Archives of Oral Biology 81 (2017) 15–20

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Expression of cancer cell-derived IgG and extra domain A-containing fibronectin in salivary adenoid cystic carcinoma

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ARTICLE INFO

Keywords:
Salivary adenoid cystic carcinoma
Cancer cell-derived IgG
Extra domain A-containing fibronectin

ABSTRACT

Objective: Cancer-IgG is a newly-discovered molecule, mainly derived from epithelial carcinoma cells and is significantly correlated with differentiation, metastasis, local invasion, and poor prognosis of many cancers. In our previous study we detected IgG expression in oral epithelial carcinoma, including salivary adenoid cystic carcinoma (SACC), using an IgG-specific commercial antibody. Here, we explored the correlation between cancer-IgG and clinicopathological features of SACC.

Design: A total of 68 human SACC tissue specimens and 2 siRNAs were used to analyze the correlation between cancer-IgG and extra domain A (EDA^+)-containing fibronectin using the cancer-IgG-specific monoclonal antibody, RP215.

Results: We found an unexpected correlation between cancer-IgG and EDA^+ fibronectin, both of which showed aberrant expression in SACC tissue samples. Both were highly expressed in SACC with nerve invasion. In our previous study, EDA^+ fibronectin overexpression in SACC cells decreased N-cadherin expression. In the present study, we used SACC-83 cells, wherein EDA^+ fibronectin is overexpressed and cancer-IgG is knocked down. EDA^+ fibronectin expression was reduced with cancer-IgG knockdown, while cancer-IgG expression did not affect EDA^+ fibronectin overexpression. Furthermore, knockdown of non-B cell-derived IgG in SACC cells decreased cellular motility ($P < 0.05$) as well as increased E-cadherin and alpha-smooth muscle actin levels.

Conclusion: The results suggest that cancer IgG potentially regulates EDA^+ fibronectin expression, thereby suggesting possible new therapeutic approaches for SACC.

1. Introduction

The complexity of the immunoglobulin (Ig) family has increased over the past 500 million years. To date, almost all previous studies have focused on B cell-derived Ig (B-Ig). It was widely believed that Ig is only secreted by B cells and plasma cells. However, recent studies showed that a variety of non-B cells such as lung, liver, renal, and breast cancer cells express high levels of IgG (Chen & Gu, 2007; Jiang et al., 2014; Qiu et al., 2003). IgG derived from cancer cells was also found to promote tumor cell growth (Qiu et al., 2003). Using immunohistochemistry (IHC), we previously found that IgG is expressed in tissues of several types of oral epithelial carcinoma, including salivary adenoid cystic carcinoma (SACC) (Zhu et al., 2008). However, commercial antibodies against IgG used in this previous study, were specific for B cell-derived IgG and failed to recognize cancer-IgG.

RP215 is a monoclonal antibody originally produced by Lee and Ge using the cell lysate of the ovarian cancer cell line, OC-3-VGH as an immunogen (Lee & Ge, 2009). Liao et al. verified that the RP215 antibody specifically recognized a glycosylated epitope of the non-B cell-expressed IgG heavy chain (Liao et al., 2015). In this study, we

Abbreviations: ECM, extracellular matrix; EDA^+ fibronectin, extra domain A-containing fibronectin; FBS, fetal bovine serum; Ig, immunoglobulin; IHC, immunohistochemistry; PCR, polymerase chain reaction; SACC, salivary adenoid cystic carcinoma

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http://dx.doi.org/10.1016/j.archoralbio.2017.04.010
Received 6 February 2016; Received in revised form 19 March 2017; Accepted 17 April 2017

analyzed cancer-IgG expression in SACC tissues using RP215. However, we also found a potential correlation between extra domain A-containing fibronectin (EDA^+ fibronectin) and cancer-IgG in SACC.

