1 Construction of competing endogenous RNA interaction networks as prognostic
2 markers in metastatic melanoma

3 Zan He ^{Equal first author, 1, 2}, Zijuan Xin ^{Equal first author, 3, 4}, Yongfei Peng ^{3, 4}, Hua Zhao
4 ^{Corresp., 1, 2}, Xiangdong Fang ^{Corresp. 3, 4}

5 1 Department of Dermatology, General Hospital of People's Liberation Army, Beijing,
6 CHINA, CHINA

7 2 Medical School of Chinese People's Liberation Army, Beijing, CHINA, CHINA

8 3 Beijing Institute of Genomics/China National Center for Bioinformation, Chinese
9 Academy of Science, Beijing, CHINA, CHINA

10 4 College of Life Sciences, University of Chinese Academy of Sciences, Beijing,
11 CHINA, CHINA

12 Corresponding Authors: Hua Zhao, Xiangdong Fang

13 Email address: hualuck301@163.com, fangxd@big.ac.cn

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31 **Abstract**

32 Malignant melanoma (MM) is a highly aggressive, metastatic cancer originating from
 33 melanocytes. These tumors have an extremely poor prognosis. MM accounts for 4%
 34 of skin cancers with an 80% mortality rate. The median survival of patients with
 35 metastatic melanoma is approximately six months, with a five-year survival rate of
 36 less than 10%. In recent years, the incidence of melanoma has gradually increased and
 37 has become one of the deadliest cancers. Competitive endogenous RNA (ceRNA)
 38 models the mechanism by which long chain non-coding RNAs (lncRNAs) play a
 39 regulatory role in the disease. lncRNAs can act as a sponge, competitively attracting
 40 small RNAs (microRNAs; miRNAs) and interfering with miRNA function. This can
 41 affect the expression of target gene messenger RNAs (mRNAs), ultimately promoting
 42 tumorigenesis and tumor progression. Bioinformatics analysis may identify
 43 potentially prognostic and therapeutically-relevant differentially expressed genes in
 44 MM, as well as lncRNAs, miRNAs and mRNAs that are interconnected through the
 45 ceRNA network. This may provide further insight into gene regulation and the
 46 prognosis of metastatic melanoma. Weighted co-expression networks were used to
 47 identify lncRNA and mRNA modules associated with the metastatic phenotype, as
 48 well as the co-expression genes contained in the modules. We used 17 lncRNAs, six
 49 miRNAs, and 11 mRNAs to construct a ceRNA interaction network with a regulatory
 50 role in patients with metastatic melanoma. The prognostic risk model was used as a
 51 sorter to classify the survival prognosis of melanoma patients. Four groups of ceRNA
 52 interaction triplets were obtained, and miR-3662 may be used in the treatment of
 53 metastatic melanoma patients. Experiments confirmed the regulating relationship and
 54 phenotype of this assumption. This study provides new targets to regulate the
 55 metastatic process, predict metastatic potential and determine that miR-3662 may be
 56 used in the treatment of melanoma.

57 **INTRODUCTION**

58 Malignant melanoma is derived from melanocytes, which are typically cutaneous. It is
 59 the third most common malignant tumor of the skin, but is the most malignant. The

60 incidence of melanoma has continued to rise over the years, with approximately
61 300,000 new cases and 60,000 deaths from melanoma worldwide annually (Swick &
62 Maize 2012). In 2016, there were 76,380 new cases of melanoma and 10,130 patient
63 deaths from melanoma in the United States alone (Reddy et al. 2017). Cutaneous
64 malignant melanomas can be classified as malignant freckled nevus-like melanoma,
65 superficial disseminated malignant melanoma, extremity malignant melanoma, and
66 nodular malignant melanoma based on their clinical and pathologic manifestations.
67 Metastatic melanoma accounts for 1% of all cancer cases, but many patients
68 experience recurrence after surgery, resulting in a poor long-term prognosis and a
69 5-year survival rate of less than 10% (McKean & Amaria 2018; Wahid et al. 2018).
70 The outcomes from current adjuvant therapies based on interferon use are not
71 satisfactory.

72 A better understanding of the molecular level of MM has brought attention to the use
73 of molecular targeted therapy targeting specific genes and signaling pathways. For
74 example, research has led to significant results in the treatment of melanoma (Eroglu
75 et al. 2015), lymphoma (Robert et al. 2015), Merkel cell carcinoma (Engels 2019) and
76 other tumors from relieving the immunosuppression of CD8+T cells in the
77 microenvironment (Topalian et al. 2016), immunotherapy drugs for cytotoxic T
78 lymphocyte antigen-4 (CTLA-4), and programmed cell death-1 (PD-1). However in
79 general, the current clinical data from immunotherapy shows that its response rate is
80 limited, and some patients have an initial drug resistance (Lei et al. 2020). The
81 occurrence and development of the tumor is due to the combined effect of multiple
82 gene mutations and the activation of multiple signaling pathways, thus, it is difficult
83 to inhibit tumor proliferation simply by targeting a single site. A combined targeted
84 therapy that focuses on multiple sites and pathways should be the focus of research in
85 the future.

86 LncRNAs are non-coding RNAs with a transcript sequence length of 200 bp or more.
87 These are closely related to the cell cycle and differentiation, development,
88 reproduction, gender regulation, aging and numerous human diseases. MiRNAs are
89 non-coding RNAs of 18-24 nt in length that are mainly involved in the regulation of

individual development, apoptosis, proliferation and differentiation through complete or incomplete pairing with the 3'UTR of the target genes to degrade them or inhibit their translation. LncRNAs can competitively bind miRNAs through an miRNA response element (MRE) to inhibit the negative regulation of mRNAs by miRNAs (Ala et al. 2013; Qi et al. 2015). lncRNA is of great interest among the ceRNAs and there is growing evidence that lncRNA is involved in a wide range of biological processes. LncRNAs act as molecular sponges, competing to attract miRNAs, leading to gene silencing and are involved in a variety of human diseases (Prensner & Chinnaiyan 2011). miRNAs regulate the expression of target genes by binding to messenger RNAs (mRNAs) on target mRNAs.

