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CCT α is a novel biomarker for diagnosis of laryngeal squamous cell cancer

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Choline phosphate-based delivery systems can target the acidic tumor microenvironment. In this study, we set out to evaluate the diagnostic value of Choline phosphate cytidyltransferase- α (CCT α) in laryngeal squamous cell cancer (LSCC). The expression of CCT α was detected using immunohistochemistry in 50 LSCC patients' tissues and 16 vocal polyps as control group. Then, clinical data was collected and we used receiver operating characteristic curve (ROC) to estimate the potential of CCT α as diagnostic biomarker. We found CCT α levels to be significantly high in the tissues derived from LSCC patients, ($p < 0.001$). Further, we observed a positive correlation of CCT α with tumor size ($p < 0.001$), TNM stage ($p < 0.001$), lymph node metastasis ($p < 0.001$) as well as the grade of LSCC malignancy ($p < 0.001$). Furthermore, AUC was determined to be 0.939 by ROC, and the optimal cutoff value 3.100, with 76.0% sensitivity and 100% specificity. We also found an epigenetic basis of CCT α over-expression in LSCC tissues with significantly reduced methylation of CCT α in LSCC tissues, compared to vocal polyps ($p < 0.001$). These results support epigenetically-induced over-expression of CCT α as a potential diagnostic marker for LSCC.

Laryngeal cancer accounts for 2–5% of new malignancies worldwide¹. Approximately, 12410 new cases of laryngeal cancer are estimated to be reported in the United States, as reported for 2019 recently² and in China, the incidence of laryngeal cancer is quite alarming^{3,4}. Laryngeal squamous cell carcinoma (LSCC) is the major malignant tumor of the larynx, accounting for 85–90% of all laryngeal tumors¹. The progression of LSCC is a complex and not completely understood⁵. Despite tremendous therapeutic advances in recent decades, improvements in the patients' 5-year survival rate are still minor^{2,5}, which could be probably attributed to late-stage diagnosis and other complex factors.

Phospholipids, being prominent in cell membranes, play an important role in cell division by supplying membrane components. Further, induction of phosphatidyl choline biosynthesis is critical for cell proliferation⁶. Choline phosphate cytidyltransferase- α (CCT α) is involved in phosphatidyl choline synthesis⁷. It was reported that CCT α may be a promising biomarker for few other cancers⁸. However, the diagnostic role of CCT α in LSCC, if any, has never been reported. We, therefore, designed this study to evaluate CCT α in LSCC through immunohistochemistry (IHC) and to evaluate the association of CCT α with clinicopathology of LSCC patients. Meanwhile, the epigenetic basis of differential expression of CCT α was also determined to complete our understanding of how CCT α expression might differ between control populations and LSCC patients.

Materials and Methods

Ethical considerations. Approval for this retrospective analysis was obtained from the institutional research ethics board of the Jilin University prior to enrolment (1106/2015). All the methods were performed in accordance with the relevant guidelines and regulations.

Patient characteristics. 50 blocks of paraffin-embedded LSCC samples were obtained from the Department of Otolaryngology, Head and Neck Surgery, The Second Hospital of Jilin University, operated and treated in 2009–2012. 16 vocal polyp blocks served as control. Informed consent was obtained from all patients. Patients with recurrence or a previous history of cancer were excluded. This study comprised of 40 male and 10

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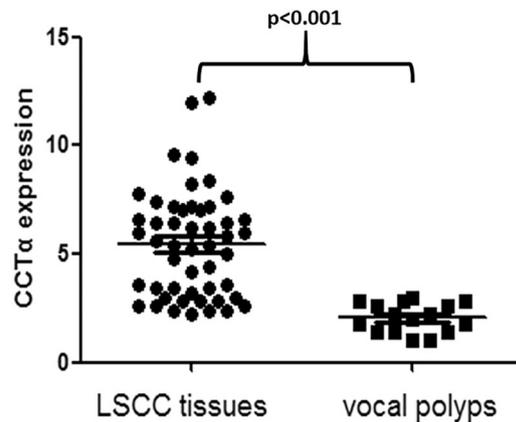


Figure 1. Detection of expression levels of CCT α in LSCC tissues and vocal polyps. The expression of CCT α in LSCC tissues was significantly higher than in controls ($p < 0.001$).

female patients with an average age of 59.8 years (age range: 44–79 years). Stage categories were based on TNM Classification of Malignant Tumours.

