Identification of a five-miRNA signature predicting survival in cutaneous melanoma cancer patients

Tao Lu[1,2], Shuang Chen[1], Le Qu[1], Yulin Wang[1], Hong-duo Chen[1], Chundi He[1]

[1] Department of Dermatology, No.1 Hospital of China Medical University, Shenyang, Liao Ning, China
[2] Department of Dermatology, Affiliated Hospital of Chifeng University , Chifeng, Inner Mongolia, China

Corresponding Author:
Chundi He[1]
Shenyang, Liaoning, China
Email address: cdhe@cmu.edu.cn

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45   **Abstract**

46   **Background.** Cutaneous melanoma (CM) is the deadliest form of skin cancer. Numerous studies

47   have revealed that microRNAs (miRNAs) are expressed abnormally in melanoma tissues. Our

48   work aimed to assess multiple miRNAs using bioinformatic analysis in order to predict the

49   prognoses of cutaneous melanoma patients.

50   **Methods.** The microarray dataset GSE35579 was downloaded from the Gene Expression

51   Omnibus (GEO) database to detect the differential expression of miRNAs (DEMs), including 41

52   melanoma (primary and metastatic) tissues and 11 benign nevi. Clinical information and miRNA

53   sequencing data of cutaneous melanoma tissues were downloaded from the Cancer Genome

54   Atlas database (TCGA) to assess the prognostic values of DEMs. Additionally, the target genes

55   of DEMs were anticipated using miRanda, miRmap, TargetScan, and PicTar. Finally, functional

56   analysis was performed using selected target genes on the Annotation, Visualization and

57   Integrated Discovery (DAVID) website.

58   **Results.** After performing bioinformatic analysis, a total of 185 DEMs were identified: 80

59   upregulated miRNAs and 105 downregulated miRNAs. A five-miRNA (miR-25, miR-204, miR-

60   211, miR-510, miR-513c) signature was discovered to be a potential significant prognostic

61   biomarker of cutaneous melanoma when using the Kaplan-Meier survival method ($P = 0.001$).

62   Univariate and multivariate Cox regression analyses showed that the five-miRNA signature

63   could be an independent prognostic marker (HR = 0.605, $P = 0.006$) in cutaneous melanoma

64   patients. Biological pathway analysis indicated that the target genes may be involved in PI3K-

65   Akt pathways, ubiquitin-mediated proteolysis, and focal adhesion.

66   **Conclusion.** The identified five-miRNA signature may serve as a prognostic biomarker, or as a

67   potential therapeutic target, in cutaneous melanoma patients.

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73   **Introduction**

74   Melanoma is the result of the malignant transformation of melanocytes. It accounts for

75   approximately only 5% of all skin malignancies, but is thought to be the most invasive and lethal

76   form (Dimitriou et al. 2018). When compared with non-melanoma skin cancer, melanoma has

77   higher invasiveness and a worse prognosis, and its incidence has increased significantly in recent

78   years (Ross et al. 2018). The histopathological features of melanoma are not as distinguishing as

79   its molecular heterogeneity, and its formation is based on the continuous alteration of specific

80   genes and pathways that control metabolism or regulate key cellular functions (Palmieri et al.

81   2018). Although new treatments for melanoma are constantly being developed, their

82   effectiveness is still unsatisfactory. If metastasis occurs, the results could be life-threatening,

83   prompting clinicians to pursue new predictive markers and targeted therapeutic genes (Falzone et

84   al. 2018; Leonardi et al. 2018; Perera et al. 2013).

85   MicroRNAs (miRNAs) are small single-stranded non-coding RNAs with oncogenic or tumor-
86   suppressive roles, consisting of 20-26 nucleotides. There is growing evidence that miRNAs play
87   key roles in multiple developmental stages of human cancers, including cutaneous melanoma
88   (Gulyaeva & Kushlinskiy 2016). These molecules can affect gene transcription by
89   complementing the promoter region of a particular gene or by directly regulating the activity of a
90   gene (Piletic & Kunej 2016). miRNAs can even be released into the bloodstream by a tumor and
91   can be detected in melanoma cells. Dysregulated miRNAs can also be used as survival markers
92   in cutaneous melanoma patients (Riefolo et al. 2019), as seen in the signature of 18 miRNAs
93   identified by Segura MF et al. (Segura et al. 2010) and the miRNAs identified by Caramuta, S et
94   al. (Caramuta et al. 2010).
95   With the development of sequencing technology and various omics (genomics, transcriptomics,
96   proteomics, etc.), a large amount of biological data have been acquired, leading to the
97   development of bioinformatics to mine, utilize and integrate these data (Manzoni et al. 2018).
98   Bioinformatics can be defined as the practical discipline of applying informatics technology
99   (including applied mathematics, computer science and statistics) to understand and process
100   biological tissue information related to macromolecules on a large scale (Luscombe et al. 2001).
101   It has been applied in various fields of biological research, such as in the identification or
102   diagnosis of disease biomarkers (Shergalis et al. 2018), individualized treatment of cancer, the
103   preparation of tumor vaccines (Hu et al. 2018), among others. Various large public
104   bioinformatics databases have been developed, such as the Gene Expression Omnibus (GEO,
105   https://www.ncbi.nlm.nih.gov/geo/) database, which is a functional genomics data repository that
106   help users download experiments and curate gene expression profiles (Barrett et al. 2013), and
107   the Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), which is the most useful tumor
108   genomics program with at least 30 cancer types included (Chandran et al. 2016). These two
109   databases play a wide range of roles in the research of cancers including melanoma. For
110   example, the data provided by GEO are used in the screening of melanoma prognostic factors
111   (Hu et al. 2019; Xin et al. 2019). Robertson et al. conducted an in-depth analysis of UM (uveal
112   melanoma) samples from TCGA project in order to gain a deeper understanding of the biological
113   processes of UM tumors with distinct prognoses (Robertson et al. 2017). Even anti-tumor drugs
114   in melanoma patients with or without anti-PD-1 treatment have been analyzed using the GEO
115   and TCGA databases (Wu et al. 2019).
116   Previous studies have shown that multiple miRNAs can be used as diagnostic and prognostic
117   markers, but the number of cases is relatively small with inconsistent results (Jayawardana et al.
118   2016; Ross et al. 2018). In our study, we screened microarray data from GEO, then downloaded
119   the clinical information and expression profiles of cutaneous melanoma patients from TCGA.
120   Our aim was to use bioinformatics methods on a large sample from TCGA database, evaluate the
121   prognostic value of the differential expression of miRNAs (DEMs) by analyzing the high-
122   throughput sequencing data, and establish a five-miRNA predictive signature of patient survival.
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Materials & Methods

Acquisition of a microarray data source and DEMs

Variations in sample collection, storage, batch testing and detection platform methods can lead to data deviation, and so we could not simply directly combine different datasets. When selecting datasets, the inclusion criteria were: 1) datasets including more than 40 samples; 2) datasets with tumor tissue samples; and 3) datasets containing melanoma (including primary and metastatic melanoma) and pigmented nevi tissue simultaneously. Ultimately, GSE35579 (cutaneous melanoma = 41, nevus = 11) was the one that met our requirements. After microarray data were downloaded from the GEO of the National Center for Biotechnology Information (NCBI), we preprocessed the miRNA expression data using the quantile normalized method, then identified DEMs between melanoma and benign nevi. The "limma" package in software R (version 3.5.2), which can be operated flexibly offline, was selected to process the differential expression data. $P<0.05$ was selected as the statistically significant cut-off criterion.

