Effects of abnormal expression of CD73 on malignant phenotype in nasopharyngeal carcinoma

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Abstract

Background Cluster of differentiation (CD) 73, encoded by the *NT5E* gene, plays important enzymatic and non-enzymatic roles in cells. There is growing evidence show that CD73 is a key regulator in the development of tumor. Nasopharyngeal carcinoma (NPC) is one of the most common cancers in east and southeast Asia. It is urgent to know more about the mechanism of NPC development and find diagnose markers for the patients.

Methods and Results Western blot, IHC and qRT-PCR were used to investigate the expression level of CD73 in NPC and we found NPC tissues had higher expression of CD73 than normal tissues. We also detected the relationship between its expression level with the clinicopathology and prognosis of NPC patients. The results showed CD73 expression was related to the clinical stages, lymph node metastasis and survival state of NPC patients. More importantly, patients with higher expression of CD73 had poorer prognosis. Then, CD73 was knocked down in NPC cells (CNE2 and CNE1), and the effects of CD73 on cell proliferation and migration were investigated by CCK8, colony formation, Transwell and wound-healing assays. We found knocking down the expression of CD73 in NPC cells could inhibit cells malignant phenotype.

Conclusion CD73 plays important roles in NPC malignant behavior and might be used as a novel target for the diagnose and treatment of NPC.

Background

Nasopharyngeal carcinoma (NPC), an epithelial tumor derived from the nasopharyngeal mucosal lining, is one of the most common malignancy in head and neck. Over 120000 new cases of NPC were reported per year[1]. NPC is characterized by its distinct geographical distribution and > 70% of new cases are reported in east and southeast Asia[2]. With the breakthrough and development of treatments, the mortality has been reduced these years. However, NPC has a high tendency to metastasize, more than 70% of newly diagnosed patients are locoregionally advanced, which has a low overall survival rate[3]. Tumor metastasis and recurrence remain the main challenges for NPC treatment[4, 5]. Studies have showed that there are genetic susceptibility genes and genomic changes participate in the development of NPC. It is urgent to know more about the mechanism of NPC development, which might help to find accurate diagnose and prognostic prediction markers for patients.

Evidence has showed that purinergic signaling plays pathophysiological roles with therapeutic potential in tumor. Adenosine triphosphate (ATP), adenosine monophosphate (AMP) and adenosine act as vital extracellular signaling molecules and take important parts in tumor progression[6]. Cluster of differentiation (CD) 73, also known as ecto-5'-nucleotidase (ecto-5'-NT, EC 3.1.3.5), is a member of the glycosylphosphatidylinositol(GPI) anchored protein family and encoded by the *NT5E* gene[7]. CD73 plays an important role in purinergic signaling and is a major and rate-limiting enzyme that catalyze extracellular AMP into adenosine[8, 9]. It has been regarded to impart cell-type-specific functions to

regulate tissue homeostasis[10]. CD73 is widely expressed in a variety of different tissues at a low level, including colon, brain, lung, heart, kidney, liver and some immune system cells [11, 12]. However, under some pathological situations, such as inflammation, tissue injury and hypoxia, CD73 has been identified to be overexpressed by the regulation of proinflammatory cytokines and then hydrolyzes AMP into adenosine[8, 13, 14].

As studies have reported, adenosine has immunosuppressive and pro-angiogenic functions[15]. In tumor microenvionment, adenosine accumulated and activated G protein-coupled receptors to regulate immune response and stimulate angiogenesis, which could further facilitate tumor progression. So CD73 plays a vital role in tumor development with its main enzymatic function[16, 17]. Besides the catalytic function, CD73 also has nonenzymatic function by participating in cell interactions. It could prevent tumor cells adhering to extracellular matrix (ECM) and inhibit tumor invasion and migration[18–20]. Taken together, the key function of CD73 makes it important role in tumor. It is abnormally expressed in breast cancer, colorectal cancer, ovarian cancer, melanoma, prostate cancer and so on. More importantly, in these cancers, high expression of CD73 was also associated with poor prognosis of patients and could act as a novel treatment target[21].

In the present research, we investigated the role of CD73 in NPC. We found that CD73 was highly expressed in NPC tissues and associated with patients' clinicopathology features and prognosis. Moreover, knocking down the expression of CD73 in NPC cells could inhibit cell proliferation and migration. Our research suggested that CD73 took an important role in NPC and might act as a potential target in NPC treatments.

Methods

Human NPC specimens

We obtained the paraffin-embedded NPC specimens from Affiliated Tumor Hospital of Nantong University. All the samples were confirmed by pathological diagnosis as nasopharyngeal squamous carcinoma. Patients in group were newly diagnosed without any special treatments (including radiation and chemotherapy), excluding organ dysfunction (including liver, kidney, cardiovascular, etc.) or concurrent with other malignant tumors. Non-cancerous samples in nasopharynx were used as controls. Patients' clinical information were shown in table 1. Fresh biopsy samples were reserved under -80 °C and all specimens got patients' informed consent. The research was approved by the Ethics Committee of Affiliated Hospital of Nantong University.