SACC has a tendency for prolonged clinical survival, but with early blood vessel and nerve invasion. A previous study with a cohort of 160 SACC patients showed that the overall survival declines to 40% at 15 years (Fordice, Kershaw, El-Naggar & Goepfert, 1999). Recent studies have focused on a series of possible molecules, including epidermal growth factor receptor, Ki-67, and proliferating cell nuclear antigen (Carlinfante, Lazzaretti, Ferrari, Bianchi, & Crafta, 2005; Cho, Lee, & Lee, 1999; Clauditz et al., 2012). EDA^+ fibronectin is an alternatively spliced form of the extracellular matrix protein, fibronectin, which is predominantly expressed in multiple malignancies and not in normal tissues (Kornblihtt et al., 1996). We previously found that high expression of EDA^+ fibronectin was associated with tumor recurrence and metastatic and nerve invasion in SACC and also affected patient survival; patients with high EDA^+ fibronectin expression exhibit shorter survival duration compared with those showing low EDA^+ fibronectin expression (Wang et al., 2015).

After analyzing the expression of cancer-IgG and EDA^+ fibronectin and their correlation with clinicopathological features, we found significant correlations between the two in SACC.

2. Materials and methods

2.1. Patient samples

A total of 68 human SACC tissue specimens were obtained from the Peking University School of Stomatology, between January 2004 and December 2009. Data for statistical analysis of clinicopathological features and patient characteristics were collected via a review of medical records. All experiments were approved by the ethics committee of Peking University.

2.2. Cell culture

The adenoid cystic carcinoma cell line, SACC-83, was obtained from Prof. Li Shengling, Peking University School of Stomatology (Li, 1990), and the cells were cultured as previously described by Li, 1990.

2.3. Immunohistochemistry (IHC)

RP215 mAb was obtained from Gregory Lee (University of British Columbia, Vancouver, Canada). The tumor tissue blocks were sectioned into 4-μm-thick slices, de-paraffinized and rehydrated through graded ethanol washes. Antigen was retrieved by boiling in 10 mM citrate buffer (pH 9.0) for 2 min. The sections were incubated with 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase and blocked with 10% normal goat serum at room temperature for 10 min. After removing excess blocking buffer, IHC staining was performed with RP215 (5 μg/ml) and mouse monoclonal antibody (IST-9) anti-EDA^+ Fibronectin (Abcam Ltd., Cambridge, MA, USA) indirectly at 4 °C overnight. Immunodetection was conducted using the Envision ABC Kit (Gene Tech Company, Shanghai, China). Both, EDA^+ fibronectin and RP215-IgG were estimated using a 4-tier intensity score (0, none; 1, weak; 2, moderate; 3, strong) and the percentage (0%–100%) of positive cells (0, negative; 1, 1%–25%; 2, 26%–50% and 3, 51%–100% cells). The final staining score was calculated by multiplying the intensity and percentage of positive cells present among all tumor cells. The score for each staining was described as negative (−) when the score was 0–1. "Low expression" included scores of 2–3 (+), and "high expression" included scores of 4–6 and 7–9 (++ and +++, respectively). All evaluations were conducted using a LEICADM4000B/M microscope.

2.4. Overexpressed EDA-containing vectors

EDA-negative (control) and EDA-positive plasmids were confirmed by DNA sequencing as described previously (Wang et al., 2015). When SACC-83 cells grew to 40%–60% confluence, the EDA-negative and EDA-positive plasmids were transfected with Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) in RPMI 1640 without FBS for 6 h. Subsequently, the medium was replaced with complete medium without streptomycin and penicillin. After 48 h, the efficiency of transfection was determined by observing the expression of green fluorescent protein and the recombinant EDA peptide was probed using an anti-Flag antibody.

2.5. Protein extraction and western blot

Protein was extracted using lysis buffer (RIPA) with a protease-inhibitor cocktail (Roche, Basel, Switzerland). The Pierce BCA Protein Assay Kit (Thermo, Rockford, Illinois, USA) was used to assess the protein concentration. Protein samples (40 μg) were separated using electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels and were then transferred onto a nitrocellulose membrane (Millipore, Bedford, Massachusetts, USA).

2.6. SiRNA synthesis and IgG knock-down assay

The siRNAs against the Ig gamma chain constant region (siRNA1, 5′-GGUGGACAAGACAGUUGAG-3′ and siRNA2, 5′-AGUGCAAGGUCUCCA ACAA-3′) and control siRNA (NC, 5′-UUCUCCGAACGUGUCACGU-3′) were purchased from Shanghai GenePharma Corporation (Shanghai, China). SACC-83 cells were seeded in a 6-well culture plate with a density of 1×10^6 cells/well. After 24 h, the siRNAs were transferred into the SACC-83 cell line by Lipofectamine 2000 transfection reagent according to the manufacturer protocol. The knockdown efficiency of IgG was assessed by western blot analysis and real-time polymerase chain reaction (PCR).