Identification of the relationship between DEMs and overall survival (OS) in melanoma patients

DEMs obtained from the GEO database were matched in TCGA's database for further analysis of their relationship to patient prognosis. After removing patients without completed clinical information and with an OS (overall survival) time <1 month, the remaining 428 patients were divided into high‐risk and low‐risk groups according to the median score of DEM expression level. We used 120 months as the end of our observation time, and patients with a survival period of more than 120 months were considered survivors. The DEM expression profiles acquired from TCGA, after being log2 transformed, were assessed with the Kaplan-Meier method and a log-rank test to preliminarily identify the miRNAs that were associated with patient survival. $P<0.05$ was considered to indicate statistically significant differences. Considering that multiple miRNAs have more reliable predictive effects, we used different combinations of several prognosis-related miRNAs to calculate the risk score for each melanoma patient in accordance with a high or low level of expression. Using this method, we classified the patients into two groups, high and low score groups, further using Kaplan-Meier analysis to assess these miRNA combinations. To make the experiment more objective, we only selected a meaningful combination of DEMs for follow-up study. The Kaplan-Meier results were visualized by the GraphPad Prism 5.0. Finally, univariate and multivariate Cox regression analyses were conducted to verify the prognostic role of the screened DEM signature.

Prediction of the DEM signature's target genes

TargetScan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/), DIANA (http://www.microrna.gr/microTCDS), and miRmap (https://mirmap.ezlab.org/app/) were used to predict these candidate target genes. The FunRich (Functional enrichment analysis

164 (http://www.funrich.org/)) tool was used to intersect the results of the four prediction tools for
165 further research.
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167 **Analysis of functions and pathways of target genes**
168 GO term and KEGG pathway analysis was conducted on the overlapping terms in the Database
169 for Annotation, Visualization and Integrated Discovery (DAVID,
170 https://david.ncifcrf.gov/home.jsp) websites, providing a comprehensive set of functional
171 annotation tools to help determine the biological meaning of the genes. The results were
172 visualized by the R "ggplot2" package. A *P*-value< 0.05 was set as the cut-o☐ criterion.
173
174 **Statistical analysis**
175 To compare the miRNA expression data between melanomas and nevi, we used unpaired *t*-tests.
176 The relationship between expression data and the melanoma patients' clinical information was
177 assessed with chi-square and *t*-tests. Kaplan-Meier survival analysis and univariate/multivariate
178 Cox proportional hazard regression analyses were performed using IBM SPSS Statistics 25.0 to
179 assess each DEM and miRNA signature prognostic function. A *P*-value<0.05 was considered
180 statistically significant.
181
182 # Results
183 **DEM identification and construction of a five-miRNA signature**
184 We first acquired 185 (80 upregulated and 105 downregulated) differentially expressed miRNAs
185 between melanomas (primary and metastatic) and benign nevi from the miRNA dataset
186 GSE35579, according to the standard of *P* <0.05. To make the results more intuitive, we used
187 volcano maps (Fig. 1) to show these DEMs. To find out whether differential miRNAs could
188 distinguish a cancer sample from a nevus sample, we selected the top 100 miRNAs with the most
189 obvious differences in expression to perform a hierarchic cluster heat map based on Euclidean
190 distance (as shown in Fig. 2), and the results were acceptable.
191 DEMs were matched in TCGA, and combined with the clinical information of melanoma
192 patients (shown in Table 1). DEMs that could not be found in TCGA were excluded, and each
193 miRNA was analyzed to determine whether it was related to the prognoses. According to our
194 statistical analysis, there were eight genes (miR-25, miR-100, miR-204, miR-211, miR-19b,
195 miR-510, miR-511, miR-513c) closely related to the prognoses of the patients.
196 We integrated the expression of these eight miRNAs and applied different combinations
197 calculating the risk score for each patient (the details of which can be found in the Methods
198 section), and we found a very obvious prognostic significance when five of the miRNAs (miR-
199 25, miR-204, miR-211, miR-510, miR-513c) were combined. Among these miRNAs, miR-204
200 correlated positively with prognosis, while miR-25, miR-211, miR-510, and miR-513c correlated
201 negatively with prognosis. These details are listed in Table 2. We then established a five-
202 miRNA-based prognostic model. The prognostic characteristics of these five miRNAs are shown
203 in Figure 2 (Figs. 3A-3E). According to the median risk score, 428 patients were divided into a

