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Two polymorphisms of CD44 3'UTR weaken the binding of miRNAs and associate with nasopharyngeal carcinoma in a Chinese population

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Abstract. – OBJECTIVE: CD44 is a member of adhesion molecule families whose function is closely associated with cancer cell metastatic spread and drug resistance. CD44 is expressed as a wide variety of isoforms but 3' untranslated region (3'UTR) is conserved relatively and it is confirmed that more than ten miRNAs regulate the expression of CD44. The aim of this paper is to investigate the association between polymorphisms existed in CD44 3'UTR and nasopharyngeal carcinoma (NPC).

PATIENTS AND METHODS: We scanned 1.5kb of CD44 3'UTR in a 287 patients and 507 controls Chinese Han population. After statistical analysis we found that the minor alleles of rs13347 C/T and rs115214213 T/C are associated with NPC (OR = 1.94 95% CI = 1.44-2.62; OR = 2.07 95% CI = 1.65-2.60). By using bioinformatics tools, we found that rs115214213 may exist in the target region of miR-590-3p. Subsequently, we confirmed that miR-590-3p can repress CD44 expression and promote the apoptosis of CNE2 cells induced by cisplatin in vitro. After that, our transient transfection focusing on reporter gene expression modulated by CD44 3'UTR demonstrated that the presence of an rs13347T allele and rs115214213C allele led to greater transcriptional activity than the rs13347C allele and rs115214213T allele. Similarly, more CD44 expression was shown in minor allele carriers in our western blotting results.

CONCLUSIONS: All these findings suggest that CD44 rs13347C>T and rs115214213T>C polymorphisms may affect NPC development by improving CD44 expression.

Keywords:
CD44, Nasopharyngeal carcinoma, Polymorphism, microRNA.

Introduction

Nasopharyngeal carcinoma (NPC) is a disease with distinct ethnic and geographic distribution which is extremely common in South China with a reported annual incidence of about 20 per 100,000 people in endemic regions[1]. This phenomenon is partially due to the Southeast Asian diet, which typically includes consumption of salted vegetables, fish and meat[2]. Besides, the etiology of nasopharyngeal cancer also includes Epstein-Barr virus (EBV) infection and genetic susceptibility[3,4].

CD44 is a major hyaluronan (HA) receptor and HA- CD44 interactions have a central role in receptor tyrosine kinase (RTK)-induced activation of anti-apoptotic pathways and actively promote tumor cell and possibly cancer initiating cells (CICs) survival through their associations with multidrug resistance genes. By cell-extracellular matrix and cell-cell adhesive interactions, CD44 plays an indispensable role in tumor pathology, involved in cell differentiation, invasion and metastasis[5-7]. Also, strong association between CD44 expression and NPC risk has been reported in some studies (8,9). Recently, Cai et al[10] reported that CD44 was upregulated in NPC patients and a normal polymorphism in 3' untranslated region (3'UTR) of CD44 is associated with NPC development which gives evidence about genetic association between CD44 and NPC.

CD44 is expressed as a wide variety of isoforms but 3'UTR is conserved relatively between isoforms which is about 3000 nucleotides long. The 3'UTRs of genes are the main regions recognized by microRNAs and there are reports that the expression of CD44 is regulated by miR-509, miR-373, miR-520c, miR-34a, miR-216, miR-330, miR-608 and miR-328[10-13]. So we hypothesize that CD44 3'UTR genetic variations can theoretically disturb the expression of CD44 may affect individuals' risk of NPC.

In this study we carried out a hospital-based case-control study including 287 patients with NPC and 507 cancer-free controls to investigate association between these polymorphisms and

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NPC risk. After scanning the 1.5kb part of 3'UTR of CD44, we found two polymorphisms are associated with NPC significantly. These two site reduce the expression suppression effect of miR-509-3p and miR-590-3p and increase CD44 protein expression. These findings suggest that CD44 3'UTR polymorphisms may affect NPC development by improving CD44 expression.

