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Original Research Article

ANP32E promotes the proliferation and glycolysis of melanoma cells by regulating the Wnt/β-catenin pathway

Yun Zhang[1], Yannan Jiang[2]*
[1]Department of Dermatology, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, Jiangsu 225012, [2]Department of Dermatology, The Fourth Affiliated Hospital of Nantong University, The First People's Hospital of Yancheng, Yancheng, Jiangsu 224000, China

For correspondence: **Email: jiangyannan0624@163.com, **Tel:** +86-0515-88508612*

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Abstract

Purpose: *This study aimed to explore the related molecular mechanism of acidic leucine-rich nuclear phosphoprotein 32 family member E (ANP32E) in melanoma cell proliferation and glycolysis.*
Methods: *Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assays were employed to determine mRNA and protein expression of ANP32E in four melanoma cell lines (A375, G361, SK-MEL-3, and A2058) and normal human immortalized keratinocytes (HACAT), while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide cell proliferation and clone formation assays were used to detect cell proliferation of A375 cells after ANP32E knockdown. Enzyme-linked immunosorbent assay and western blotting were used to assess the role of ANP32E in glucose consumption and the levels of lactate, adenosine triphosphate (ATP), and glycolytic metabolism-related proteins. Protein expressions of β-catenin and c-Myc in melanoma cells after ANP32E knockdown were determined by Western blotting.*
Results: *Protein expression and mRNA levels of ANP32E were higher in the four melanoma cell lines than in normal human immortalized keratinocytes ($p < 0.001$). ANP32E suppression inhibited the proliferation of melanoma cells ($p < 0.05$). Additionally, ANP32E suppression decreased glucose consumption ($p < 0.001$), lactate ($p < 0.05$), ATP ($p < 0.001$), and glycolytic metabolism-related protein expression in melanoma cells ($p < 0.05$). Finally, ANP32E was confirmed to regulate Wnt/β-catenin signaling pathway ($p < 0.05$).*
Conclusion: *ANP32E promotes the proliferation and glycolysis of melanoma cells by regulating Wnt/β-catenin pathway, thus facilitating the potential discovery of advanced therapeutic targets in melanoma.*

Keywords: *ANP32E, Melanoma, Wnt, β-catenin, Cell proliferation, Glycolysis*

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INTRODUCTION

Melanoma is a malignant tumor that develops in the skin but can also develop in the mouth, intestines, or eyes. The incidence of melanoma has been increasing in recent years. Today, melanoma is a leading cause of skin-cancer-related deaths because of its high degree of metastasis. The prognosis of melanoma patients is disappointing. Radiotherapy and chemotherapy have shown limited improvement in patients, resulting in high mortality rates. Early

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diagnosis and treatment can improve the cure rate. Therefore, the identification of new molecules and the development of new effective therapeutic targets are crucial to improve the prognosis and survival of melanoma patients [1]. Wnt/β-catenin signaling is associated with tumorigenesis and progression. Wnt signaling is activated in tumors, increasing the expression level of β-catenin and binding to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. The β-catenin-TCF/LEF complex then induces the transcription of downstream cancer target genes, such as c-MVC and c-Myc [2].

Acidic leucine-rich nuclear phosphoprotein 32 family member E (ANP32E) is a protein encoded with abundant leucine repeats. As a histone chaperone of H2A.Z, ANP32E can remove H2A.Z from the chromatin of target genes and regulate its expression. Several studies have reported that ANP32E regulates DNA repair and cerebellar development. Recent studies have shown that ANP32E is involved in cancer development. For example, ANP32E promotes tumor development in triple-negative breast cancer cells by upregulating E2F1 [3]. Downregulation of miRNA-141 promotes the growth and metastasis of breast cancer cells by enhancing ANP32E [4]. Additionally, ANP32E promotes the proliferation and migration of thyroid cancer cells by enhancing Akt/mTOR/HK2-mediated glycolysis [5]. The upregulation of ANP32E in lung adenocarcinoma is significantly associated with adverse survival [6]. ANP32E activates β-catenin/c-Myc signaling to promote the proliferation and migration of pancreatic cancer cells [7].

TCGA showed that ANP32E expression in metastatic melanoma was higher than that in primary melanoma, but the report on ANP32E in melanoma was unclear. Therefore, we investigated the effect of ANP32E on melanoma cells to provide a reference to identify treatment targets of melanoma.