204 high-risk group (n=222) and a low-risk group (n=206). Survival analysis was performed using
205 the Kaplan-Meier method with the log-rank test. The results showed that the survival rate of the
206 high-risk group was significantly lower than that of the low-risk group (P = 0.001, Fig. 3F). To
207 explore whether these five miRNAs were related to clinical features, we conducted correlation
208 analysis and found that, except for miR-25, these miRNAs might be related to T stage and
209 Breslow depth value (see Table 3 for details).
210   Univariate and multivariate Cox regression analyses were used to test the effects of the five
211 miRNA features (high-risk and low-risk) combined with the clinical features of melanoma
212 patients (including age, T stage, N stage, M stage, and clinical stage) on OS (overall survival).
213 Univariate analysis showed that T stage (HR = 0.443, *P* <0.000), N stage (HR = 0.517, *P*
214 <0.000), clinical stage (HR = 0.543, *P* < 0.000), age at diagnosis (HR=1.563, *P* = 0.003),
215 Breslow depth value (HR = 0.370, *P* < 0.000) and the five-miRNA characteristics (HR = 0.613,
216 *P* = 0.001) were associated with OS in melanoma patients. In the multivariate analysis, the five-
217 miRNA characteristics (HR = 0.605, *P* = 0.006), N stage (HR = 0.246,*P* = 0.008) and Breslow
218 depth value (HR = 0.509, *P* = 0.011) were all shown to be independent prognostic factors for
219 patients with cutaneous melanoma (Table 4).
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221 **GO and KEGG pathway analysis of target genes of the five-miRNA signature**
222 Using TargetScan, miRDB, DIANA, and miRmap, we predicted five miRNA target genes (miR-
223 25, miR-204, miR-211, miR-510, and miR-513c). The four prediction tools' intersecting genes
224 were visualized using the FunRich tool (Figs. 4A-4E). A total of 367 (228 miR-25, 28 miR-204,
225 31 miR-211,77 miR-510, 37 miR-513c, and deleted duplicate values) overlapping genes were
226 entered into DAVID, and assessed by GO and KEGG pathway analyses. The biological process
227 (BP) analysis results showed that the genes were concentrated in the transcription regulation,
228 angiogenesis, protein phosphorylation and ubiquitination (Fig. 5A). Cellular component (CC)
229 analysis showed that these genes were concentrated in the cytoplasm, nucleus, focal adhesion
230 and cell-cell adherens junctions (Fig. 5B). Molecular function (MF) analysis showed that genes
231 were concentrated in protein binding and ubiquitin-protein transferase activity (Fig. 5C). KEGG
232 pathway analysis showed that these genes were concentrated in the PI3K-Akt signaling pathway,
233 ubiquitin-mediated proteolysis, and focal adhesion pathway (Fig. 5D).

234 **Discussion**
235 The melanocyte pedigree is derived from the neural ridge, originating in the neural tube, and
236 these cells migrate to specific locations in the skin , hair follicle and other parts of the body
237 during embryonic development. In patients with vitiligo, the pigment "island" appears first in a
238 location around the hair follicles after UVB treatment, which suggests that melanocytes have
239 stem cell properties. These characteristics may contribute to the melanoma-derived invasion
240 (Richard L. Mort 2015). Caucasian populations have had a stable CM mortality rate since the
241 1990s, while the age-standardized mortality rate of cutaneous melanoma patients in East Asian
242 populations has significantly increased over the past six decades (Chen & Jin 2016). As the aging
243 population grows, the incidence of melanoma is predicted to also increase (Karimkhani et al.

244 2017). Early detection and initial care are still crucial for treatment (Schadendorf et al. 2018).
245 Recent studies have shown that epigenetic mechanisms play a very complex role in the
246 development and progression of melanoma, including its methylation, chromosomal changes and
247 remodeling, and regulation of the active function of various non-coding RNAs (Sarkar et al.
248 2015). Research on microRNAs is more developed compared to that of other non-coding RNAs,
249 and these molecules play a significant role in nearly every biological process in nevi and
250 melanomas, including proliferation, invasion, and apoptosis. Because of their chemical stability,
251 these molecules can resist the degradation of RNase and can distinguish between different types
252 of cancers, specifically those that can be secreted into the serum by tumor cells. Therefore, these
253 molecules can be used to predict the prognosis and to perform the initial diagnosis in melanoma
254 patients (Ross et al. 2018). Previous studies have explored the relationship between miRNAs and
255 the prognoses of melanoma patients, but these studies were usually small sample studies without
256 uniform results, that only looked at the primary melanoma or metastatic stage of the tumor, and
257 only focused on single miRNAs (Galasso et al. 2018; Jayawardana et al. 2016; Sanchez-Sendra
258 et al. 2018). TCGA's database contains an abundance of cancer information, making access to
259 cancer expression profile data easy and cost-effective. However, unlike for other cancers,
260 TCGA's database only contains skin melanoma samples without normal control tissue nor nevi
261 tissue information, and we were unable to obtain differentially expressed miRNAs. Thus, we
262 obtained the DEMs from the GEO database, performed prognostic analysis on the DEMs with
263 expression profiles and clinical information sourced from TCGA's database, and gained eight
264 prognosis-associated miRNAs. Considering that the main purpose of our experiment was to
265 create a multi-gene-based reliable prognostic signature, and the different combinations of these
266 miRNAs were not all meaningful, we will only discuss the five-miRNA signature that can be
267 used as a prognostic factor to make our results more clear and purposeful. Through additional
268 analysis, we established a five-miRNA (miR-25, miR-204, miR-211, miR-510, miR-513c)
269 signature to make the results more reliable. This five-miRNA based signature associated with the
270 prognosis of cutaneous malignant melanoma will provide a theoretical basis for later studies, and
271 can be used to non-invasively predict clinical prognoses in melanoma patients.
272 We used previous miRNA studies to verify the reliability of our results. In our study, miRNA-25
273 was the sole upregulated microRNA of the five miRNAs. Zhu et al. found that miRNA-25 can be
274 secreted into serum and can be used as a marker for the early diagnosis of gastric cancer (Zhu et
275 al. 2014). Kim et al. found that miRNA-25 directly regulates P57 (a tumor suppressor gene), and
276 the abnormal expression of this miRNA in gastric cancer patients can advance cancer cells from
277 G1 to S phase (Kim et al. 2009). Liu et al. tested the serum of patients with pancreatic cancer
278 found that miRNA-25 levels were considerably higher than that of the normal control group,
279 indicating that miRNA-25 can be used in the prognosis of pancreatic cancer (Liu et al. 2012).
280 Zoni et al. observed that miR-25 is a key regulator of human prostate cancer invasiveness, and
281 that it interacts directly with α(v) - and α(6) - integrins. Interestingly, Zoni et al. also found that
282 miR-25 was a tumor suppressor in highly aggressive prostate cancer (Zoni et al. 2015). Huo et al.
283 found that the expression of miR-25 increased in melanoma tissue and melanocyte cell lines, and