Patients and Methods

Study population, tissue samples and cell Lines

This study contains 287 Han-Chinese NPC patients and 507 ethnically matched cancer-free controls. Patients with newly diagnosed primary NPC and healthy controls are all from Zhejiang Province in Eastern China and consecutively recruited from 2011 to 2013 at the Second Affiliated Hospital Zhejiang University School of Medicine. EBV capsid antigen immunoglobulin A antibodies (EB-VCA-IgA) and immunoglobulin-A antibodies to EBV early antigen were confirmed by serologic testing at the time of study enrolment.

Samples of NPC tissues were obtained from patients undergoing surgery and were frozen immediately after surgical resection. Western blotting assays were performed to analyze the correlation between rs13347 C>T and rs115214213 T>C polymorphisms in 3'UTR of CD44 and the protein expression levels in NPC tissues. NPC tissues were washed with phosphate-buffered saline (PBS), and total tissue extracts were made by using a detergent lysis buffer. Sixty micrograms of total proteins were used for western blotting assays.

Human NPC cell lines CNE1 (human nasopharyngeal high differentiated squamous epithelium carcinoma cell), CNE2 (human nasopharyngeal low differentiated squamous epithelium carcinomas cell) and EBV-positive NPC cell line C666-1 were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 5% glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

DNA collection and genotyping

DNA from blood samples were extracted by using TIANamp Blood DNA Kit (TIANGEN, Beijing, China). DNA specimens were amplified by using standard PCR protocols. The PCR products were sequenced in forward direction with the ABI 3730xl sequencing platform. The sequencing results were analyzed by using DNAMAN and Chromas Lite software.

Dual luciferase assay

1500 nucleotide long CD44 3'UTR contain miR-509-3p and miR-590 were cloned into downstream of firefly luciferase coding region in pmirGLO vector (Promega, Madison, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, CNE1, CNE2 and C666-1 cells were seeded in 24-well plates. MiR-509-3p, miR-590 mimics and negative controls were co-transfected by using lipofectamine 2000 (Invitrogen, Carlsbad, CA USA). Two days later, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI, USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

Western blotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 60 μg samples were loaded into each lane of 8% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-E6 polyclonal antibody (Abcam, Cambridge, MA, USA), mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 37°C. The specific protein antibody complex was detected by using horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-mouse IgG. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

MTT assay

CNE2 cells were seeded in 96-well plates at low density (5x103) in Dulbecco's Modified Eagle Medium (DMEM) culture, and allowed to attach overnight. The cells were then transfected with miR-590-3p mimic or with nonsense short RNA as control. Twenty microliters 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

bromide (MTT) (5mg/ml) (Sigma, St. Louis, MO, USA) were added into each well 48h after transfection, and the cells were incubated for further 4h. The absorbance was recorded at A570 nm with a 96-well plate reader after the dimethyl sulfoxide (DMSO) addition

Detected the serum CD44 concentration by enzyme-linked immunosorbentassay (ELISA)

Serum CD44 levels were detected by using sandwich ELISA method and rabbit and mouse anti-hCD44 antibodies (Santa Cruz Biotechnology Inc.). The relative concentrations were compared using optical density (OD) value directly.

Statistical analysis

Chi square tests were used to examine the differences in the distributions of genotypes between cases and controls. The association between the CD44 polymorphisms and risk of NPC was estimated by odds ratios (ORs) and their 95% confidence intervals, which were calculated by unconditional logistic regression models.

The differences in the luciferase reporter activity and normalized expression values of CD44 in cancer tissue between each allele were analyzed by one-way ANOVA. The tests were all two-sided and analyzed using SPSS statistical Package version 16 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistical significant.

Results

Genotypes and risk of NPC

According to the dbSNP database (build 137), there are more than forty SNPs in the 3'UTR of CD44. The confirmed miRNAs recognized regions mainly exist in the 3' end of CD44 mRNA, so we scanned the 1500bp region near the end of CD44 mRNA.

