DOI: 10.1002/elan.201500426

Screening for oral cancer using electrochemical telomerase assay

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Abstract: Electrochemical telomerase assay (ECTA) developed by our group was evaluated in an oral cancer screening using exfoliated oral cells and tissues obtained from patients of oral cancer, mucosa associated disease, or healthy volunteers. Telomerase activity from ECTA is correlated with *hTERT* mRNA expression level using a real-time PCR and was increasing in the following order: healthy volunteer group < mucosa associated disease group < oral cancer group. Sensitivity and specificity of ECTA were 88% and 72%, respectively when used 17% of the threshold value based on the receiver operating characteristic curve in ECTA data.

Keywords: telomerase · *hTERT* gene · oral cancer screening · electrochemical assay

1. Introduction

Telomeric DNA consists of repetitive DNA sequences (TTAGGG) at the ends of chromosomes and contributes to genomic stability. Telomerase is a ribonucleoprotein composed of the catalytic subunit of human telomerase reverse transcriptase (*hTERT*) and telomerase RNA component and elongates telomeric DNA [1]. It has been known that telomerase is involved in establishing cellular immortalization [2] and is described to contribute to oncogenic event because of expression of telomerase in cancer cell. Its activity is observed in more than 80% of clinical samples from head and neck squamous cell carcinomas using telomeric repeat amplification protocol (TRAP) assay [3–7]. TRAP was tested in tissue or mouthwash from patients carrying leukoplakia as a precancerous lesion [4,5]. Moreover, *hTERT* mRNA expression is elevated in cancer cells having telomerase activity [6]. Therefore, telomerase activity is expected to be a useful marker in cancer detection.

Although TRAP assay is conventional method to detect a telomerase activity, this method requires several tedious steps and it is difficult to provide objective evaluations of its activity [8]. To overcome this problem, novel telomerase detecting methods have been developing and these are containing PCR- and/or gel electrophoresis-free technique [9]. For example, telomerase activity detection was monitored by the wavelength change of Surface Plasmon Resonance on the telomerase substrate (TS) primer-immobilized on silicon microsensing chip before and after telomerase treatment [10]. Since electrochemical technique is expected to provide a simple and rapid method [11], electrochemical telomerase assay has been studied by several researchers [9,12,13]. It has been reported that electrochemical signal was decreased after telomerase elongation of TS primer-immobilized electrode hybridized with its complementary ferrocenyloligonucleotide [12]. The former or latter technique provides high sensitivity of 10 HeLa cells/μL or 0.1 HeLa cells/μL (100 HeLa cells/mL), respectively. We have been developing an electrochemical telomerase assay (ECTA), which consists TS primer-immobilized electrode coupled with ferrocenylnaphthalene diimide as telomere DNA ligand (Scheme 1) [13]. This method provides simple and rapid screening with the detection limit of 0.4 HeLa cells/μL (10 cells in 25 μL of sample solution) [13]. Exfoliated cell and tissue from healthy and cancer individuals were tested by ECTA and high positive rate of 85% and 90%, respectively. In clinical site, it is required the discrimination of cancer patient from patient suffered from oral disease rather than healthy one evaluating by patients with a disease who test positive and patients without a disease who test negative. For example, detection of gastric and cervical cancers is generally carried out using gastric photofluorography with barium [14] and cytology [15], respectively. These detection methods have a relative high sensitivity and specificity from 80% to 95%.

One benefit of diagnosing oral lesions for other internal one is to achieve by gross inspection and palpation. Since oral cancer recognizes by patient as well as specialist, this finding is often delayed for the following two reasons: (i) Patient doesn't have a distinguishing subjective symptoms before his cancer progresses [16] and (ii)