2.7. Wound-healing assay

The wound-healing assay was performed to assess cell migration. Briefly, after SACC-83 cells were transfected with siRNA for 24 h, they were seeded and cultured as confluent monolayers in RPMI 1640 medium with 0.5% FBS for 6 h, and then wounded by a 300–400 μm strip across the well. Images at 10 × magnification (TE-2000 U, Nikon, Japan) were captured at 0 and 24 h after wounding. The results of wound healing were assessed by measurement of the average linear speed of movement of the wound edges over 24 h.

2.8. Statistical analysis

Correlations between cancer-IgG expression, EDA^+ fibronectin and clinicopathological features including age, gender, local invasion, recurrence and metastasis, were assessed using a Chi-square test (for positive rate) and Mann-Whitney test (for staining score). Experimental data were compared to determine the difference between knockdown or overexpression versus control SACC cells. Each experiment was performed in triplicate. A two-sided P value < 0.05 was considered statistically significant. All analyses were performed using the SPSS 19 software.

3. Results

3.1. EDA^+ fibronectin and cancer-IgG are expressed in SACC

We previously (Wang et al., 2015) showed that high EDA^+ fibronectin expression is associated with tumor metastasis, recurrence, and nerve invasion in SACC; in addition, patients with high EDA^+

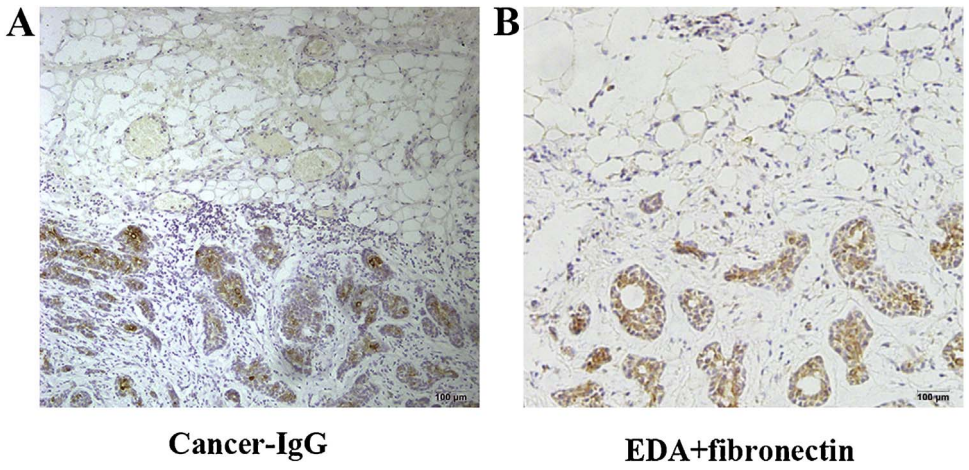

Fig. 1. The expression of cancer-IgG and EDA+ fibronectin in the adjacent non-cancerous tissues. Cancer-IgG(A) and EDA+ fibronectin(B) were specifically expressed in tumor tissues and not in normal tissues.