The ceRNA hypothesis has attracted extensive attention in the study of the molecular biological mechanisms of cancer development. Nevertheless, a comprehensive analysis of the lncRNA-miRNA-mRNA ceRNA regulatory network based on high-throughput sequencing and large samples are still lacking in the MM field. We sought to show the specific ceRNA regulatory mechanisms in MM patients compared to non-metastatic patients, construct a ceRNA network of melanoma marker prognostic model, and provide a new perspective for elucidating the regulatory mechanisms and prognosis of metastatic melanoma by extracting the lncRNA, miRNA, and mRNA expression matrices of skin cutaneous melanoma (SKCM) in situ and metastatic samples from the TCGA database, and obtaining the hub genes.

110

111 **MATERIALS & METHODS**

112 All bioinformatics analyses were conducted using RStudio (R-4.0.0). Our analyses and study design are shown in Figure 1A. The interaction networks of Figure S2 and Figure S3 were drawn using Cytoscape 3.7.

115 **Patient cohort information and data processing**

116 The melanoma patient cohort data were obtained from the Cancer Genome Atlas (TCGA), which is deposited on the GDC website (<https://portal.gdc.cancer.gov/repository>). Three files were downloaded containing 118 miRNA, isoform, and clinical information, respectively. A total of 470 melanoma 119

120 samples were stored in the database, including 103 in situ melanoma samples and 367
121 metastatic melanoma samples. The data were downloaded using the "download"
122 command from the recommended download tool gdc-client
123 (<https://gdc.cancer.gov/access-data/gdc-data-metastatic-tool>). The clinical information
124 and miRNA expression matrix were obtained using the R package "XML". mRNA
125 and lncRNA were extracted separately according to the genome annotation file. The
126 mRNA extraction parameters were type = "gene", gene_biotype = "protein_coding",
127 and gene_biotype = "protein_coding", the lncRNA extraction parameters arguments
128 were type = "transcript", transcript_biotype = "lncRNA".
129 "3prime_overlapping_ncRNA", "bidirectional_promoter_lncRNA", "sense_".
130 overlapping", "sense_intronic" and "antisense_RNA".

131 **Identification of differentially expressed genes**

132 We used the R package "edgeR" to identify differential genes between metastatic and
133 in situ melanoma samples (Robinson et al. 2010). All q-values were corrected for
134 statistical significance of multiple testing using FDR, with screening thresholds of
135 $|\log_2FC| > 1.0$, $FDR < 0.05$, and retention of significantly different genes (Lai 2017;
136 McCarthy et al. 2012).

137 **Functional enrichment analysis**

138 ClusterProfiler was used for GO/KEGG analysis (Gene Ontology/ Kyoto
139 Encyclopedia of Genes and Genomes) (Ashburner et al. 2000; Kanehisa et al. 2010;
140 Yu et al. 2012). GO was used to describe gene functions along three aspects:
141 biological process (BP), cellular component (CC), and molecular function (MF).
142 KEGG was searched for pathways at the significance level of $p < 0.05$.

143 **Weighted correlation network analysis**

144 WGCNA is an algorithm used for the identification of gene co-expression networks
145 for mRNAs or lncRNAs with different characteristics by high-throughput spectrum of
146 expression. Neighborhood matrix assessment of weighted co-expression relationships
147 between all datasets was performed using Pearson's correlation analysis. We used
148 WGCNA to analyze mRNAs and lncRNAs to obtain the mRNAs and lncRNAs with
149 which the metastatic melanoma samples were most associated.

150 **Construction of a ceRNA regulatory network**

151 Some authoritative databases have provided relationship pairs such as
152 lncRNA-miRNA and miRNA-mRNA, which can assist in the construction of a
153 ceRNA network centered on miRNA, based on an experimental module and
154 prediction module. MirDIP (<http://ophid.utoronto.ca/mirDIP/index.jsp>) was used to
155 predict interactions between lncRNAs and miRNAs. LncBase
156 ([http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2](http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex)
157 Findex) databases were used to explore target mRNAs.

158 **Construction of metastatic melanoma survival risk prognosis model**

159 Metastatic melanoma samples with low expression of miRNA, high expression of
160 lncRNA, and mRNA in the ceRNA network were used to perform three LASSO
161 regression modeling filters. Factors that fit the normalized lambda value model were
162 included in the subsequent analysis. A prognostic model was constructed using
163 multiple COX regression analyses, and a comprehensive prognostic scoring system
164 was obtained based on these factors. The risk score was calculated as follows: risk
165 score = $\sum \beta_i \times \text{expRNA}_i$, where expRNA is the expression level of RNA and β is the
166 regression coefficient derived from the multivariate Cox regression model. Melanoma
167 patients were classified into high-risk or low-risk groups based on the median risk
168 score cutoff value based on this risk score formula and other factors we determined.
169 Kaplan-Meier analysis was used to determine the overall survival between the groups
170 and there was a difference in overall survival (OS) between the two groups. $P < 0.05$
171 was considered significant unless otherwise stated.

172 **Cell line and cell culture**

173 A375 (ATCC, Manassan, VA, USA, Catalog number: CRL-1619) cells were cultured
174 in an incubator in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island,
175 NY, USA) containing 10% fetal bovine serum (AusGeneX, Molendinar, Qld,
176 Australia) and 1% penicillin–streptomycin (P/S; Invitrogen, Carlsbad, CA, USA) with
177 a humidity of 5% CO₂ at 37°C.