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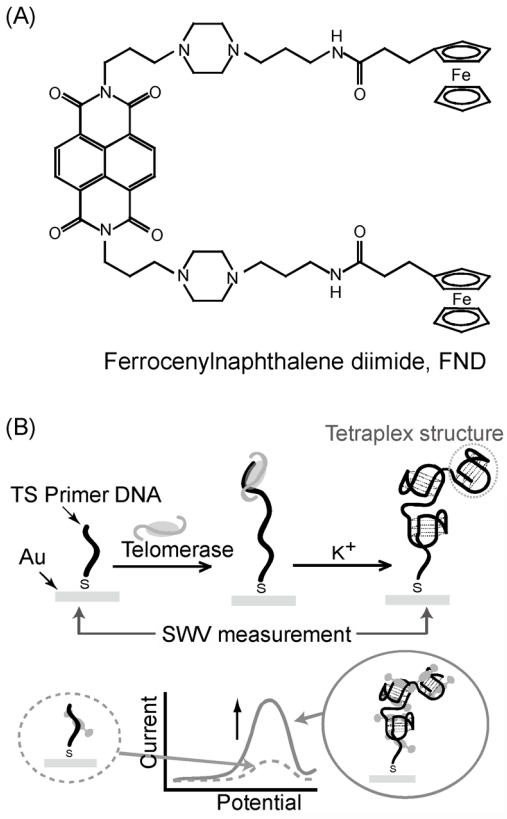

Scheme 1. FND (A) based electrochemical telomerase assay, ECTA (B).

Aspect of oral cancer or precancerous lesions was often confused with oral leukoplakia or oral lichen planus because of similarity of their color and form [17]. Therefore, it is difficult to distinguish oral cancer or precancerous lesions under early stage. Delayed cancer diagnosis leads to a delayed treatment resulting in a severe surgical stress, dysfunction, and decreasing the 5-year survival rates [18]. Therefore, necessity in the cancer detection for various oral lesions is growing and the detection method with simple, rapid, and minimal invasive procedure.

Our previous researches succeed cancer diagnosis for cancer and healthy individuals. However, it is important to detect cancer patients from patients suffered from mucosa-associated disease including precancerous lesion [19,20]. Here, we analyzed the expression levels of *hTERT* mRNA and evaluated telomerase activity with ECTA for same clinical sample. ~~Furthermore, we~~ aimed to detect oral cancer using ECTA as a simple, rapid, and minimal invasive technique with clinical samples obtained from patients with oral cancer or mucosa-associated disease and healthy volunteers. Furthermore, we evaluated the usefulness of cancer diagnosis with calculation of sensitivity and specificity.

2. Experimental

2.1 Collection of clinical samples

This study was approved by the ethics committee of Kyushu Dental University (approval no. 12–37). Samples were collected at the Department of Oral and Maxillofacial Surgery, Kyushu Dental University Hospital (Fukuoka, Japan) between 2010 and 2013 from 30 patients with oral cancer, 30 patients with mucosa-associated disease (leukoplakia or oral lichen planus), and 30 healthy volunteers after obtaining informed consent. All patients with oral cancer were diagnosed with squamous cell carcinoma (SCC), and patients with mucosa-associated disease were diagnosed by histopathological analysis (Tables S1–6).

Exfoliated oral cells (EOCs) and tissues were collected from each participant as clinical samples. The EOCs were collected by scratching the buccal mucosa and the tongue on the right and left sides five times each with a sponge-type brush regardless of the location of the lesions. The collected cells were suspended in 20 mL saline and then centrifuged at 10,000 rpm (91,000 × *g*) for 5 min at ~~4~~°. The obtained pellets were mixed with 500 μL of lysis buffer (10 mM ~~Tris-HCl~~ (pH7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5 % CHAPS, 10 % Glycerol) and stored at ~~−80~~°. This collection method is similar to a self-screening method that anyone can perform easily, even if the individual is not aware of the presence of lesions. Tissues measuring about 1.0 × 1.0 × 1.0 mm were collected by surgical resection or biopsy. In healthy volunteers, samples of the same size were collected from the buccal mucosa or the tongue. After collection, 500 μL of lysis buffer was added to each sample, and samples were stored at ~~−80~~°. Before the assay, lysates were centrifuged at 10,000 rpm (91,000 × *g*) for 10 min at ~~4~~°, and supernatants were collected. The protein concentrations of the supernatants were determined based on absorbance at 280 nm using an ND-1000 instrument (NanoDrop Technologies, Wilmington, DE, USA).