Immunohistochemistry

IHC was implemented as previously described[22]. Tissue sections were incubated with anti-human CD73 (Sangon Biotech, D121879, 1:50) and immunoreactivity of CD73 was visualized with 3, 39-diaminobenzidine tetrachloride (DAB) chromogenic solution. IHC scores were evaluated by two

pathologists without knowing patients' clinicopathological outcomes. The intensity of CD73 staining was scored as 0, no staining;1, weak staining;2, medium staining;3, strong staining. The percentage of immunopositive cells was scored as 0, <10%; 1,10–25%; 2,26–75%; 3, >75%. Then we summed the previous two scores and classified as: low expression group: 0-3, high expression group:4-6.

Cell lines

Cell lines we used included NPC cells CNE1(high differentiation), CNE2(low differentiation), 5-8F(high tumorigenesis and high metastasis), 6-10B(low tumorigenesis and low metastasis) and normal nasopharyngeal epithelial cells NP69. They were generously given by the Sun Yat-Sen University and Xiang-Ya School of Medicine. All the tumor cells were growing in RPMI 1640 (Biological Industries Israel Beit-Haemek, 01–100-1ACS) with10% fetal bovine serum (Biological Industries Israel Beit-Haemek, 04–001-1ACS), NP69 was cultured in Keratinocyte-SFM (Thermo Fisher Scientific, 17005–042). Cells were cultured at 37 °C in 5% CO_2 incubator.

Western Blot

We took western blot to detect protein expression of CD73. Tissues and cells were lysed with RIPA Lysis Buffer and protease inhibitors. After quantified the concentration of proteins with a Pierce Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific), the proteins were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then they were transferred to polyvinylidene difluoride membranes (Millipore, ISEQ00010, Bedford, MA). Membranes were incubated in fat-free milk and incubated with anti-CD73(Sangon Biotech, D121879, 1:500) overnight. In the next day, after incubating the membrane with HRP-tagged secondary antibody, we visualized the band with ECL reagent (Millipore).

Quantitative RT-PCR

Total RNA was extracted with Trizol reagent (Thermo Fisher Scientific, 15596018) and then it was reverse transcribed into cDNA. We performed qRT-PCR with SYBR Green PCR Master Mix (Roche, 04913914001) in a Real-Time PCR System (StepOne Plus, Applied Biosystems, Grand Island, NY) with the following primers:CD73: forward: 5'- TGCCCAGTTATGACCCTCTC -3', reverse: 5'- GGAACCCATCTCCACCATTG -3'. The results were normalized with GAPDH and relative mRNA expression were quantified by $2^{-\Delta\Delta Ct}$ method.

Transfection with siRNAs

Cells were cultured in plates at an appropriate density, then they were transfected with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. CD73-siRNAs were designed and obtained from Biotechnologies Co, Ltd (Nantong, China). CD73-siR1, Sense: 5'-GGAUACACUUCCAAAGAAAdTdT-3', Antisense: 5'-UUUCUUUGGAAGUGUAUCCdTdT-3'; CD73-siR2: Sense: 5'-CCUGAAGUAGAUAAGUUAAdTdT-3', Antisense: 5'-UUAACUUAUCUACUUCAGGdTdT-3'; CD73-siR3: Sense:

5'-GAAGAUCGAGUUUGAUGAAdTdT-3', Antisense: 5'-UUCAUCAAACUCGAUCUUC dTdT-3'; CD73-siR4: Sense: CGGAUGAAAUGUUCUGGAA dTdT-3', Antisense: 5'-UUCCAGAACAUUUCAUCCG dTdT-3'.

CCK8

NPC cells were transfected with CD73-siRNA or non-specific siRNA, then they were seeded into a 96-well plate (Corning inc, Corning NY) at the density of 5000 cells per well. At different time points, 10ul CCK8 solution (Beyotime Institute of Biotechnology, China) was added to each well. Optical density(OD) was accessed with a microplate reader at 450 nm.

Colony formation assay

500 cells which had transfected with si-CD73 or si-NC were seeded on 6-well plates. After incubation for 10 days, cells were fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet solution. Colonies that contain more than 50 cells were counted.

Transwell

5×10^4 pretreated cells (transfected with si-CD73 or si-NC) were resuspended in 200 ul serum-free medium and seeded into the upper chamber of an 8mm polycarbonate flitter (Millipore). The lower chamber was filled with culture medium with 10% FBS. Following the incubation of 18 h, cells in the upper surface were removed and cells adhering on the lower surface were fixed in 4% paraformaldehyde. The migration cells were visualized under a microscope after staining with 0.1% crystal violet.

