Original

Elevated CREPT Expression Enhances the Progression of Salivary Gland Adenoid Cystic Carcinoma

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Abstract: The elevated expression of Cell cycle-Related and Expression-elevated Protein in Tumor (CREPT) is reported to promote the growth of several tumors by enhancing Wnt/β-catenin signaling and cell cycle. However, the relevance of CREPT to the malignancy of salivary gland adenoid cystic carcinoma (SACC) remains unclear. The samples from 51 SACC patients were exploited in this study. We found that SACC samples exhibited a noticeably robust CREPT expression than the para-cancerous tissues. Statistical analysis suggested that CREPT expression was significantly correlated with the T classification of SACC. To up- or down-regulating CREPT expression, the specific shRNA or full length of CREPT was delivered into SACC cell lines to examine the cell proliferation, migration, colony formation and implanted xenograft survival. Western blot assay and immunohistochemistry were applied to evaluate the expression of CREPT, cyclin D1, c-Myc and CDK4. Up-regulated CREPT in the low metastatic SACC line significantly promoted proliferation and colony formation, as well as cyclin D1, c-Myc and CDK4 expression. While knocking down of CREPT in the high metastatic SACC line remarkably reduced above effects. Furthermore, the SACC xenograft in mice confirmed that down-regulation of CREPT inhibited the in vivo tumor growth. Our study indicated that the elevated CREPT expression promoted the cell proliferation and tumor size of SACC by enhancing the expression of cyclin D1, c-Myc and CDK4, suggesting that CREPT contributed to SACC progression by stimulating cell proliferation, and might act as a potential target in future SACC therapy.

Key words: CREPT, Salivary gland adenoid cystic carcinoma, Oncogene, Cell cycle, Cyclin D1

Introduction

Salivary adenoid cystic carcinoma (SACC) arises from the secretory epithelial cells of the salivary glands. It accounts for approximately 25% of malignant tumors in the major salivary glands and 50% in the minor glands1-3. SACC is characterized by the slow but relentless growth, the nerve and blood vessel invasion, and the lung metastasis with a high incidence4. However, the most unfortunate aspect of SACC is the poor prognosis. The overall survival rates at 5, 10 and 15 years after SACC diagnosis are 71%, 54% and 34%, respectively4. While the survival rate in the patients with SACC progression even decreases to 35% after 5 years, 15% after 10 years, and almost 0% after 15 years, which is primarily attributed to the late distant lung metastasis5. Therefore, it is imperative to identify an associated gene to predict the prognosis of SACC to guide individualized treatment.

Since the first report at 2012, CREPT has been proven as an accelerator in multiple tumors6. Known as the regulator of nuclear pre-mRNA domain containing protein 1B (RPRD1B), CREPT fundamentally accelerates cell cycle by activating cyclin D1, cyclin E, CDK2, CDK4 and CKD6 expression via the direct binding to their promoters. Moreover, CREPT facilitates transcription by changing DNA configuration into loops, which promotes RNA polymerase recycle to inhibit transcription termination7-11. The phosphorylation of the Ser145 in a conserved motif of CREPT by Aurora B is suggested to be critical to accelerate the G2/M transition by activating the transcription of Cyclin B1(12). Further studies revealed that CREPT could also activated cyclin D1 and c-Myc by stabilizing β-catenin/TCF4 complex on their promoters13. Latest studies found that CREPT not only increased the acetylation and stabilization of β-catenin through the cooperation with p300, but also elevated the active histone acetylation and decreased the repressive histone at the promoters of Wnt target genes14. Additionally, the promotion on cell cycle by CREPT is also reported in peripheral T cells and keratinocyte15. Consistently, by targeting CREPT, microRNA-300 (miR-300) was capable of inhibiting the proliferation of liver cancer cells, as well as suppressing the Wnt/β-catenin signaling16. Thus, CREPT is implicated to promote cell cycle via Wnt signaling pathway.

There are the increasing amount of studies suggesting that the high level expression of CREPT could be considered as an indicator for the poor prognosis of colorectal cancer, non-small cell lung cancer and gastric cancer17-19. On the other hand, the silence of CREPT induces cell cycle arrest at G0/G1 phase, which results in the decreased Cyclin D1 and CDK4 expression, and increased p53 and p21 transcription20. Interestingly, the suppressed CREPT level reduced the migration of gastric cancer cells by altering the expression of extracellular matrices, such as E-cadherin, N-cadherin, vimentin and MMP120. Therefore, CREPT is suggested to act as an oncogene in tumorgenesis. However, if the
CREPT expression could fulfill the identical role in the prognosis of SACC is still required to be elucidated.