178 **Fluorescence in situ hybridization(FISH)**

179 The A375 cells were inoculated in a 12-well plate and incubated overnight in an
180 incubator set at 37°C with 5% CO₂. The medium in the orifice plate was absorbed
181 and washed twice with PBS, and then 1 ml 4% paraformaldehyde was added to each
182 well, and fixed at room temperature for 15 min. We followed the manufacturer's
183 instructions for the RNA FISH kit (GenePharma, Suzhou, China, Catalog number:
184 F12201/50). Five probes were designed and the sequences are listed in Supplementary
185 Table 1. We added 200 µl diluted DAPI working solution to each well, and stained the
186 samples for 20 min in dark. An anti-quenching agent was added to a clean slide, and
187 these were observed under a fluorescence microscope (630×, oil lens).

188 **Plasmid, siRNA and primer synthesise**

189 Plasmids of miRNA-has-miR-3662 and miRNA-shNC, siRNA of siRP11-594N15.3
190 and si-NC were bought from GenePharma. The overexpressed plasmids of the
191 miR-3666 sequence are shown in Supplementary Table 2. The primers of
192 RP11-594N15.3, ZNF831, PKIA, CSF2RB, miR-3662, GAPDH, U6 and reverse
193 universal primer were designed by Primer Premier 5 software and were purchased
194 from the Beijing Genomics Institute (Supplementary Table 3).

195 **Expression vectors transfection**

196 A375 cells were cultured in 10cm petri dishes at a concentration of 7×10^6 /8 ml the
197 day after transfection. Cells were stably transduced with the miR-3662 and shNC
198 expression vectors, using Lipofectamine 2000 (Invitrogen) according to the
199 manufacturer's protocol. Each plate was added to 12µg plasmids and 24µl
200 Lipofectamine 2000. The medium was replaced after 6 h of transfection. Each plate
201 was added to 80µM siRNA for the transfection of siRNA and additional steps were
202 performed as described above.

203 **RNA isolation and quantitative real-time PCR (qRT-PCR)**

204 We used TRIzol® Reagent (Life Technologies) to extract RNA from cells. After
205 wiped off DNA, equal amounts (2µg) of each RNA were separately reverse
206 transcribed to their corresponding cDNAs using a RevertAid™ First Strand cDNA
207 Synthesis Kit (Thermo Fisher Scientific). All the opeations are subject to the

208 manufacturer's instructions. CFX96 Real-Time PCR detection system (Bio-Rad) was
209 used to perform qRT-PCR with Maxima® SYBR Green/ROX qPCR Master Mix
210 (Thermo Fisher Scientific). U6 and GAPDH were used as internal control for
211 miR-3662 and other mRNAs respectively.

212 **Cell counting kit-8 (CCK-8) assay**

213 2,000 cells/well were seeded into 96-well plates, and were cultured for one, two, three,
214 or four days. The condition of cell proliferation was assessed with a Cell Counting
215 Kit-8 assay (C0038, Beyotime Biotechnology, China). According to manufacturer's
216 instruction, cells were treated with 10 μ L CCK-8 (100 μ L culture medium per well)
217 solution for 2 h at 37 °C after a period of incubation. Absorbance at 450 nm was
218 measured by using a microplate reader.

219 **Transwell migration and invasion assays**

220 The cells were resuspended in serum-free medium the day before experiments. For
221 Transwell invasion assays, Transwell upper chambers(8 mm, BD Biosciences,
222 Franklin Lakes, BJ, USA) were coated with Matrigel matrix (dilution 1:7; 356234,
223 BD Biosciences) and 2×10^5 cells were placed in. For Transwell migration assays,
224 2×10^4 cells were seeded in the upper chamber of the Transwell without Matrigel
225 matrix. Then, a medium containing 20% serum was added to lower chambers. Cells
226 were incubated at 37 °C and 5% CO₂ for 18 h, and then those cells remaining in
227 upper chambers were removed using a wet cotton swab. We used 4%
228 paraformaldehyde to fix the cells that had migrated and adhered to lower chambers for
229 10 min. Then hematoxylin (ZLI-9610, ZSGB-Bio) and eosin (ZLI-9613, ZSGB-Bio)
230 were used to stain cells adhered to lower chambers for 20 min and then imaged. The
231 number of cells was counted in ten separated high power fields with vertical cross
232 distribution.

233

234 **RESULTS**

235 **Identification of differential genes in metastatic and in situ melanoma samples**

236 There were 103 samples of melanoma in situ and 367 samples of metastatic

melanoma in the TCGA database (Supplementary Table 4, Supplementary Table 5). We identified 1,413 differential lncRNAs, of which 1,059 were highly-expressed in metastatic samples and 354 in the in-situ samples. We identified 2,251 differential mRNAs, of which 1,442 were highly-expressed in the metastatic samples and 809 in the in-situ samples. We identified 81 differential miRNAs, of which 60 were highly expressed in metastatic samples and 21 in the in-situ samples (Figure 1B-1D, Figure S1 A-C). The differentially expressed mRNAs in the metastatic group were enriched for GO function and KEGG pathway, immune cell activity-related terms on biological processes (BP), transmembrane transduction structures on cellular composition (CC), G protein-coupled receptor activity, passive transport and other entries on molecular function (MF) (Figure 1E). The KEGG pathway was enriched for the PI3K-Akt signaling pathway, chemokine signaling pathway, JAK-STAT signaling pathway, cell adhesion molecules (CAMs), allograft rejection, NF-kappa-B signaling pathway, and other tumor-related pathways (Figure 1F).