Wound healing assay

CNE2 or CNE1 cells transfected with si-CD73 or si-NC were seeded on 6-well plates. When cells confluence reached about 80%, the scratches were created with a 100 ul pipette tip. Then cell culture medium was replaced with serum-free medium. At different time points, the migration distance of cells was captured. Percentage of wound width was calculated by the wound width/the distance measured at 0 h.

GEPIA2 Analysis

To analyze the expression level of CD73 (*NT5E*) mRNA in Head and Neck squamous cell carcinoma (HNSC), we used GEPIA2(Gene Expression Profiling Interactive Analysis) database (http://gepia2.cancer-pku.cn/), which is a publicly available web server for analyzing RNA sequencing expression from TCGA[23].

Statistical Analysis

All the experiments were repeated 3 times and data presented as mean±standard deviation (SD). Statistical analysis was performed with GraphPad Prism 6 and SPSS17.0. Two-tailed student's t-tests were used to determine statistical significance in groups and $\chi 2$ test tests were used to evaluate the correlation of CD73 expression level with clinicopathological features. Kaplan-Meier curves was

constructed for survival analysis, and log-rank test was performed. *P* value less than 0.05 was considered statistically significant.

Results

High expression of CD73 in NPC tissues

First of all, we detected the expression of CD73 in NPC by IHC. The results showed that in normal nasopharyngeal epithelial tissues, CD73 was absent or lower expressed, while in NPC tissues, it was highly expressed (Figure 1A). Then we obtained fresh tissues to evaluate the protein and mRNA levels of CD73. Western blot and qRT-PCR showed that it was notably higher in NPC tissues (Figure 1B-C). In addition, as NPC is one of the most common tumors in head and neck, we also analyzed high throughput Head and Neck squamous cell carcinoma (HNSC) RNA expression profile datasets from The Cancer Genome Atlas (TCGA). And in accordance with our research in NPC, CD73 was also highly expressed in HNSC than normal tissues, the difference is statistically significant ($P<0.05$) (Figure 1D). Taken together, CD73 was overexpressed in NPC.

Clinicopathologic significance of CD73 expression in NPC

We then analyzed IHC staining data from 50 NPC samples. It showed that CD73 could be observed in membrane and cytoplasm of tumor cells. In most NPC tissues, CD73 was highly expressed, and with the increasing of tumor stage, its expression level was increased (Figure 2A). Next, we further explored the clinicopathologic significance of CD73 in NPC. 50 samples were divided into high(n=29) and low(n=21) groups according to the staining level of CD73. The clinicopathologic features of patients were summarized in Table 1. Our statistical results showed that CD73 expression was related to patient's clinical stages, lymph node metastasis and survival status ($P < 0.05$), while there was no significant with gender or age ($P > 0.05$) (Table 1).

High expression of CD73 indicated poor prognosis of NPC patients

Survival analysis with follow-up data from 50 NPC patients was taken to evaluate the survival difference. Kaplan-Meier overall survival curve showed that the prognosis of patients with high CD73 expression were poorer than patients with low CD73 expression (Figure 2B). Moreover, we also checked the GEPIA based on the follow-up data and TCGA datasets of HNSC RNA expression, the results showed that high *NT5E* expression was associated with poor overall survival of HNSC patients (Figure 2C).

Knocking down the expression of CD73 inhibited proliferation of NPC cells

Cell proliferation and migration play key roles in tumor progression and are closely related to the prognosis of tumor patients, we further explored the role of CD73 on NPC cells. Firstly, we took western blot and qRT-PCR to detect CD73 expression in 4 NPC cell lines (CNE1, CNE2, 5-8F, 6-10B) and normal nasopharyngeal epithelial cells NP69. The results showed that all NPC cell lines had higher expression of CD73 than NP69, both in protein and mRNA levels. Moreover, the highest expression level was found in

CNE2 cells, which was the most common pathological pattern of NPC. We did further research on both CNE2 and CNE1 cells (Figure 3A-B). To detect the function of CD73 on tumor cells, we designed siRNAs targeting CD73 and siRNA2 had the highest knocking down efficiency in both CNE2 and CNE1 cells, so we choose it for subsequent experiments (Figure 3C).

CCK8 showed that when CD73 was knocked down in NPC cells, cell proliferation was inhibited compared with cells transfected with negative control siRNA (Figure 3D). Moreover, we also found the number of cell colonies was reduced when CD73 was downregulated (Figure 3E-F). It suggested that knocking down the expression of CD73 notably decreased cell growth.

