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Original Article

Rapid diagnosis of cisplatin-sensitive and resistant cervical squamous cell carcinomas by reverse transcription loop-mediated isothermal amplification

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Abstract: Cisplatin is a widely used platinum anticancer drug in cervical cancer chemotherapy; however, its use is limited by the development of drug resistance, which is often rationalized via effects on cellular uptake. Human copper transporter 1 (hCTR1) was proven as crucial regulator for cellular platinum uptake in cancer cells and improving effect of cisplatin treatment. Thus, methods to detect expression of hCTR1 in patients in biopsy specimens with certain rapidity and accuracy are essential to evaluate platinum drug sensitivity and perform further individualized treatment. In this study, a total of 46 normal cervical and primary cervical squamous cell carcinoma tissues were collected and tested by reverse transcription loop-mediated isothermal amplification (RT-LAMP) analysis targeted hCTR1 mRNA. Sensitivity and specificity of RT-LAMP results were calculated and compared to immunohistochemical (IHC) examination. Overexpression of hCTR1 RNA was detected in 12 samples using RT-LAMP within 45 minutes reaction, which indicated positivity with cisplatin resistance. This new technique is suggested to be alternative for rapid diagnosis of cisplatin sensitive patients whose hCTR1 is prevalent.

Keywords: Cervical squamous carcinoma, human copper transporter 1 (hCTR1), reverse transcription loop-mediated isothermal amplification (RT-LAMP)

Introduction

Cervical carcinoma is one of the most frequent malignancy occur in women with second leading mortality rate in worldwide [1]. Currently, radical operation plus chemotherapy is the most common treatment in clinics. Cisplatin (cDDP), and other platinum based antitumor drugs, such as carboplatin (CBP), Sulfatodiaminocyclohexane Platinu (SHP) and oxaliplatin (L-OHP), have been used as first-line chemotherapeutic agents for cervical cancer treatment. However, overcoming the rapid development of drug resistance is still important for improving cisplatin outcomes [2-4]. Because once it has developed, other effective treatment options are limited, and no effective strategy for combating cisplatin resistance is currently available.

One mechanism associated with cisplatin resistance in cancer cell is that reduced intracellular accumulation owing to impaired drug intake and enhanced outward transport. Human copper transporter 1 (hCtr1), the major copper influx transporter, has been convincingly demonstrated to transport platinum drugs, including cisplatin and its analogues [5-8]. Moreover, clinical evidences indicated that expression of hCTR1 were associated with chemo-sensitivities in platinum-based therapy in several cancers [3, 9, 10]. So, an early diagnosis of hCTR1 in cervical carcinoma patients would be effective to evaluate cisplatin sensitivity and perform further individualized treatment.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a novel RNA amplification technique with high specificity,

Table 1. Primer design for the detection of hCTR1 mRNA by RT-LAMP

Primer	Sequence
F3	ACTTCAGCCAGGATTGATGG
B3	CGGGGTTTCCCCATGTTG
FIP (F1C+F2)	TCCGGCCCTGGACCTTATTTTC-AGGGAAATTCTTGCCCAACT
BIP (B1C+B2)	GGCCCATGCCTGTAATCCCA-CAGACTGGTCTCGAACTCCT

efficiency and rapidity under isothermal conditions [11]. Amplification of a targeted gene is detectable in real-time fashion by an increase of turbidity of the solution derived from a side product of the reactions. Thus, the reaction could be completed in a single test tube and within 1 h. In this study, clinical specimens were precisely analyzed by RT-LAMP and the diagnostic potential of this new technique was evaluated to detect cisplatin resistant cervical squamous carcinomas.