Only five SNPs were found in this population in this region and the genotype frequencies of these five polymorphisms in controls conformed to the Hardy-Weinberg equilibrium ($p = 0.64$ for rs115214213, $p = 0.91$ for rs13347, 0.66 for rs79220468, 0.35 for rs11821102 and 0.85 for rs10836347). After statistical analysis, we found that the rare alleles of these two SNPs (rs13347 T>C and rs115214213 C>T) are associated with human NPC significantly (OR = 1.94 [1.44, 2.62] $p < 0.01$; OR = 2.07 [1.65, 2.60] ($p < 0.01$) (Table I).

Rs115214213 exist in the binding site of miR-590-3p which represses CD44 expression

Because there are reports that the allele C of rs11347 can reduce the repression effect of miR-509 on CD44 expression and associated with breast cancer, so we focus on the function of rs115214213. Predicted by using bioinformatics tools, we found that rs115214213 existed in the predicted binding region of miR-590-3p (Figure 1A), but there are no reports that miR-590-3p can repress CD44 expression. So the first hypothesis we examined was that miR-590-3p may repress the expression of CD44 by directly binding to the 3'UTR and rs115214213 exist in the binding region.

As shown in Figure 1A, the 1.5kb 3'UTR of CD44 that contain the predicted miR-590-3p binding region was cloned into the pGL3 control plasmid, behind firefly luciferase coding region, for dual luciferase assay. HEK293T cells were co-transfected with pGL3-CD44 and miR-590-3p mimics or inhibitor (Figure 1B left). Compared with the miRNA control, the luciferase activity was significantly suppressed by the miR-590-3p, about 46.4% ($p < 0.01$). Furthermore, the luciferase activity was significantly upregulated by the miR-590-3p inhibitor compared with the anti-miR control, about 41.1% ($p < 0.05$). These results indicate that miR-590-3p targets the 3'-UTR of CD44, leading to the change of firefly luciferase translation.

Seed sequence mutation clone was also used to further confirm the binding site for miR-590-3p (Figure 1A). Putative miR-590-3p binding region in the 3'-UTR of CD44 with 3 mutant nucleotides (designated as pGL3-CD44-Mu) was constructed. The histogram in Figure 1B (right) showed that the enzyme activity was not different between miR-590-3p mimic and miRNA control. These data indicate that miR-590-3p may suppress CD44 expression through binding to seed sequence at the 3'-UTR of CD44 directly.

To further confirm that miR-590-3p can repress endogenous CD44 expression, CNE2 cells were transfected with miR-590-3p mimics or inhibitor to see whether the dysregulation of miR-590-3p expression affected endogenous CD44 expression. Compared with corresponding control, the level of CD44 protein was significantly suppressed by miR-590-3p mimics and up-regulated by miR-590-3p inhibitor (Figure 1C), which indicated that CD44 is a target gene of miR-590-3p.

Table I. Genotype frequencies of the five SNPs in the CD44 gene in patients and controls and their association with NPC.

