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Aberrant Wnt-1/beta-catenin signaling and WIF-1 deficiency are important events which promote tumor cell invasion and metastasis in salivary gland adenoid cystic carcinoma[1]

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Abstract. This study investigates whether Wnt components play a role in carcinogenesis, or the invasion and metastasis of salivary glands, also referred to as adenoid cystic carcinoma (sAdCC). Several sAdCC cell lines with low invasive potential (ACC-2), high metastatic potential (ACC-M), and higher invasive potential (T-ACC-M) were examined to determine whether Wnt components correlate with tumors' invasive and metastatic behavior. Immunohistochemistry was performed in a sAdCC tissue array. ACC-M expressed higher levels of Wnt-1, beta-catenin and lower WIF-1 compared to ACC-2 ($P<0.05$). T-ACC-M exhibited increased mRNA of Wnt-1 and beta-catenin, and decreased WIF-1 compared to ACC-2 and ACC-M. Immunohistochemistry showed up-regulation of Wnt-1 and down-regulation of WIF-1 in sAdCC compared with normal salivary glands. Beta-catenin was found in the cytoplasm and nuclei of sAdCC. Dislocation of E-cadherin in sAdCC was observed. These results suggest that sAdCC exhibits diverse expressions of Wnt components. It has an important relationship with the invasive phenotype of these cells.

Keywords: Adenoid cystic carcinoma; Wnt-1; WIF-1; Beta-catenin; E-cadherin;

[1] No potential conflicts of interest were disclosed.

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1. Introduction

Salivary gland malignant neoplasms represent 3% to 7% of all head and neck cancers, and these numbers are steadily rising [1, 2]. Adenoid cystic carcinoma (sAdCC) is one of the most common malignant tumors in salivary glands, and is characterized by heterogeneous histological features. It invades the nerves, but infrequently causes metastasis via the lymphatic system. The neoplasm exhibits a protracted clinical course with local recurrences, hematogenous metastases, and poor responses to classical chemotherapeutic approaches [3-5]. The survival rate for patients is 40% even after surgery and radiation therapy [6, 7].

Wnt signaling pathways play an important role in the regulation of cell differentiation, proliferation, and epithelial-mesenchymal transition (EMT). Down-regulation of Wnt antagonists and/or overexpression of Wnt proteins have also been implicated in the pathogenesis of human cancer. Wnt-1 overexpression might induce beta-catenin accumulation. This promotes beta-catenin translocation to the nucleus, where it activates the LEF/TCF family of transcription factors, inducing and mediating the transactivation of target genes involved in the tumor progression, invasion, and metastasis [8]. In particular, down-regulation of the Wnt inhibitory factor1 (WIF1) was recently reported in several human tumors, correlating with aberrant Wnt/beta-catenin signaling [9]. However, few published papers identify the role of the Wnt family in this case.

In this study, we examine the effects of Wnt/beta-catenin on the carcinogenesis, invasion, and metastasis of sAdCC. To do this, we determined the expression profiles of Wnt components including Wnt-1, beta-catenin, e-cadherin and WIF-1 in different invasive phenotypes of sAdCC cell lines. We also aim to investigate the potential relationship between Wnt-1/beta-catenin pathways, E-cad and WIF-1, as well as the biological behaviors of sAdCC.

2. Materials and methods

2.1. Tissue specimens and tissue microarray (TMA) construction

Archived FFPE tissue blocks, contributed by the Dept. of Oral Pathology at West China College of Stomatology, Sichuan University, were used for histopathologic and immunohistochemical analysis. The histological diagnosis of sAdCC was based on the criterions of the 2005 WHO classification of salivary glands tumors, including cribriform, tubular and solid patterns.

Representative areas of these sAdCC cases were carefully selected on H & E-stained sections and marked on individual paraffin blocks. One tissue core with a diameter of 2-mm was obtained from each specimen. In addition, three normal salivary gland tissue samples were included as control samples. Finally the TMA used in this research consisted of fifty-two 2-mm diameter sAdCC tissue samples and three normal salivary gland tissue samples.

The study protocol has been approved by the Ethics Committee of West China College of Stomatology, Sichuan University in Chengdu, China.

