with high risk score represented a low survival ability compared with low level risk score. Then, calculate the median of all patients and use the median as the criterion, which means patients with higher score were defined as high risk and vice versa. The distribution of survival time was analyzed by Kaplan-Meier model and the significance of different classification with the same variable was judged by log-rank testing.

Target genes analysis of miRNA

Target genes of differentially expressed miRNA was extracted from miTarBase [9] and then

Table 1. 6 significantly differentially expressed miRNA

Type	miRNA ID	logFC	*P* value	FDR
up regulated miRNA	hsa-mir-202	2.801956	2.09E-50	1.10E-47
	hsa-mir-206	2.375182	1.38E-47	4.80E-45
	hsa-mir-3681	1.551125	4.45E-17	1.16E-14
Down regulated miRNA	hsa-mir-122	-3.92206	9.36E-64	9.79E-61
	hsa-mir-1246	-1.49909	7.30E-11	1.09E-08
	hsa-mir-129-2	-1.15581	1.02E-10	1.34E-08

The first column is the type of regulation and the second column is the name of miRNA and the third column is the differentially expressed logFC value of normal samples and cancer samples. The forth column is the significant degree of *P* value and the fifth column is the FDR of adjusted *P* value.

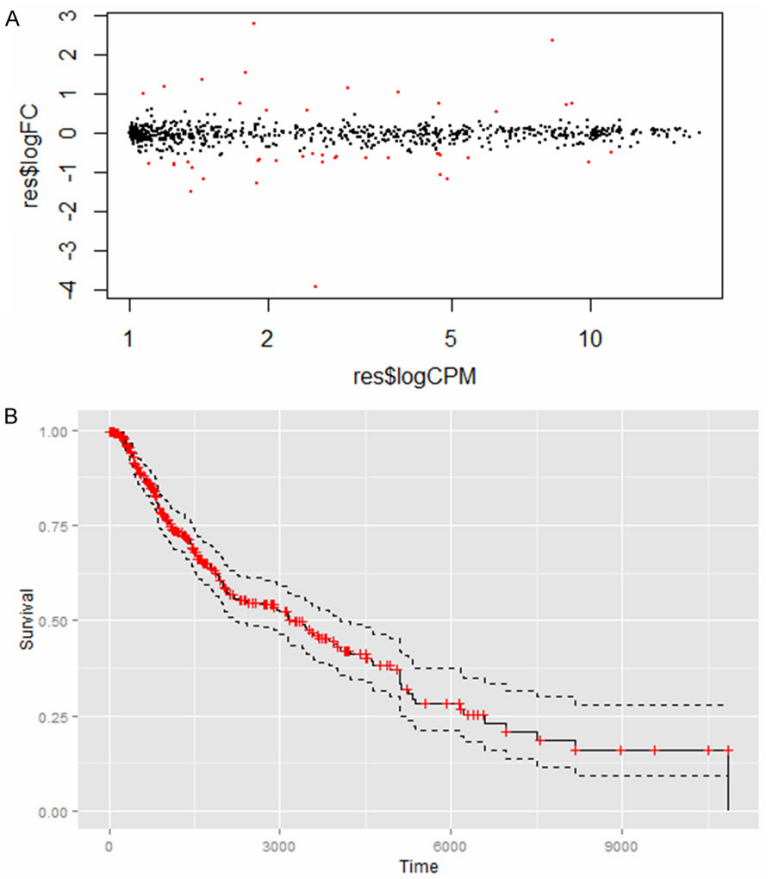


Figure 2. A. Differentially expressed miRNA distribution figure. The horizontal axis is the expression value of every miRNA and the vertical axis represents the fold change of expression value. B. The overall survival (OS) of patients. The survival curve is painted according to the survival time of patients. The horizontal axis is survival time and the vertical axis is survival rate. The red-cross in the curve is censoring rate. As can be seen from the figure, the survival ability of patients decrease as time grows.

Gene Ontology (GO) [10] and KEGG [11] analysis were performed by DAVID [12] Bioinformatics Resources. The enriched *P* valued was calculated out and corrected by multi-test Benjamini.

Interactions network analysis of miRNA and target genes

Topology property of protein-protein interaction network was analyzed by Network Analyzer plug of Cytoscape [13] software. The top 5 modules with *P*-value less than 1.0e-05 were selected out for the function analysis.

Results

Different expression levels of miRNA

There were total 1046 miRNA expression values in the 448 samples dataset, including 307 SCM samples and 162 normal samples. Through the PCA and clustering analysis, we found that normal samples can be well separated from those cancer specimens by the altered expression levels of miRNA (**Figure 1**).