Genotype	Patients (287), n%	Controls (507), n%	OR (95% CI)	p
rs115214213 T>C				
C	99 (0.18)	100 (0.10)	OR = 1.94 [1.44, 2.62]	<0.01
T	453 (0.82)	888 (0.90)	OR = 0.52 [0.38, 0.70]	
C C	16 (0.058)	6 (0.012)	OR = 5.01 [1.94, 12.95]	<0.01
C T	67 (0.24)	88 (0.18)	OR = 1.48 [1.03, 2.12]	
T T	193 (0.70)	400 (0.81)	OR = 0.55 [0.39, 0.77]	
rs13347 C>T				
C	334 (0.61)	750 (0.77)	OR = 0.48 [0.39, 0.61]	<0.01
T	210 (0.39)	228 (0.23)	OR = 2.07 [1.65, 2.60]	
CC	104 (0.38)	288 (0.59)	OR = 0.43 [0.32, 0.59]	<0.01
CT	126 (0.46)	174 (0.36)	OR = 1.56 [1.16, 2.11]	
TT	42 (0.15)	27 (0.055)	OR = 3.12 [1.88, 5.20]	
rs79220468 G>A				
G	508 (0.91)	875 (0.89)	OR = 1.32 [0.92, 1.88]	0.13
A	48 (0.086)	109 (0.11)	OR = 0.76 [0.53, 1.08]	
GG	233 (0.84)	390 (0.79)	OR = 1.35 [0.92, 1.99]	0.30
GA	42 (0.15)	95 (0.19)	OR = 0.74 [0.50, 1.11]	
AA	3 (0.011)	7 (0.014)	OR = 0.76 [0.19, 2.95]	
rs11821102 G>A				
G	531 (0.95)	932 (0.94)	OR = 1.18 [0.75, 1.86]	0.48
A	29 (0.052)	60 (0.061)	OR = 0.85 [0.54, 1.34]	
GG	252 (0.90)	439 (0.89)	OR = 1.17 [0.72, 1.88]	0.77
GA	27 (0.096)	54 (0.11)	OR = 0.87 [0.54, 1.42]	
AA	1 (0.0036)	3 (0.0060)	OR = 0.59 [0.061, 5.69]	
rs10836347 C>T				
C	525 (0.94)	931 (0.94)	OR = 1.07 [0.69, 1.68]	0.65
T	31 (0.056)	59 (0.060)	OR = 0.93 [0.60, 1.46]	
CC	249 (0.90)	438 (0.88)	OR = 1.12 [0.70, 1.79]	0.90
CT	27 (0.097)	55 (0.11)	OR = 1.79 [0.25, 12.75]	
TT	2 (0.0072)	2 (0.0040)	OR = 0.86 [0.53, 1.40]	

MiR-590-3p promotes the apoptosis of CNE2 cells induced by cisplatin in vitro

Since CD44 plays an important role in tumor pathology and drug resistance, to explore the biological function of miR-590-3p on NPC, we examined the effects of miR-590-3p on apoptosis in NPC cell lines by MTT and western blot. As shown in Figure 2, the CNE2 cell viability was significantly reduced after miR-590-3p transfection in the cisplatin treated group. In additon, the levels of activation of caspase 3 and PARP, the two typical characteristics of cell apoptosis, were examined by western blot analysis in CNE2 cells. As illustrated in Figure 2B, the cleaved caspase 3 and PARP were markedly increased in CNE2-miR-590-3p cells compared to that in CNE2-miR-control cells after treatment. These data revealed that miR-590-3p promotes cisplatin-induced apoptosis in NPC cells.

Two SNPs reduce miRNAs expression suppression effect on CD44

To detect the effect on the expression of CD44 by those two NPC related SNPs, we employed the dual-luciferase assay systems. Compared with pmirGLO-CD44-rs13347-C group, the relative firefly luciferase activity of pmirGLO-CD44-rs13347-T is up-regulated significantly in the presence of miR-509-3p in CNE1 cells ($p < 0.01$) (Figure 3A left). Meanwhile, compared with pmirGLO-CD44-rs115214213-T, the relative firefly luciferase activity of pmirGLO-CD44-rs115214213-C is also increased when co-transfected with miR-590-3p in CNE1 cells ($p < 0.05$) (Figure 3A right). These results indicated that miR-509-3p and miR-590-3p can reduce CD44 expression by target the 3'UTR of CD44 but the rs13347 C>T and rs115214213 T>C variation can reduce the expression suppression effect of these two miRNAs.