Knocking down the expression of CD73 inhibited migration of NPC cells

We then detected the role of CD73 on cell migration by Transwell and wound-healing assays. As shown in Figure 4A-B, migrated cells were reduced in CD73-downregulated group compared with cells transfected with negative control siRNA. Similarly, the wound was harder to heal when CD73 was knocked down (Figure 4C-D). EMT is a process that plays important roles in tumor metastasis, we next detected EMT-associated markers in CD73-downregulated cells. We found in CNE2 and CNE1 cells, when CD73 was knocked down, epithelial marker E-cadherin was up-regulated, while mesenchymal marker N-cadherin was down-regulated. To sum up, CD73 played important roles in accelerating migration of NPC cells.

Disscussion

Nasopharyngeal carcinoma is one of the most common malignance in head and neck. Easy to metastasize in early stage is the main feature of NPC[3, 24]. Although with the development of radiation and chemotherapy, local control of NPC has been improved obviously, the molecular mechanism is still need to be further investigated. At present, studies have shown that abnormal gene expression participates in tumor progression, thus, exploring these abnormal expression genes might help us find markers for NPC diagnose and the judgment of prognosis.

CD73 is regarded as an important regulatory molecule in tumor progression, it is highly expressed in a variety of tumors, including breast cancer, glioblastoma, colorectal cancer and so on[25]. One of the most important results of this research was that we found CD73 was highly expressed in NPC tissues, both in protein and mRNA levels (Fig. 1A-C). What's more, we also analyzed data from 50 patients and found that CD73 expression was associated with clinical stages, lymph node metastasis and the survival status of patients (Fig. 2A, Table 1). These findings were consistent with the previous research. In a large cohort of papillary thyroid carcinoma patients, IHC was taken on tissue microarrays of 511 patients. The results showed that high expression of CD73 was associated with the aggressive histologic variant, extra thyroidal extension and lymph node metastasis of the patients[26]. Similarly, CD73 expression was correlated with clinicopathologic characteristics of renal cell carcinoma and breast cancer[27].

In a previous research in HNSC, CD73 was significantly overexpressed, it might act as a poor prognostic marker of patients and could activate EGF/EGFR signaling to promote tumorigenesis[28]. In our research, we detected *NT5E* expression with TCGA datasets of HNSC RNA expression, and found *NT5E* was increased in HNSC (Fig. 1D). The prognosis was also poorer in CD73 high-expression group (Fig. 2C). As NPC is one of the most common tumors in head and neck, CD73 was also found to be highly expressed in it in a recent research, scientists reported that mesenchymal stem cells-derived IL-6 could promote NPC progression via upregulating CD73 expression [29]. But it remains elusive whether CD73 plays a role in NPC progression. In our research, we took analysis of 50 NPC patients' prognosis and found the survival time of patients with higher expression of CD73 was decreased (Fig. 2B). These findings indicated CD73 might act as a potential biomarker in NPC.

Tumor malignant behaviors such as cell proliferation took a vital role in tumor progression. CD73 is a key enzyme to produce adenosine and studies have reported that adenosine may promote cancer cell proliferation[30]. In breast cancer, the accumulation of adenosine could interact with adenosine receptors and accelerate tumor cells proliferation[31]. We also investigated the function of CD73 in NPC in vitro. After knocking down CD73, cells proliferation rate was inhibited (Fig. 3D-E). So CD73 might promote cells proliferation in NPC cells.

Metastasis is a vicious feature of tumor and could dominate cancer-related deaths[32]. In gastric cancer, highly expression of CD73 was found in patients with more progressive pathological features or metastatic properties. Further mechanism study also showed that CD73 could promote tumor metastasis by modulating RICS/RhoA signaling and epithelial-to-mesenchymal transition process[33]. In melanoma, scientists found that the enzymatic activity of CD73 might influence the invasion of tumor cells, while cell migration could be influenced by the non-enzymatic function[19]. In our research, we took transwell and wound healing assays and found CD73 could promote the metastasis of NPC cells (Fig. 4A-C). Moreover, we found CD73 could regulate cell migration via EMT (Fig. 4E), but the internal mechanism is still need to be further investigated.

Several agents have taken to target CD73 in tumors. Scientists used small molecular inhibitors or monoclonal antibodies targeting CD73. In ovarian cancer, blocking CD73 could increase cytotoxicity of alloreactive primed T cells by blocking adenosine-dependent immune evasion[34]. Azambuja et al. constructed a cationic nanoemulsion to nasal delivery of siRNA CD73 (NE-siRNA CD73) and the results showed that nasal administration of NE-siRNA CD73 exhibits higher anti-glioma effects[35]. In a word, targeted blockade of CD73 might be a therapeutic strategy for tumor patients.

Conclusion

Our study verified that CD73 was highly expressed in NPC and associated with poor prognosis of patients. Besides, it played important roles in NPC malignant behaviors. So it might be a novel molecular target for the diagnose and treatment of NPC.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by BJ, MMT, SS, HJX, LZ, JPS. SP performed statistical analysis. The first draft of the manuscript was written by BJ, MMT and SS. JPS and LZ contributed to the project discussion and manuscript revision. All authors read and approved the final manuscript.