2.2 Analysis of the expression levels of ~~hTERT~~ mRNA

Total RNA was extracted from clinical samples using an RNeasy kit (Qiagen GmbH, Hilden, Germany), and cDNAs were synthesized using a QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol. cDNAs were then subjected to polymerase chain reaction using a QuantiTect SYBR GREEN PCR kit (Qiagen) and MX3005P instrument (Agilent, Santa Clara, CA, USA). Thermal cycling was carried out under the following conditions: ~~95~~° for 15 min; 40 cycles of ~~94~~° for 15 s, ~~55~~° for 30 s, and ~~72~~° for 30 s. The expression level of *hTERT* mRNA was calculated using the following formula (1).

$$\Delta Ct = Ct(hTERT)/Ct(GAPDH) \quad (1)$$

Reverse transcription (RT)-PCR primers were custom synthesized by Genenet (Fukuoka, Japan). The sequences were as follows [21]: ~~hTERT~~, forward primer 5′-GGAGCAAGTTGCAAAGCATTG-3′ and reverse primer 5′-TCCCACGACGTAGTCCATGTT-3′; glyceraldehyde phosphate dehydrogenase (*GAPDH*), forward primer 5′-ATGGAAATCCCATCACCATCTT-3′ and reverse primer 5′-CGCCCCACTTGATTTTGG-3′ [14].

2.3 ECTA

ECTA was carried out using an ALS/CHI Electrochemical Analyzer Model 650A (CH Instrument, Austin, TX, USA). Briefly, 25 μL elongation reaction solution (40 ng/μL lysate, 50 mM Tris(hydroxymethyl)aminomethane-HCl [pH 8.0], 1.0 mM $MgCl_2$, 50 mM KCl, 0.10 mM 2-mercaptoethanol, 0.10 M spermidine, and 20 μM dNTP mixture) was placed on a T8TS1 immobilized electrode manufactured by Sato et al. [22] after multipotential step, SWV measurements as ~~i_0~~ measurements. The electrode was then incubated at 37 °C for 30 min and washed with 350 μL Milli-Q water, 1× phosphate-buffered saline (PBS), and 0.1 M acetic acid-potassium acetate (AcOK-AcOH) containing 0.1 M KCl. Next, 350 μL of 20 μM FND (N,N′-bis[[4-(3-ferrocenepropionamidopropyl)-piperazin-1-yl]propyl]-naphthalene- 1,4,5,8-tetracarboxylic acid diimide) [23] (Scheme 1A), 0.1 M AcOK-AcOH, and 0.1 M KCl solution was placed on the electrode. Electrochemical measurements were carried out using multipotential step, SWV measurements (~~i~~ measurements) [13] (Scheme 1B). The data were evaluated according to the following formula (2):

$$\Delta i\,\% = (i - i_0)/i_0 \quad (2)$$

2.4 Statistical analysis

The results of *hTERT* mRNA expression and ECTA were analyzed using Kruskal Wallis tests and the Steel-Dwass method. All analyses were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Differences with p values of less than 0.05 were considered significant.

3. Results and Discussion

First, we analyzed the expression levels of *hTERT* mRNA obtained from 15 patients with oral cancer, 15 patients with mucosa-associated disease, and 15 healthy volunteers (Tables S1–3). As shown in Figure 1A, the relative expression of *hTERT* mRNA in EOCs was highest in patients with oral cancer, followed by patients with mucosa-associated disease. Significant differences in expression were observed between healthy volunteers and patients with mucosa-associated disease ($p < 0.01$) and between healthy volunteers and patients with oral cancer ($p < 0.01$). Similar trends were observed for tissue samples, with expression levels highest in patients with oral cancer and lowest in healthy volunteers (Figure 1B). Significant differences were observed between healthy volunteers and patients with oral cancer ($p < 0.01$) and between patients with mucosa-associated disease and patients with oral cancer ($p < 0.05$).

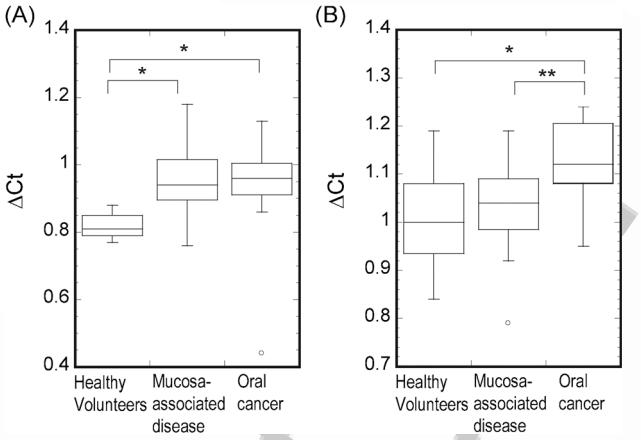

Fig. 1. ~~hTERT~~ mRNA expression in EOCs (A) (*: $p < 0.01$) and tissue (B) (*: $p < 0.01$, **: $p < 0.05$).