Material and methods

Patients and surgical specimens

This study was approved by the Ethics Committee of West China Second Hospital of Sichuan University, and the written informed consent was obtained. Study participants consisted of 23 patients with primary cervical squamous cell carcinoma between June 2015 and June 2016, whose ages range from 33 to 56 (mean age 45.4 ± 5.5 y). Patients were followed up for 6 to 12 months after surgery and the recurrence was evaluated by liquid-based cytology test (LCT). Cervical malignancy and adjacent normal tissues from same patient were obtained in surgery. Each sample was divided in half immediately after resection. One half was frozen within 30 min after surgery in liquid nitrogen and stored at -80°C until molecular analysis, while the other half was fixed with 10% buffered formalin and embedded in paraffin for routine histopathological examination with H&E staining and IHC examination with monoclonal anti-hCTR1 antibody (Novus, Littleton, USA). Each slide was evaluated using light microscopy and the staining was scored semi-quantitatively by assessing the intensity (on a 1-4 scale) and by estimating the percentage of positive cytoplasmic or membranous staining cells (on a 1-4 scale: 1, 1-25% staining; 2, 26-50% staining; 3, 51-75% staining; or 4, >75% staining). With respect to both intensity and frequency, overexpression was defined as hCTR1 positive tumors with diffuse cytoplasmic staining of moderate/strong intensity (≥25% cells and intensity score ≥2) in this study.

RT-LAMP reaction

Frozen clinical specimen was ground down with liquid nitrogen and total RNA was extracted by RNA assay kit (Qiagen, Hilden, Germany) in accordance with manufacture's protocol. RT-LAMP primers targeting hCTR1 mRNA were designed by Primer ExplorerV4 (Eiken Chemical Co, Tokyo, Japan) as indicated in **Table 1**. To quantify and prove the integrity of isolated RNA, β-actin was performed as control. The RT-LAMP reaction was carried out on 25 μL of the total reaction mixture with a Loopamp RNA amplification kit (Eiken Chemical Co, Tokyo, Japan) containing 40 pmol each of the forward and backward inner primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 35 pmol dNTPs, 20 μM Betamine, 0.5 μM Tris-HCl (pH 8.8), 0.25 μM KCl, 0.25 μM $(NH4)_2SO_4$, 0.2 μM $MgSO_4$, 0.2% Tween-20, 1.0 μl Enzyme Mix (Bst DNA polymerase and avian myeloblastosis virus reverse transcriptase), and 5 μl RNA at a constant temperature for 60 min. A fundamental experiment was performed to determine the sensitivity of the RT-LAMP method for detecting hCTR1 mRNA. Cervical squamous carcinoma C-33A cells were serially diluted from 1×10^6 cells to one cell in 1 mL of water, and the extracted RNA reacted as the same method.

The DNA fragments synthesized by LAMP reaction were detected based on the production of a white precipitate of magnesium pyrophosphate. The real-time monitoring of amplification of the dengue RT-LAMP assay was observed with Loopamp real-time turbidimeter (LA-320, Teramecs, Co., Ltd., Japan). In the case that reaction solution is saturated with magnesium pyrophosphate or solution contains lipids, decline of the turbidity was observed over time. Therefore, declines of the turbidity of solution in each measurement interval were estimated as zero increments, and cumulative increments of turbidity at 6 sec intervals were plotted to estimate the gene amplification. The cut-off value for positivity of gene amplification was set when the turbidity reached 0.1 within 60 min at 650 nm, according to the data indicated elsewhere.