Ethics approval

The research was approved by the Ethics Committee of Affiliated Hospital of Nantong University and in accordance with the 1964 Declaration of Helsinki.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publish

The authors affirm that human research participants provided informed consent for publication of the images resulted from statistical analysis.

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Tables

Table 1
The association between the expression of CD73 and clinicopathological parameters of NPC

Clinicopathological parameters	Total	CD73 Expression		*P*
		Low	High	
Gender				0.724
Male	37	15	22	
Female	13	6	7	
Age (year)				0.126
60	32	16	16	
≥ 60	18	5	13	
Clinical stages				0.046*
1	3	2	1	
2	19	12	7	
3	21	6	15	
4	7	1	6	
Lymph node metastasis				0.047*
N0-N1	30	16	14	
N2-N3	20	5	15	
Survive				0.007*
Death	23	5	18	
Alive	27	16	11	
*Statistical analyses were performed by the Pearson χ2 test. $p < 0.05$ was considered significant.				

Figures

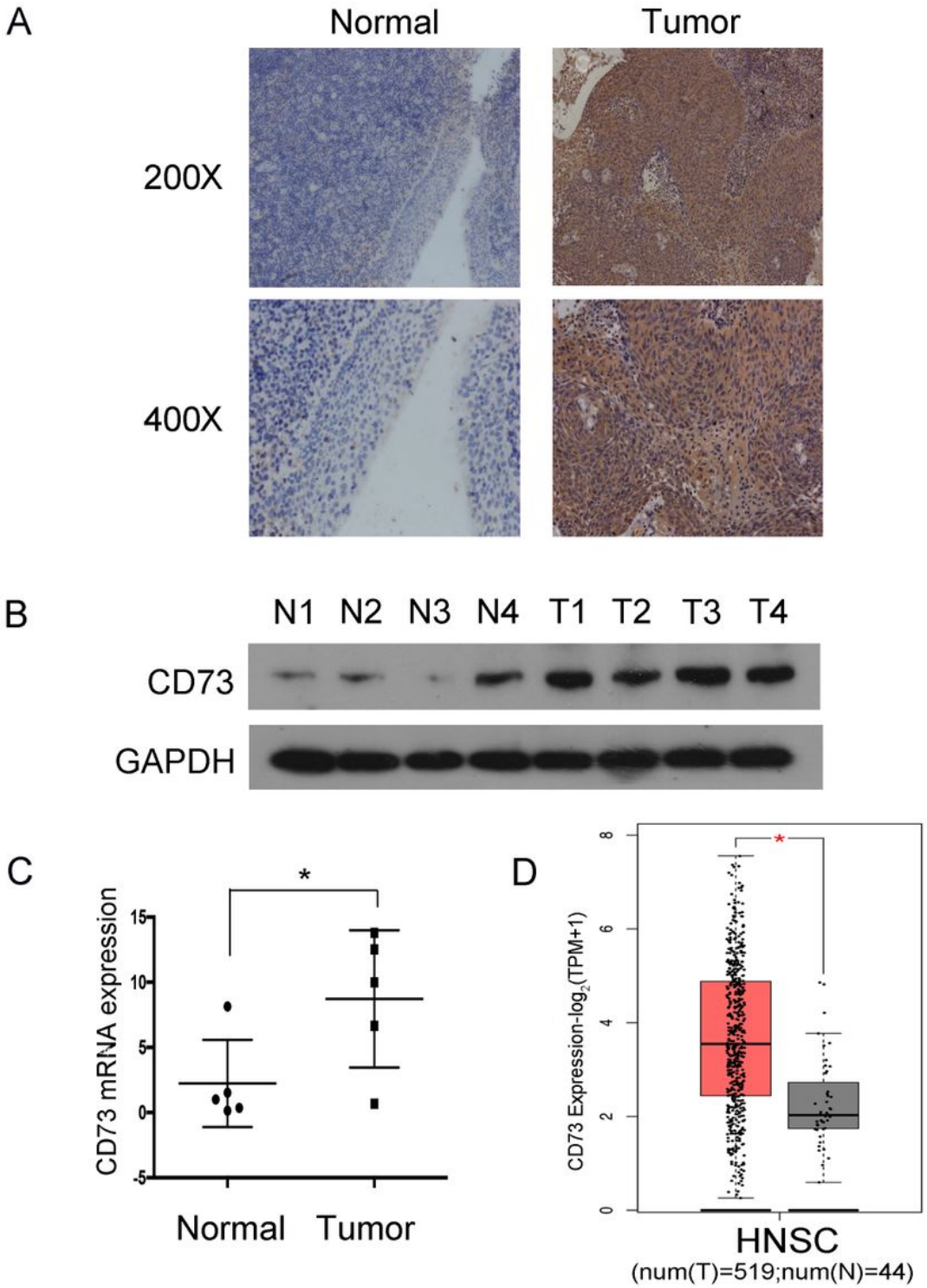

Figure 1

High expression of CD73 in NPC tissues. A: Immunohistochemical detection of CD73 expression in NPC tissues and nasopharyngeal epithelial tissues. Normal: nasopharyngeal epithelial tissues. Tumor: nasopharyngeal squamous carcinoma tissues. B: Western blot was used to detect CD73 protein expression. T: nasopharyngeal carcinoma tissues. N: nasopharyngeal epithelial tissues. C: qRT-PCR showed the mRNA expression in cases of NPC tissues and nasopharyngeal epithelial tissues. D: The