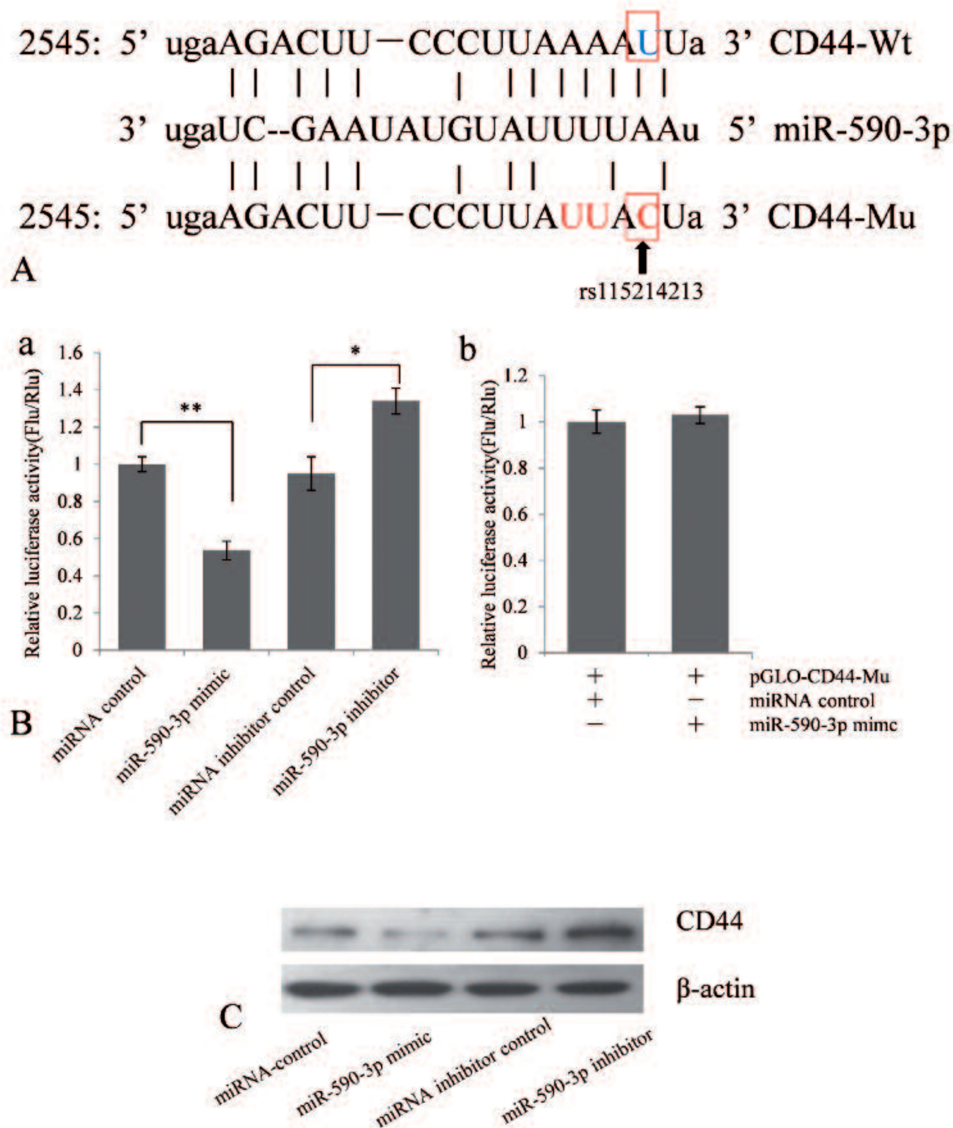

Figure 1. The expression of CD44 was repressed by miR-590-3p. **A**, Predicted miR-590-3p target site in CD44 3'UTR and mutant plasmid construction. **B**, Confirmation of the target gene of miR-590-3p. (a) HEK293T cells were co-transfected with miRNA control, miR-590-3p mimic, anti-miR control or miR-590-3p inhibitor and pGL3-CD44 for dual-luciferase assay. pRL-TK containing Renilla luciferase was co-transfected with 3'-UTR of CD44 for data normalization. (b) Mutation analysis of the miR-30a binding site. When 3 nucleotides of the binding site of miR-590-3p in the 3'-UTR of CD44 was mutated (pGL3-CD44-Mu), the luciferase activity was no significantly difference in HEK293T cells co-transfected with miR-590-3p mimics and pGL3-CD44 compared with pGL3-FOXL2-Mu.

To confirm the theory above, we test the relative luciferase activities when transfect different genotypes CD44 3'UTR containing reporter vectors into three different nasopharyngeal carcinoma cell lines. Except the result of rs115214213 variation genotype vector in CNE1 cells, these two sites variation (rs13347 C->T variation rs115214213 T->C) can increase the relative luciferase activity in these three nasopharyngeal carcinoma cells lines (Figure 3B, C). The result suggests that these two sites variation can increase CD44 expression by reducing the expression suppression effect of endogenous miRNAs.