Screening of the most altered miRNA expressed in SCM samples

There were 96 most altered miRNAs between normal samples and SCM samples by the SAMR analysis in R package. Expressed levels of 17 miRNA, including hsa-mir-202, has-mir-206 and has-mir-3681, were found to be increased while 79 which includedhsa-mir-122, hsa-mir-1246 and hsa-mir-129-2 were found to be decreased, accounting for 17.7% and 82.3% respectively (**Table 1**, **Figure 2**).

Correlations between miRNA expression and survival of SCM patients

Next, we performed survival analysis between miRNA expression profiles and patient (**Table 2**)

Table 2. Clinical information summary of SCM patients

Covariates	Category	Total
Age, years, no (%)	<60	245
	≥60	224
Gender, no (%)	Male	289
	Female	180
Vital status	Alive	149
	Dead	162
Lymph node involvement, no (%)	N0	232
	N1	73
	N2	48
	N3	59
	NX	36
M stage, no (%)	M0	417
	M1	24
T stage, no (%)	T0	23
	T1	42
	T2	78
	T3	88
	T4	155
Tumor_stage	Stage I	76
	Stage II	138
	Stage III	173
	Stage IV	23
	I or II NOS	14

The first column is the information of patients; the second column is different types and the third line is the number of patients.

survival using Kaplan-Meier and log-rank survival methods. Different variants as age, gender, T stage, M stage, N stage and tumor stage were validated in this study (**Figure 3**). Our analysis showed that there was strong correlation between different tumor stages and overall survival (OS) of patients. By Cox proportional hazard regression model, expression levels of miRNA in different tumor stages of patients and survival time were identified. The result was illustrated in the form of heatmap (**Figure 4**). Then, biomarkers of miRNA were screened out according to the significance of *P* value (P<0.1) and significant result in at least two batches.

Establishment of predictive model of SCM

A univariate Cox proportional hazard regression model was carried out to construct the model of miRNA and OS. The formula is Prognostic score = (-7.922 × expression level of hsa-mir-122) + (-10.499 × expression level of hsa-mir-1246) + (+1.745 × expression level of hsa-mir-1295) + (2.766 × expression level of hsa-mir-200b) + (3.602 × expression level of hsa-mir-3156-3). 2 miRNA was found to be risky in the progression of tumor while 3 miRNA were protective. Patients were divided into two groups: patients with high risk and those with low risk. Log-rank test was used to verify the differences (**Figure 5**). There were significant difference between high-risk patients and low-risk ones in both training and testing groups, indicating its feasibility in clinical prognosis. Furthermore, we performed the analysis of predicted role of miRNA in SCM patients and the results were listed in **Figure 6**. Different expression levels of miRNA in high and low risk patients were listed in the heatmap. The result showed that the lower the risk coefficient was, the longer survival time was.

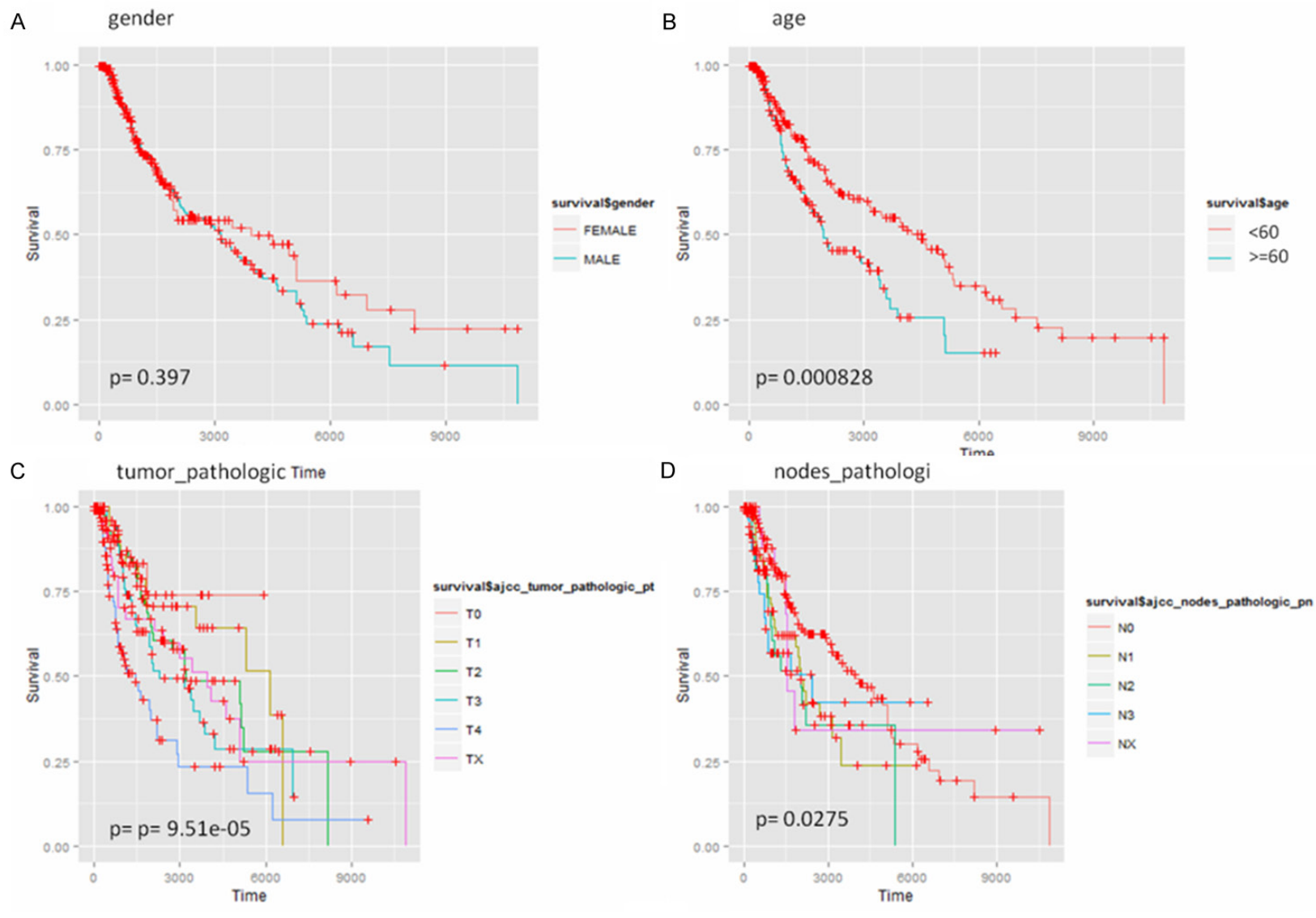
Functional analysis of the 5 interested miRNA in SCM