Materials and Methods

Ethics statements

This study was approved by the Ethical Committee of Chinese PLA (People’s Liberation Army) General Hospital and conducted in accordance with the guidelines of the Declaration of Helsinki. All of the patients participated in this study have been informed of the nature and purpose of the research and agreed to use their medical records and tissue specimens for research by signing the written consent. The follow-up data were collected by directly interviewing with or calling the patients or their relatives. The animal experiments were approved by the Animal Research Committee at Beijing Laboratory Animal Research Center and carried out in accordance with the authorized Protocol (P2015015).

Samples collection

All of the 51 samples of salivary adenoid cystic carcinomas (SACCs) were collected from the patients in Chinese PLA general Hospital from 2006 to 2010. All the SACC samples were originated from the parotid, sub-lingual and sub-mandibular glands, and the para-cancerous tissues (PCT) were also collected as the controls. None of the patients received preoperative therapy before surgical resection. The SACC diagnosis was histopathologically confirmed and staged according to the 2009 UICC-TNM Classification of Malignant Tumors. The demographic and clinic-pathological information for each patient is listed in Table 1.

Immunohistochemistry

The preparation of paraffin-embedded sections, the reagents and procedure of immunohistochemistry followed the protocol previously described\(^{[21]}\). The anti-CREPT antibody (gifted by Pro. Zhijie Chang at the State Key Laboratory of Biomembrane and Membrane Biotechnology in Tsinghua University) was used as primary antibody in the dilution of 1:60\(^{[9,17]}\). For the mouse xenografts, the primary antibody against Ki67 was diluted in 1:500 (Wuhan Boster Biological Technology, Ltd., Wuhan, China). The secondary antibody was the HRP-conjugated anti-rabbit/mouse IgG (MXB Biotechnologies Inc. Fujian, China). The DAB substrate kit (MXB Biotechnologies Inc. Fujian, China) was applied for color development. Hematoxylin was used for counter-staining.

SACC scoring

The CREPT scoring was performed as previously described\(^{[21]}\). According to the percentage of the positive tumor nuclear staining, the proportion score was assigned as 0 (less than 5%), 1 (6–25%), 2 (26-50%), 3 (51-75%) and 4 (more than 75%). Similarly, the intensity score was assigned as 0 (none), 1 (weak), 2 (moderate) and 3 (strong) based on the average intensity of CREPT positive cells. The expression score was the sum of the percentage and intensity scores, which ranged from 0 to 12. CREPT expression was categorized as - (negative; =0), +, ++ (>4 to ≤8), and +++ (>8 to ≤12), among which - and + were defined as low-expression, while ++ and +++ were defined as high-expression\(^{[22]}\). Five sites in each sample were evaluated to calculate the average score.

Cell culture

The SACC cell line, SACC-83 was established from a patient with SACC in the sub-lingual gland. The SACC-LM cell line was derived from the in vivo selection of the lung metastatic foci from SACC-83, which has a higher rate of lung metastasis compared to SACC-83\(^{[23,24]}\). Both cell lines were gifted by Dr. Sheng-Lin Li of Peking University School and Hospital of Stomatology, Beijing, China. Cells were cultured in RPMI 1640 medium (GIBCO Co., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, GIBCO Co., Carlsbad, CA, USA) at 37°C in 5% CO₂ atmosphere.

Stable transfection

SACC-83 was transfected with the lentivirus vector pLKO-puro encoding CREPT or lentivirus vector pLKO-puro (con). SACC-LM was transfected with the lentivirus vector pLKO-puro encoding shCREPT1/2 or sh-control (con) (Lentivirus vector pLKO-puro encoding CREPT, shCREPT1/2 or shcontrol is constructed by Pro. Zhijie Chang at the State Key Laboratory of Biomembrane and Membrane Biotechnology in Tsinghua University). The shRNA sequences have been described previously\(^{[21]}\). SACC-83 and SACC-LM grew in 24-well plates to 80% confluence, and then, medium containing CREPT, shCREPT1/2 or sh-control lentiviral particles supplied with Polybrene (8 μg/ml, sc-134220, Santa Cruz Ltd., CA, USA) was added to these cells, respectively. The medium was refreshed after 12h transfection, and the stable clones were selected by Puromycine dihydrochloride (1 μg/ml, sigma) after 72 h of transfection. The CREPT expression in the selected clones was assessed by Western blot as described above after 3 weeks of selection to confirm the successful transfection.

