Ultrasensitive Quantification of Oncoprotein CREPT Using Quartz Crystal Microbalance Sensor

Yonggan Xue, Wenchen Wang, Mu Ke, Hao Li, Feng Tan, Jianguo Hu, *Member, IEEE*, and Tian-Ling Ren, *Fellow, IEEE*

***Abstract*—A novel and ultrasensitive strategy to detect protein CREPT (Cell Regulated and Expression-evaluated Protein in Tumor) in cancer cells using quartz crystal microbalance (QCM) sensor is developed in this study. CREPT is an oncoprotein and plays vital roles in cancer initiation, growth and metastasis via mediating oncogene transcription, and the content of CREPT can reflect the degree of carcinogenesis of tissues and organs. However, there is no rapid, low-cost and ultrasensitive procedure for the quantitative detection of CREPT content in cancer tissues. In order to solve this critical problem, we established a swift and ultrasensitive QCM detection system that achieves an inexpensive and quantitative detection of CREPT. The experimental results showed that CREPT is upregulated in cancers and the weight of CREPT in 1μL extraction of colorectal cells was 0.180064mg. Our work suggests that QCM enables rapid and efficient quantification of proteins and holds promise to apply to quantitative diagnosis and accurate prediction of the degree of canceration of human organs.**

***Index Terms*—QCM, CREPT, quantitative detection, carcinogenesis**

This work was supported by the National Natural Science Foundation of China (62301297). (*Corresponding author: Jianguo Hu, jianguohu@uestc.edu.cn, Tian-Ling Ren,* rentl@tsinghua.edu.cn. Yonggan Xue and Wenchen Wang contribute equally to the study.).

Yonggan Xue and Mu Ke are with the Department of General Surgery, The First Medical Centre, Chinese PLA General Hospital, Beijing 100853, China.

Wenchen Wang is with the Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China.

Hao Li, Feng Tan and Jianguo Hu are with the School of Automation Engineering, University of Electronic Science and Technology of China, Chengdu 611731, China (e-mail: jianguohu@uestc.edu.cn).

Tian-Ling Ren is with the School of Integrated Circuits & Beijing National Research Center for Information Science and Technology (BNRist), Tsinghua University, Beijing 100084, China. (e-mail: rentl@tsinghua.edu.cn).

I. Introduction

MOUNTING evidence has suggested that CREPT (Cell Regulated and Expression-evaluated Protein in Tumor) plays an essential role in tumorigenesis and cancer progression [1-3]. CTEPT expression is significantly elevated in a variety of cancer tissues, relative to the paired cancerous adjacent normal tissues [1]. Moreover, the expression level of CREPT is also indicative of prognostic outcomes of several carcinomas, such as pancreatic, colorectal and renal cell carcinomas [4-6]. Additionally, accumulating research has revealed the predictive value of CREPT during adjuvant therapy and chemotherapy [7, 8]. Those findings indicate that precise detection and quantification of CREPT are significant not simply for early diagnosing cancers but for predicting prognostic outcomes and therapeutic responses of the patients.

Current methods for detecting CREPT largely rest on conventional techniques, like Western blotting and immunohistological staining, which are hard to quantify the absolute amount of the protein. Mass spectrometry has been introduced in protein analysis and has shown excellent performance in terms of sensitivity and versatility, accurate protein quantification remains a significant challenge due to the unstable ionization efficiency [9]. Thus, there is still an unmet need to develop new methods to precisely quantify the mass of a protein in a complex biological sample.

Recent advances in quartz crystal microbalance (QCM) sensors have captivated scientists in analytical fields [10-14]. The QCM sensor has been increasingly used in the detection and analysis of small mass with nanogram [15-18]. The device has a simple structure of two gold electrodes deposited on a thin quartz wafer and utilizes the piezoelectricity of quartz crystal to obtain the mass change information. Sauerbrey developed a model to establish a relationship between subtle mass change on the sensing electrode and resonance frequency change [19]. Considering that the resonance frequency of QCM sensor can reach above dozens of MHz or even hundreds of MHz, it means that the QCM efficiently detects a mass change of sub-nanogram. Moreover, it can be used to determine the biochemical process of mass change in real time. These advantages of high mass sensitivity and ultra-low mass detection ability prompt us to introduce the QCM sensor in the field of cancer protein detection.

In this study, we integrate the ultra-sensitive mass detection

capacity of the QCM sensor with immunobiological principles to quantify the absolute mass of CREPT protein in cancer cells. Our work establishes a framework for targeted protein quantification in a mixed sample and suggests an alternative approach for cancer diagnose.