2.2. Immunohistochemistry (IHC)

Immunohistochemistry staining was performed by the Labeled Streptavidin Biotin method with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in boiling 10 mM sodium citrate at pH6.0 for 3 minutes in a pressure cooker in order to revive the antigens. The antibod-

ies used in the study are as followed: Wnt-1, RB-9264-P, Lab Vision, working concentration 1:100. WIF-1, sc-25520, Santa Cruz, 1:200. beta-Catenin, sc-7199, Santa Cruz, 1:200. E-Cadherin, sc-7870, Santa Cruz, 1:200.

Two pathologists simultaneously evaluated the immunohistochemical staining. The percentage of the cell with cytoplasmic staining was scored for Wnt-1, WIF-1, and beta-Catenin. The percentage of stained nuclei, independent of the intensity, was scored for beta-Catenin. Three categories for semi-quantification analysis were defined for Wnt-1, WIF-1 and beta-catenin: 0 (negative and less 5% positive staining), 1 (between 5% and 50% positive staining), and 2 (more than 50% positive staining). For the beta-catenin translocated in cell nuclei, the following categories were defined: 0 (0–5%), 1 (6–25%), and 2 (more than 25% of stained nuclei).

2.3. Cell culture

The human salivary adenoid cystic carcinoma ACC-2 cell line, procured from adenoid cystic carcinoma, and the ACC-M cell line, adenoid cystic carcinoma cell clones highly metastatic to the lung, were donated by the State Key Laboratory of Oral Diseases at the West China School of Stomatology, Sichuan University. They were then cultured in RPMI1640 (Sigma, St. Louis, MO, USA) with 10% fetal calf serum (Sigma), 50ug/mL streptomycin (Sigma), and 50IU/mL penicillin (Sigma).

Transwell in vitro invasion assay:

The invasion assays were performed in a BioCoat™ Matrigel™ Invasion Chamber (Becton-Dickinson, Bedford, MA, USA) according to the protocol provided by the manufacturer. ACC-M cells were briefly suspended in a basal medium without serum. The cells were then seeded onto Matrigel-coated Transwell filters (8-μm pore size coated with 200μL of solid growth factor reduced Matrigel) in 6-well Transwell invasion chambers (Costar Inc.). The cells were seeded at 1.5×10^4 per well. 600μL of the normal medium with 10% fetal calf serum was added to the lower well. The chambers were incubated at 37°C for 48 hours. The cells on the filter side of the upper chambers were cleaned with a cotton swab. Within regular procedure, they were scanned by a scanning electric microscope (HITACHI S-3000N). For another two Transwell chambers, we collected the migrated ACC-M cells in the lower chambers, then cultured them in the normal medium with 10% fetal calf serum, and passaged them as they became confluent. These ACC-M cells, selected by Transwell coated with Matrigel, had both high migration and strong invasion ability, and were therefore were renamed T-ACC-M.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The total RNA of the ACC-2, the ACC-M and the T-ACC-M (ACC-M Transwell-selected cells) was extracted with a TRIzol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA concentrations were then quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The first strand of cDNA was synthesized in 20ul reactions using Oligo$(dT)_{18}$ Primer (TaKaRa, D511, Dalian, CHN), Reverse Transcriptase M-MLV(TaKaRa, D2639A), and dNTP Mixture (TaKaRa, D4030RA). Reactions were performed according to the manufacturer's guide. The cycling conditions for the experiment were 40 cycles of 94°C for 30 seconds, 55°C (for beta-actin), 60°C (for E-cad, beta-cat and WIF-1), 63°C (for Wnt-1) 45 seconds, and 72°C for 60 seconds. PCR products were visualized by 1% agarose gel electrophoresis, ethidium bromide staining, and UV light illumination. The data were analyzed by the imaging analysis software Quantity One V4.2.2.

The primers sequences (5'-3') used in this study are as followed: *E-cad: Forward: CGTAGCAGTGACGAATGTGGTA, Reverse: GATGTATTGGGAGGAAGGTCTG, production 513bp. β-cat: GCAGTTCGCCTTCACTATGGA, ATCTTGTGGCTTGTCCTCAGAC, 558bp. Wnt-1: CTCCACGAACTGCTTACAGACT, GTTGACGATCTTGCCGAAGAG, 229bp. WIF-1: GACTAGAGGGAGAGCAGTGTGAA, GAGGCTGGCTTCGTACCTTTTA, 236bp. β-actin: ATCAGCAAGCAGGAGTATGA, GACTTCCTGTAACAACGCAT, 342bp.*