There were 2147 target genes of the 5 interested miRNA using the Targetscan software. The enriched GO terms were ion binding and metal ion bind. What's more, analysis of KEGG pathway showed the interested miRNA were mainly involved in neurotrophin signal pathway and insulin signal pathway (**Figure 7**).

Discussion

In this study, we identified 96 differentially expressed miRNAs from the most significantly altered miRNAs using data from TCGA dataset. Kaplan-Meier survival and Cox multivariate proportional hazard model confirmed that expression of hsa-mir-516a-1, hsa-mir-519a-1, hsa-mir-675, hsa-mir-323b, hsa-mir-935 were significantly correlated with OS of patients. The target genes of the interested miRNA were identified by Targets can software. The functional enrichment analysis of the target genes revealed that the genes were mostly involved in the pathway of protein phosphorylation. Our results suggest a potential application of miRNA profiles and their enriched pathways in development and improvement of prognostic tools and treatments.

Presently, the early diagnosis of tumor, especially the type of non-invasive tumor has drawn much attention. Nuclear acid or protein detec-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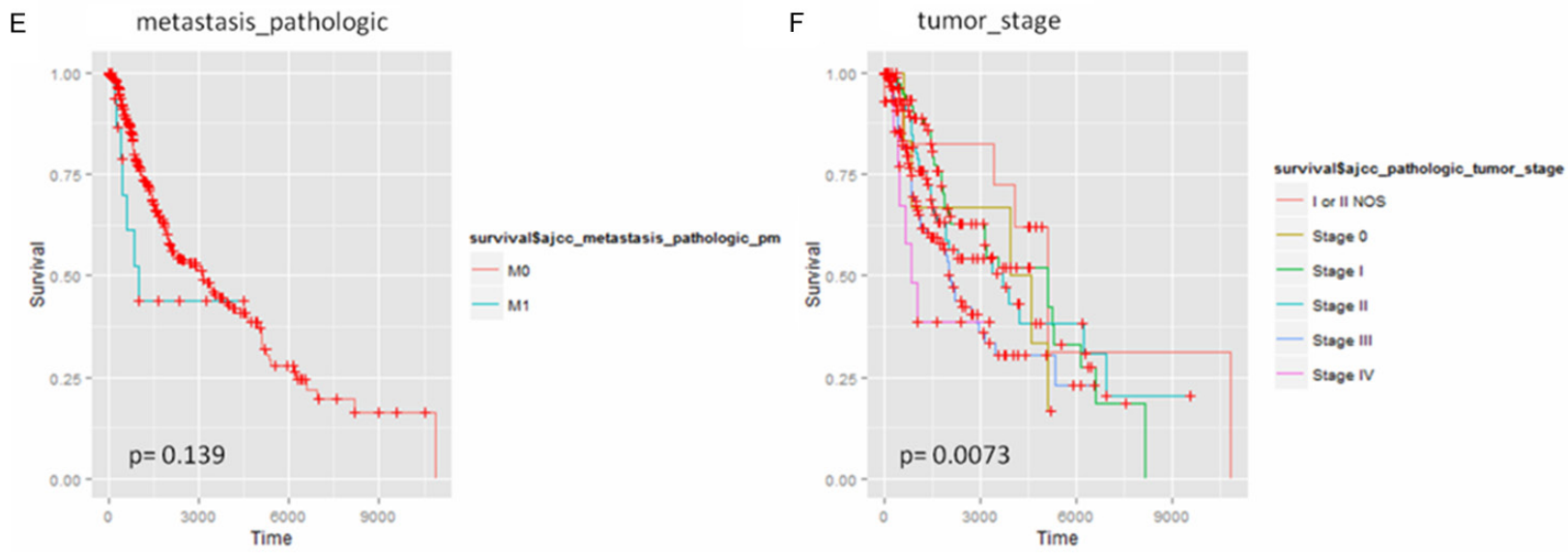