II. Methods And Materials

A. Practicality and Significance

Fig. 1 shows the process of the QCM detection system for detecting CREPT protein. d1 and d2 represent quartz diameter and gold electrode diameter. TE and TW represent quartz thickness and gold electrode thickness. In real detection of CREPT, we chose the pure water as the reference sample. The theoretical expression of QCM sensor in liquid phase can be calculated by[20]

$$\rho_L = \frac{nD(\Delta f_2 - \Delta f_1)}{K_{Pf} C_{Pf} \Delta V f_0^2} \quad (1),$$

where n is the overtone number of quartz crystal resonator; D is the diameter of electrode; ΔV is the volume increment from second liquid addition; Δf1 and Δf2 are the resonance frequency change after the first liquid addition and after the second liquid addition, respectively; f0 is the resonance frequency of quartz crystal resonator; KPf is the pressure-frequency sensitivity coefficient and KPf = f0Kf/nD 21; Kf is pressure-frequency coefficient and Kf = -23.3 × 10-15 (ms/N) 22; CPf is the pressure-frequency coefficient of quartz resonator in liquid. Then, the density of HA-tag protein in pure water is obtained. Lastly, the density of CREPT protein in HA-tag protein and pure water can be calculated by equation (1), and the mass of CREPT protein is measured. It is noted that the resonance frequency of AT-cut quartz crystal resonator in this system is 10MHz.

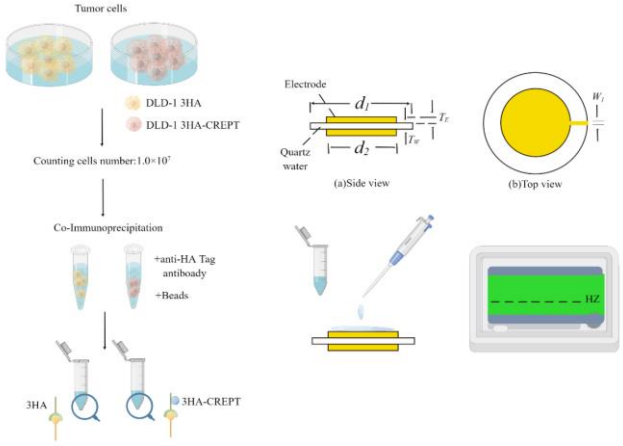

Fig.1. Process of QCM sensor system for detecting CREPT protein. A. protein sample preparation. B. QCM sensor. C. Mass measurement of CREPT protein. DLD-1: A commonly used colorectal adenocarcinoma cell line. 3HA: 3x Hemagglutinin (HA) Tag.

CREPT plays a vital role in driving tumorigenesis and malignant evolution, which makes CREPT an attractive target to be measured [23]. To validate the significance of CREPT in cancers, we first leveraged the public dataset to compare the expression of CREPT between tumor lesions and matched normal tissues at the protein level. As it is shown in Fig. 2A, the expression level of CREPT is remarkably upregulated in tumors in comparison with normal tissues. This finding was further evidenced by the histological staining results that we conducted in multiple cancer contexts including gastric colon and breast cancers (Fig. 2B). In addition, we investigated the prognostic relevance of CREPT in colon cancer patients and found that CREPT expression level was significantly related to survival outcomes (Fig. 2C). These results collectively demonstrate the tumor-driving role of CREPT and the hiding significance of precisely quantifying CREPT in malignancy development.

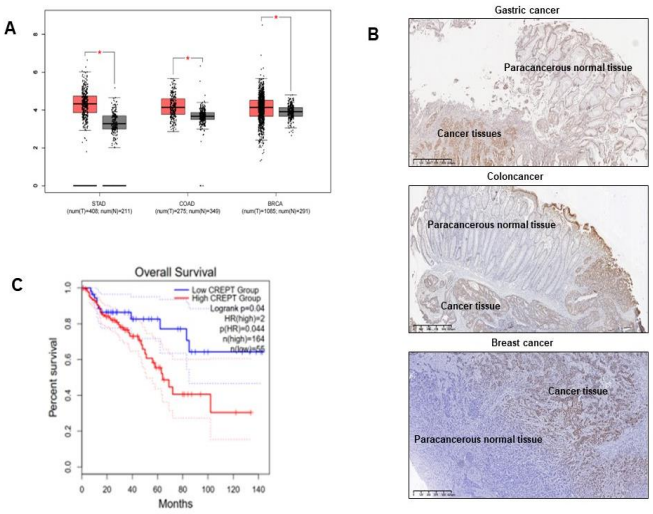

Fig.2. CREPT is overexpressed in cancer tissues and is correlated with poor prognosis. A. TCGA dataset analysis indicate that CREPT is upregulated in cancer tissues when compared to normal tissues. B. histological staining shows overexpression of CREPT in cancer tissues. C. the expression level of CREPT is related to the prognosis of colon cancer patients. STAD: Gastric cancer. COAD: Colorectal cancer. BRCA: Breast cancer.