Western blotting

The reagents and procedure of Western Blotting have been described previously\(^{[21]}\). The primary antibodies included mouse anti-human CREPT antibody (1:500 dilution in TBS-T)\(^{[8,13]}\), anti-cyclin D1 antibody (rabbit IgG, 1:1,000 dilution in TBS-T; Cell Signaling Technology Ltd., Beverly, CA), anti-c-Myc antibody (rabbit IgG, 1:1,000 dilution in TBS-T; Cell Signaling Technology Ltd., Beverly, CA), anti-CDK4 antibody (rabbit IgG, 1:1,000 dilution in TBS-T; Cell Signaling Technology Ltd., Beverly, CA) and anti-β-actin (mouse IgG, 1:2,000 dilution in TBS-T; ZSGB-BIO Ltd., Beijing, China), respectively. The secondary antibodies were anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (ZSGB-BIO Ltd., Beijing, China). All the Western Blotting were repeated three times to ensure the accuracy.

Cell proliferation assay

SACC cell lines were seeded at 5×10⁴ cells/well with complete medium. Cell Counting Kit-8 (CCK-8) was used to assess cell proliferation rate as instructed in the manufacturer’s protocol (Dojindo Ltd., Kyoшу, Japan). The absorption peak at 450 nm was measured by microplate reader (Thermo Multiskan MK3 Co., MA, USA). In the mouse xenografts, the percentages of cell proliferation were determined by the numbers of Ki67 positive nuclei to the numbers of the total nuclei.

Colony formation assay

1×10⁴ cells were plated into a well of the 6-well plate with complete medium. After 2 weeks, the cells were fixed by methanol and stained by crystal violet to count the colony numbers.

Flow cytometry

The percentages of cells in G1, S and G2 phases were calibrated by flow cytometry. SACC cells were digested with trypsin, washed twice with phosphate buffer saline (PBS) and then, fixed with cold 70% ethanol at 4°C overnight. The fixed SACC cells were incubated with RNase A for 1 h at 37°C, and stained with propidium iodide (PI) for 30 mins...
(Cell cycle and Apoptosis Analysis Kit, Beyotime Biotechnology Ltd., Dalian, China). The flow cytometer (BD FACS Calibur™) was utilized for cell cycle analysis as the manufacturer instructed (Becton, Dickinson and Company, USA).

**SACC xenografts**

All animal experiments were approved by the Animal Research Committee at Beijing Laboratory Animal Research Center and were carried out in accordance with established International Guiding Principles for Animal Research (Protocol NO. P2015015). SACC-LM-con and SACC-LM-shCREPT2 treated cells (5×10^6 cells /mouse) were subcutaneously injected into the right flank of to 5-week old female athymic nude mice. Twenty mice were assigned in each group. After 28 days, the SACC xenografts were harvested for immunohistochemistry assay.

**Statistical analysis**

The data were input into SPSS 17.0 software (IBM, NY, USA) for Mann-Whitney U test, the Student’s t-test, the X^2 test and the Fisher’s exact test. The statistical difference was though significant when p value is less than 0.05.

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**Results**

**The implicated correlation of CREPT level to SACC malignancy**

To verify if the CREPT expression is elevated in SACC as in other tumors, the CREPT expression in 51 paraffin-embedded SACC and para-cancerous samples were evaluated by immunohistochemical staining (Fig. 1A; Table 1). The immunohistochemical scores ranged from 0 to 10.8 in the SACC samples (median=5.294), but from 0 to 2.6 in the normal counterparts (median=0.710). The average CREPT expression in SACC samples was obviously higher than that in the normal counterparts, as the statistical significance confirmed (Fig. 1B). Thus, we further analyzed the relationship between clinicopathologic characteristics and CREPT expression levels in SACC individuals (Table 1). Although there was no significant correlation of CREPT levels to patients’ age, gender or SACC stage, the elevated expression level of CREPT was markedly associated with the larger tumor size, hence a higher T classification. To further verify the correlation between CREPT level and malignancy of SACC, the CREPT expression was analyzed in the SACC-83 and the SACC-LM, respectively. Western blotting showed that the CREPT level in SACC-83 was significantly lower than that in SACC-LM, implicating the correlation of CREPT level to metastasis (Fig. 1C, D).