Next, we analyzed telomerase activity by ECTA using clinical samples from 30 patients with oral cancer, 30 patients with mucosa-associated disease, and 30 healthy volunteers (Tables S4–6). In EOCs, telomerase activity was highest in patients with oral cancer (27%), followed by patients with mucosa-associated disease (17%) and healthy volunteers (8.3%; Figure 2A). Comparisons between groups demonstrated that all differences were statistically significant. Similar trends were observed in tissues, with highest telomerase activity observed in patients with oral cancer (24%), followed by patients with mucosa-associated disease (13%) and healthy volunteers (12%; Figure 2B). Significant differences were observed between healthy volunteers and patients with oral cancer and between patients with mucosa-associated disease and patients with oral cancer ($p < 0.01$). These results were

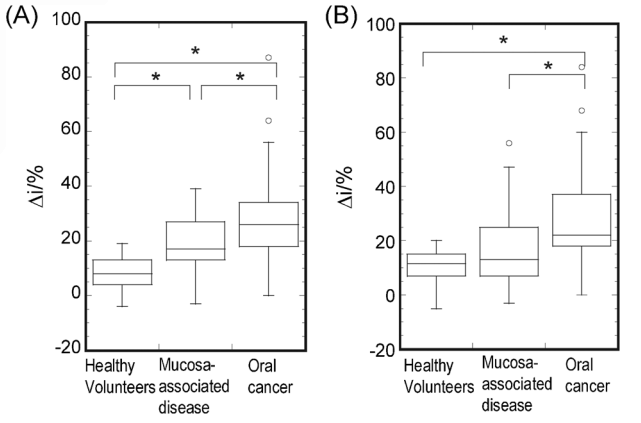

Fig. 2. Current increases in EOCs (A) (*: $p < 0.01$) and tissues (B) (*: $p < 0.01$).

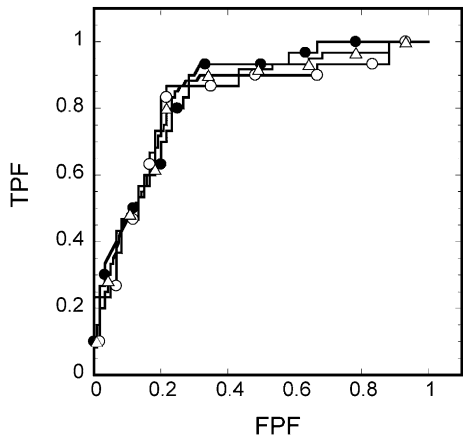

Fig. 3. ROC analysis of EOCs (●), tissues (○), and all samples (Δ).

consistent with the results of *hTERT* mRNA expression levels in the different groups.

Finally, based on the results of ECTA, we performed receiver operating characteristic (ROC) curve analysis to detect oral cancer from the clinical samples obtained in this study. High effectiveness is indicated by higher values for the area under the ROC curve (AUC; nearing 1). The results for the AUC were 0.85 in EOCs (Figure 3, ●), 0.82 in tissues (Figure 3, ○), and 0.83 in all samples combined (Figure 3, Δ). The threshold value determined using ROC curves was 17% telomerase activity as detection of oral cancer was achieved with 16.2% activity in EOCs, 17% activity in tissues, and 16.5% in all samples. Thus, less than 17% activity was considered negative, while more than 17% activity was considered positive. As shown in Table 1, the sensitivities for detection of telomerase activity in EOCs and tissues using ECTA were 90% and 87%, respectively, and the specificity was 72%. From these data, the false-positive rate was 28%, and the false-negative rates were 10% and 13% for EOCs and tissues, respectively. Furthermore, in all samples, the sensitivity was 88%, the specificity was 72%, the false-positive rate was 28%, and the false-negative rate was 12%.