Immunohistochemistry. Specimens of laryngeal carcinomas and benign lesions were removed and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C, embedded in paraffin and sectioned at 4 μ m. Deparaffinisation, hydration and epitope demasking were carried out with a microwave antigen retrieval procedure in sodium citrate buffer for 5 min. Then sections were treated with 3% H₂O₂ for 20 min to quench endogenous peroxidase activity and blocked with 5% bull serum albumin (BSA) for 20 min. The primary antibody used were goat anti-rabbit anti-CCT α (1:50, ab109263, Abcam, Cambridge, UK). Slides stained with primary antibodies were incubated at 4°C overnight. Then the slides were incubated with an anti-rabbit IgG antibody at room temperature for 30 minutes. The binding of the primary antibody to the sections was visualized by using DAB Substrate-Chromogen Solution (Dako Cytomation, Carpinteria, CA, USA), the sections were then counterstained with hematoxylin (Beyotime Biotechnology).

Evaluation of immunohistochemical reaction. Two pathologists, blinded to the study, carried out the evaluation independently at x1000 magnification, using Eclipse Ni-U (Nikon, Japan) upright microscope coupled with visual circuit and NIS Elements F (Nikon) software for computer image analysis. The evaluation of CCT α expression was determined with the use of five-point evaluation scale⁹, taking into account the intensity of colour reaction (score 0–3) and percentage amount of positive cancer cells in a given specimen (score 0–4). The final result was the product of scores obtained for the evaluation of both parameters and values from 0 to 12 were considered⁹.

Bisulfite conversion of FFPE samples. To quantitate the differential methylation of FFPE samples, we used the FFPE Bisulfite conversion kit from Active Motif (Shanghai, China) and performed the analyses exactly as recommended by the manufacturer's instructions. Primer design for evaluating CCT α -specific methylation was done using published method¹⁰.

Statistical analysis. All data were analyzed with SPSS statistical software, version 13.0 (SPSS, Inc, Chicago, IL). To evaluate the relationship between the intensity of CCT α expression and LSCC grade, Mann-whitney U test and Kruskal-wallis test were used. And Fisher's exact test was used to determine the association between CCT α expression level and clinical and pathological factors. In all analyses, values were considered significant at P -value < 0.05 .

Results

Evaluation of CCT α in LSCC tissues. We started the investigation by detecting the differential expression of CCT α in LSCC patients, compared to controls. For this, we analyzed the expression of CCT α by Immunohistochemistry in LSCC tissues and vocal polyps. As seen in Fig. 1, CCT α was significantly up-regulated in tissues derived from LSCC patients, as compared to vocal polyps, the controls ($p < 0.001$).

CCT α and clinicopathological characteristics. Next, we assessed the correlation between CCT α expression and the clinicopathological characteristics, using Fisher's exact test. As evident from the data presented in Table 1, CCT α significantly correlated with tumor size ($P = 0.013$), TNM stage ($P = 0.000$) and lymph node metastasis ($P = 0.029$). A majority of high CCT α expressing tumors (22 of 38) corresponded to T3-T4 while a majority of low CCT α expressing tumors (10 of 12) corresponded to T1-T2. For the TNM stage, a majority of high CCT α expressing tumors (27 of 38) correlated with advance stage III-IV tumors while a majority of low CCT α expressing tumors (11 of 12) correlated with low grade stage I-II tumors. Similarly, while low CCT α expressing tumors had no lymph node metastasis, majority of high CCT α expressing tumors were associated with lymph node metastasis. However, we did not find any correlation between CCT α and gender ($p = 0.934$) or age ($p = 0.631$).