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**Table 1. Correlation between CREPT expression and clinical classification in SACC**

| CREPT expression level | total | Low | High | p-value |
|------------------------|-------|-----|------|---------|
|                        | N     | N (%) | N (%) |         |
| Age at surgery (year)  |       |       |       |         |
| <60                    | 28    | 13 46.43 | 15 53.57 | 0.8331 |
| ≥60                    | 23    | 10 43.48 | 13 56.52 |         |
| Gender                 |       |       |       |         |
| Male                   | 30    | 15 50.00 | 15 50.00 | 0.4004 |
| Female                 | 21    | 8 38.10 | 13 61.90 |         |
| T-primary tumor        |       |       |       |         |
| 1+2                    | 33    | 19 59.38 | 14 40.62 | 0.0331* |
| 3+4                    | 18    | 4 21.05 | 14 78.95 |         |
| Clinical stage         |       |       |       |         |
| I+II                   | 25    | 15 60.00 | 10 40.00 | 0.0694 |
| III+IV                 | 26    | 8 38.31 | 18 61.69 |         |
| N-regional lymph node  |       |       |       |         |
| -                      | 48    | 22 44.68 | 26 55.32 | 0.8604 |
| +                      | 3     | 1 50.00 | 2 50.00 |         |
| Nerve invasion         |       |       |       |         |
| -                      | 20    | 10 50.00 | 10 50.00 | 0.572  |
| +                      | 31    | 13 41.94 | 18 58.06 |         |
| Local regional recurrence |   |       |       |         |
| -                      | 16    | 9 56.25 | 7 43.75 | 0.1392 |
| +                      | 35    | 12 34.29 | 23 65.71 |         |
| Distant metastasis     |       |       |       |         |
| -                      | 27    | 13 48.15 | 14 51.85 | 0.5403 |
| +                      | 24    | 10 41.67 | 14 58.33 |         |

*p values are obtained from X^2 test, For Gender p values are obtained from Fisher’s exact test, significant difference, p<0.05
Up-regulating CREPT enhanced cell proliferation, colony formation and cell cycle in SACC

To confirm the oncogenic role of CREPT in SACC, the lower CREPT-expressing SACC cell line, SACC-83 was selected for the gain-of-function assay. Stable transfection and expression of CREPT in SACC-83 cells were accomplished by infection of lentivirus carrying CREPT encoding sequence (Fig. 2A). As expected, the capabilities of cell proliferation and colony formation were both significantly elevated in the CREPT transfected group (Fig. 2B, C). Moreover, the cell cycle assay demonstrated that the increased CREPT expression attracted more cells into S phase, suggesting the promotion of DNA synthesis by CREPT (Fig. 2D). Coincided with the cell cycle distribution, the expression of cyclin D1, c-myc and CDK4, which were associated with S phase, were also dramatically increased by CREPT transfection (Fig. 2E).

Down-regulated CREPT expression suppressed proliferation, colony formation and cell cycle in SACC

To perform the loss-of-function assay, the SACC-LM cell line, which possessed a high CREPT expression, was transfected with shCREPT1/2. Western blot analysis revealed that the protein levels of CREPT in shCREPT1/2 transfected cells were significantly lower than those in the control cells (Fig. 3A). The proliferation capability of shCREPT1/2-transfected SACC-LM cells decreases approximately 39% and 50%, respectively, after 6 days (Fig. 3B). Colony formation assay was also exhibited an approximately 3.1 and 3.78 folds decrease in the colony number of CREPT-shRNA1/2 transfected cells compared with the SACC-LM and shcon- transfected SACC-LM, respectively (Fig. 3C). Cell cycle assay disclosed that the suppression on CREPT by shCREPT transfection arrested cell cycle in G0-G1 phase, though the G2-M phase seemed no impact (Fig. 3D). Moreover, the obviously decreased ratio in S phase implicated that the suppression of CREPT was correlated with the reduced DNA synthesis (Fig. 3D). Consistent with the results in the gain-of-function assay, western blot assay showed that knocking down the endogenous CREPT expression by shCREPT1/2 transfection in SACC-LM also decreased the amounts of cyclin D1, c-Myc and CDK4 proteins noticeably (Fig. 3E), suggesting that the promoted cell cycle or DNA synthesis by CREPT through cyclin D1, c-Myc and CDK4.