Figure 3. Changes of patients in different states of survival. A-F. Stands for the relationship of age, gender, T stage, M stage, N stage and tumor stage with survival time. *P* value of Log rank test is on the bottom left of each figure.

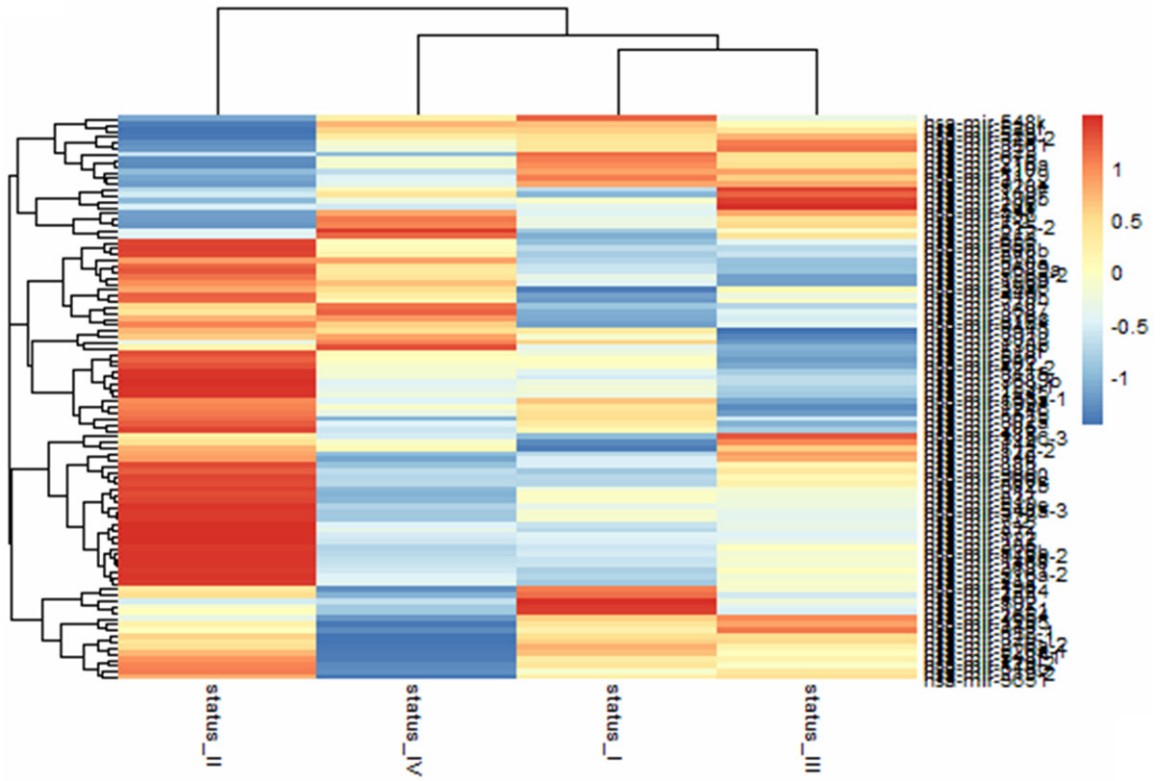

Figure 4. Predictive power of miRNA in different states of tumor. The horizontal axis stands for different stages of tumor while the vertical axis represents different types of miRNA. The heatmap reveals 140 tumor-oriented miRNA's ability in affecting survival ability in different states of tumor. The deeper the red color is, the stronger of the positive correlation of miRNA expression with the survival time of patients. The green color represents the negative correlation.

tion in patients' blood has become a new application in clinical classification study of SCM patients [8, 14]. Meanwhile, it's beneficial for the classification of tumor and specific therapy, as well as judgment of the prognostic effect by the non-invasive method. Thus, the role of miRNA in the process of anti-tumor has been increasingly emphasized. No convincing prognostic or predictive factors have been prevalent in clinical management of SCM patients.