expression of CD73 in HNSC tissues from TCGA (T=519; N=44). The results were obtained from the GEPIA web tool. Data represented as mean ± SD. * $P < 0.05$.

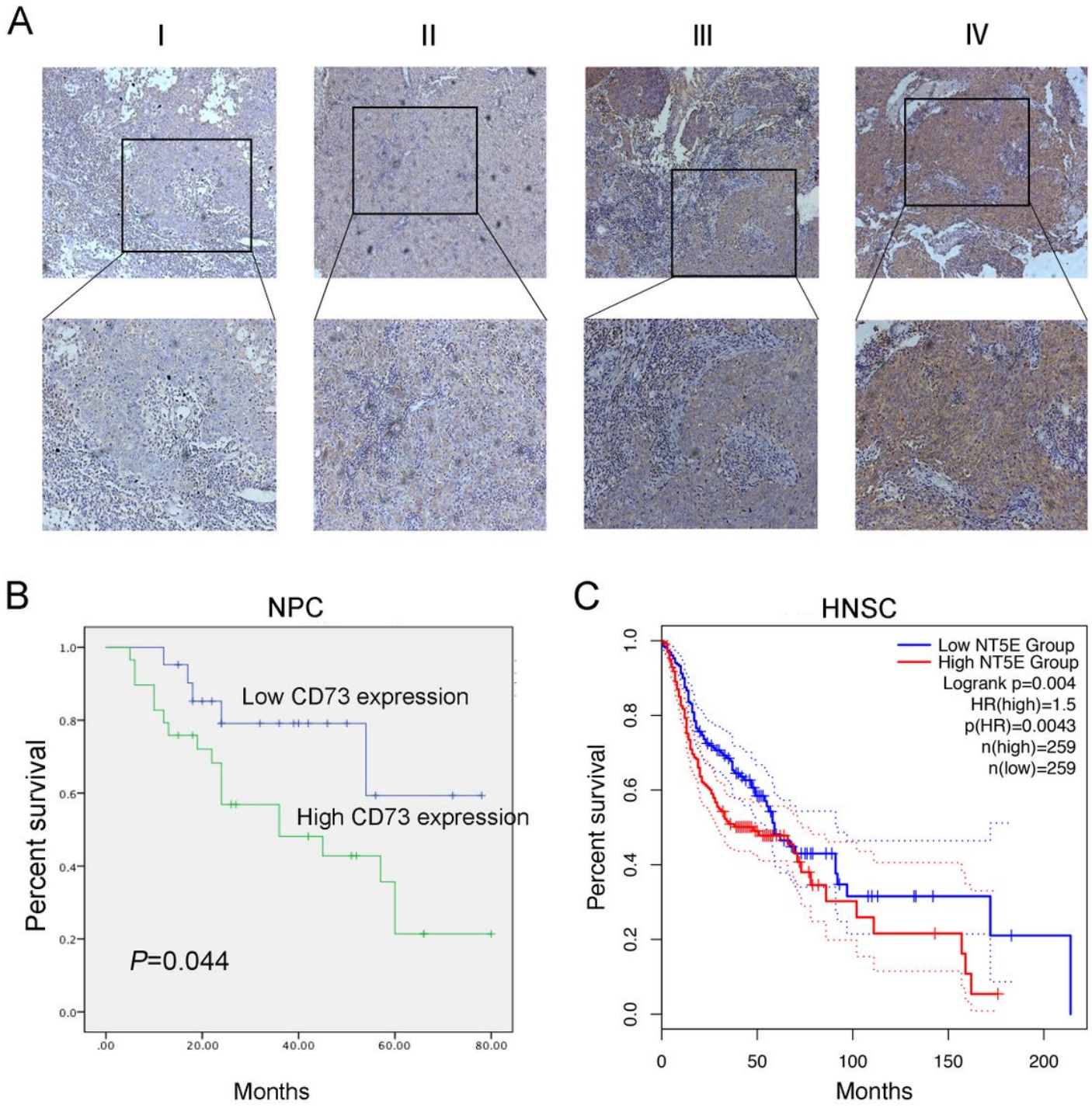

Figure 2

High expression of CD73 indicated poor prognosis of NPC patients. A. Immunohistochemical detection of CD73 expression in NPC tissues of different clinical stages (Ⅰ, Ⅱ, Ⅲ, Ⅳ). Down line: High-powered view of the

rectangular areas in the up line. B: Survival analysis of CD73 in NPC by Kaplan–Meier overall survival curve with log-rank test. Survival differences are compared between patients with high and low CD73 expression: high(n=29) and low(n=21). C: Survival curves for the HNSC patients with different CD73 expression: high(n=259) and low(n=259). The result was obtained from GEPIA based on TCGA datasets.