EXPERIMENTAL

Cell culture

Four malignant melanoma cell lines (A375, G361, SK-MEL-3, and A2058) and the normal human immortalized keratinocytes (HACAT) were obtained from the Chinese Academy of Sciences. The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA, USA) with 10 % fetal bovine serum (Gibco), and 1 % penicillin-streptomycin solution (Gibco) in an incubator with 5 % CO_2.

Real-time quantitative polymerase chain reaction (qPCR)

Trizol reagent (Invitrogen, Grand Island, NY, USA) was applied to collect the total RNA from cells. A Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used to evaluate the quantity and integrity of RNA. SYBR Premix EX Taq (Takara, Japan) was used to detect the expression of ANP32E. The primer sequences are shown in Table 1.

Table 1: Primers for ANP32E and reference genes

Gene	Primer	Sequence (5'→3')
ANP32E	Forward	GGAGGAGGTGACAGAGTTAG
	Reverse	GGGCCAGCGAACTTAGTTCC
GAPDH	Forward	GACTCATGACCACAGTCCATGC
	Reverse	AGAGGCAGGGATGATGTTCTG

Western blotting

Cells were first washed three times in phosphate-buffered saline, and total protein was extracted using radio-immunoprecipitation assay buffer (Beyotime). The BCA protein assay kit (CoWin Biotechnology) was used to detect protein concentration. Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Next, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Boston, MA, USA) and then incubated with 5 % non-fat milk for 1 h. The membranes were then incubated with the following specific primary antibodies: ANP32E (ab5993; 1:1000; Abcam), LDHA (ab125683; 1:5000; Abcam), GLUT1 (ab115730; 1:3000; Abcam), HK2 (ab104836; 1:1200; Abcam), β-catenin (ab6302; 1:3,000; Abcam), c-Myc (ab152146; 1:3,000; Abcam), and GAPDH (ab9485; 1:1,200; Abcam) overnight at 4°C. After that, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (ab205718; 1:2,000; Abcam) and the protein bands on the membranes were visualized using ECL chemiluminescence reagent (Beyotime).

Cell transfection

For ANP32E knockdown, si-ANP32E and si-NC (both synthesized by GenePharma, Shanghai, China) were each cloned into the pSuper-retro-puro vector. After the cells were cultured in 6-well plates for 24 h, si-ANP32E or si-NC was transfected into cells with Lipofectamine 3000 (Invitrogen).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

To assess the extent of cell proliferation, the MTT assay was used. Briefly, the cells were plated into 96-well plates in triplicate and were treated as indicated in each experiment. Following treatment, a final concentration of 0.5 mg/mL of MTT solution (Beyotime) was added, and the cells were incubated for another 4 h. Subsequently, the culture medium was discarded and 100 μL of DMSO (Sigma) was added to visualize the cells. The optical density value of each sample was detected at 490 nm using a microplate reader (BioTek, Winooski, VT, USA).

Cell cloning assay

To assess the extent of cell proliferation, crystal violet staining was used. Briefly, 4.5×10^3 cells were plated into 6-well plates in triplicate and were treated as indicated in each experiment. After culture for 3 weeks, the supernatant was removed and 4 % formaldehyde was added for 15 min. Following treatment, the cells were stained with 0.25 % crystal violet solution for 25 min. The number of cells was counted after drying the culture plate.

Enzyme-linked immunosorbent assay (ELISA)

To detect glucose uptake (ab136955; Abcam), lactate (ab65331; Abcam), and ATP (83355; Abcam), ELISA kits were used according to the manufacturer's instructions.

Statistical analysis

All the data were shown as the means ± standard error of the mean of three independent experiments. Differences between two groups were compared using Student's t test, and differences among multiple groups were compared using one-way analysis of variance (ANOVA). $P < 0.01$ (two-tailed) was considered statistically significant.

RESULTS

ANP32E is highly expressed in melanoma cells

To investigate the correlation between the expression of ANP32E and melanoma, mRNA and protein expression levels of ANP32E in normal human immortalized keratinocytes (HACAT) and four malignant melanoma cell lines (A375, G361, SK-MEL-3, and A2058) were determined using qRT-PCR and western blotting, respectively. ANP32E mRNA and protein expression levels were markedly higher in all four melanoma cell lines than in HACAT cells (Figure 1 A and B). Thus, ANP32E was highly expressed in melanoma cells.