284 promoted melanoma cell proliferation and invasion, in part, by targeting Dickkopf-associated
285 protein 3 (DKK3) (Huo et al. 2016). This result is consistent with the poorer prognosis in
286 melanoma patients with increased miRNA-25 expression. In our experiments, miRNA-204 was
287 expressed at low levels in melanoma cells, and K-M survival analysis showed that the higher the
288 expression, the longer the OS time. This finding is consistent with previous studies, such as Xin
289 Chen et al.'s mechanistic study on the LINC01234-miR-204-5p-CBFB axis in gastric cancer
290 patients where miR-204 was found to have low expression in gastric cancer tissues. In a gastric
291 cancer cell miR-204 over exposure assay, miR-204 was found to inhibit the proliferation of
292 cancer cells and to induce apoptosis and cell cycle arrest in G1 -G0 phase, and survival analysis
293 showed that patients with higher miR-204 levels had a better prognosis (Chen et al. 2018). In
294 studies of colorectal cancer, nasopharyngeal carcinoma, and melanoma, miRNA-204 was
295 suggested to have an inhibitory effect on cancer cells and to possibly be associated with cancer
296 cell resistance or anti-radiation mechanisms (Bian et al. 2016; Diaz-Martinez et al. 2018; Lu et
297 al. 2016). Marco et al. suggested that the low expression or loss of miR-204 play a key role in the
298 progression of melanoma. In this study, miR-204 was associated with a better prognosis in
299 cutaneous melanoma patients (Galasso et al. 2018). Previously published studies and data from
300 existing miRNA databases indicate that miR-204-5p and miR-211-5p share some common
301 targets, and, as a matter of fact, miR-204 and miR-211 have very similar nuclear targets. The
302 nucleotide sequence has only two different nucleotides in the entire sequence and in the same
303 seed region, which may be why these molecules share some common targets. In preceding
304 studies, these miRNAs acted as tumor suppressors in melanoma and inhibited cell invasion
305 (Diaz-Martinez et al. 2018; Levy et al. 2010). The most important function of miR-211 is the
306 direct or indirect targeting of many other genes that may affect melanoma invasiveness and
307 adhesion (Bell et al. 2014). In our study, both miR-204 and miR-211 were down regulated in
308 melanoma tissues, but miR-204 was positively associated with tumor progression , miR-211
309 showed the opposite results. This result may be due to the small sample size of the GEO data set
310 we selected. The reason for this situation needs further exploration. Early research of miR-510
311 did not examine its relationship with cancer, only its aberrant expression in irritable bowel
312 syndrome (Kapeller et al. 2008). Researchers later discovered its relationship to cancer; for
313 example, Patnaik et al. found that miR-510 can predict the survival of patients with local stage I
314 non-small cell lung cancer after surgical resection (Patnaik et al. 2010). miR-510 also plays a
315 role in promoting tumor growth and invasion in breast cancer (Guo et al. 2013). In our study, we
316 found that the higher the expression of miR-510, the poorer the prognoses of melanoma patients.
317 Mairinger et al. proposed that the expression of miR-513c in pulmonary neuroendocrine tumors
318 was involved in tumor grade, and Wang et al. studied African-American patients with prostate
319 cancer and acquired similar results (Mairinger et al. 2014; Wang et al. 2015). Previous studies
320 also included miR-513c in the establishment of a nine-miRNA based uveal melanoma prognostic
321 model (Xin et al. 2019). However, we missed including a related study on the mechanism of
322 action of miR-510 and miR-513c in cutaneous melanoma.

323 To further verify the active mechanisms of these five miRNAs, we performed miRNA target
324 gene predictions and found a total of 367 overlapping target genes. Interestingly, 222 of them
325 were target genes derived from miR-25 (mainly enriched in the PI3K-Akt signaling pathway), 77
326 were target genes derived from miR-510 (mainly enriched in ubiquitin-mediated proteolysis),
327 and the few remaining genes were target genes of other miRNAs. KEGG pathway analysis
328 revealed that these target genes were mainly involved in the PI3K-Akt pathway, ubiquitin-
329 mediated proteolysis, and cell adhesion. The most abundant genes, including PHLPP2, SGK3,
330 CREB5, COL5A3, PTEN, ITGA5, ITGAV, COL27A1, CREB3L2, COL1A2, PPP2R5E,
331 PIK3AP1, GNG2, and PIK3R3, were enriched in the PI3K-Akt pathway. The PI3K-Akt pathway
332 (phosphoinositide 3-kinase-RAC-alpha serine/threonine-protein kinase) is involved in a variety
333 of cellular processes in both normal cells and cancer cells, including cell survival, metabolism,
334 transmigration, and proliferation (Schadendorf et al. 2018). For example, abnormal PI3K-Akt-
335 mTOR signaling is one of the most common dysfunctions present in human cancers (Janku et al.
336 2018). The use of PI3K-Akt as a target in the treatment of cancer has been extensively
337 researched (Hamzehzadeh et al. 2018). Phosphatase and tensin homolog (PTEN), a tumor
338 suppressor gene, has been the focus of many studies. PI3K-Akt-PTEN signaling has been
339 confirmed in previous studies of breast cancer and prostate cancer (Jamaspishvili et al. 2018;
340 Schadendorf et al. 2018; Yang et al. 2016). In cutaneous melanoma, PTEN is downregulated,
341 and this type of PTEN loss can increase T cell-mediated immunotherapy resistance (Peng et al.
342 2016). In our study, we showed that miR-25 may promote the development of cutaneous
343 melanoma by downregulating the expression of PTEN, thus affecting the prognoses of
344 melanoma patients. Feng et al. increased the sensitivity of hepatoma stem cells to TRAIL
345 (Tumor necrosis factor (TNF) -related apoptosis-inducing ligand), reducing apoptosis by
346 knocking out miR-25. This effect is also achieved through the PTEN/PI3K/Akt signal pathway
347 (Feng et al. 2016). The adhesion-related genes obtained by KEGG analysis included ACTB,
348 ITGA5, ITGAV, COL27A1, COL1A2, RAP1B, SHC1, COL5A3, PIK3R3, PPP1CC, and PTEN,
349 and we found that most of these genes were also involved in the PI3K signaling pathway.
350 Degradation of the basement membrane and the extracellular matrix (ECM) is critical for the
351 invasion and metastasis of malignant cells. ECM ligands not only control cell adhesion,
352 migration, and actin cytoskeleton structure through signaling pathways, but they also control
353 anchorage dependence, a key group of survival mechanisms (Ciolczyk-Wierzbicka & Laidler
354 2018; Multhaupt et al. 2016). While COL27A1, COL1A2, and COL5A3 are encoded ECM
355 components, experiments have found that the upregulation of COL1A2 in cancer can serve as a
356 molecular basis for metastasis development (Lin et al. 2016). Integrins are the major cell-
357 adhesive receptor; are key components of signaling molecules, mechanical transducers, and
358 cellular migration mechanisms; and are involved in many aspects ranging from primary tumor to
359 metastatic cancer progression (Hamidi & Ivaska 2018) Integrin-alpha-5 (ITGA5) and integrin-
360 alpha-V (ITGAV) are members of the integrin receptor gene family, closely related to the
361 regulation of cancer growth and metastasis (Chernaya et al. 2018; Morandi et al. 2016).
362 Ubiquitination is a widespread post-transcriptional modification, a selective marker that binds to