Characteristic	Cases	CCT α expression		x ²	p-values
		Low N = 12	High N = 38		
Gender					
Male	40	9	31	0.007	0.934
Female	10	3	7		
Age (years)					
≤60	28	6	22	0.231	0.631
>60	22	6	16		
Tumor size					
T1–T2	26	10	16	6.211	0.013
T3–T4	24	2	22		
TNM stage					
Stage I/II	22	11	11	14.560	0.000
Stage III/IV	28	1	27		
Lymph nodes					
N0	28	10	18	4.788	0.029
N1–N3	22	2	20		

Table 1. Relationship between CCT α expression and clinicopathological characteristics in LSCC. Expression intensity: low, 0–3; high, 3–12. Significant differences for the chi-square test are indicated in bold.

Expression of CCT α in LSCC with different degrees of differentiation. IHC revealed nuclear expression of CCT α in LSCC (Fig. 2) in histopathological specimens. The higher the grade of the analyzed LSCC, the greater level of expression of CCT α marker, as evident by brown staining. Grade 1 had the lowest expression among LSCC samples (Fig. 2B), even though it was higher than the control (Fig. 2A). Grades 2 (Fig. 2C) and 3 (Fig. 2D) had substantially more CCT α expression than Grade 1, indicating that CCT α expression correlated with increasing grade of LSCC. Additionally, CCT α was found to be expressed statistically differently among the three grades tested ($p < 0.01$) (Fig. 3). Combined, the results presented in Figs 2 and 3 support the major conclusion that CCT α expression increases with increasing tumor grade of LSCC.

CCT α as a diagnostic biomarker. We established ROC curve for the estimation of diagnostic value of CCT α , if any, for LSCC (Fig. 4). The AUC value was determined to be 0.939, with 76.0% sensitivity and 100% specificity and a 3.100 cutoff value. This observation supports an interpretation that CCT α is a potential biomarker for differentiating LSCC patients from the controls.

Methylation of CCT α in LSCC samples. In order to assess the basis of increased expression of CCT α in LSCC, we performed CCT α -specific methylation evaluation and found that the methylation of CCT α was significantly reduced in LSCC samples (Fig. 5), which may define the underlying cause of increased CCT α expression. It is well known that reduced methylation correlates with increased expression of genes¹¹ and therefore our methylation analysis provides a direct evidence supporting epigenetic regulation of CCT α in tumor microenvironment.

Discussion

LSCC is the predominant laryngeal cancer characterized by long asymptomatic latency and poor prognosis. There is an urgent need for finding and validating novel biomarkers to aid in the time diagnosis of patients. A number of bio-markers, such as cell motility protein 3 (ELMO3)¹², minichromosome maintenance proteins (MCM)⁹, nucleus protein Ki-67⁹, cytokeratin-18 fragment (CK18)¹³ have been proposed for LSCC by different researchers, however, there is almost no clinical data to verify their possible utility in clinics.

CCT α is a rate-limiting enzyme involved in the biosynthesis of phosphatidyl choline that localizes to the nucleus^{7,8,14}. It is involved in the formation of nuclear membrane phospholipid bilayer and the nucleoplasmic reticulum¹⁵, and is critical for cell viability and embryonic development^{15,16}. CCT α plays a role in Ras-transformed cells' anchorage-independent growth¹⁷, apoptosis resistance¹⁸, cell proliferation¹⁹ and synthesis of phosphatidyl choline²⁰, all of which have connection with cancer.

Previous reports have demonstrated that CCT α is another nuclear antigen recognized by 8F1, which happens to be a commonly used monoclonal antibody for the evaluation of ERCC1 expression⁸. However, there is no information on the diagnostic and prognostic value of CCT α , particularly in LSCC patients. To fill the void in our understanding and knowledge, we undertook this study and compared the expression of CCT α in tissues derived from LSCC patients representing different grades. As a control, we used the benign lesions from vocal polyps. Our results speak for themselves as a very clear difference in expression of CCT α was found in the LSCC patients, when compared with controls. Interestingly, increased CCT α expression was noted in higher grade LSCC patients, as compared to low grade LSCC patients, which in itself supports a possible use of CCT α in differential diagnosis of LSCC.