Suppression of CREPT reduced SACC xenografts' volume and weight in vivo

To verify the oncogenic role of CREPT in vivo, the shCREPT2-transfected SACC-LM cells were injected into nude mice to generate SACC xenografts. The results showed that the tumor xenografts from the shCREPT2-transfected SACC-LM group were significantly smaller than those from the control group (Fig. 4A). Moreover, the body weight and tumor volume were also significantly reduced in the shCREPT2-transfected group (Fig. 4B). These results indicated that CREPT played an important role in the tumorigenicity of SACC.

Figure 1. Expression levels of CREPT in SACC samples and SACC cell lines. (A) Immunohistochemistry assay of CREPT in primary SACC samples. (a,c) The negative CREPT control in normal salivary gland tissue; (b, d) The positive expression of CREPT in salivary adenoid cystic carcinoma; (a and b in the magnification of 10 folds, with the black scale bar of 100 μm; c and d in 40 folds, with the red scale bar of 25 μm). (B) The Mann-Whitney U test showed the statistical comparison of CREPT protein levels in SACC and para-cancerous tissues (*p<0.05). (C) Western blotting analysis compared the original levels of CREPT protein in SACC-83 and SACC-LM lines. (D) Densitometric CREPT protein data were normalized to the β-actin protein levels (p<0.002). (PCT stands for para-cancerous tissue, 83 for SACC-83 and LM for SACC-LM)
Figure 2. CREPT increased proliferation and colony formation of SACC. (A) Western blotting showed that CREPT-transfection increased CREPT expression in SACC-83 cells (*p<0.05, Mann-Whitney U test). (B) CCK-8 assay showed the increased absorption in the CREPT-transfected SACC-83 group compared with the con-transfected cells. (C) Violet Staining showed that CREPT-transfection remarkably increased the colony number of SACC-83 cells to 271±21, from the 98±15 and 95±11 in the SACC-83 group and SACC-83 control group, respectively. (*p<0.05, Mann-Whitney U test). (D) Flow cytometry indicated that more CREPT-transfected SACC-83 cells were located in the S-phase (G0-G1: 75.6%; S: 19.41%; G2-M: 4.99%) compared with the normal (G0-G1: 83.56%; S: 9.93%; G2-M: 6.43%) and control groups (G0-G1: 75.6%; S: 7.22%; G2-M: 5.25%). (E) The expression of cyclin D1, c-myc and CDK4 in SACC-83 cells were greatly elevated by CREPT transfection (*p<0.05, Mann-Whitney U test).
Figure 3. Down-regulating CREPT decreased proliferation and colony formation in SACC. (A) Western blotting showed that transfection with shCREPT1/2 significantly suppressed CREPT expression in SACC-LM cells (*p<0.05, Mann-Whitney U test). (B) CCK-8 assay showing that the cell proliferation of shCREPT1/2 transfected SACC-LM cells were significantly weaker than those in original and sh-con transfected cells (*p<0.05, Mann-Whitney U test). (C) Violet staining showed that SACC-LM cells formed less colonies after with shCREPT1/2 transfection in comparison with normal and control cells (LM: 287±45; LM-con: 266±39; LM-shCREPT1: 89±16; LM-shCREPT2: 67±10) (*p<0.05, Mann-Whitney U test). (D) Flow cytometry exhibited the retention in G0-G1 phase and decreased ratio in S phase of shCREPT1/2 transfected SACC-LM cells (shCREPT1: G0-G1: 86.46%; S: 9.25%; G2: 4.3%; shCREPT2: G0-G1: 83.25%; S: 7.22%; G2: 5.25%) compared with normal (G0-G1: 75.0%; S: 20.57%; G2: 4.43%) and sh-con transfected SACC-LM (G0-G1: 77.83%; S: 18.96%; G2: 3.21%) (*p<0.05, Mann-Whitney U test). (E) The cyclinD1, c-Myc and CDK4 levels in con- or shCREPT1/2-treated cells was evaluated by Westernblotting with β-actin normalization showed that the reduced cyclinD1, c-Myc and CDK4 expression in shCREPT1/2-transfected SACC-LM cells compared with the control groups. (*p<0.05, Mann-Whitney U test).
xenografts. After 28 days of implantation, the volumes of shCREPT2 treated SACC-LM xenografts were obviously smaller than those in shcon treated SACC-LM xenografts (Fig. 4A). Consistent with the reduced cell proliferation by repressing CREPT, the average weight of shCERPT2-transfected SACC xenografts was found to be 0.2215 g, which is almost 7.22-fold less than the average weight of control xenografts (Fig. 4B). The growing curvature revealed that the shcon treated SACC xenografts started the rapid growth from day 15, while the shCERPT2 treated SACC xenografts showed no evident increase in growth rate during the entire period (Fig. 4C). The histological and statistical assay of cell proliferation indicated that Ki67 was less activated in the shCREPT2 treatment SACC xenografts (Fig. 4D, E), which attributed the reduced volume and weight of SACC xenografts to the suppressed cell proliferation by the down-regulated CREPT.