363   protein aggregates and dysfunctional organelles, thereby promoting autophagy-dependent
364   degradation. Autophagy can promote the growth of tumor cells by maintaining arginine-serine
365   circulation (Grumati & Dikic 2018; Poillet-Perez et al. 2018). Yang et al. showed that ubiquitin-
366   mediated proteolysis by bioinformatics analysis may have potential as a prognostic and
367   predictive marker for survival in patients with uveal melanoma (Yang et al. 2019).
368   In summary, the five screened miRNAs and their target genes were closely related to the
369   occurrence and development of tumors, and the results proved reliable. Our established five-
370   miRNA signature theoretically predicted the survival of melanoma patients, but further
371   experiments are needed to determine the mode of action and mechanism of these miRNAs, and
372   whether they can be used in a non-invasive diagnostic method.
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375   ## Conclusion
376   The five screened miRNAs and their target genes were closely related to the occurrence and
377   development of tumors, and the results proved reliable. The identified five-miRNA signature
378   may serve as a prognostic biomarker, or even as a potential therapeutic target, in cutaneous
379   melanoma patients.
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384   ## Abbreviations
385
386   **miRNAs** microRNAs
387   **DEMs** the differential expression of miRNAs
388   **GEO** Gene Expression Omnibus
389   **TCGA** The Cancer Genome Atlas
390   **UM** Uveal Melanoma
391   **DAVID** Annotation, Visualization and Integrated Discovery
392   **GO** Gene Ontology
393   **OS** overall survival
394   **HR** hazard ratio
395   **PI3K-Akt** phosphoinositide 3-kinase-RAC-alpha serine/threonine-protein kinase
396   **CM** cutaneous melanoma
397   **ECM** extracellular matrix
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Figure 1

Volcano maps

DEMs in GSE35579 between melanoma and nevi: green represents down regulation in melanoma (logFC<-1) ; red represents up regulation in melanoma (logFC>1) ; P<0.05.

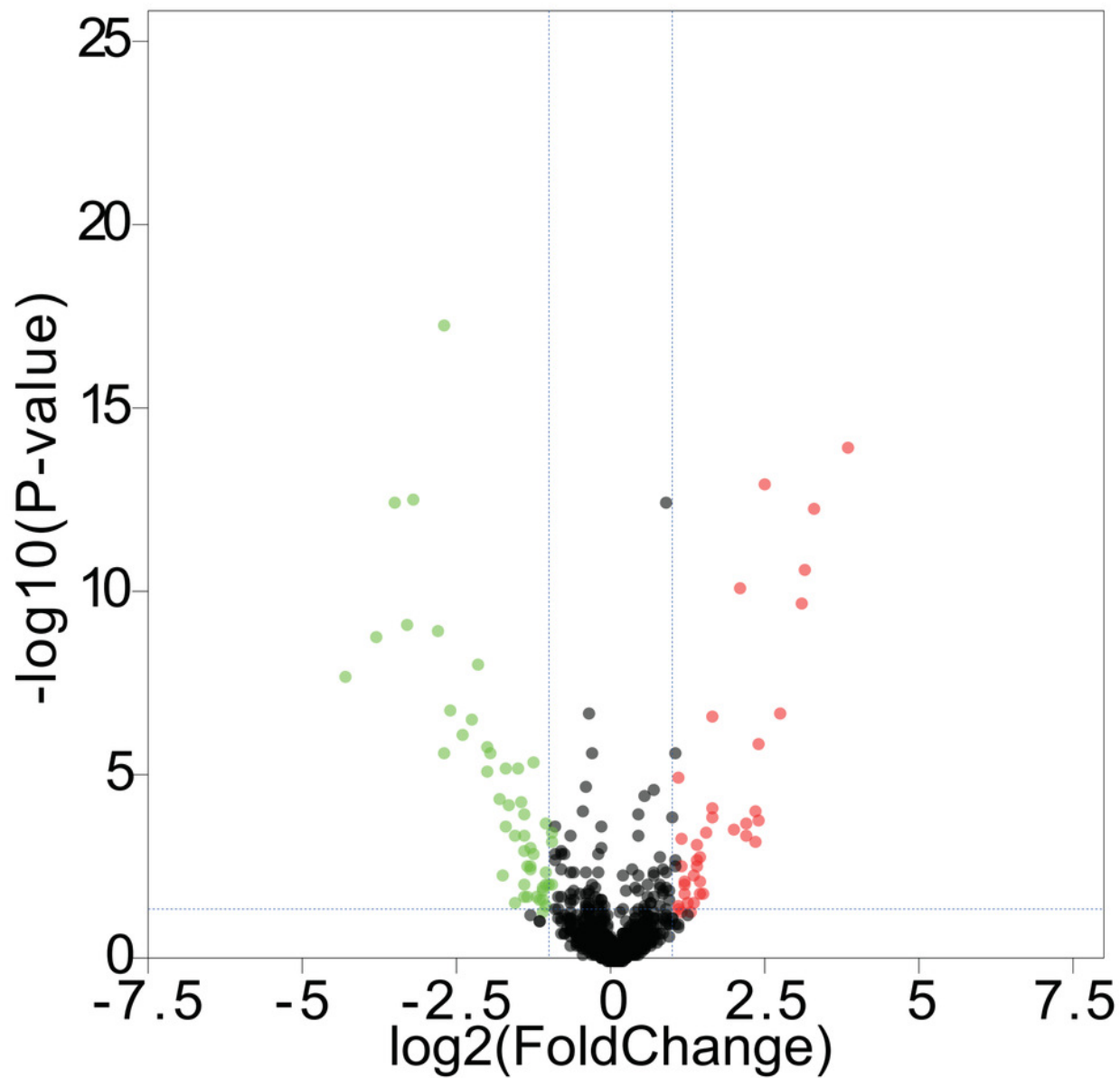



Figure 2

A hierarchic figure

Hierarchic analysis: green, black and red indicate the top 100 downregulated, nonsignificantly differentially expressed and up regulated DEMs, Respectively