Further, we elucidated the possible mechanism of up-regulation of CCT α in LSCC patients. In recent years, there has been a push towards a better understanding of epigenetic regulations that control cancer²¹. A survey of literature revealed that a few tumor suppressors have recently been reported to epigenetically silenced in

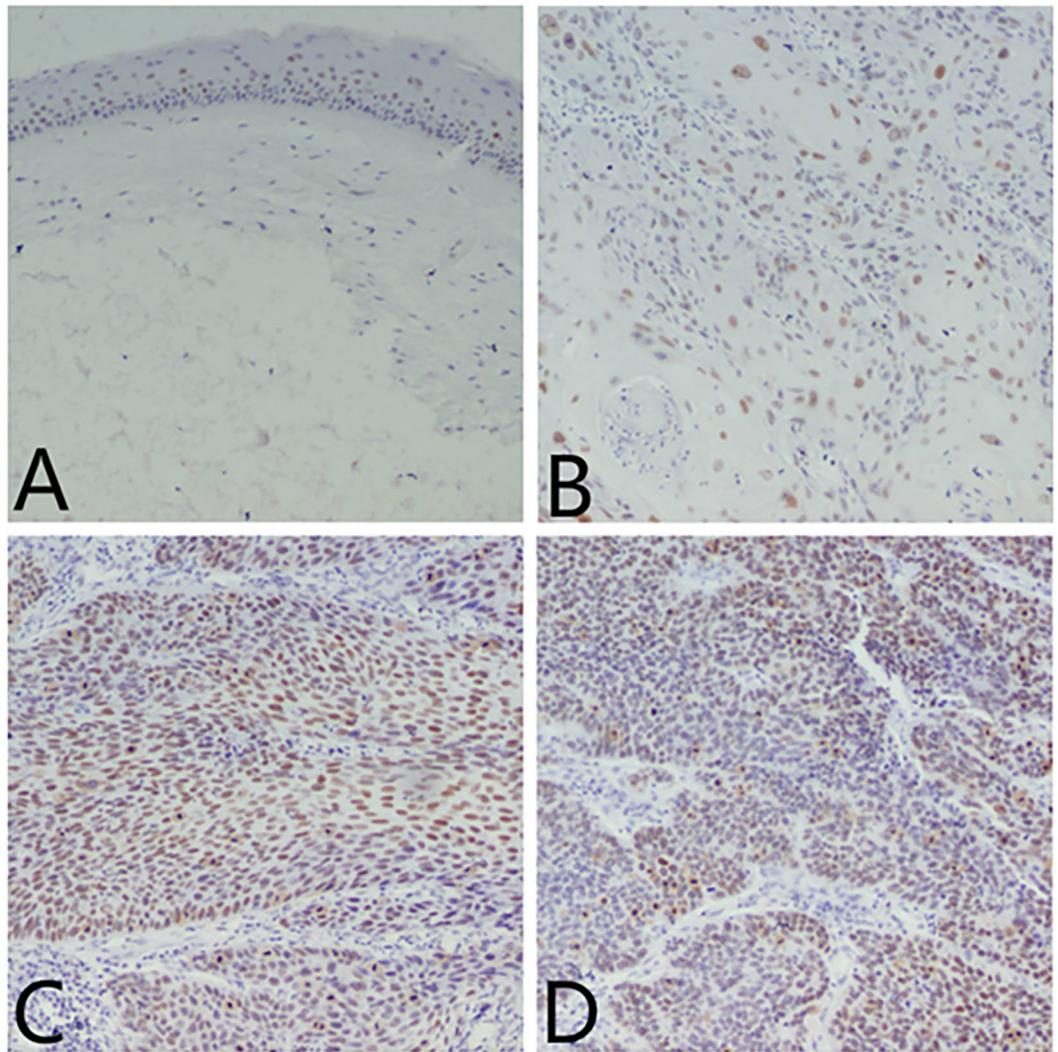


Figure 2. Positive immunohistochemical reaction (brown nuclei) indicating CCT α antigen expression in laryngeal benign lesions and in different histological grades of LSCC. CCT α expression - benign lesion (A) and LSCC ((B) Grade 1, (C) Grade 2 and (D) Grade 3).