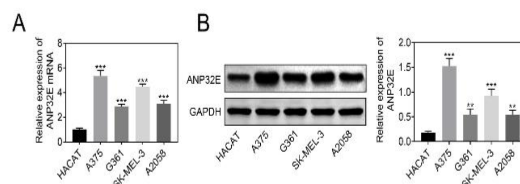

Figure 1: ANP32E is highly expressed in melanoma cells. (A) mRNA expression levels of ANP32E in normal human immortalized keratinocytes (HACAT) and four malignant melanoma cell lines (A375, G361, SK-MEL-3, and A2058). (B) Protein expression levels of ANP32E in human normal human immortalized keratinocytes and four malignant melanoma cell lines. Data are presented as the means ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}p < 0.001$ versus the HACAT group

ANP32E knockdown inhibits the proliferation of melanoma cells

To determine the effects of ANP32E on melanoma cell proliferation, the melanoma cell line (A375) was transfected with the empty vector (Control), siRNA control (si-NC), or ANP32E-targeting siRNA (si-ANP32E). After transfection, the mRNA expression level of ANP32E in the melanoma cell line was determined. Cells transfected with ANP32E-targeting siRNA showed strikingly lower ANP32E expression at the mRNA level than that in the si-NC or control group, confirming the transfection efficiency (Figure 2 A). The MTT assay revealed that compared with the si-NC or control group, ANP32E suppression markedly inhibited the cell proliferation of melanoma cell lines (Figure 2 B). Furthermore, the clone formation assay was used to identify the proliferation of melanoma cell lines *in vitro*, revealing that down-regulated ANP32E expression decreased the number of cell clones (Figure 2 C). Thus, ANP32E suppression inhibited the proliferation of melanoma cells.

ANP32E knockdown inhibits the glycolysis of melanoma cells

ELISA was applied to investigate glucose consumption and levels of lactate and ATP in melanoma cells in each group to determine the effect of ANP32E on glycolysis in melanoma cells. ELISA demonstrated that compared with the si-NC or control group, glucose consumption and the levels of lactate and ATP in melanoma cells were markedly decreased in the si-ANP32E

group, indicating that ANP32E suppression inhibited the glycolysis of melanoma cells (Figure 3 A). Western blotting was performed to determine the effect of ANP32E on glycolysis in melanoma cells. Compared with the si-NC or control group, ANP32E suppression markedly inhibited the expression of glycolytic metabolism-related proteins, such as IDHA, GLUT1, and HK2 (Figure 3 B). These results indicated that ANP32E suppression inhibited the glycolysis of melanoma cells.

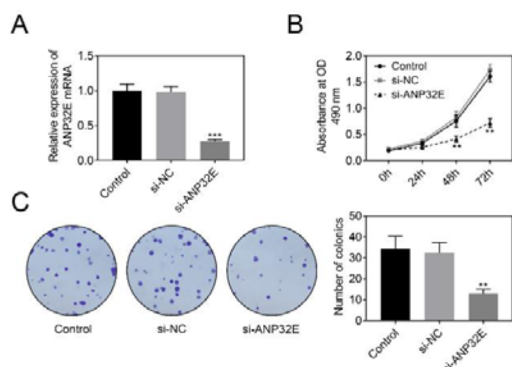

Figure 2: ANP32E knockdown inhibits the proliferation of melanoma cells. (A) ANP32E knockdown in A375 cells was verified by qPCR. (B) Serial MTT cell proliferation assays detected the proliferation of A375 cells after ANP32E knockdown. (C) Serial clone formation assays detected the proliferation of A375 cells after ANP32E knockdown. The data are expressed as the means ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}p < 0.001$ versus the si-NC or control group

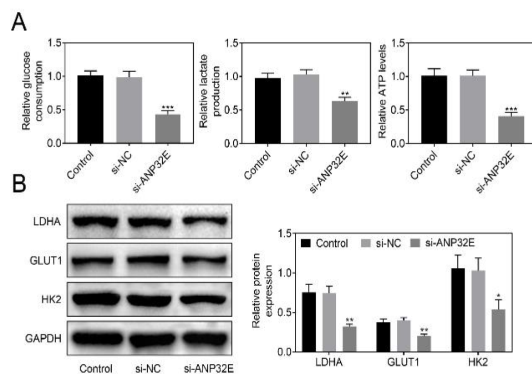

Figure 3: ANP32E knockdown inhibits the glycolysis of melanoma cells. (A) Glucose consumption and levels of lactate and ATP in melanoma cells in each group. (B) Western blotting to detect the expression of glycolytic metabolism-related proteins (IDHA, GLUT1, and HK2). The data were presented as the means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the si-NC or control group

ANP32E regulates Wnt /β-catenin signaling pathway

To explore the mechanism of ANP32E in melanoma cell proliferation and glycolysis, western blotting was used to detect the expression of β-catenin and c-Myc in melanoma cells. Compared with the si-NC or control group, protein expression of β-catenin and c-Myc were significantly decreased in the si-ANP32E group (Figure 4). Thus, ANP32E promoted proliferation and glycolysis of melanoma cells by regulating the Wnt/β-catenin pathway.