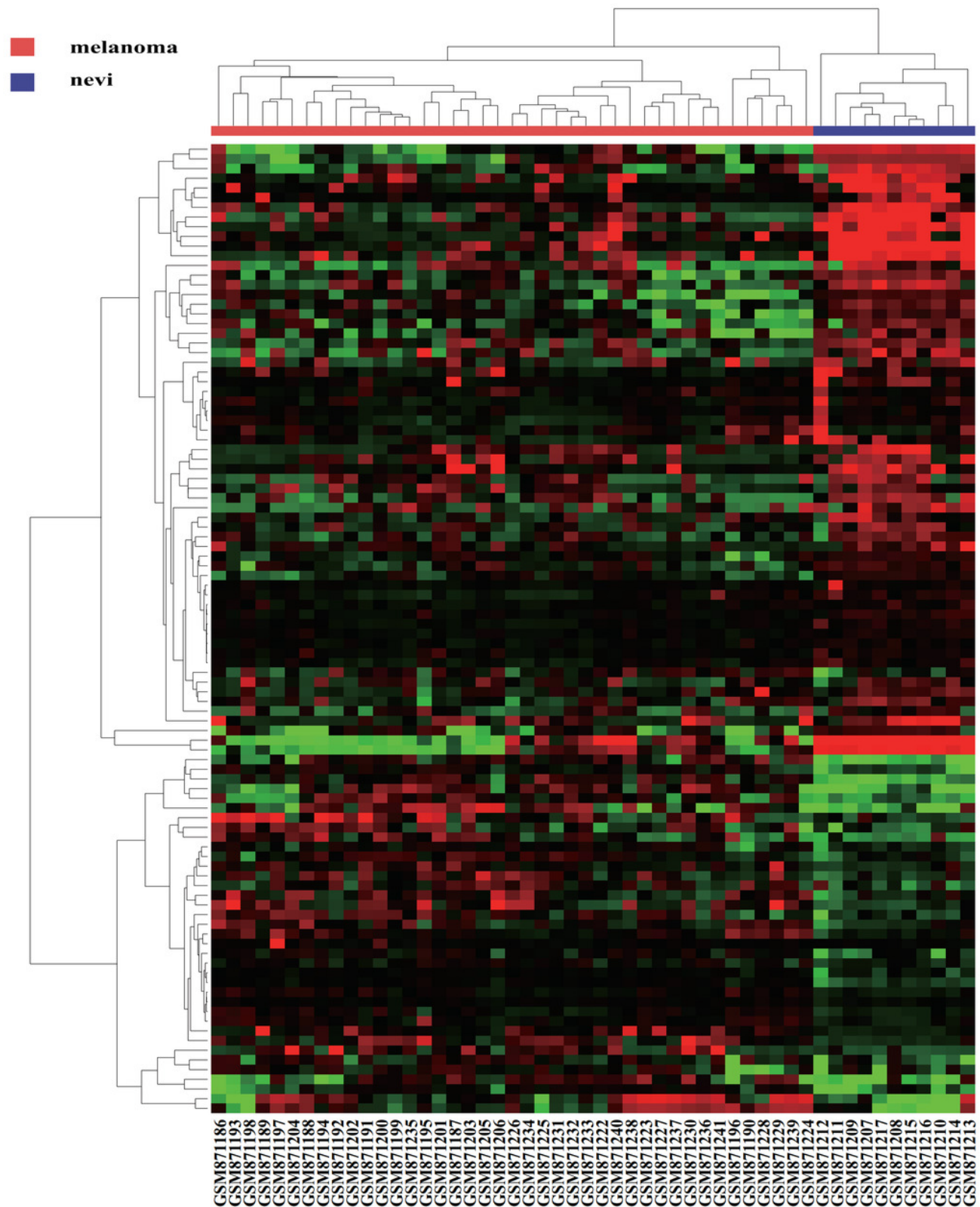



Figure 3

survival analysis and prognosis of cutaneous melanoma patients from TCGA.

Fig.3A-3E show that the prognostic characteristics of these five miRNAs(miR-25,miR-204,miR-211,miR-510,miR-513c); Fig.3F show that the prognostic characteristics of a 5-miRNA signature (miR-25,miR-204,miR-211,miR-510,miR-513c). Blue curves represent the low expression group, and red curves represent the high expression group

A **miR-25**

B **miR-204**

C **miR-211**

D **miR-510**

E **miR-513c**

F **a five-miRNA**

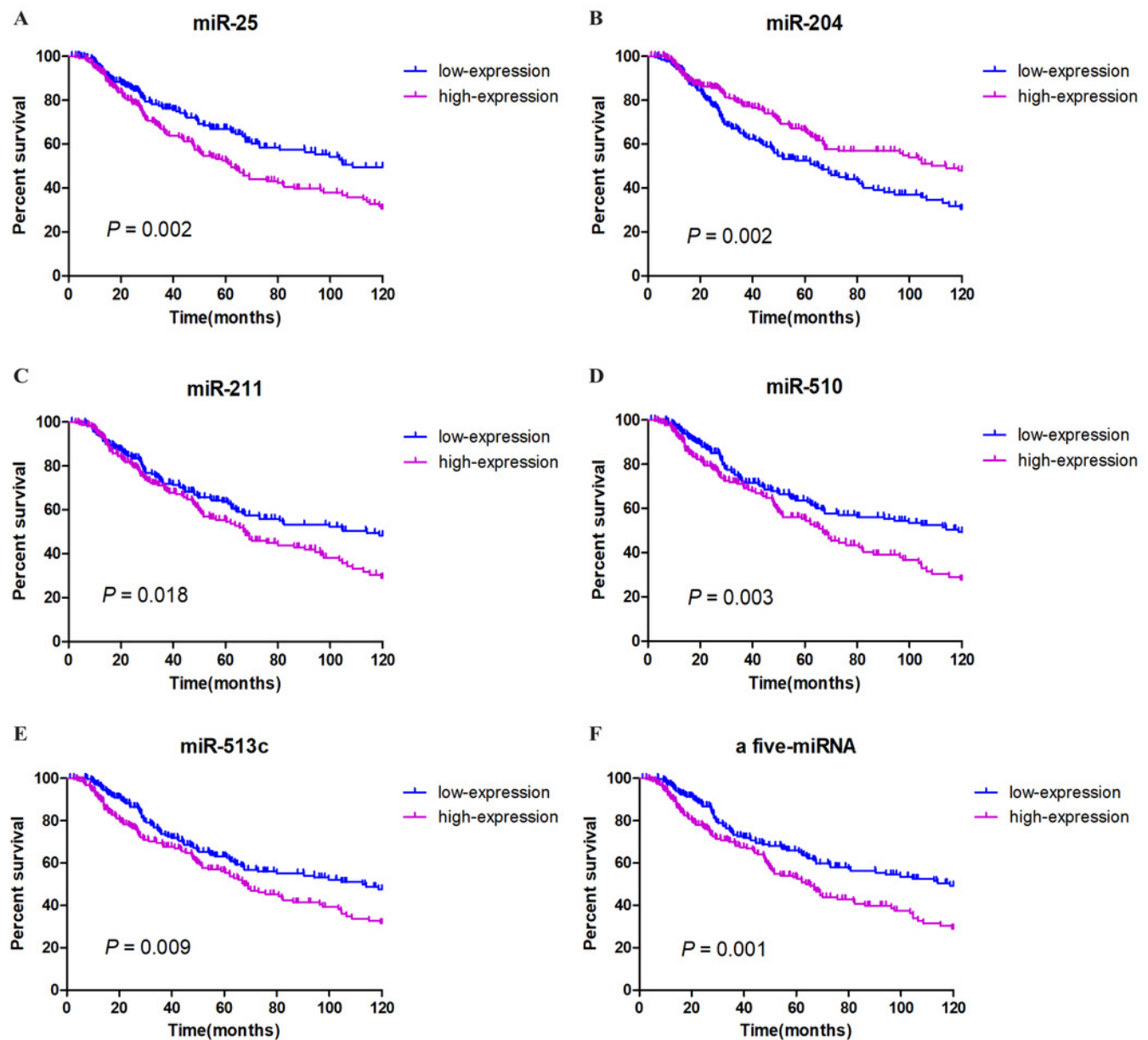



Figure 4

Venn diagrams

Target genes of the five DEMs were identified by four software programs, including