Identification of mRNA and lncRNA co-expression modules by weighted gene co-expression network analysis

We used weighted gene co-expression network analysis (WGCNA) to analyze the gene expression patterns of mRNA and lncRNA expression matrices extracted from metastatic and in-situ samples. Genes with similar expression patterns were clustered into a co-expression module (Langfelder & Horvath 2008). We identified nine mRNA co-expression modules and 10 lncRNA co-expression modules in the two groups, and the number of genes contained in each module is shown in Supplementary Table 6. The significantly related co-expression modules were selected according to the clustering of linkage levels and the number of co-expression associations. The mRNA clustering results showed that the black module and yellow module of in-situ samples, and the turquoise module of metastatic samples were identified as highly-associated co-expression modules (Figure 2A-C). The lncRNA clustering results showed that the pink and red module of in-situ samples, and the turquoise and brown modules of the metastatic samples were identified as highly correlated co-expression modules (Figure

266 2D-F). The corresponding interactions of the first 20 co-expressed genes in the above
 267 modules are shown in Figure S2 A-G. GO functional enrichment of co-expression
 268 genes in the turquoise module in mRNA showed that the module genes were
 269 associated with immune responses, and were also enriched for entries associated with
 270 immune negative regulation and cell adhesion (Figure 2G). We removed the turquoise
 271 module in mRNA and the brown/turquoise module in lncRNA for the next step of the
 272 analysis.

273 **Construction of ceRNA regulation network**

274 The mRNAs from the turquoise module obtained after WGCNA analysis were
 275 intersected with the highly expressed mRNAs in the metastatic samples, and 319
 276 mRNAs were obtained. lncRNAs from the brown/turquoise module were intersected
 277 with the highly expressed mRNAs in the metastatic samples, and 287 lncRNAs were
 278 obtained (Fig. 3.A-B). The above 319 mRNAs were subjected to Pearson's correlation
 279 coefficient calculation with 287 lncRNAs. The results showed that 138 mRNAs had
 280 potential interactions with 89 lncRNAs when $cor > 0.5$ and $pvalue < 0.05$ (Figure S3).
 281 Using the mirDIP database to predict the target genes, the low expression miRNAs in
 282 the 21 metastatic samples were predicted to score "very high" of 3,771 mRNA target
 283 genes. The 3,771 target genes were cross-linked to 138 mRNAs by correlation
 284 analysis to obtain 11 mRNAs (CD53, CD96, CSF2RB, CXCL9, MS4A1, PIM2,
 285 PKIA, PTPN22, SH2D1A, SLA, ZNF831) (Figure 3C). The 11 mRNAs and their
 286 corresponding six miRNAs (has-miR-346, has-miR-3662, has-miR-429,
 287 has-miR-891b, has-miR-892a, has-miR-944) were analyzed and mapped for the
 288 interaction network (Figure 3D). These six miRNAs were used to predict 3,760
 289 related lncRNAs in the lncBase database. The 3,760 lncRNAs were intersected with
 290 89 lncRNAs screened in correlation analysis to obtain 17 lncRNAs (AC007386.4,
 291 AC104024.1, GTSCR1, LINC00402, LINC00861, MIAT, PRKCQ-AS1,
 292 RP11-121A8.1, RP11-164H13.1, RP11-202G28.1, RP11-203B7.2, RP11-594N15.3,
 293 RP11-598F7.3, RP11-693J15.5, RP11-768B22.2, RP5-887A10.1) (Figure 3E).
 294 Interaction networks were mapped for the above 17 lncRNAs and six miRNAs
 295 (Figure 3F). The final selection of 11 mRNAs and lncRNAs, the data tSNE

downscaling analysis, were better able to distinguish the in-situ samples from the metastatic samples (Figure 3G), resulting in the construction of lncRNA-miRNA-mRNA ceRNA network with the final selection of six miRNAs (Figure 3H). The factors of our network were found to be highly expressed in metastatic samples in another melanoma cohort (GEO database; accession number: GSE65904) (Figure 3I).

Construction of metastatic melanoma survival risk prognosis model

We clustered the 34-factors included in our ceRNA interaction network, and 17 lncRNAs, 6 miRNAs, and 11 mRNAs were subjected to risk prediction by LASSO regression, respectively. The regularization term controlled for the lncRNA, miRNA, and mRNA models having minimal likelihood error when $\lambda = 7, 1$, and 2, respectively. We screened lncRNAs, one miRNA, and two mRNAs for high correlation with prognosis (Figure 4A-F). The above 10 factors were analyzed using multiple COX regression to construct risk models. There were five factors considered to be risk factors for prognosis and the other five factors were protective factors for prognosis. lncRNA AC104024.1, MIAT, RP11-598F7.3, and miRNA hsa-mir-429 could be used as significant risk markers ($P < 0.05$). The concordance index of the regression model was 0.753 (Figure 4G). In the cohort, the expressions of AC104024.1, AC007386.4, and has-mir-429 were gradually up-regulated as the risk scoring increased, while the expressions of RP11-598F7.3, PKIA, CXCL9 and MIAT were down-regulated (Figure 4H). The KM curves of melanoma patient survival were plotted according to the expression profiles of risk and protective factors in the multivariate COX regression model. These showed that the altered expression of factors in the model was significantly correlated with the follow-up prognosis of the cohort of patients ($P < 0.0001$) (Figure 4I). Four groups of ceRNA interacting triplets were obtained (RP11-594N15.3-miR3662-CSF2RB, RP11-594N15.3-miR3662-ZNF831, RP11-594N15.3-miR3662-PKIA, AC104024.1-miR346-PKIA), of which miR3662 may have potential for the treatment of metastatic melanoma (Figure 4J).

miR-3662 down-regulates its target mRNAs and suppresses melanoma cell proliferation, migration and invasion in vitro