Two sites variation altered the serum CD44 concentration in NPC patients

To investigate whether the two normal polymorphism sites can change the CD44 expression *in vivo*, we detected the CD44 concentration in different genotype NPC patients by ELISA. As

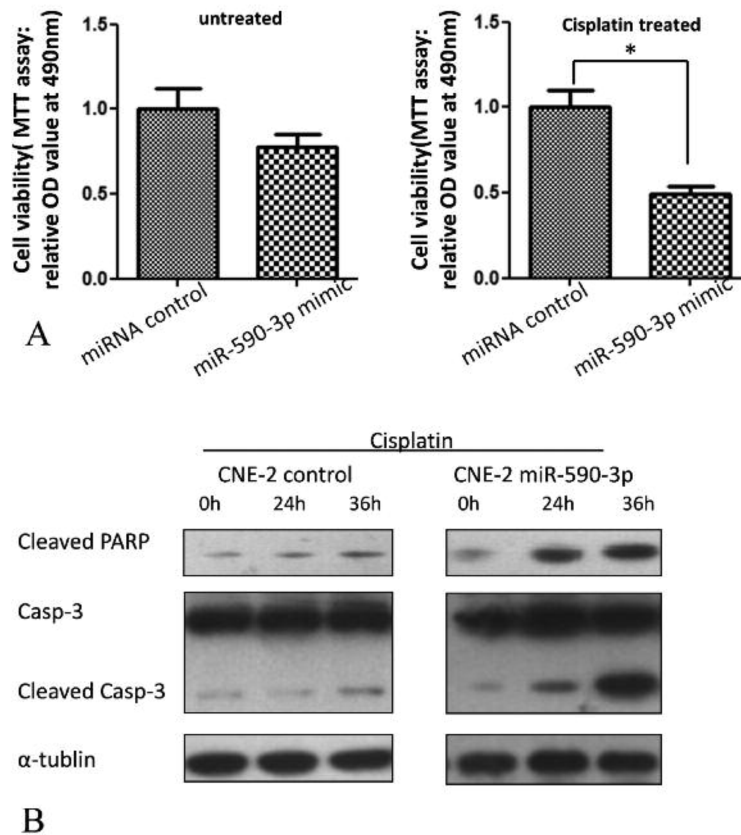

Figure 2. miR-590-3p promotes the apoptosis of CNE2 cells induced by cisplatin *in vitro.* (A) miR-590-3p transfected and negative control CNE2 cells were treated with cisplatin (IC50). MTT assay was used to detect the cell viability. (B) After treatment with cisplatin for the indicated times, the cleavages of caspase 3 and PARP was detected in miR-590-3p transfected and negative control CNE2 cells by Western blot analysis. $*p < 0.05$.

shown in Figure 4, the levels of CD44 protein in these two SNPs homozygous genotype people (CD44: CC-TT) was significantly low compared with heterozygous genotypes people (CD44: CT-TT or CD44: CC-CT or CD44: CT-CT) which indicated that the two SNPs: rs13347 and rs115214213 are related to controlling the serum CD44 concentration *in vivo*.

Discussion

MicroRNAs are a kind of short, non-coding RNA molecules whose function is mainly as post-transcriptional expression suppressors of protein-coding genes. Bioinformatics researchers estimated that >60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs[14]. Recent studies showed that miRNAs are involved in most biological and pathological processes, including development timing, cellular differentiation, proliferation, apoptosis, and especially tumorigenesis[15-17].

miRNAs disturb the expression of genes mainly by targeting 3'UTR of them directly. So theoretically, SNPs within a miRNA target could either weaken or reinforce the binding with miRNAs, thereby, altering the normal regulation of a given gene. And more and more evidence have been reported that variations in 3'UTR of genes are associated with human tumorigenesis and survival rate[18-20].