MicroRNAs, are a family of small, sing-stranded, non-coding RNAs that target the 3'-untranslated region of about 30% of all human genes, and they're negatively involved in gene regulation. They are recognized as important intervention targets and predictive tools since it can be easily detected in several diseases [15]. Over the past decades, the study of miRNA-mediated regulation of cancers has grown more important and meaningful [16, 17]. In Giles' study [18], miR-7-5p was found to be a novel tumor suppressor since it can inhibit melanoma cell migration and invasion. In another study, miR-200c, miR-205 and miR-211 was found to act as tumor suppressors of melanoma [19]. However, few markers have proven to be of clinical significance in understanding the genetic defects in melanoma and no single molecular marker has been informative over a wide range of lesion types.

In the present study, hsa-mir-516a-1, hsa-mir-519a-1, hsa-mir-675, hsa-mir-323b, hsa-mir-935 were found to be significantly down-regulated in the SCM patients compared to normal specimens. Early reports showed that miRNA involved in the progression of melanoma were of great significance in clinical management [20, 21]. Thus, we believe that the miRNA targeting important genes and signaling pathways involved in the progression and development of melanoma could contribute to the miRNA-oriented therapy in the future.

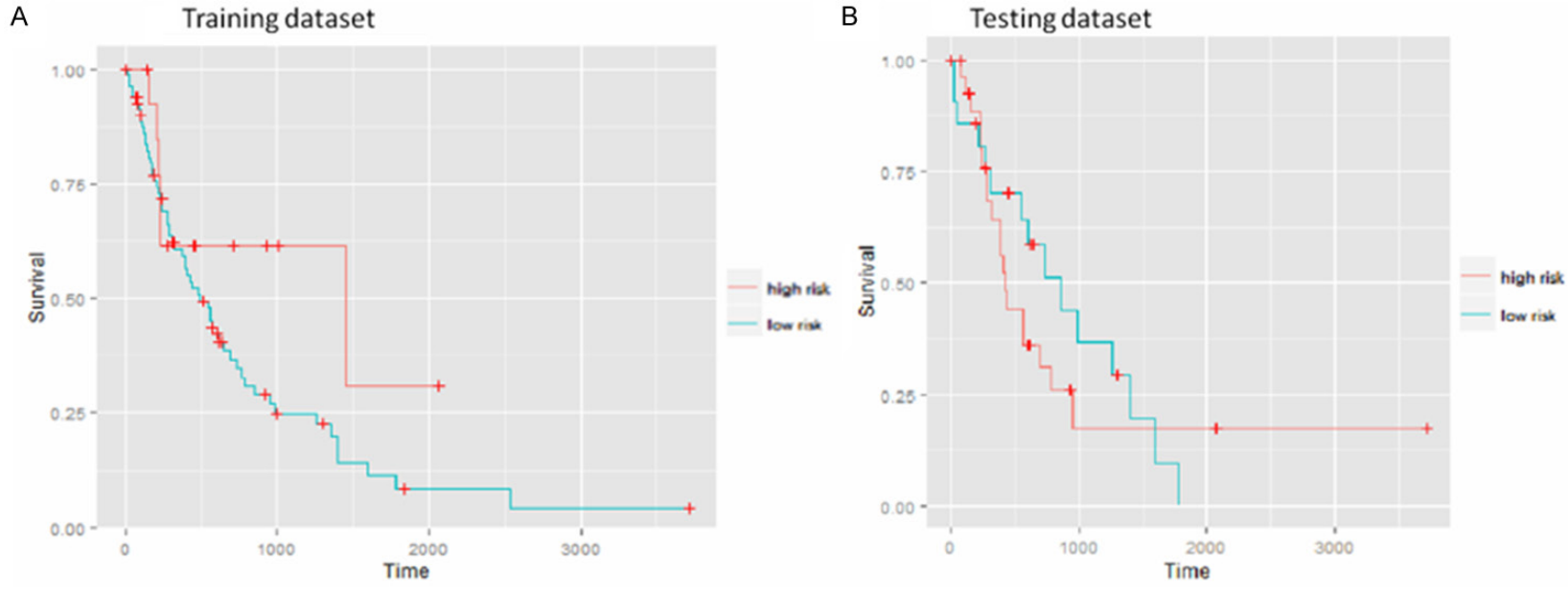

Figure 5. Judgment of survival risk of patients with expression value of 5 miRNA by Kaplan Meier survival analysis. The horizontal axis is the survival time of patients while the vertical axis is the survival rate. The red stands for patients with high risk while the blue one represents low-risk patients. The *P* value on the bottom left indicates the differences of two degree of risk.