TargetScan, miRDB, DIANA and miRmap, to acquire overlapping genes by Funrich tool.

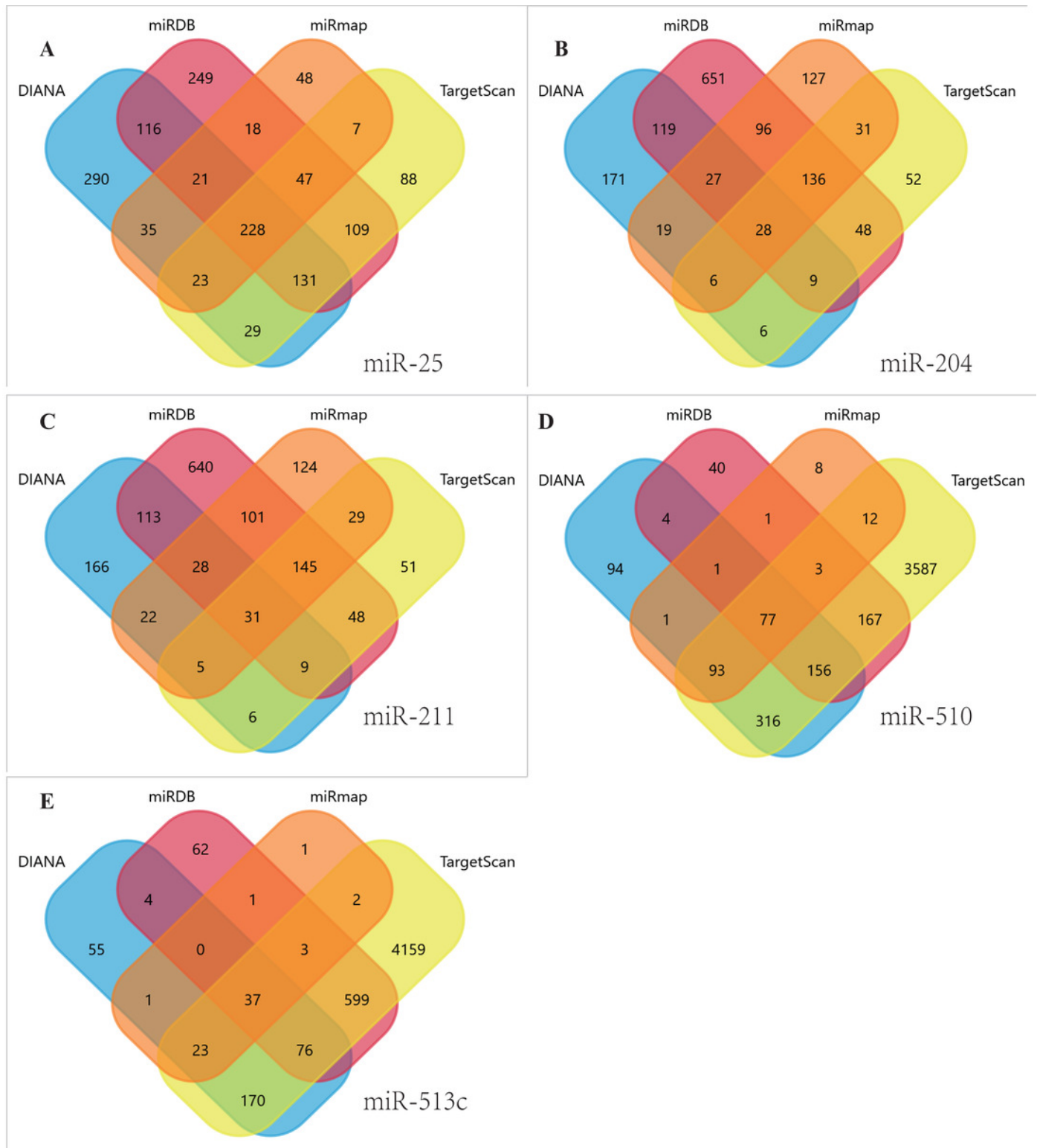



Figure 5

GO and KEGG pathway analyses

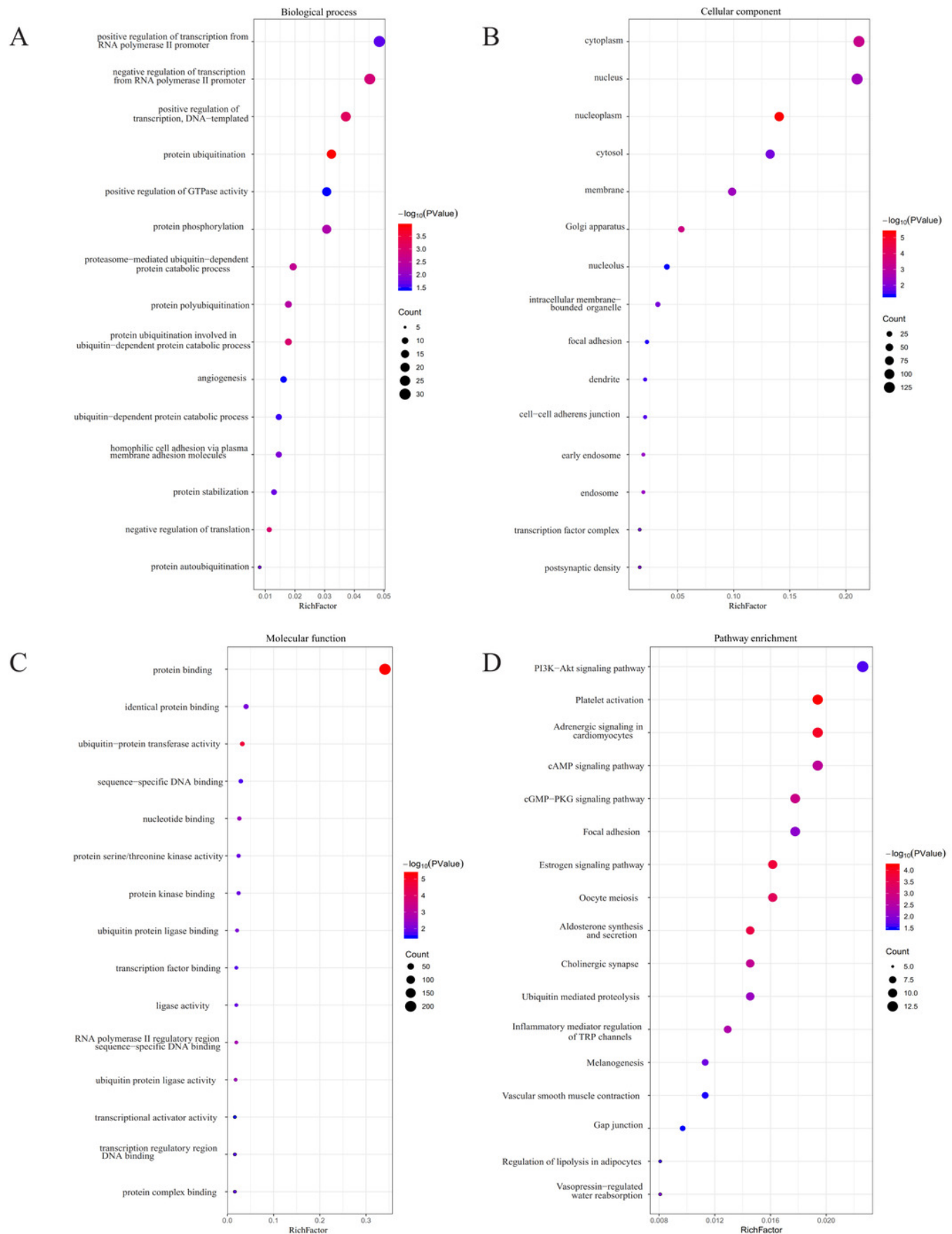
(A) The biological process (BP) analysis; (B) Cellular component (CC) analysis; (C) Molecular function (MF) analysis; (D) KEGG pathway analysis.

Clinicalfeature of melanoma patients from TCGA

Table 1 Clinical feature of melanoma patients from TCGA

Variables	Case,n(%)
Age at diagnosis(yr)	
<60	241
≥60	221
NA	8
Gender	
Male	290
Female	180
T stage	
T0	23
T1(a+b)	42
T2(a+b)	78
T3(a+b)	90
T4(a+b)	143
Tis	8
TX	47
NA	29
Pathologic stage	
Stage 0	7
Stage I	77
Stage II	140
I/II NOS	14
Stage III	171
Stage IV	23
NA	38
Node status	
N0	235
N1-3	178
Nx	36
NA	21
Metastasis	
M0	418
M1	24
NA	28

1   **NA:Not available**

The prognosticrelated differentially expressed miRNAs identifed between melanoma and nevi

1 **Table 2 The prognostic related differentially expressed miRNAs identifed between melanoma and nevi**

Down-regulation DEMs	P-value	up-regulation	P-value
hsa-miR-211	0.033	hsa-miR-25	0.004
hsa-miR-204	0.001		
hsa-miR-510	0.007		
hsa-miR-513c	0.014		

2 **DEMs: Differentially expressed miRNAs**

Associationbetween the five miRNAs and melanoma cancer clinical characters

1   **Table 3 Association between the five miRNAs and melanoma cancer clinical characters**

Variables	miR-25 expression			miR-204 expression			miR-211 expression			miR-510 expression			miR-513c expression		
	low	high	*P*	low	high	*P*	low	high	*P*	low	high	*P*	low	high	*P*
Age															
<60	124	106	0.064	92	138	<0.000*	122	108	0.175	119	111	0.379	115	115	0.755
≥60	89	109		118	80		92	106		94	104		96	102	
gender															