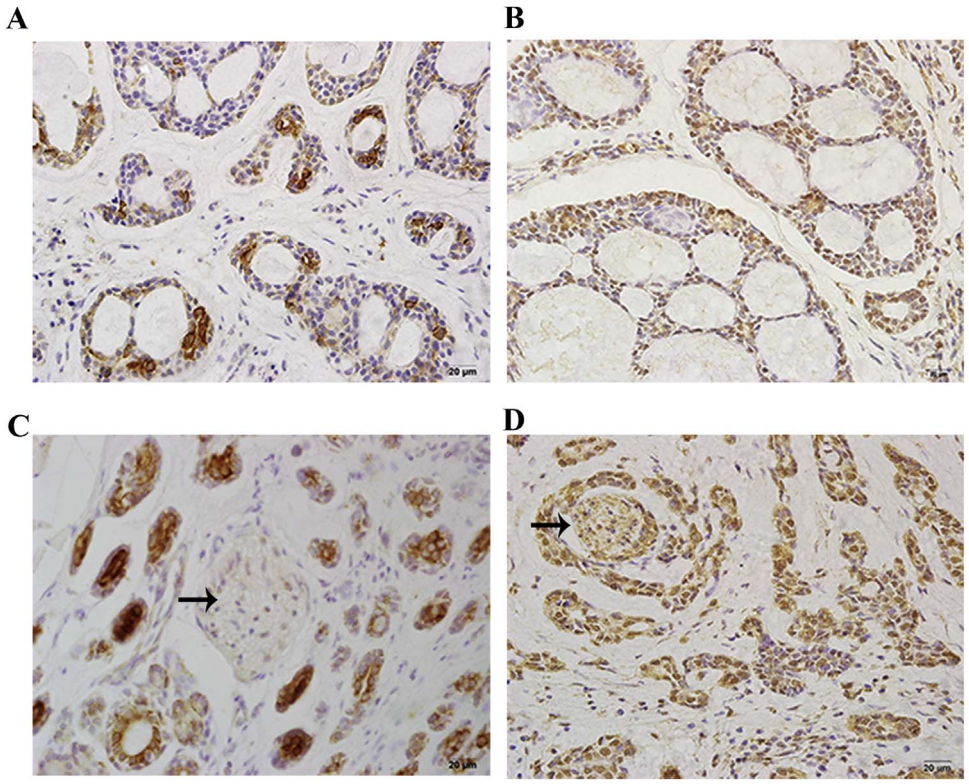

Fig. 2. The expression of cancer-IgG and EDA+ fibronectin in the salivary adenoid cystic carcinoma tissues. A, Cancer-IgG was expressed in the SACC tumor cells, strong positive staining in cytomembrane of some cells; B, EDA+ fibronectin appeared as brown particles in cytoplasm of SACC, occasionally in cancer stroma. C and D, The strong positive staining in SACC tissues with nerve invasion.

fibronectin expression survival exhibit shorter survival than controls with low EDA^+ fibronectin expression. In the current study, IHC of adjacent non-cancerous tissues using RP215 (anti-cancer- IgG antibody) and IST-9 (anti- EDA -fibronectin antibody) revealed that cancer-IgG and EDA+ fibronectin were not detected in normal gland tissue compared with tumor tissue (Fig. 1A, B). What's more, we found that both, cancer-IgG and EDA^+ fibronectin were expressed in tumor cells in SACC samples (Fig. 2A, B). Patients with high IgG expression also expressed high levels of EDA^+ fibronectin in SACC tissues with nerve invasion (Table 1).

3.2. Overexpression of recombinant EDA fragment did not affect cancer-IgG expression

EDA-negative and EDA-positive plasmids were transfected into SACC-83 cells. After 36 h of transfection, more than 40% cells expressed green fluorescence in both groups (Fig. S4A–D). Western blot analysis and real time PCR results indicated that SACC cells transfected with EDA-positive plasmids overexpressed recombinant EDA, with no changes in cancer-IgG expression (Fig. 3A, B).

3.3. Knockdown of IgG decreased the expression of EDA^+ fibronectin in SACC cells

To determine whether cancer-IgG has any effects on EDA^+ fibro-

Table 1
Association between the expression of cancer-IgG and EDA+ fibronectin and clinicopathological parameters in patients with SACC.

characteristics	Case No.	Strong positive RP215 No. (%)	P Value	Strong positive EDA No.	P Value	Both Strong positive	P Value
Sex							
M	34	17(50%)	0.12	16(47.1%)	1	14(41.2%)	0.239
F	52	35(67.3%)		25(48.1%)		14(26.9%)	
Location							
Major salivary	54	38(70.4%)	0.80	29(53.7%)	0.26	20(37.1%)	0.482
Minor salivary	32	24(75%)		12(37.5%)		9(28.1%)	
Histologic subtype							
Tubular or solid	46	32(69.6%)	0.48	21(45.7%)	0.829	14(30.4%)	0.503
Cribriform structure	40	30(75%)		20(50%)		15(37.5%)	
Nervous symptom							
Negative	31	16(51.6%)	0.00**	12(37.5%)	0.002*	5(16%)	0.01**
Positive	55	45(81.8%)		39(70.9%)		24(43.6%)	
Recurrence							
Negative	58	43(74.1%)	0.00**	28(48.3%)	0.041	20(34.5%)	0.264
Positive	28	19(67.9%)		13(46.4%)		9(32.1%)	
Metastasis							
Negative	61	33(54.1%)	0.08	32(52.5%)	0.035	20(32.8%)	0.105
Positive	25	19(76%)		9(36%)		9(36%)	

* $p < 0.05$.
** $p < 0.01$.

nectin, we knocked down IgG in SACC-83 cells using two siRNAs and an negative control siRNA(NC). The expression of EDA$^+$ fibronectin decreased considerably when IgG was knocked down, as shown by western blot and real-time PCR assays (Fig. 4A, B).