2.5. Western blot

Cells were lysed in a RIPA lysis buffer (50mM Tris-cl pH 7.4; 150mM NaCl; 1% NP40; 0.25% Na-deoxycholate), with a fresh addition of 100mM of PMSF (Beyotime, Changzhou, Jiangsu, CHN) 100ul per ml and Halt protease inhibitor cocktail (Thermo, Rockford, IL, USA) 10ul per ml. Twenty-five micrograms of lysate were run on 10% SDS-Page 2-2.5hr with 100 volts and transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA) overnight. Membranes were blocked with 5% fat-free milk, then immunoblotted with specific antibodies and revealed using a chemiluminescent (ECL) western blot detecting system (Millipore). The antibodies were against Wnt-1(1:1000; Lab Vision), WIF-1(1:500; Santa Cruz), beta-catenin (1:500; Santa Cruz), E-cadherin (1:500; Santa Cruz) and GAPDH (1:5000; Cell Signaling).

2.6. Statistical analysis

The statistical significance was determined by Fisher's exact test for categorical variables, and a two-sided Student's t test for continuous variables. A P value of less than 0.05 is considered statistically significant. The SPSS for Windows statistical program (SPSS, Inc., Chicago, IL, USA) was used for this analysis.

3. Results

3.1. sAdCC cell lines of high metastatic and invasive phenotypes expressed higher levels of Wnt-1, suggesting that Wnt-1 may play a role in the invasion and metastasis of sAdCC

We found that ACC cell lines of highly metastatic to the lung (ACC-M) displayed elevated levels of Wnt-1 protein compared with ACC-2 cell lines (Figure 1, Wnt-1, Figure 2, Wnt-1). The more invasive types of ACC-M cells (T-ACC-M) exhibited increased mRNA levels of Wnt-1 (3.3-fold and 2-fold) compared with ACC-2 cells separately. In addition, immunohistochemistry detection of TMA displayed that positive staining (moderate and strong positive) of Wnt-1 was detectable in sAdCC, while only moderate or negative staining was detected in normal salivary gland tissues (Figure 3, Wnt-1). Moreover, all 8 metastasis cases and 9 out of 10 recurrent cases showed moderate to strong Wnt-1 positive staining. Notably, Wnt-1 has a strong expression in 4 cases with both metastasis and recurrence (Figure 4, A, E, F).

IHC results showed that up-regulation of Wnt-1 in sAdCC was a frequent event compared with normal salivary gland tissues, suggesting a possible role of Wnt-1 in the carcinogenesis of sAdCC. In addition, we observed higher levels of Wnt-1 in both metastatic and recurrent sAdCC cell lines. These

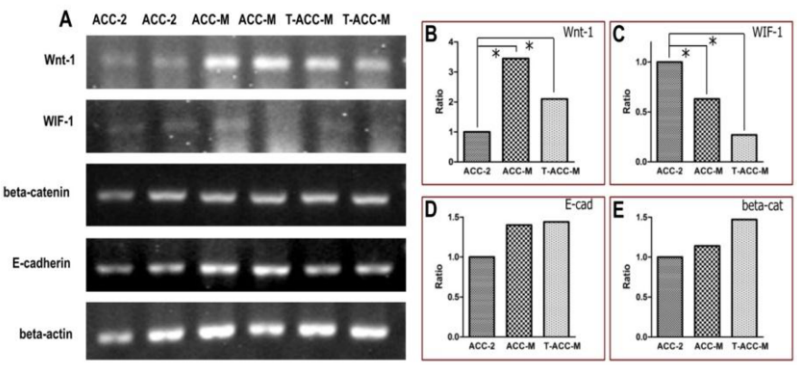

Fig. 1. Reverse Transcriptional PCR demonstrating the expression levels of Wnt-1, E-cadherin, beta-Catenin and WIF-1 in human sAdCC cell lines with different invasion and metastatic behavior: (B) sAdCC cell lines with high metastasis behavior(ACC-M cells and T-ACC-M cells) exhibited elevated expression of mRNA levels of Wnt-1 compared with ACC-2 cells ($P<0.05$). (C) sAdCC cell lines with higher invasion and metastatic behavior exhibited significantly decreased mRNA levels of WIF-1.