Female	80	81	0.980	83	78	0.424	73	88	0.134	76	85	0.411	73	88	0.203
Male	133	134		127	140		141	126		137	130		138	129	
T stage															
T1+T2	75	62	0.157	54	83	0.004*	84	53	<0.000*	79	58	0.003*	75	62	0.037*
T3+T4	103	116		121	98		89	130		91	128		95	124	
Lymph node status															
N0	106	108	0.758	107	107	0.955	107	107	0.955	109	105	0.811	103	111	0.516
N1-2	82	89		86	85		85	86		85	86		88	83	
Mestasis															
M0	192	193	0.841	189	196	0.769	188	197	0.243	193	192	0.823	188	197	0.458
M1	10	11		11	10		13	8		10	11		12	9	
Clinical stage															
I + II	107	95	0.166	102	100	0.715	97	105	0.512	97	105	0.738	93	109	0.250
III+IV	84	99		89	94		94	89		91	92		95	88	
Breslow depth value(mm)															
<3	83	77	0.255	70	90	0.041*	91	69	<0.000*	91	69	<0.000*	84	76	0.015*
≥3	78	93		94	77		61	110		61	110		67	104	

4   ***P* <0.05 was considered statistically significant**

TCGA univariable and multivariable Cox regression analysis

1 **Table 4 TCGA univariable and multivariable Cox regression analysis**

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
age at diagnosis (≥60 vs. <60)	1.563 (1.166-2.096)	0.003*	1.199(0.839-1.714)	0.319
T stage (T3+T4 vs. T1+T2)	0.443(0 .316-0.622)	<0.000*	0.825(0.468-1.456)	0.507
N stage (N1–2 vs. N0)	0.517(0.380-0.703)	<0.000*	0.246(0.087-0.699)	0.008*
M stage (M1 vs. M0)	0.554(0.292-1.051)	0.071	0.616(0.218-1.741)	0.361
Clinical stage (III+IV vs. I + II)	0.543(0.399-0.738)	<0.000*	1.986(0.691-5.708)	0.203
Gender (Male vs. Female)	0.997(0.734-1.355)	0.986	1.040 (0.726-1.491)	0.830
Breslow depth value (≥3mm vs.＜3mm)	0.370(0.265-0.518)	<0.000*	0.509(0.302-0.859)	0.011*
Five-miRNA signature (high risk vs. low risk)	0.613(0.456-0.823)	0.001*	0.605(0.424-0.863)	0.006*

*P <0.05 was considered statistically significant