3.4. Cancer cell-derived IgG promoted the motility of SACC cells via initiating epithelial–mesenchymal transition

In this study, we found that the motility of SACC cells decreased when IgG was knocked down (Fig. 5A, B). We also observed changes in the expression of a series of genes that reflected epithelial–mesenchymal transition (Fig. 5C). E-cadherin and alpha-smooth muscle actin (α-SMA) expression increased and N-cadherin expression decreased.

4. Discussion

SACC accounts for approximately 10% of all salivary gland tumors; the clinical characteristics include high perineural invasion, high metastasis rates to lung, and high rate of recurrence. The primary treatment approach for SACC includes surgery and postoperative radiotherapy. However, SACC patients still have a poor long-term survival rate. Therefore, it is essential to identify new potential approaches for the management of metastasis and recurrence in SACC.

Tumor cell-stroma interactions play a critical role in regulating tumor evolution and progression (De Wever & Mareel, 2003; De Wever, Demetter, Mareel, & Bracke, 2008). Carcinoma cells are surrounded in the microenvironment by extracellular matrix (ECM) proteins such as laminin, collagens, tenascins, and fibronectin (Pupa, Menard, Forti, & Tagliabue, 2002). Fibronectin, a major ECM protein, is generated by 3 alternative splicing combinations, including EDA/EIIIA, extra domain B (EDB/EIIIB), and connecting segment III (V). EDA$^+$ fibronectin has been shown to promote tumorigenesis by stimulating lymphangiogenesis and angiogenesis (Ou et al., 2011, 2014). Furthermore, in our previous study, we found that EDA$^+$ fibronectin is involved in tumor invasion and metastasis in SACC and is significantly correlated with poor prognosis (Wang et al., 2015).

Cancer-IgG has been reported to be overexpressed in many forms of cancer, including SACC. In the present study, we aimed to investigate if cancer cell-derived IgG is correlated with the clinicopathological features in SACC. Intriguingly, we found that cancer-IgG may be involved in the expression of EDA$^+$ fibronectin in SACC. These results suggest that strong positive staining for both, cancer-IgG and EDA$^+$ fibronectin in tissue samples from SACC patients was significantly related to tumor recurrence, metastasis, and nerve invasion. Moreover, patients with strong positive staining for both had shorter survival periods than patients with low positive staining for both. These

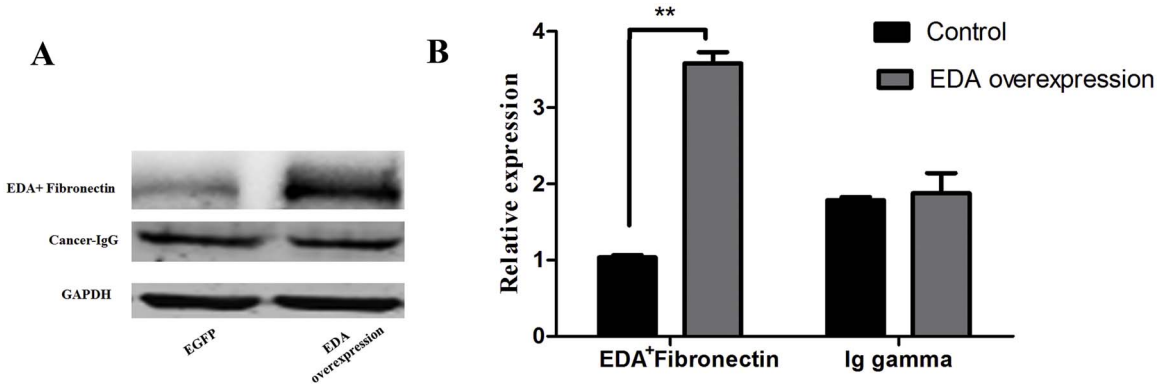

Fig. 3. The IgG expression was detected after over expressing EDA+ fibronectin. Picture A and B was reflected that IgG expression was no changes when over expressed EDA+ fibronectin in SACC cell line by western blotting and real-time PCR respectively.