B. Sample Preparation

CREPT over-expression cell line was produced according to the previous protocol from our group 7. Human colorectal adenocarcinoma cell line DLD1 was obtained from American Type Culture Collection and was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The expression lentivirus vector for CREPT was marked with 3HA-tag and was constructed in our laboratory. 3HA-tag served as an empty control. 3HA and 3HA-CREPT were then inserted into a lentivirus vector, respectively, which were subsequently used to infect DLD1 cells and yielded 3HA and 3HA-CREPT DLD-1 cells accordingly. The transfection efficiency was confirmed by conventional western blotting analysis. Anti-HA antibody was purchased from proteintech.

Protein samples were obtained by virtue of co-Immunoprecipitation (co-IP), as it was described previously. Briefly, 3HA and 3HA-CREPT DLD-1 cells were harvested and lysed with the RIPA buffer (50mM Tris-HCl, 150mM NaCl,0.5% Na- deoxycholateoxy, 0.1%SDS, 1% NP-40). Then, the anti-HA antibody was added to the cell lysates, which were incubated overnight at 4 ℃ to form the antibody-3HA or antibody-3HA-CREPT complexes. Subsequently, the protein A beads were added to the lysates to immobilize antibody-3HA or 3HA-CREPT complexes. After a series of washes, unspecific protein was removed. We further examined the presence or absence of over-expressed CREPT using western blotting assay

(Fig. 3).

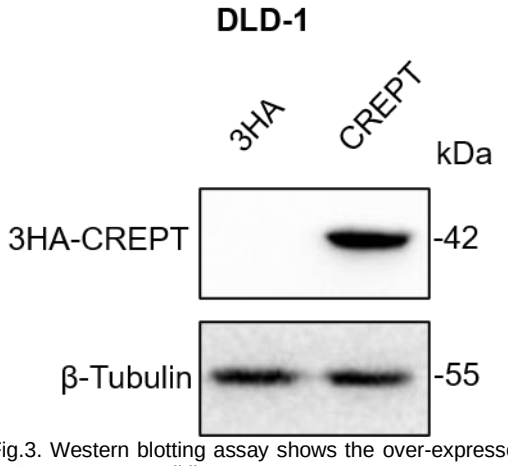

Fig.3. Western blotting assay shows the over-expressed CREPT in 3HA-CREPTDLD-1 cell line.

III. Results And Discussions

A. QCM Measurement System Standardization

Sauerbrey first proposed an equation to describe the quantity relationship between small mass change on QCM surface and its resonance frequency shift and presented that QCM can be used to measure micro-nano gram level mass change in gas phase 19. Unfortunately, Sauerbrey's equation is limited to the gas phase. Kanazawa and Gordon studied the resonance frequency change of quartz resonator when it was in liquid environment and found that the resonance frequency change is determined by the viscosity and density of the liquid 23. Tan proposed a method to measure the viscosity and density of liquid separately based on a QCM sensor, and this method contributed to expanding the application of QCM in liquid 20.

To quantify the absolute mass of CREPT in cancer cells, we propose a practical method to achieve this goal based on a QCM sensor. First, pure water is used as a reference sample and the validity of the model is determined. Then, the density of the 3HA is obtained by QCM sensor. Therefore, the HA protein mass was calculated. Last, the density of the mixture of 3HA-CREPT was measured. The quality of the 3HA-CREPT was obtained accurately by using a QCM sensor, and the construction of the whole measurement system is simple and low-cost.

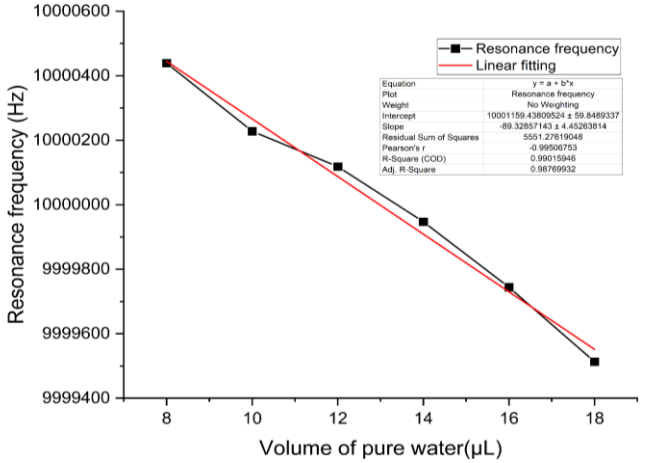

Fig.4. The resonance frequency changes with the volume of pure water added to the electrode surface.