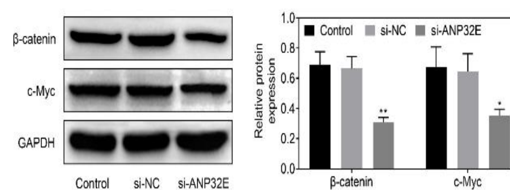

Figure 4: ANP32E regulates the Wnt /β-catenin signaling pathway. (A) Protein expression levels of β-catenin and c-Myc in melanoma cells in each group. The data were presented as the means ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}p < 0.01$ versus the si-NC or control group

DISCUSSION

Despite recent advances, the molecular mechanisms of melanoma are still unclear. Recently, some studies have suggested that ANP32E plays an essential role in tumor development [8,9]. However, ANP32E is rarely studied in melanoma, and understanding the molecular mechanism of ANP32E in melanoma may provide novel therapeutic targets for melanoma. However, the molecular mechanism of ANP32E in melanoma fate determination remains poorly understood.

The ANP32 family plays vital roles in modulating cell proliferation and apoptosis [10]. Many ANP32 family proteins are highly expressed in tissues or organs, contributing to the maintenance of normal tissue functions and homeostasis, and affecting cell proliferation and apoptosis [11]. Additionally, many ANP32 family proteins are implicated in cancer-associated cell growth and apoptosis, and disorders of ANP32 family proteins play a vital role in the occurrence and progression of cancer. Specifically, studies have found that ANP32E promotes tumorigenesis in lung adenocarcinoma [6].

Recently, ANP32B was reported to be associated with the occurrence of hepatocellular carcinoma [10]. Additionally, ANP32E was found

to be involved in breast cancer cell migration and invasion [4]. Targeting the many actions of ANP32E could lead to the development of new and safe drugs. However, few studies have concentrated on the pharmacological value of ANP32E in melanoma, and its potential mechanism has not been reported yet. This study found that mRNA and protein expression levels of ANP32E were higher in four melanoma cell lines than in normal human immortalized keratinocytes. ANP32E suppression inhibited the proliferation of melanoma cells. Additionally, ANP32E suppression decreased glucose consumption and the levels of lactate, ATP, and glycolytic metabolism-related proteins in melanoma cells. Thus, ANP32E may promote proliferation and glycolysis in melanoma cells.

A growing number of studies have hinted that ANP32E regulates the expression of target mRNAs. A previous study showed that ANP32E was associated with cell proliferation and migration in pancreatic cancer by regulating β-catenin [7]. Additionally, ANP32E induced the tumorigenesis of triple-negative breast cancer cells by upregulating E2F1 [3]. Furthermore, ANP32E promoted glycolysis, cell proliferation, and migration in thyroid carcinoma by activating AKT/mTOR/HK2 [5]. Another novel discovery of this study was that β-catenin and c-Myc protein are targets of ANP32E. β-Catenin is a dual function protein involved in regulating and coordinating cell–cell adhesion and gene transcription. Mutations and overexpression of β-catenin are associated with many cancers. c-Myc is a regulatory gene that encodes transcription factors and plays a role in cell cycle progression, apoptosis, and cell transformation. In our study, protein expression levels of β-catenin and c-Myc were significantly decreased in the si-ANP32E group, indicating that ANP32E promotes proliferation and glycolysis in melanoma cells by regulating the Wnt/β-catenin pathway.

CONCLUSION

The findings of this study show that the mRNA and protein expression levels of ANP32E are higher in the four melanoma cell lines assessed than in normal human immortalized keratinocytes. Additionally, ANP32E suppression inhibits the proliferation of melanoma cells. Furthermore, suppression of ANP32E inhibits glucose consumption and the levels of lactate, ATP, and glycolytic metabolism-related proteins in melanoma cells. Finally, ANP32E regulates Wnt /β-catenin signaling pathway. These results demonstrate the role of ANP32E/Wnt/β-catenin signaling pathway in promoting proliferation and glycolysis in melanoma cells, thus facilitating the identipotential for discovering advanced therapeutic targets in melanoma.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yun Zhang and Yannan Jiang designed the study and supervised the data collection. Yun Zhang analyzed and interpreted the data. Yannan Jiang prepared the manuscript for publication and reviewed the draft of the manuscript. All the authors have read and approved the manuscript.

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