CD44 is a multiple function molecule that not only participates in some fundamental biological processes including lymphocyte activation and homing, cell migration, haematopoiesis, embryonal development and apoptosis but also is involved in neoplasm invasion, metastasis, clinical stage and drug resistance[5]. It has been reported that tumor cells with relatively high concentrations of CD44 proteins were capable of forming more aggressive tumors in animal experiments[21,22].

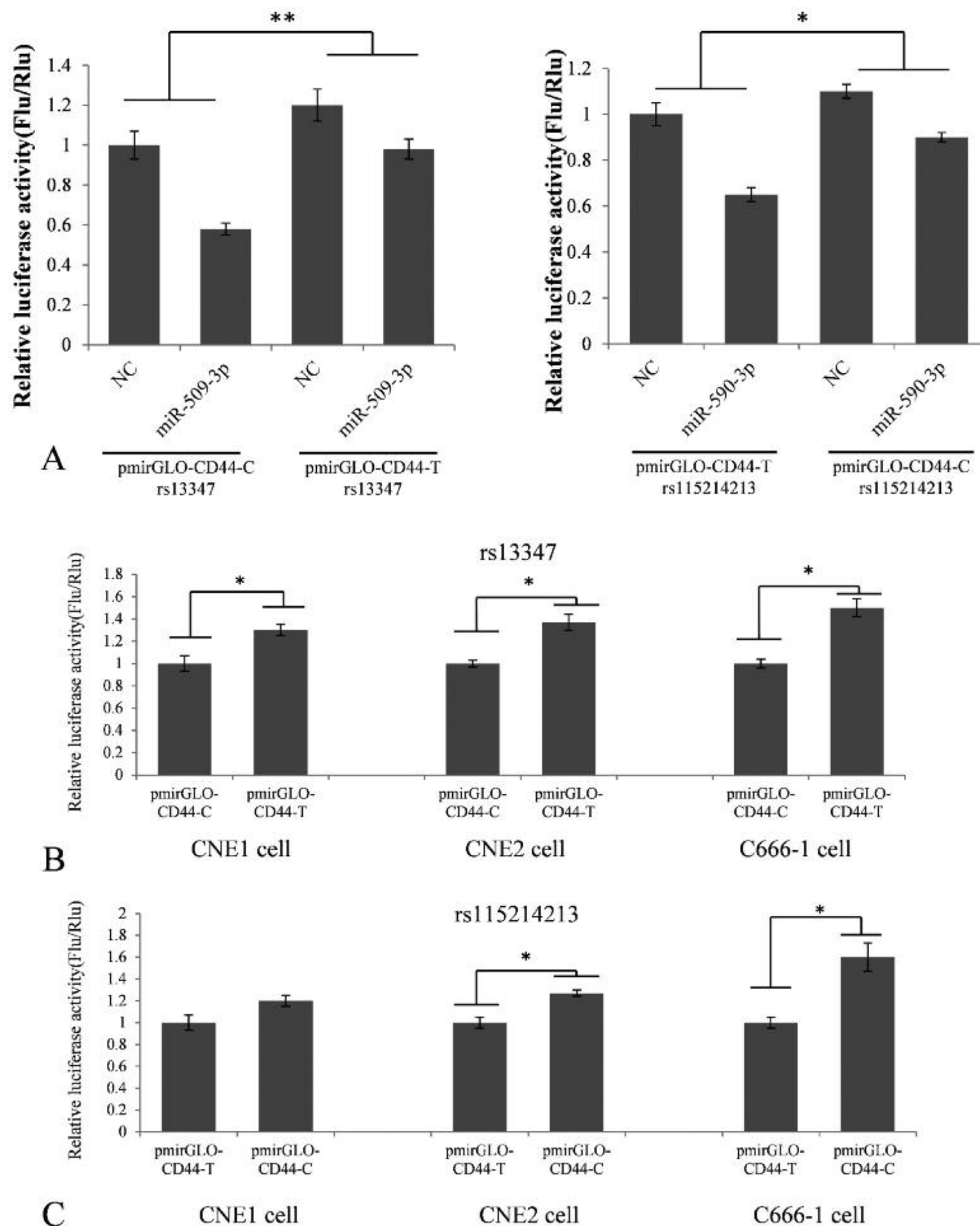

Figure 3. Detect the effect of two polymorphisms on CD44 expression by using dual-luciferase assay. (A) miR-509-3p or miR-590-3p were co-transfected with different genotype reporter plasmids into CNE1 cells, luciferase activities were detected 48h after transfection. (B) pmirGLO-CD44-rs13347C or pmirGLO-CD44-rs13347T plasmid transient transfected into three NPC cell lines. The luciferase activity represents the suppression effect on CD44 expression by endogenous miRNAs. (C) pmirGLO-CD44-rs115214213C or pmirGLO-CD44- rs115214213T plasmid transient transfected into three NPC cell lines. The luciferase activity represents the suppression effect on CD44 expression by endogenous miRNAs. Three replicates for each group and the experiment repeated at least three times.

Drug resistance is one of the major drawbacks in cancer therapy, and several lines of experimental evidence support the idea that apoptosis resistance is a feature of cancer initiating cells (CICs) rather than the mass of tumor cells[23,24]. In addition, there is a lot of evidence that the interaction of CD44, predominantly of CD44v with hyaluronan (HA), contributes to apoptosis resistance of CICs[25]. CD44 mediated activation of anti-apoptotic proteins is mainly initiated through the association with receptor tyrosine kinases (RTKs). It is confirmed that the association of CD44 with ERBB2 and ERBB3 mediates heterodimerization and the activation of the receptor in response to

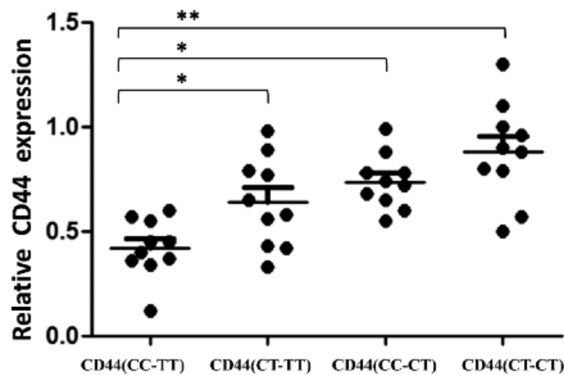