Fig. 4 shows that the resonance frequency changes with the volume of pure water added to the electrode surface. The values of R-Square and Adj. R-Square are 0.990 and 0.988, respectively. This shows that with the addition of the same amount of pure water, the change of resonant frequency decreases linearly, indicating that the contribution of viscosity to the resonator frequency response remains unchanged after the pure water is in contact with the electrode. The decrease in resonant frequency is caused by the increase in the quality of pure water. The pressure-frequency coefficient C_{Pf} equals 1.13×10^{-7}.

B. Precise Measurement

Fig.5 shows that the resonance frequency shifts increase with the increase of volumes of 3HA and 3HA-CREPT. It can be seen from the results that when the volume of 3HA or 3HA-CREPT added to the electrode surface is less than 2 microliters, the change of resonant frequency is not linear with the liquid volume, which indicates that 3HA or 3HA-CREPT does not completely cover the electrode surface. The change in frequency is the result of mass effect and viscosity effect. When the volume of 3HA or 3HA-CREPT added to the electrode surface is more than 2 microliters, there is a linear relationship between the change of resonant frequency and the liquid volume, which shows that 3HA or 3HA-CREPT have completely covered the electrode surface, and the frequency change is the result of 3HA or 3HA-CREPT mass.

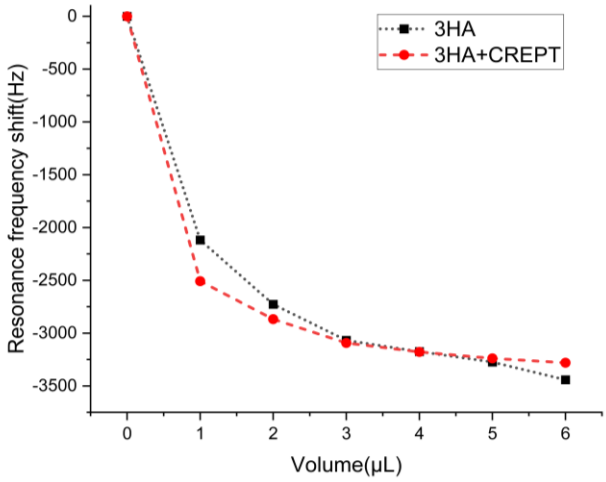

Fig.5. The resonance frequency shift with the volume of 3HA protein and 3HA-CREPT added to the electrode surface.

According to the above experimental analysis, we can measure the change of resonant frequency when the protein of 3HA and 3HA-CREPT is more than 2 microliters. With the increase of protein mass, the change of resonance frequency is mainly caused by the increase of mass. Then the CREPT mass is calculated quantitatively by equation (1), and the 0.180064mg CREPT in 1μL extraction of colorectal cancer cells is obtained.

C. Discussions

Expanding evidence underpins the fact that CREPT participates in the process of cancer initiation and evolution. Our group first reported that CREPT was preferentially over-expressed in various human cancers and, subsequently,

determined that CREPT up-regulates the expression of cell-cycle-related gene cyclin D1, facilitating tumorigenesis 1. The ensuing study revealed that CREPT renders colorectal cancer progression via mediating the Wnt/β-catenin pathway, which plays vital roles in carcinoma development as well as tissue regeneration, among others 2, 25. Recent research suggested that CREPT is implicated in STAT3 signaling pathway and leads to multiple tumorigeneses 3. Moreover, increasing studies demonstrate that the expression level of CREPT is closely related to the cancer aggressiveness, treatment responses and prognosis of cancer patients 6, 26, 27. Therefore, precisely measuring CREPT expression is of great significance in the cancer biological field.

QCM sensor can quantitatively detect the CREPT biomarker of cancer due to its ability to sub-nanogram mass detection and ultrahigh mass sensitivity. However, Traditionally, QCM in liquid phase detection measures the coupling of liquid viscosity and density parameters, so it is difficult to measure the relationship between them separately. In this paper, we have carried out experiments based on the viscosity effect of liquid, proposed a decoupling method, and successfully measured the density separately. Additionally, this method can be popularized in other liquid detection. Seeking to improve the mass sensitivity of the QCM sensor and detection precision of CREPT biomarker, we can utilize the enhancement sensitization mechanism of the QCM sensor overtone mode in the future.

IV. Conclusions

In this study, we introduce a swift strategy to precisely quantify protein concentration. As the concept of liquid biopsy upwardly gaining popularity in cancer management, our work provides an alternative neat solution for precision oncology, starting from protein quantification and potentially extending to DNA and RNA fragment detection. This approach is helpful to improve oncology management and outcomes, including early detection of cancer, precise medicine treatment and prognosis prediction.

Acknowledgment

We gratefully acknowledge funding by the National Natural Science Foundation of China (62301297) and China Postdoctoral Science Foundation (No. 2022M721896).

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