326 In order to verify whether the regulatory relationship of ceRNA was objective, we
 327 chose three groups of ceRNA interacting triplets (RP11-594N15.3-miR3662-CSF2RB,
 328 RP11-594N15.3-miR3662-ZNF831, RP11-594N15.3-miR3662-ZNF831,
 329 RP11-594N15.3-miR3662-PKIA) for experimental verification. The localization of
 330 lncRNA in the cytoplasm is the premise of its spongy function. The database “lncBase”
 331 was used to predict the interaction of lncRNA-miRNA based on a base experimental
 332 module and prediction module. The binding sites on the sequence between lncRNAs
 333 and miRNAs, and the interaction between lncRNAs and miRNAs was tested in other
 334 studies. Since miRNAs only exist in the cytoplasm, the lncRNAs predicted by the
 335 database must also theoretically exist in the cytoplasm. We conducted FISH
 336 experiment in melanoma A375 cells and selected lncRNA RP11-594N15.3 for
 337 localization to ensure that our lncRNAs conformed. Our results show that
 338 RP11-594N15.3 may be localized in the cytoplasm (Figure 5A). Since
 339 RP11-594N15.3 is an RNA with a length of more than 3 KB, it is very difficult to
 340 construct its overexpression vector. Therefore, we decided to knock down the
 341 expression of RP11-594N15.3 with siRNA in A375 cells to observe the changes in the
 342 expression of miR-3662, CSF2RB, PKIA and ZNF831 (Figure 5B). miR-3662 was
 343 found to be significantly overexpressed, PKIA and ZNF831 were down-regulated, and
 344 CSF2RB was not significant (Figure 5C, D). Our experiments proved that the sponge
 345 function of lncRNA was objective.

346 In order to confirm whether the regulation of miR-3662 on target mRNAs in ceRNA
 347 regulatory triplets was objective, we transfected the miR-3662 overexpression
 348 plasmid vector and the NC plasmid vector into melanoma A375 cells, obtained
 349 A375-miR3662 and A375-NC cells, and verified the high expression of miR-3662 at
 350 the transcriptional level (Figure 5E). The qRT-PCR of the downstream target mRNAs
 351 shows the transcriptional differences of the three targets. The transcriptional levels of
 352 PKIA and ZNF831 were down-regulated by miR-3662, but there was no significant
 353 change on CSF2RB (Figure 5F). The overexpression of miR-3662 significantly
 354 promoted cell proliferation (Figure 5G). We used the Transwell chamber assay to
 355 explore the functions of miR-3662 on the invasion and migration of melanoma cells.

356 The overexpression of miR-3662 significantly suppressed cell migration through a
357 permeable filter and invasion through Matrigel Matrix (Figure 5H). Our results
358 indicate that miR-3662 may repress melanoma cell proliferation, migration and
359 invasion by down-regulating PKIA and ZNF831.

360

361 **DISSCUSSION**

362 The clinical treatment of melanoma poses a significant health and economic burden.
363 There is a need to develop new chemotherapy drugs, surgical procedures and patient
364 care (Meng et al. 2018). The ability of tumor cells to migrate and metastasize is an
365 important feature in the pathogenesis of melanoma. The accurate diagnosis of early
366 primary melanoma and timely surgical resection can significantly improve patient
367 survival. However, the long-term survival of patients with metastatic melanoma is
368 very low (Filippi et al. 2016). In this complex regulatory process, mRNAs in the
369 tumor cell and the proteins they encode are aberrant, and the non-coding RNAs and
370 miRNAs involved as upstream regulators also show specificity in their expression
371 profiles, while lncRNAs, as non-coding RNAs, are involved in regulating gene and
372 protein expression at the epigenetic level (Wilusz et al. 2009). It has been
373 phenotypically demonstrated that miRNAs can influence the progression of melanoma
374 by inhibiting mRNA. For example, miR-331 can inhibit the proliferation and
375 metastasis of melanoma cells by downregulating AEG-1 (Chen et al. 2018a).

376 We used The Cancer Genome Atlas (TCGA) database to obtain bioinformatics that
377 were used to construct a differentiated endogenous competitive RNA network among
378 patients with melanoma metastasis. We conducted a risk assessment of key factors in
379 the network to find prognostic markers. The Weighted Gene Co-expression Network
380 Analysis (WGCNA) was used to find gene modules for collaborative expression and
381 to explore the relationships between gene networks and phenotypes of interest. We
382 selected the highly expressed ceRNA in the metastatic samples after the intersection
383 of the genes and differential genes in the co-expression module recognized by
384 WGCNA. The lncRNA and miRNA were predicted through the mirDIP and lncBase
385 database, and the interactive relationship between miRNA and mRNA was established

386 to construct the overall ceRNA interaction network of
 387 DElncRNA-DEmiRNA-DEmRNA in the metastatic melanoma samples. The 17
 388 highly expressed DElncRNAs were selected from metastatic melanoma patients and
 389 triggered the down-regulation of the expression of their corresponding 6 DEmiRNAs
 390 by exerting their sponge function. This led to the high expression of the 11 target gene
 391 DEmRNAs in metastatic patients and exerted a regulatory effect on the proliferation
 392 and invasion of melanoma cells in the process of transcription. We provided a new
 393 perspective by elucidating the upstream molecular mechanisms of melanoma invasion
 394 and migration.

395 We conducted three LASSO regression modeling screening of 34 factors according to
 396 lncRNA, miRNA and mRNA, respectively, in order to explore the correlation between
 397 factors in the ceRNA network and the prognosis of the cohort samples. The 10 factors
 398 were screened for best fit to the model with regularized lambda values. The risk
 399 factors of the 10 factors were modeled using multiple COX regression, and the results
 400 showed that three lncRNAs (AC104024.1, RP11-594N15.3, MIAT), one miRNA
 401 (miR-429), one mRNA (PKIA) were identified as molecular markers with high
 402 correlation with patient prognosis by constructing the risk model. AC104024.1,
 403 RP11-594N15.3, and miR-429 were risk factors for prognosis; MIAT PKIA was a
 404 protective factor for prognosis. The above factors may be used as markers to predict
 405 metastatic potential in melanoma patients and may also serve as a target to distinguish
 406 the survival prognosis of melanoma patients. Finally, we screened four groups of
 407 ceRNA regulatory triplets present in metastatic melanoma patients, in which
 408 AC104024.1 and RP11-594N15.3 served as key endogenous sponges that upregulated
 409 the expression level of target genes CSF2RB, ZNF831, and PKIA by competitive
 410 adsorbing miR-364 and miR-3662, respectively. Our assays in the A375 cell line
 411 confirmed that the phenotype of miR-3662 was consistent with the anticipation of
 412 bioinformatics analysis. The candidate genes above were studied related to cancer
 413 progression.