Figure 4. Detect serum CD44 concentration in different genotypes NPC patients by using ELISA. Serum CD44 level in 40 NPC patients from individuals who carried different rs13347 and rs115214213 genotypes were detected by ELISA. $*p < 0.05$. $**p < 0.01$.

neuregulin, which strongly promotes CIC apoptosis resistance[26,27]. The CD44–HA interaction initiates ERBB2 phosphorylation and the stimulation of HA production induces assembly of a lipid raft-integrated complex of ERBB2, CD44, ezrin, PI3K and the chaperone molecules HSP90 and CDC37.

The human CD44 gene is located in chromosomal locus 11p13. There at least l8 isoforms of CD44 coded by this region but the 3'UTR of CD44 mRNAs are relatively conserved. There are reports that expression of CD44 was regulated by miRNAs and rs11347 C>T variation can increase CD44 expression and associated with human NPC. In this study, we scanned 1.5kb of CD44 3'UTR which contains the most miRNAs recognition sites by sequencing. We find two normal polymorphism sites are associated with human NPC turmorigenesis. Except rs13347 C>T variation, which reported by Cai's group, we found rs115214213 T>C variation was also related to human NPC. After detecting dual-luciferase activity in three NPC cell lines and CD44 expression value in NPC tissues, we found that these two site variation can reduce the expression suppression effect of miR-509-3p and miR-590-3p. The minor alleles can increase CD44 protein expression.

Conclusions

Our study demonstrated a significant association between the CD44 rs13347 C/T, rs115214213 T-C polymorphism and risk of NPC. Moreover, larger, preferably population-based case-control studies, as well as well-designed mechanistic studies, are warranted to validate our findings in Chinese population or to investigate the association between this polymorphism with different tumor in different ethnicity.

Conflict of interest

The Authors declare that they have no conflict of interests.

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