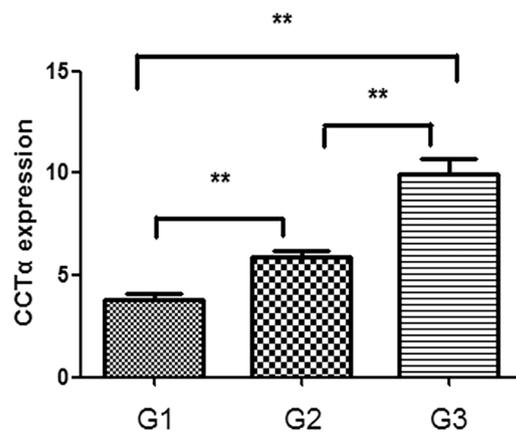


Figure 3. Correlation of expression of CCT α with the grade of LSCC malignancy. ** $p < 0.01$.

LSCC^{22,23}. Looks like methylation is the major epigenetic mechanism for the regulation of genes in LSCC^{22,23}. We, therefore, evaluated methylation of CCT α in our samples and found significantly reduced methylation of CCT α in LSCC patients which can surely lead to its over-expression. Our results bear resemblance to the report

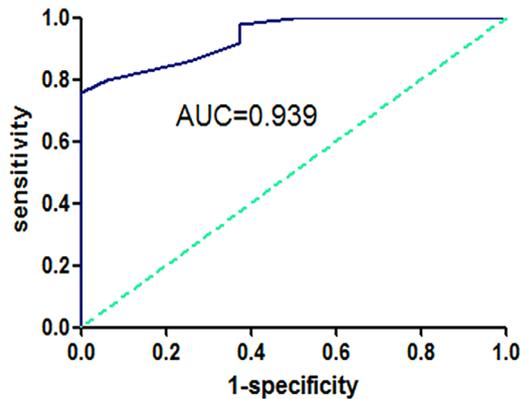


Figure 4. Diagnostic value of CCT α via ROC which had a AUC of 0.939, combining with a sensitivity of 76.0% and a specificity of 100.0%.

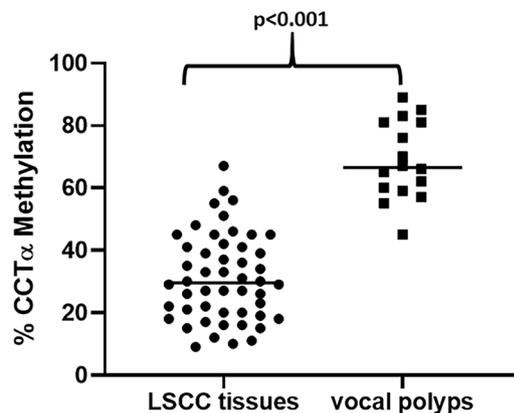


Figure 5. Comparative percentage of CCT α methylation in control (vocal polyps) Vs. tissues from LSCC patients. FFPE samples were used for the analyses. The methylation of CCT α in LSCC tissues was significantly lower than in controls ($p < 0.001$).

on *TREX2* in laryngeal cancer recently that also correlated reduced methylation with increased gene and protein expression²⁴. These results provide a new direction to the research on LSCC and need to be pursued further.

Based on the results presented here, we conclude that the methylation of CCT α is reduced, leading to its over-expression in LSCC patients. Ours is the first report on epigenetic regulation of CCT α in LSCC and its possible diagnostic importance. We argue that these interesting findings need to be further corroborated in larger clinical studies with a focus on mechanistic aspects and the possible therapeutic targeting of CCT α .

Data Availability

All the data collected for this study has been reported here.

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Acknowledgements

This research was supported by a grant from the Development and Reform Commission of Jilin Province (2014G072) and Science and Technology Department of Jilin Province (3D515R933429).

Author Contributions

J.Y., Z.Z., Y.Z. and Z.C. performed experiments; J.Y., Z.Z., Y.Z., Z.C. and C.Z. analyzed results; J.Y. and Z.W. prepared first draft; All authors edited manuscript draft; ZW provided resources and funding, and approved the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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