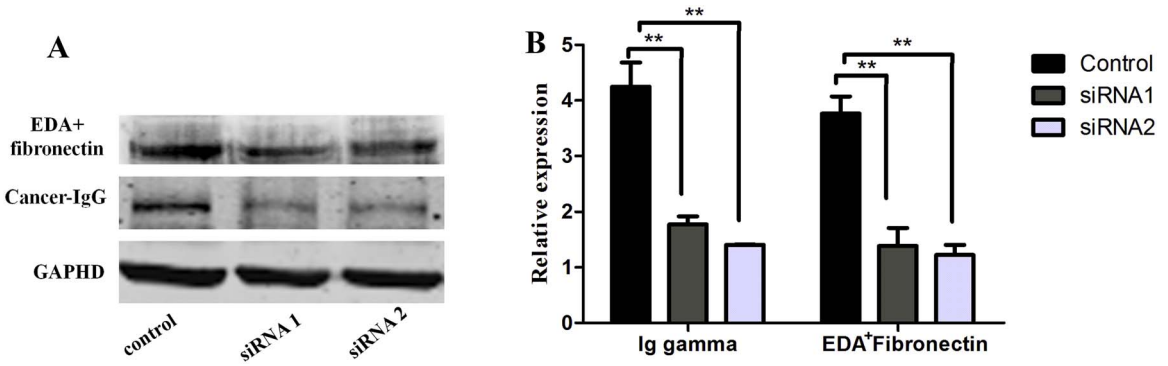

Fig. 4. IgG knockdown significantly decreased the level of EDA+ fibronectin in SACC cell line. Picture A was reflected the EDA+ fibronectin expression decreased when knock down IgG by western blotting assay; similarly, the same trend was detected by real-time PCR (B).

unexpected results led us to investigate if EDA$^+$ fibronectin and cancer-IgG interact in SACC and promote tumor evolution and progression, which suggests a possible therapeutic approach for SACC.

To determine the correlation, if any, between cancer-IgG and EDA$^+$ fibronectin, we knocked down IgG expression and overexpressed EDA+ fibronectin in the SACC cell line. We found that EDA$^+$ fibronectin