414 Zhu et al. (2019) expressed miR-3662 through a lentiviral vector, which could
 415 significantly reduce the ZEB1 protein level and inhibit the growth of A375 cells in

416 vitro and in vivo. The expression of ZEB1 induced by miR-3662 decreased, the EMT
417 expression of A375 cells was inhibited, and the relative expression of metastatic genes
418 was reduced. Down-regulation of ZEB1 expression by a miR-3662 lentivirus vector
419 may significantly reduce in vitro and in vivo growth of the highly invasive melanoma
420 cell line A375. miR-3662 was considered to be a therapeutic target in adult T-cell
421 leukemia/lymphoma (ATLL) studies. miR-3662 knockdown inhibited the proliferation
422 of ATLL cells, and the expression of miR-3662 was related to the antiviral resistance
423 of ATLL cells (Yasui et al. 2018). miR-3662 in hepatocellular carcinoma tends to be
424 downregulated, and the high expression of miR-3662 inhibits hepatocellular
425 carcinoma growth by inhibiting HIF-1 α (Chen et al. 2018b). miR-429 also plays a
426 regulatory role in the development of various tumors. For example, it promotes the
427 proliferation of bladder cancer cells by inhibiting CDKN2B (Yang et al. 2017). ZEB1
428 expression was regulated to promote the migration, invasion and EMT of pancreatic
429 cancer (Shen et al. 2019). miR-429 may also promote the proliferation of non-small
430 cell lung cancer by targeting DLC-1 (Xiao et al. 2016). Our study showed that in
431 patients with metastatic melanoma, the low expression of microRNAs, miR-3662 and
432 miR-429, led to the upregulation of the expression of target genes, CSF2RB,
433 ZNF831 and PKIA, while the transcription of differentially expressed mRNA played a
434 specific role in the tumor phenotype.

435 CSF2RB (Colony Stimulating Factor 2 Receptor Subunit Beta) is involved in multiple
436 signaling pathways, and promotes survival, proliferation, and differentiation through
437 JAK2/STAT5, PI3K/mTOR, MEK/ERK, and other signaling pathways (Hercus et al.
438 2013). Cimas et al. (2020) identified CSF2RB mutations in patients with high
439 PD-1/PD-L1 expression using the RNA-seq of basal-like breast tumors, combined
440 with the TCGA database, suggesting good prognosis and correlation with immune
441 infiltration. In a public database study of lung adenocarcinoma, Xu et al. (2020)
442 identified nine genes, including CSF2RB, from three independent LUAD cohorts (Xu
443 et al. 2020). In colorectal cancer patients, CSF2RB was matched with 10 miRNAs
444 that were involved in regulating apoptosis (Slattery et al. 2018). Normal NF- κ B
445 activation in leukemia patients positively regulated the expression of IL-3 and

446 granulocyte/macrophage CSF2RB, thereby promoting the proliferation and survival of
 447 CML stem cells. No studies have been conducted, to our knowledge, on CSF2RB in
 448 melanoma. The protein encoded by PKIA (CAMP-Dependent Protein Kinase
 449 Inhibitor Alpha) is a member of the cAMP-dependent protein kinase (PKA) inhibitor
 450 family. Zhang et al. (2020) analyzed the gene transcriptome profiles of eight GEO
 451 cohorts in thyroid cancer, performed LASSO regression models, and identified seven
 452 genes, including PKIA, that were highly correlated with recurrence data in the TCGA
 453 database (Zhang et al. 2020). In the field of cervical cancer research, PKIA is
 454 considered an important marker for stage III cervical cancer (Banerjee & Karunagaran
 455 2019). Hoy et al. (2020) found that the amplification of PKIA is common in prostate
 456 cancer and was associated with reduced progression-free survival. Depletion of PKIA
 457 in prostate cancer cells results in reduced migration, increased sensitivity to nest loss
 458 apoptosis, and reduced tumor growth. By altering the activity of protein kinase A,
 459 protein kinase I can act as a molecular switch that drives the gPCR-GMAP1 s-cAMP
 460 signal toward activation of α -RAP1 and MAPK, ultimately regulating tumor growth
 461 (Hoy et al. 2020). As a transcription factor, ZNF831 (Zinc Finger Protein 831) is
 462 thought to regulate the immune response network with phenotypes in breast cancer
 463 (da Silveira et al. 2017). The co-expression network was also identified in the
 464 H-immune subtype of triple negative breast cancer (He et al. 2018). ZNF831 may
 465 regulate tumor immune infiltration in melanoma and plays an important role in
 466 infiltration and immune escape from melanoma.

467 There are some limitations to our study although we provided a complete analysis,
 468 used reasonable analytical tools, verified our results by phenotypic assays, and the
 469 results are clinically important for elucidating the regulatory mechanism and
 470 prognosis of melanoma metastasis. We predicted the interaction network of ceRNA
 471 through the database, selected RP11-594N15.3-miR-3662 and its target mRNAs for
 472 verification, however, we failed to verify all the regulatory relationships in the
 473 network objectively in vitro. We constructed a risk classification model, but did not
 474 verify the universality of the model in the verification set because it was difficult to
 475 find another melanoma queue containing lncRNA and miRNA sequencing data.

476 Finally, 2-lncRNAs in the four groups of triplets lacked the evidence of previous
477 relevant studies in tumors. Future studies should explore its function and mechanism.