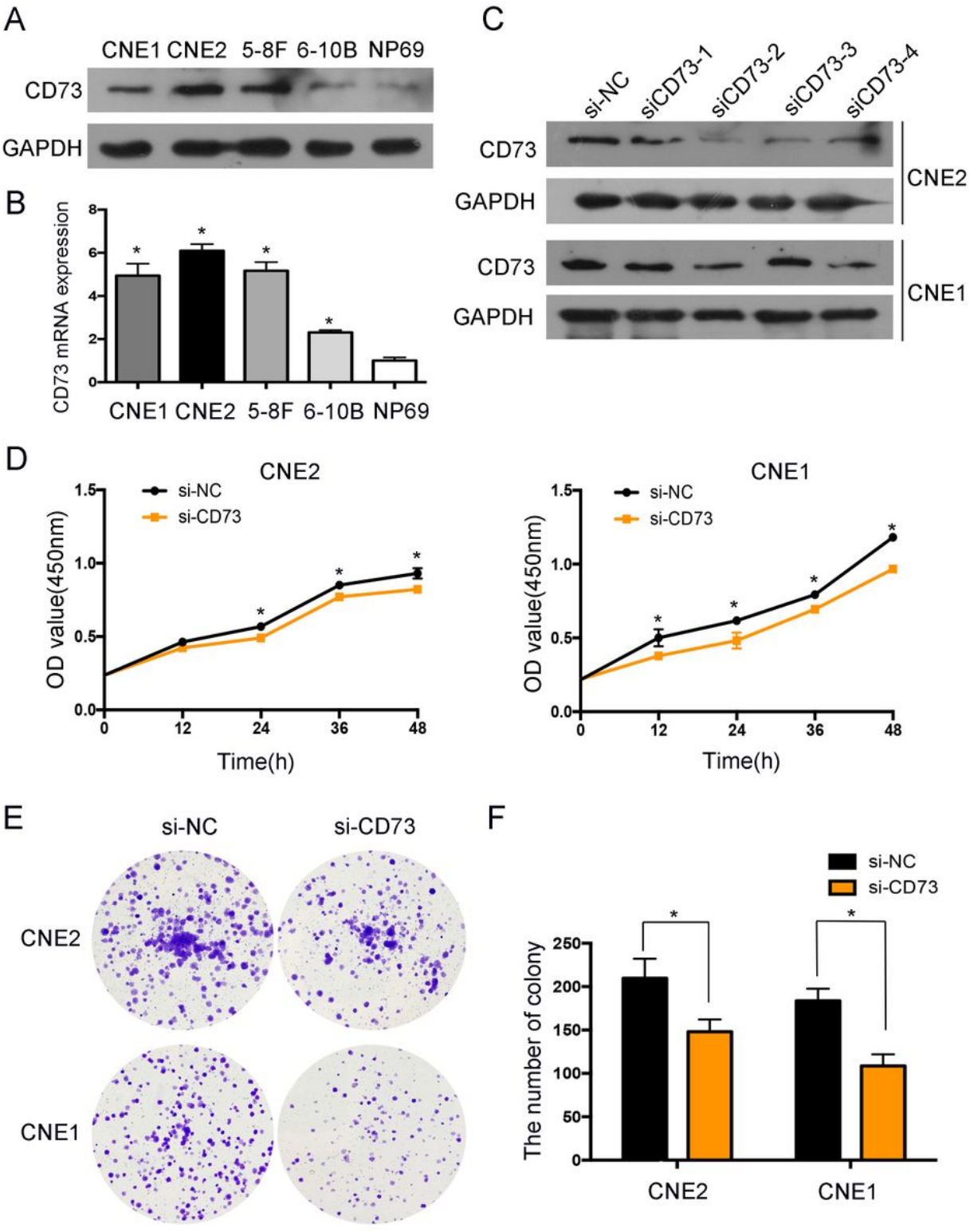

Figure 3

Knocking down the expression of CD73 inhibited proliferation of NPC cells. A: Western blot was used to detect the expression of CD73 in NPC cell lines (CNE1, CNE2, 5-8F, 6-10B) and NP69. B: qRT-PCR showed the mRNA expression of CD73 in NPC cell lines (CNE1, CNE2, 5-8F, 6-10B) and NP69. C: Western blot showed the knockdown efficiency after the treatment of different siRNAs in CNE2 and CNE1 cells. D: CCK8 assay was used to detect cell proliferation after NPC cells transfected with si-CD73or si-NC. E: Colony formation were performed after NPC cells transfected with si-CD73or si-NC. F: The histogram showed the number of colonies in colony formation assay. Data represented as mean ± SD. * $P < 0.05$.