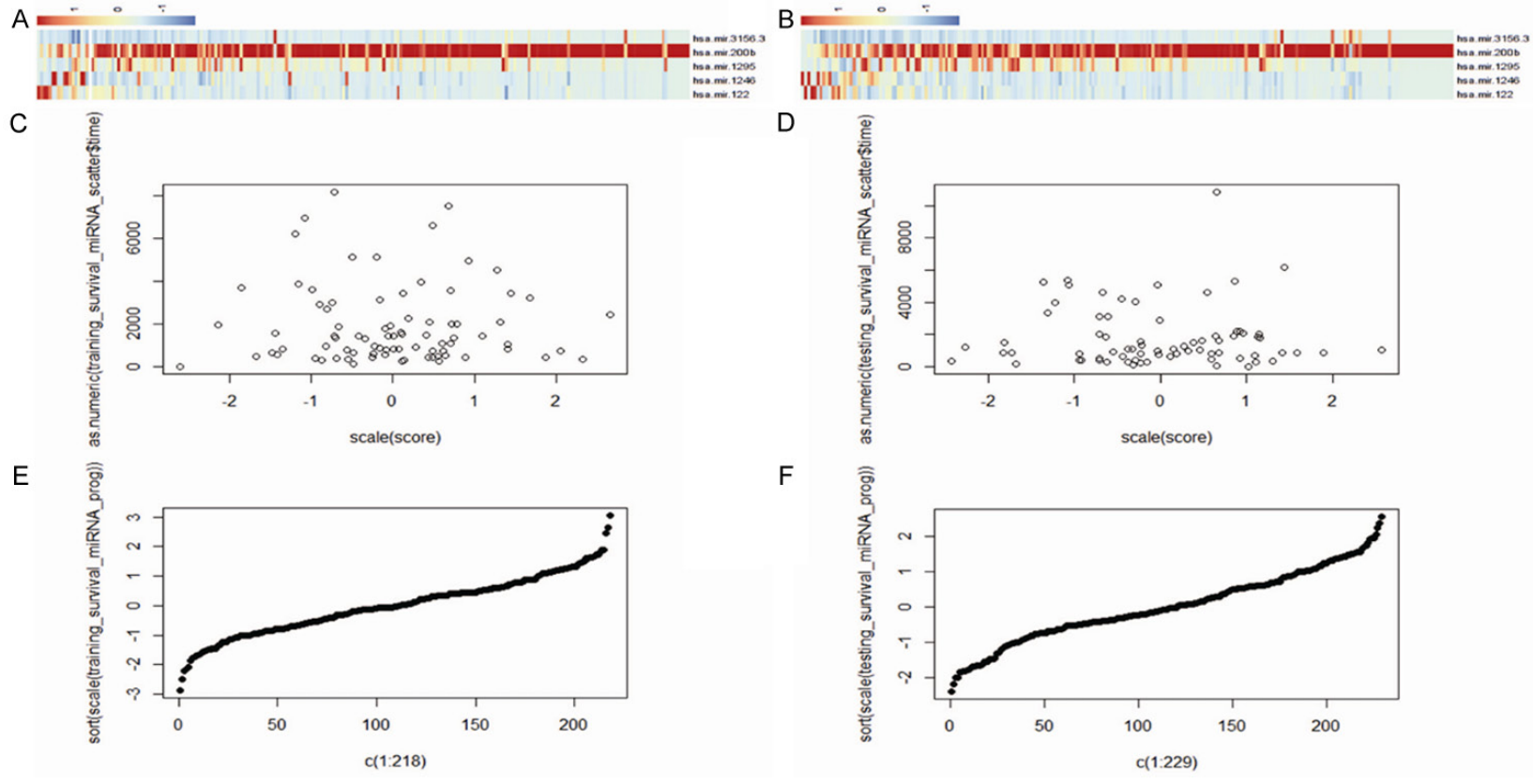

Figure 6. Analysis of predicted risk coefficient of 5 miRNA. A is the risk coefficient analysis of data in the training group while B is the heatmap of the expression value of 5 miRNA. C and D is the correlation of survival time and risk coefficient. E and F is the distribution of risk coefficient.