478

479 **CONCLUSIONS**

480 We used the differentially expressed genes in situ and metastatic samples of
481 melanoma, as well as the phenotypic co-expression module of metastatic melanoma to
482 construct a ceRNA interaction network that may regulate melanoma metastasis. We
483 established a classifier model that can predict the prognosis of melanoma patients
484 through LASSO regression and multivariate COX regression. We found that
485 AC104024.1 and RP11-594N15.3 can be used as markers in patients with melanoma,
486 indicating a poor prognosis. We obtained four groups of ceRNA regulatory triplets
487 present in metastatic melanoma patients, in which AC104024.1 and RP11-594N15.3
488 served as key endogenous sponges that upregulated the expression level of target
489 genes CSF2RB, ZNF831, and PKIA by competitive adsorbing miR-346 and
490 miR-3662, respectively. These may regulate the metastasis of melanoma and lead to a
491 worse prognosis. Further experiments have shown that miR-3663 can reduce the
492 transcription of its target mRNAs, and that it inhibits the proliferation, invasion and
493 migration of melanoma cells.

494

495

496

497 **References**

- 498 Ala U, Karreth F, Bosia C, Pagnani A, Taulli R, Léopold V, Tay Y, Provero P, Zecchina R, and
499 Pandolfi P. 2013. Integrated transcriptional and competitive endogenous RNA
500 networks are cross-regulated in permissive molecular environments. *Proceedings of*
501 *the National Academy of Sciences of the United States of America* 110:7154-7159.
502 10.1073/pnas.1222509110
- 503 Ashburner M, Ball C, Blake J, Botstein D, Butler H, Cherry J, Davis A, Dolinski K, Dwight S,

504 Eppig J, Harris M, Hill D, Issel-Tarver L, Kasarskis A, Lewis S, Matese J, Richardson J,
505 Ringwald M, Rubin G, and Sherlock G. 2000. Gene ontology: tool for the unification of
506 biology. The Gene Ontology Consortium. *Nature genetics* 25:25-29. 10.1038/75556

507 Banerjee S, and Karunakaran D. 2019. An integrated approach for mining precise RNA-based
508 cervical cancer staging biomarkers. *Gene* 712:143961. 10.1016/j.gene.2019.143961

509 Chen L, Ma G, Cao X, An X, and Liu X. 2018a. MicroRNA-331 inhibits proliferation and
510 invasion of melanoma cells by targeting astrocyte-elevated gene-1. *Oncology*
511 *research*. 10.3727/096504018x15186047251584

512 Chen Z, Zuo X, Zhang Y, Han G, Zhang L, Wu J, and Wang X. 2018b. MiR-3662 suppresses
513 hepatocellular carcinoma growth through inhibition of HIF-1 α -mediated Warburg effect.
514 *Cell death & disease* 9:549. 10.1038/s41419-018-0616-8

515 da Silveira W, Palma P, Sicchieri R, Villacis R, Mandarano L, Oliveira T, Antonio H, Andrade J,
516 Muglia V, Rogatto S, Theillet C, du Manoir S, and Tiezzi D. 2017. Transcription Factor
517 Networks derived from Breast Cancer Stem Cells control the immune response in the
518 Basal subtype. *Scientific reports* 7:2851. 10.1038/s41598-017-02761-6

519 Engels EA. 2019. Epidemiologic perspectives on immunosuppressed populations and the
520 immunosurveillance and immunocontainment of cancer. *Am J Transplant*
521 19:3223-3232. 10.1111/ajt.15495

522 Eroglu Z, Kim DW, Wang X, Camacho LH, Chmielowski B, Seja E, Villanueva A, Ruchalski K,
523 Glaspy JA, Kim KB, Hwu WJ, and Ribas A. 2015. Long term survival with cytotoxic T
524 lymphocyte-associated antigen 4 blockade using tremelimumab. *Eur J Cancer*
525 51:2689-2697. 10.1016/j.ejca.2015.08.012

526 Filippi A, Fava P, Badellino S, Astrua C, Ricardi U, and Quaglino P. 2016. Radiotherapy and
527 immune checkpoints inhibitors for advanced melanoma. *Radiotherapy and oncology :*
528 *journal of the European Society for Therapeutic Radiology and Oncology* 120:1-12.
529 10.1016/j.radonc.2016.06.003

530 He Y, Jiang Z, Chen C, and Wang X. 2018. Classification of triple-negative breast cancers
531 based on Immunogenomic profiling. *Journal of experimental & clinical cancer*
532 *research : CR* 37:327. 10.1186/s13046-018-1002-1

533 Hercus T, Dhagat U, Kan W, Broughton S, Nero T, Perugini M, Sandow J, D'Andrea R, Ekert P,
534 Hughes T, Parker M, and Lopez A. 2013. Signalling by the βc family of cytokines.
535 *Cytokine & growth factor reviews* 24:189-201. 10.1016/j.cytogfr.2013.03.002

536 Hoy J, Salinas Parra N, Park J, Kuhn S, and Iglesias-Bartolome R. 2020. Protein kinase A
537 inhibitor proteins (PKIs) divert GPCR-Gas-cAMP signaling toward EPAC and ERK
538 activation and are involved in tumor growth. *FASEB journal : official publication of the*
539 *Federation of American Societies for Experimental Biology*. 10.1096/fj.202001515R

540 Kanehisa M, Goto S, Furumichi M, Tanabe M, and Hirakawa M. 2010. KEGG for
541 representation and analysis of molecular networks involving diseases and drugs.
542 *Nucleic acids research* 38:D355-360. 10.1093/nar/gkp896

543 Lai Y. 2017. A statistical method for the conservative adjustment of false discovery rate
544 (q-value). *BMC bioinformatics* 18:69. 10.1186/s12859-017-1474-6

545 Langfelder P, and Horvath S. 2008. WGCNA: an R package for weighted correlation network
546 analysis. *BMC bioinformatics* 9:559. 10.1186/1471-2105-9-559

547 Lei Q, Wang D, Sun K, Wang L, and Zhang Y. 2020. Resistance Mechanisms of

548 Anti-PD1/PDL1 Therapy in Solid Tumors. *Front Cell Dev Biol* 8:672.
549 10.3389/fcell.2020.00672

550 McCarthy D, Chen Y, and Smyth G. 2012. Differential expression analysis of multifactor
551 RNA-Seq experiments with respect to biological variation. *Nucleic acids research*
552 40:4288-4297. 10.1093/nar/gks042