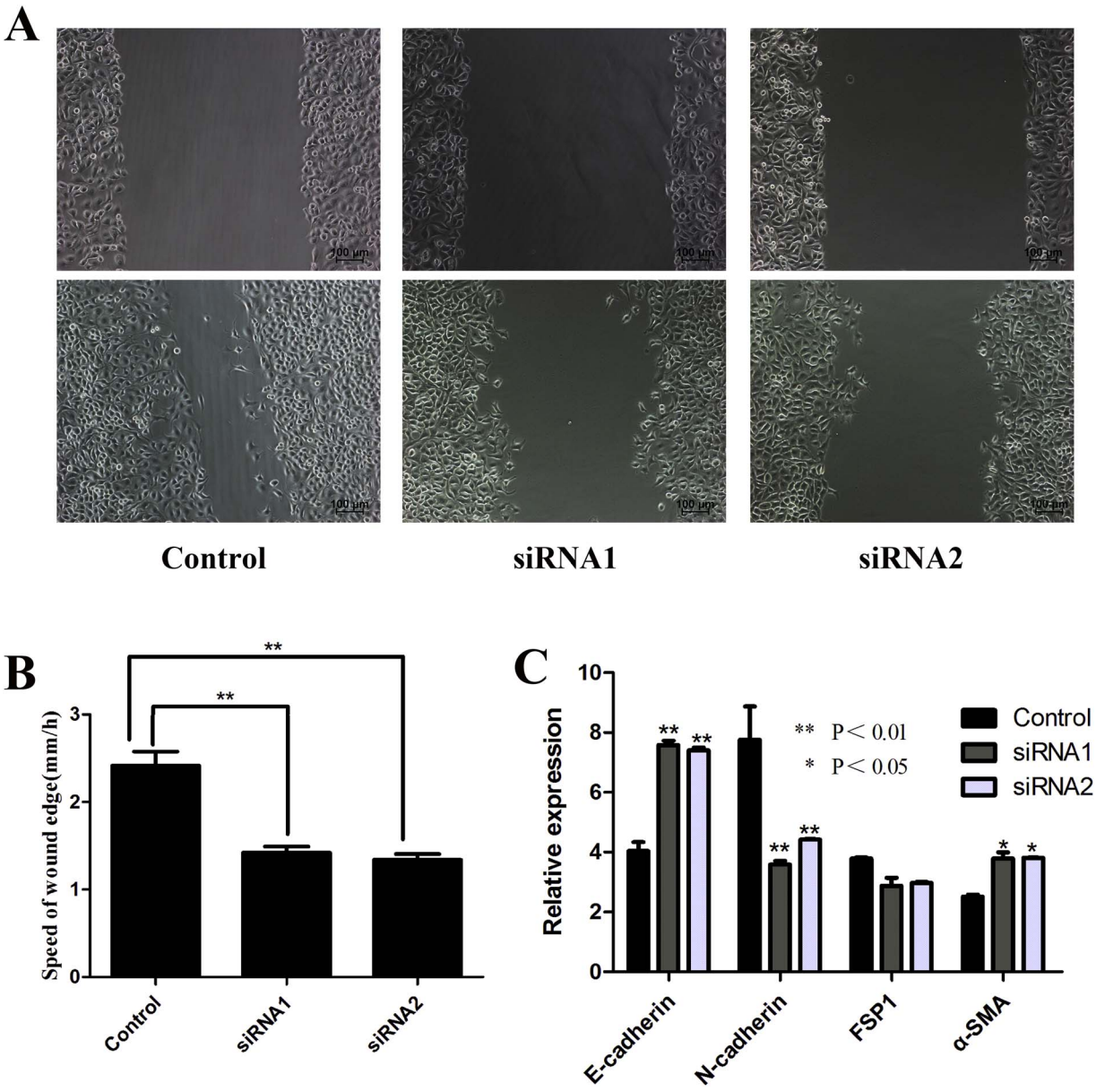

Fig. 5. The motility of cancer cells in SACC. The motility of cancer cells was obviously decreased when knockdown the expression of IgG in picture A. And the statistical significance was showed in picture B. Picture C showed the changes of gene expression of relative molecular about EMT.

expression decreased significantly on knockdown of IgG expression. However, IgG expression did not alter the overexpression of EDA^+ fibronectin. These results suggest that cancer-IgG may be a potential molecule affecting the expression of EDA^+ fibronectin. In addition, we found that, when IgG expression was knocked down, the motility of tumor cells significantly ($P_1 = 0.004$, $P_2 = 0.0032$) decreased, E-cadherin and α-SMA expression levels increased, and N-cadherin expression level decreased. This is consistent with our previous results showing that EDA^+ fibronectin regulates the motility of SACC cells via EMT (Wang et al., 2015).

It has been reported that RP215 mainly recognizes IgG derived from epithelial tumor cells, especially epithelial CSC-like cells, but not IgG derived from cells of non-epithelial origin (Liao et al., 2015). EDA^+ fibronectin was reported to sustain a CD133 + /CD44 + subpopulation of colorectal cancer cells (Ou et al., 2013). Furthermore, it was found that EDA^+ fibronectin increased the proliferation of embryonic stem cells (Losino et al., 2013). However, the precise mechanism underlying the association between cancer-IgG and EDA^+ fibronectin is not completely understood. Therefore, their molecular mechanism in SACC or other tumors needs to be studied to obtain better insight into tumor diagnosis, prognosis, and treatment.

Overall, in this study, we found a significant association between cancer-IgG and EDA^+ fibronectin, as shown by IHC analysis. Knockdown of cancer-IgG expression resulted in decrease in EDA^+ fibronectin expression, while cancer-IgG did not show any effect on EDA^+ fibronectin overexpression. These results suggest that cancer-IgG potentially regulates EDA^+ fibronectin expression, thereby suggesting possible new therapeutic approaches for SACC.

Conflicts of interest statement

None declared.

Acknowledgement

This work was supported by the National Nature Science Foundation of China (grant numbers 81072214, 30371547 to Dr. Cui-Ying Li).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2017.04.010.

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