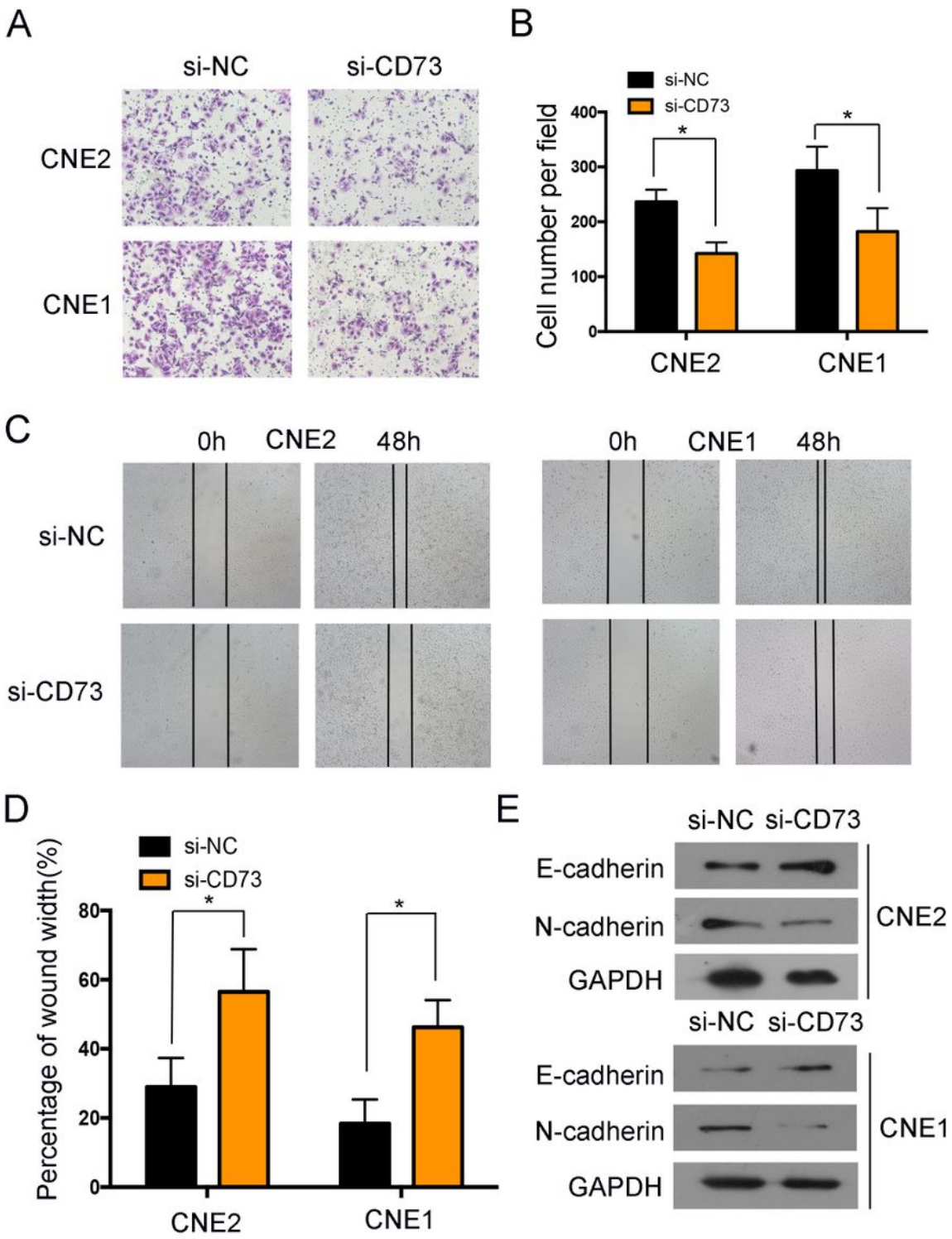

Figure 4

Knocking down the expression of CD73 inhibited migration of NPC cells. A: Transwell migration assay showed migrated cells were reduced when CD73 was knocked down. B: The histogram showed the number of migrated cells in Transwell migration assay. C: Wound healing assay showed cells treated with si-CD73 migrated slower than control group. Representative images of cells migration were shown at 0 and 48 h with a microscope. D: The histogram showed the relative migrated width. It was calculated by

the wound width/the distance measured at 0h. E: Western blot showed that the expression of E-cadherin and N-cadherin when CD73 was knocked down in NPC cells. Data represented as mean ± SD. * $P < 0.05$.