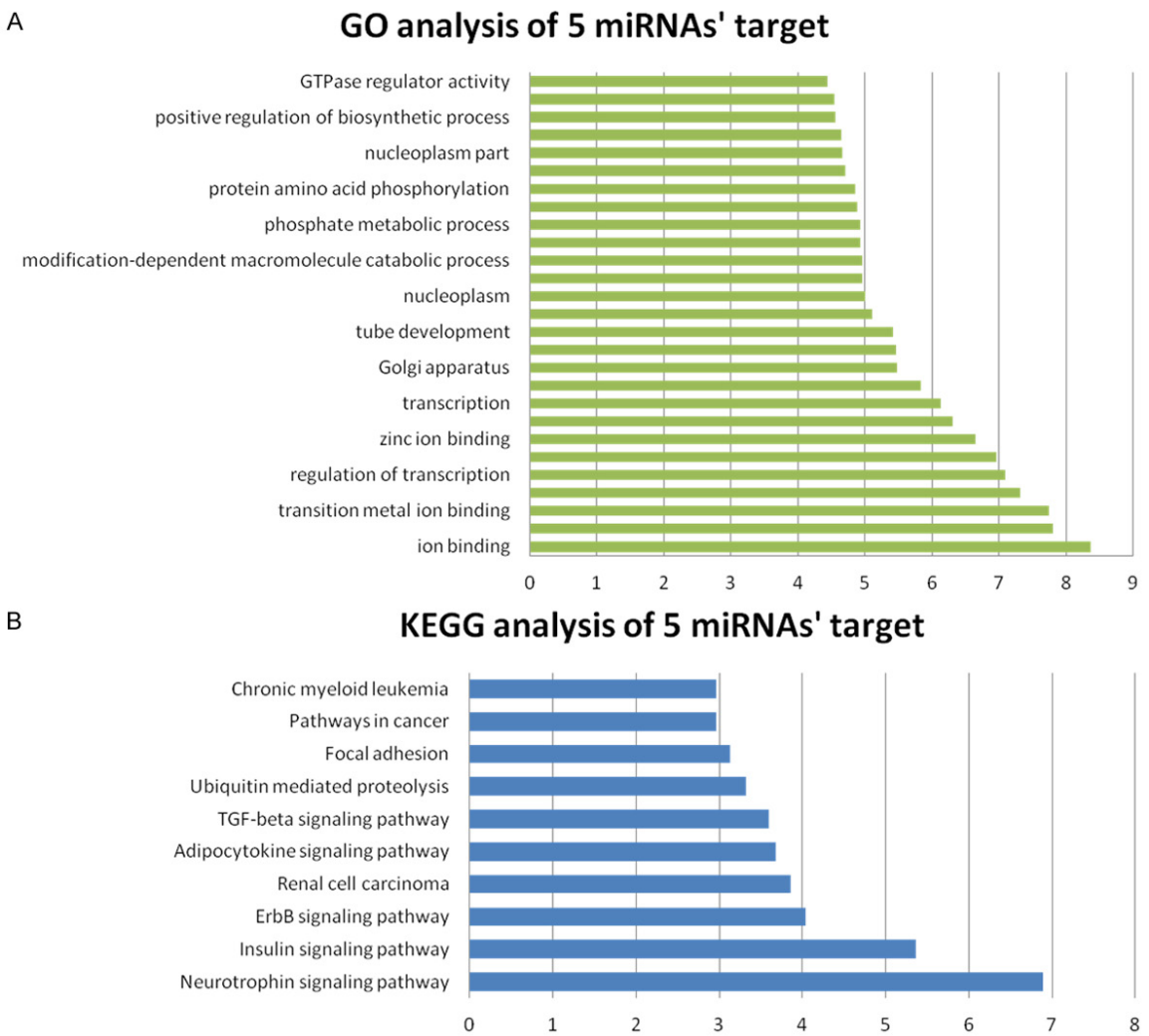

Figure 7. GO and KEGG analysis of target genes of differentially expressed miRNA. A is GO analysis of target genes while B is the KEGG analysis. The horizontal axis stands for the different degree of significance while the vertical axis stands for the functional annotation. The bigger the significance degree is, the stronger the correlation of target genes with the annotation.

The KEGG analysis revealed that P53 signaling pathway played an important role in the progression of cancer. It has been reported that more than half of all cancers may involve p53-inactivating mutations [22]. P53 gene can encode tumor inhibition factors [23] and once it's damaged, it could cause the cell division and proliferation of cells out of control [24]. P53 gene (mtp53) were involved in the process of cell cycle and can help to maintain the stability of genome, avoiding the accumulating of the damaged DNA by promoting cell apoptosis and DNA repairing [25]. What's more, in a 10-year follow-up period study including 31 patients, down-regulation of p53 gene was recognized as an appropriate marker for assessing the risk of melanoma progression and metastases, suggesting that the detection of p53 could be used as a prognostic indicator to predict the progression of metastatic disease states and poor outcome of melanoma patients [26]. Therefore, it's promising to regulate p53 signaling pathway by regulating the interested 5 miRNAs. Therefore, the regulation of p53 pathway may provide new views on diagnosis and prognosis of SCM patients.