553 McKean M, and Amaria R. 2018. Multidisciplinary treatment strategies in high-risk resectable
554 melanoma: Role of adjuvant and neoadjuvant therapy. *Cancer treatment reviews*
555 70:144-153. 10.1016/j.ctrv.2018.08.011

556 Meng Y, Hertel N, Ellis J, Morais E, Johnson H, Philips Z, Roskell N, Walker A, and Lee D.
557 2018. The cost-effectiveness of nivolumab monotherapy for the treatment of advanced
558 melanoma patients in England. *The European journal of health economics : HEPAC :
559 health economics in prevention and care* 19:1163-1172. 10.1007/s10198-018-0964-4

560 Prensner J, and Chinnaiyan A. 2011. The emergence of lncRNAs in cancer biology. *Cancer*
561 *discovery* 1:391-407. 10.1158/2159-8290.cd-11-0209

562 Qi X, Zhang D, Wu N, Xiao J, Wang X, and Ma W. 2015. ceRNA in cancer: possible functions
563 and clinical implications. *Journal of medical genetics* 52:710-718.
564 10.1136/jmedgenet-2015-103334

565 Reddy B, Miller D, and Tsao H. 2017. Somatic driver mutations in melanoma. *Cancer*
566 123:2104-2117. 10.1002/cncr.30593

567 Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassel JC, Rutkowski P, McNeil C,
568 Kalinka-Warzocha E, Savage KJ, Hernberg MM, Lebbe C, Charles J, Mihalcioiu C,
569 Chiarion-Sileni V, Mauch C, Cognetti F, Arance A, Schmidt H, Schadendorf D, Gogas

570 H, Lundgren-Eriksson L, Horak C, Sharkey B, Waxman IM, Atkinson V, and Ascierto
571 PA. 2015. Nivolumab in previously untreated melanoma without BRAF mutation. *N*
572 *Engl J Med* 372:320-330. 10.1056/NEJMoa1412082

573 Robinson M, McCarthy D, and Smyth G. 2010. edgeR: a Bioconductor package for differential
574 expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)*
575 26:139-140. 10.1093/bioinformatics/btp616

576 Shen J, Hong L, Yu D, Cao T, Zhou Z, and He S. 2019. LncRNA XIST promotes pancreatic
577 cancer migration, invasion and EMT by sponging miR-429 to modulate ZEB1
578 expression. *The international journal of biochemistry & cell biology* 113:17-26.
579 10.1016/j.biocel.2019.05.021

580 Slattery M, Mullany L, Sakoda L, Wolff R, Samowitz W, and Herrick J. 2018. Dysregulated
581 genes and miRNAs in the apoptosis pathway in colorectal cancer patients. *Apoptosis :*
582 *an international journal on programmed cell death* 23:237-250.
583 10.1007/s10495-018-1451-1

584 Swick J, and Maize J. 2012. Molecular biology of melanoma. *Journal of the American*
585 *Academy of Dermatology* 67:1049-1054. 10.1016/j.jaad.2011.06.047

586 Topalian SL, Taube JM, Anders RA, and Pardoll DM. 2016. Mechanism-driven biomarkers to
587 guide immune checkpoint blockade in cancer therapy. *Nat Rev Cancer* 16:275-287.
588 10.1038/nrc.2016.36

589 Wahid M, Jawed A, Mandal R, Dar S, Akhter N, Somvanshi P, Khan F, Lohani M, Areeshi M,
590 and Haque S. 2018. Recent developments and obstacles in the treatment of
591 melanoma with BRAF and MEK inhibitors. *Critical reviews in oncology/hematology*

592 125:84-88. 10.1016/j.critrevonc.2018.03.005

593 Wilusz J, Sunwoo H, and Spector D. 2009. Long noncoding RNAs: functional surprises from
594 the RNA world. *Genes & development* 23:1494-1504. 10.1101/gad.1800909

595 Xiao P, Liu W, and Zhou H. 2016. miR-429 promotes the proliferation of non-small cell lung
596 cancer cells via targeting DLC-1. *Oncology letters* 12:2163-2168.
597 10.3892/ol.2016.4904

598 Xu ZY, Zhao M, Chen W, Li K, Qin F, Xiang WW, Sun Y, Wei J, Yuan LQ, Li SK, and Lin SH.
599 2020. Analysis of prognostic genes in the tumor microenvironment of lung
600 adenocarcinoma. *PeerJ* 8:e9530. 10.7717/peerj.9530

601 Yang J, Liu Y, He A, Liu Y, Wu J, Liao X, Lv Z, Wang F, and Mei H. 2017. Hsa-miR-429
602 promotes bladder cancer cell proliferation via inhibiting CDKN2B. *Oncotarget*
603 8:68721-68729. 10.18632/oncotarget.19878

604 Yasui K, Izumida M, Nakagawa T, Kubo Y, Hayashi H, Ito T, Ikeda H, and Matsuyama T. 2018.
605 MicroRNA-3662 expression correlates with antiviral drug resistance in adult T-cell
606 leukemia/lymphoma cells. *Biochemical and biophysical research communications*
607 501:833-837. 10.1016/j.bbrc.2018.04.159

608 Yu G, Wang L, Han Y, and He Q. 2012. clusterProfiler: an R package for comparing biological
609 themes among gene clusters. *Omics : a journal of integrative biology* 16:284-287.
610 10.1089/omi.2011.0118

611 Zhang L, Wang Y, Li X, Wang Y, Wu K, Wu J, and Liu Y. 2020. Identification of a Recurrence
612 Signature and Validation of Cell Infiltration Level of Thyroid Cancer Microenvironment.
613 *Front Endocrinol (Lausanne)* 11:467. 10.3389/fendo.2020.00467

614

615