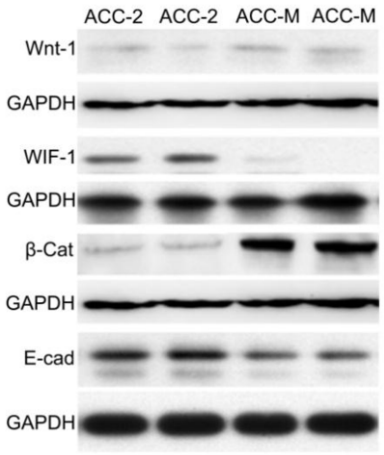

Fig. 2. Western blot demonstrating ACC-M expressed higher levels of Wnt-1, beta-catenin, lower levels of WIF-1 and E-cadherin compared with ACC-2.

results demonstrate that Wnt-1 plays a vital role in the initiation and progression of sAdCC metastasis and recurrence.

3.2. Invasive and metastatic phenotypes of ACC cells exhibited higher levels of beta-catenin, indicating its important role in sAdCC progression

Our data showed an up-regulation of beta-catenin mRNA in T-ACC-M. For the ACC-M cells, Western Blot analysis showed that beta-Catenin protein also up-regulated (Figure 2, beta-Catenin). These data demonstrated that beta-catenin activation was a common event in sAdCC and that its over-expression may promote cell invasion.

3.3. Beta-catenin stability in cytoplasm and translocation into cell nuclei demonstrates canonical Wnt signaling pathway activation and relative events were evoked

In immunohistochemistry investigation, normal salivary glands showed positive staining of beta-Catenin. This was mainly located on the membrane rather than cytoplasm staining. However, in the fifty-two sAdCC cases, beta-Catenin showed positive staining for all but two cases, mainly localized in the cytoplasm of tumor cells. In some cases, the staining of beta-Catenin also appeared in the nuclei, demonstrating that beta-Catenin had translocated into the nuclei (Figure 3, b-Cat).

Among the three patterns of sAdCC, all twelve cases of the solid pattern, which was believed to be the most invasive pattern, showed cytoplasmic positive staining. Seven of them showed strong staining, which was higher than the cribriform and the tubular pattern. Notably, in the tubular pattern, the five cases with positive staining in the nuclei had strong cytoplasmic positive staining, suggesting that beta-Catenin accumulation in cytoplasm may induce its subsequent translocation to nuclei (Figure 4, A, B, C, D). Furthermore, two case of both metastasis and recurrence of the solid pattern showed strong

positive staining in nuclei as well as in cytoplasm, suggesting that translocation of beta-Catenin is evidence of a high invasive ability in sAdCC (Figure 4, A, E, F).

3.4. Low invasive phenotype of ACC cells exhibited higher protein levels of E-cadherin, indicating its important role in sAdCC progression.

The mRNA levels of E-cadherin gradually increased from ACC-2, ACC-M, to T-ACC-M ($P>0.05$), which is consistent with beta-catenin. However, no statistic difference was observed by RT-PCR (Figure 1, E-cadherin).Contrary to this result, E-cadherin protein level exhibited a slight decrease in ACC-M when compared with ACC-2 (Figure 2, E-cad).

In the IHC analysis, the positive staining of E-cadherin showed a tendency to decrease as the invasiveness of the histomorphologic patterns increased. To be specific, eleven cases showed moderate staining while three showed strong staining out of twenty-three cases of cribriform cases. Nine cases of moderate staining and no cases of strong staining were obtained from seventeen cases of tubular patterns. Only four moderate staining cases in twelve solid pattern cases were detected (Figure 3 and

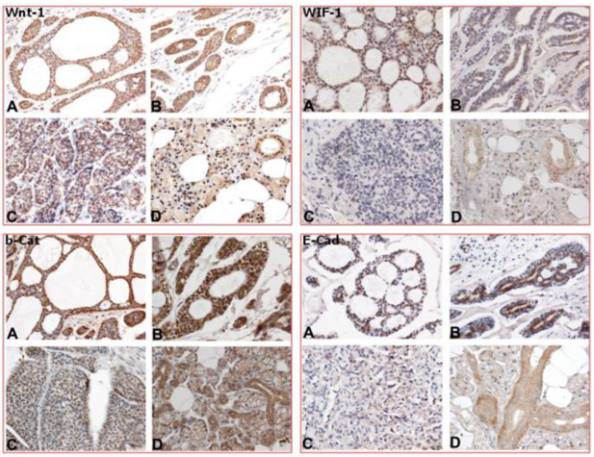

Fig. 3. Immunohistochemistry staining of Wnt-1, E-cadherin, beta-Catenin and WIF-1 in sAdCC and normal salivary glands. A: cribriform pattern of sAdCC; B: tubular pattern of sAdCC; C: solid pattern pattern of sAdCC; D: normal salivary gland.