Discussion

In this study, we reported that CREPT plays an oncogenic role in the tumorigenesis of SACC by enhancing cell proliferation, colony formation and the expression of cyclin D1, c-Myc and CDK4. Consistently, knocking down CREPT expression significantly reduced not only the cell proliferation, colony formation and the expression of cyclin D1, c-Myc and CDK4 in SACC cell lines, but also the size and weight of SACC xenografts. Surprisingly, when CREPT was over-expressed, the increased ratio of SACC in S-phase provided a new clue for the enhancement of CREPT on cell proliferation, and SACC volume and weight. Since cyclin D1 plays an oncogenic role in multiple cancers by controlling the G1/S transition\textsuperscript{25-27}, CREPT was suggested to promote SACC cells into S phase and DNA synthesis by activating cyclin D1. This speculation was supported by the latest finds that the over-expression of CREPT made the colorectal cancer more sensitive to fluorouracil, an uracil analog inhibiting DNA synthesis and thus inducing apoptosis\textsuperscript{28}. Additionally, c-myc was also regarded as an oncogene which elevation markedly increased the metastasis of tumors\textsuperscript{29-32}. Therefore, it indicated that CREPT played the oncogenic role upstream to the cyclin D1 and c-myc, which suggested that CREPT might act as a potential target for the future SACC therapy.

Initially, we hypothesized that CREPT might work as the standard for the diagnosis of SACC. However, the statistical results of CREPT expression in the clinical samples denied this hypothesis because there were 3 cases with negative CREPT expression in all 51 cases. Although the CREPT expression in the metastasized SACC was significantly higher than the local SACC, the correlation between CREPT expression and the SACC metastasis was rejected because CREPT seemed no contribution to the lymph-node and distant metastasis in SACC patients. Thus, the only correlation between CREPT expression and the malignancy of SACC was implicated to be the promotion on SACC proliferation. This hypothesis was first verified by the higher level of CREPT in SACC-LM. Second, CERPT was definitely correlated to SACC proliferation. Suppression on CREPT slowed down not only the SACC proliferation and cell cycle \textit{ex vivo}, but also the volume and weight of SACC \textit{in vivo}. Third, the up-regulated CREPT expression was closely associated with the poor differentiation and clinical stage of many malignant tumors\textsuperscript{33,34}. Our previous study also reported that the CERPT expression was hard to serve as a hallmark of oral squamous cell carcinoma (OSCC), it was strongly associated with the malignancy of OSCC by promoting proliferation\textsuperscript{35}.

In summary, our study demonstrates that CREPT expression enhanced the progress of SACC \textit{ex vivo} and \textit{in vivo} by up-regulating the expression of cyclin D1, c-Myc and CDK4, which promoted the clinical malignancy of SACC. Therefore, CREPT was supposed to work as a
potential indicator for SACC prognosis and a target for anticancer medicine.

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Conflicts of Interest
The authors have no Conflicts of interest to declare.

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