In conclusion, in this study, hsa-mir-516a-1, hsa-mir-519a-1, hsa-mir-675, hsa-mir-323b, hsa-mir-935 marker related with survival of SCM were identified out and the regulation of signaling pathway p53, which were closely

related with tumor progression, could be regulated by the 5 interested miRNAs, implying the application for diagnosis and prognostic tools and treatment of SCM.

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Disclosure of conflict of interest

None.

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References

[1] Balch C, Soong S, Shaw H, Urist M and McCarthy W. An analysis of prognostic factors in 8500 patients with cutaneous melanoma. Cutaneous Melanoma 1992; 2: 165-187.

[2] Purdue MP, Freeman LB, Anderson WF and Tucker MA. Recent trends in incidence of cutaneous melanoma among US Caucasian young adults. J Invest Dermatol 2008; 128: 2905.

[3] Friedman RJ, Rigel DS and Kopf AW. Early detection of malignant melanoma: The role of physician examination and self-examination of the skin. CA Cancer J Clin 1985; 35: 130-151.

[4] Howell PM Jr, Li X, Riker AI and Xi Y. MicroRNA in melanoma. Ochsn J 2010; 10: 83-92.

[5] Chen Y, Zhu X, Zhang X, Liu B and Huang L. Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. Mol Ther 2010; 18: 1650-1656.

[6] McLendon R, Friedman A, Bigner D, Van Meir EG, Brat DJ, Mastrogianakis GM, Olson JJ, Mikkelsen T, Lehman N and Aldape K. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008; 455: 1061-1068.

[7] Tusher VG, Tibshirani R and Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci 2001; 98: 5116-5121.

[8] Mangan ME, Williams JM, Lathe SM, Karolchik D and Lathe WC. UCSC genome browser: deep support for molecular biomedical research. Biotechnol Ann Rev 2008; 14: 63-108.

[9] Hsu SD, Lin FM, Wu WY, Liang C, Huang WC, Chan WL, Tsai WT, Chen GZ, Lee CJ and Chiu CM. miRTarBase: a database curates experimentally validated microRNA-target interactions. Nucleic Acids Res 2010; 39: D163-9.

[10] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS and Eppig JT. Gene Ontology: Tool for the unification of biology. Nat Genet 2000; 25: 25-29.

[11] Kanehisa M and Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000; 28: 27-30.

[12] Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC and Lempicki RA. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol 2003; 4: P3.

[13] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003; 13: 2498-2504.

[14] Mocellin S, Pasquali S, Rossi CR and Nitti D. Interferon alpha adjuvant therapy in patients with high-risk melanoma: a systematic review and meta-analysis. J Natl Cancer Inst 2010; 102: 493-501.

[15] Felicetti F, Errico MC, Bottero L, Segnalini P, Stoppacciaro A, Biffoni M, Felli N, Mattia G, Petrini M and Colombo MP. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. Cancer Res 2008; 68: 2745-2754.

[16] Kong YW, Ferland-McCollough D, Jackson TJ and Bushell M. microRNAs in cancer management. Lancet Oncol 2012; 13: e249-e258.

[17] Friedman RC, Farh KK, Burge CB and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19: 92-105.

[18] Giles KM, Brown RA, Epis MR, Kalinowski FC and Leedman PJ. miRNA-7-5p inhibits melanoma cell migration and invasion. Biochem Biophys Res Commun 2013; 430: 706-710.

[19] Xu Y, Brenn T, Brown E, Doherty V and Melton D. Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. Br J Cancer 2012; 106: 553-561.

[20] Caramuta S, Egyházi S, Rodolfo M, Witten D, Hansson J, Larsson C and Lui WO. MicroRNA

expression profiles associated with mutational status and survival in malignant melanoma. J Invest Dermatol 2010; 130: 2062-2070.
[21] McElligott DL. Enhanced biodistribution of oligomers. In: Google Patents; 2012.
[22] Levine AJ, Momand J and Finlay CA. The p53 tumour suppressor gene. Nature 1991; 351: 453-456.
[23] Greenblatt M, Bennett W, Hollstein M and Harris C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994; 54: 4855-4878.
[24] Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hosteller R, Cleary K, Signer SH, Davidson N, Baylin S and Devilee P. Mutations in the p53 gene occur in diverse human tumour types. Nature 1989; 342: 705-708.
[25] Tchernev G and Orfanos C. Downregulation of cell cycle modulators p21, p27, p53, Rb and proapoptotic Bcl-2-related proteins Bax and Bak in cutaneous melanoma is associated with worse patient prognosis: preliminary findings. J Cutan Pathol 2007; 34: 247-256.
[26] Bosserhoff AK. Novel biomarkers in malignant melanoma. Clin Chim Acta 2006; 367: 28-35.