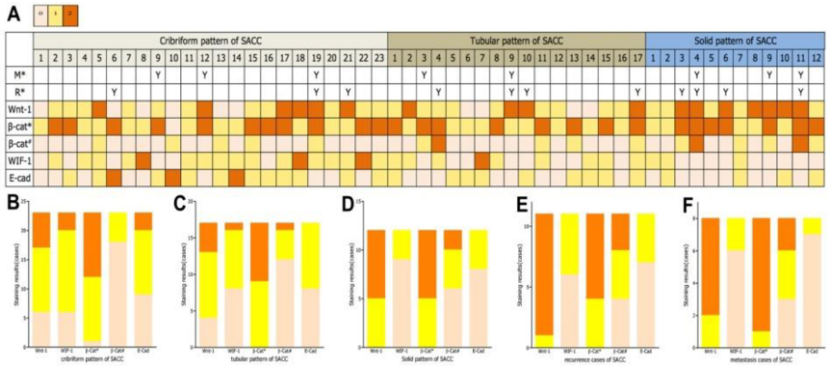

Fig. 4. Immunohistochemistry analysis demonstrated by color. 0, light orange: negative and less than 5% positive staining; 1, yellow: less than 50% positive staining in sAdCC cells; 2, orange: more than 50% positive staining in sAdCC cells. M: metastasis; R: recurrence. b-cat*: positive staining located in cytoplasm and membrane. b-cat#: positive staining located in cell nuclei. Two-sited student's t test for data analysis

Figure 4, A, B, C, D). Furthermore, dislocation of positive staining in tissues was detected. In normal salivary glands, E-cadherin is located in cyto-membranes and has continuous positive staining at cell lines. However, the continuous staining lines were discontinued in tumor cells, while more positive staining was observed in cell cytoplasm (Figure 3, E-Cad). Moreover, all four cases of both metastasis and recurrence showed E-cadherin deficiency, which implies that these tumor cells have high potential for invasion and metastasis (Figure 4, E, F).

3.5. WIF-1 down-regulation may promote tumor cells invasion and metastasis in sAdCC

WIF-1transcript levels were low in the ACC-2 cells, revealing a weak band in agarose gel. For the ACC-M and the T-ACC-M cells, the band on the gel was almost undetectable. After software analysis however, we found the WIF-1 transcript level decreased as the invasive ability of the ACC cells increased (ACC-2 > ACC-M > T-ACC-M). There were also statistic differences among them (Figure 1, WIF-1). Furthermore, the Western Blot analysis showed a significant down-regulation of WIF-1 in the ACC-M cells compared with the ACC-2 cells (Figure 2, WIF-1). These findings support our hypothesis that WIF-1 deficiency may promote tumor cell invasion and metastasis.

Further, we investigated WIF-1 expression in sAdCC TMA by IHC. WIF-1 showed positive staining in normal tissues, while the intensity of the stains generally decreased in tumor tissues. Moderate positive staining was detected in three of twelve cases in solid patterns of sAdCC and no instances of strong staining were observed. In the tubular pattern, eight of seventeen cases showed moderate staining, while one case showed strong staining. As for the cribriform pattern, fourteen of twenty-three cases showed moderate staining and three cases showed strong staining (Figure 4, A, B, C, D). Notably, in the four cases of both metastasis and recurrence, only moderate positive staining was detected in one case, and all the others were negative (Figure 4, A, E, F). Therefore, a WIF-1 deficiency may be a frequent event in sAdCC. Its down-regulation may indicate high metastasis and higher risk of recurrence.

4. Discussion

sAdCC is characterized by an invasive growth pattern and a poor long-term prognosis. Eventually, 50% or more of this tumor would disseminate to distant sites such as the patient's bones and lungs. Traditional surgery and post-operative radiotherapy have not made significant difference in the overall survival rate or the improvement of the quality of life [10, 11]. It is necessary to highlight the importance of exploring the molecular mechanisms underlying sAdCC recurrence and distant metastasis.