In this study, we analyzed *hTERT* mRNA expression levels by RT-PCR and evaluated telomerase activity with ECTA using clinical samples (EOCs and tissues) obtained from patients with oral cancer, patients with mucosa-associated disease, and healthy volunteers. Our data showed that ECTA and analysis of *hTERT* expression could predict the presence of oral cancer.

Some researchers have shown that expression of *hTERT* mRNA increases as the histological stage of oral carcinogenesis becomes more advanced [24,25]. In our study, the results showed that patients with oral cancer exhibited the highest level of *hTERT* expression, followed by patients with mucosa-associated disease and finally healthy volunteers, consistent with previous reports. Furthermore, we subsequently confirmed the correlation between telomerase activity, as measured using ECTA, and *hTERT* mRNA expression.

Similar to the results of *hTERT* expression, telomerase activity was highest in patients with oral cancer and lowest in healthy volunteers, consistent with a previous report [14]. Additionally, telomerase activity of patients with mucosa-associated disease was moderate compared with the other groups. Mucosa-associated disease may result in oral cancer, although this outcome is not always observed. Although we were not attempting to distinguish oral cancer from mucosa-associated disease, our study did reveal that ECTA could detect oral cancer from all samples based on high sensitivity and specificity. Moreover, our data indicated that telomerase activity, as measured using ECTA, may be associated with *hTERT* mRNA expression. Thus, telomerase activity in patients with mucosa-associated disease, which may transform into oral cancer in the future, can be also detected with ECTA. Furthermore, clinical samples showing dramatic increases in telomerase activity may have a greater tendency to transform in oral cancer. Therefore, these data suggest that ECTA may be applicable for evaluation and diagnosis of carcinogenesis [3,26,27].

We used both tissue samples and EOCs because we assumed that clinical samples would vary in cell numbers and contain both normal cells and cells from any lesions present in the oral cavity. However, the results of ROC analysis revealed that ECTA with EOCs showed slightly increased telomerase activity that tissues and that the method was highly sensitive and specific. Thus, these results indicates that EOCs provided a highly reliable specimen for screening, enabling simple evaluation of the entire oral cavity, regardless of whether a known lesion is present. Furthermore, both the false-negative and false-positive rates were low. The low false-negative rate showed that EOCs, which could be collected using noninvasive, simple methods, yielded results similar to those of tissues. It is generally considered necessary to collect specific tissues in order to reach a definitive diagnosis; however, such tissue collection is often invasive and painful. Therefore, this method is appropriate for self-examination screening by scratching of the entire oral cavity. On the other hand, the false-positive rate was slightly higher than 20%; this may be caused by high telomerase activity resulting from inflammation with infiltration of lymphocytes or necrotic tissues in clinical samples [28,29]. Therefore, depending on the results of such examinations, it is

Table 1. Sensitivity, specificity, false-positive rate, and false-negative rate

	Sensitivity	Specificity	False-positive rate	False-negative rate
EOC	90%	72%	28%	10%
T	87%	72%	28%	13%
~~All samples~~	88%	72%	28%	12%

EOC: exfoliated oral cells; T: tissues

Table 1: All charactors are fixed in the center of columns.

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important to pursue an accurate diagnosis by a medical specialist.

4. Conclusions

In summary, our study showed that EOCs and tissues from the oral cavity could be used for diagnosis of oral cancer by ECTA. ECTA allowed for early detection of cancer, which is imperative for improved treatment outcomes. Furthermore, ECTA represents a promising examination method which could be applied to other fluid samples, such as urine or sputum.

Acknowledgements

This study was performed as a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct), Ministry of Education, Culture, Sports, Science and Technology of Japan. Furthermore, this work was supported in part by JSPS KAKENHI, Grant-in-Aid for Young Scientists (B) Grant Number 25861969, and for Scientific Research (C) Grant Number 26463074.

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Received: June 24, 2015
Accepted: July 30, 2015
Published online: ■■ ▮▮, 0000

FULL PAPERS

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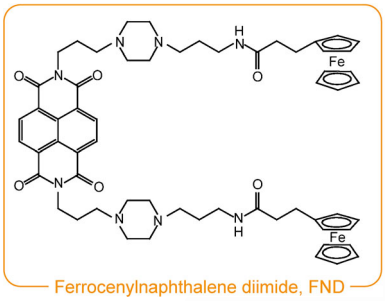

Ferrocenylnaphthalene diimide, FND

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