The Wnt family plays a critical role in human carcinogenesis. Overexpression of Wnt-1 has been reported in many cancers, such as prostate cancer, breast cancer, hepatocellular cancer, lung cancer [12-15].

To our knowledge, this study is the first to address the role of Wnt and relative factors in sAdCC invasion and metastasis. We observed the elevated expression of mRNA and protein levels of Wnt-1 and Beta-catenin in ACC-M cells compared with ACC-2 cells, indicating their role in ACC metastasis. Further we noticed an over-expression of mRNA of Wnt-1 and Beta-catenin in T-ACC-M cells compared with in ACC-2 cells and ACC-M cells, suggesting that Wnt-1/ Beta-catenin signaling activation may further promote the invasion of sAdCC cells.

Beta-catenin stability in cytoplasm and translocation into nuclei indicated canonical Wnt pathway activation and evocation of some critical downstream events. However, its effects in tumors remained

unclear. Beta-catenin might perform its functions through two main paths: the Wnt-beta-catenin signaling pathway and the E-cadherin-Catenin complex pathway [16].

Our study demonstrated the accumulation of Beta-catenin in sAdCC cells compared with normal cells by IHC. Notably, beta-Catenin was located mainly in the cytoplasm of sAdCC tumor cells, as well as in the nuclei, suggesting beta-Catenin may translocate into nuclei and evoke a canonical Wnt signaling pathway. It also revealed a possible correlation between the Wnt-1 overexpression and beta-catenin accumulation and translocation in sAdCC. Therefore, it is reasonable to say that Wnt-1 overexpression may trigger an aberrant activation of the Wnt signaling pathway, including beta-catenin stability and translocation, as well as the morphological transformation of sAdCC cells. However, T-ACC-M cells exhibited lower Wnt-1 mRNA expression levels compared with ACC-M cells. This may be because Wnt-1 up-regulation is not the sole contributor for beta-catenin activation in T-ACC-M cells.

This study revealed that E-cadherin positive staining decreased in more invasive patterns of sAdCC. Furthermore, its dislocation of positive staining in tissues was detected in sAdCC. This phenomenon, combined with the dislocation of beta-catenin in sAdCC, suggested that the E-cadherin-beta-Catenin complex dysregulation resulted in loss of intercellular adhesion. It may also consequently induce tumor cell invasion and sAdCC progression. Therefore, E-cadherin-catenin may become a significant invasive marker for sAdCC behavior.

Besides the synergistic role beta-catenin and E-cadherin play in maintaining tight intercellular adhesion, beta-catenin plays a major role in cell signaling and promotion of neoplastic growth. In our study, the protein expression level of beta-catenin increased in more invasive types of sAdCC, suggesting its dual roles in tumor progression. The interplay between E-cadherin-catenin and Wnt/beta-catenin in sAdCC progression is subject to further investigation.

Queimado et.al investigated the Wnt pathway in sAdCC and found that WIF-1 was rearranged in sAdCC. They demonstrated that WIF1 is extensively expressed in normal salivary glands and down-regulated in pleomorphic adenoma [17]. J. Mazieres et.al found that WIF-1 silencing occurred as a result of promoter hypermethylation and may be an important cause of constitutive activation of the Wnt pathway lung cancer [18]. Our study found that WIF-1 expression significantly decreased in ACC-M cells compared with ACC-2 cells at both protein and RNA levels. This suggests that it may play a role maintaining the stability of ACC cells and the loss of it may lead to enhanced invasion and metastasis ability. The IHC result showed that the intensity of WIF-1 staining decreased in sAdCC tissues compared with normal tissues, indicating that WIF-1 down-regulation may lead to aberrant Wnt/beta-catenin signaling in sAdCC.

5. Conclusion

This study showed that the expression of Wnt bears an important relationship with the metastatic and invasive phenotype of these cells. Up-regulation of Wnt and Beta-catenin, coupled with down-regulation of WIF-1 were observed in metastatic and more invasive phenotypes of sAdCC cells. This shows that these events may promote the invasion and metastasis of sAdCC. Furthermore, high throughput tissue microarray showed beta-catenin accumulation in the cytoplasm and the nuclei via translocation in sAdCC tissues. This process may occur as a result of alterations of Wnt-1 and WIF-1 and evoke canonical Wnt signaling pathway in sAdCC. These supported our hypothesis that alterations in the correlative components of canonical Wnt pathway might contribute to sAdCC oncogenesis, invasion and metastasis.

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