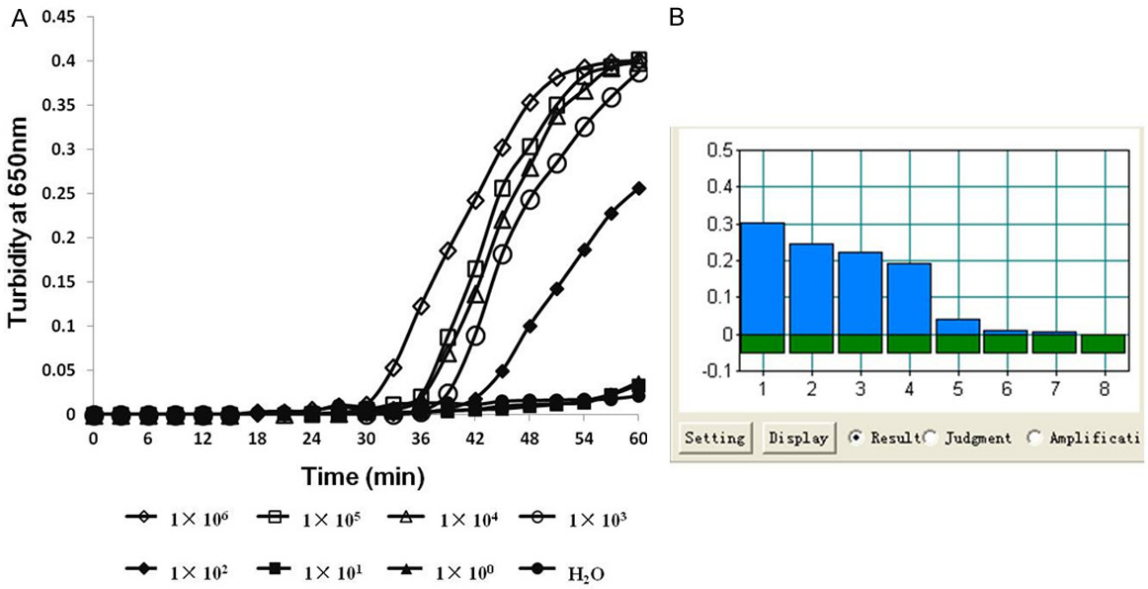

Figure 1. Sensitivity of LAMP reaction to detect hCTR1. A. The turbidity curve of LAMP technique using a known number of hCTR1 cell copies after 60 min amplification. B. The result of LAMP reaction after 45 min amplification measured by turbidimeter. Lane 1 to 7 was loaded with serially diluted hCTR1 cell copies from one million to one. Lane 8 was loaded as ddH_2O as blank control.

Statistical analysis

For RT-LAMP analysis, turbidity data was presented as mean ± SD, and paired comparison of carcinoma and normal cervix from same patient was statistically analyzed using two-tailed t-test. $P<0.05$ was considered statistically significant.

Results

Sensitivity of RT-LAMP reaction to detect hCTR1

Standard curves were produced from serially diluted C-33A cell RNA, which indicated that low limit of detection of hCTR1 by the RT-LAMP reaction was 100 copies (**Figure 1A**). This detection level was achieved within 60 min, whereas our optimized reaction time was 45 min, which required to detect at least 1×10^3 copies (**Figure 1B**). Within this amplification time, each disparity of turbidity reached 0.1 indicated dozens folds of hCTR1 expression.

Analysis of clinical specimens

A total of 46 specimens were retrieved from surgical specimens of 23 patients with primary cervical squamous carcinoma. IHC analysis revealed that 11 of 23 were hCTR1 positive and 12 were negative. **Figure 2** showed representative cases of over- and low-expression of hCTR1 in cervical tumors. A typical hCTR1 positive tumor sample manifested diffuse cytoplasmic immunohistological staining (**Figure 2A**); the average value of turbidity in LAMP reaction was certainly greater than 0.1 and statistically significant compared with adjacent normal tissues ($P<0.05$). In contrast, hCTR1 negative tumor section indicated ubiquitous low expression in both malignant and normal cervical tissues, and according RT-LAMP turbidity values were maintained near critical value (0.1) and the difference between them were not statistically significant ($P>0.05$) (**Figure 2B**). The correlation between hCTR1 expression and pathological factors are still unknown by the limited patient number. Distribution of IHC and RT-LAMP positivity of sections was shown in **Table 2**, and the concordance rate between IHC and RT-LAMP was 91.67% (11/12). Thus, the positivity of IHC and RT-LAMP were roughly identical between these two analyses.

Discussion

The combination of platinum and non-platinum agent is the standard first-line treatment of patients with cervical cancer. Recently, the high-affinity copper influx transporter, hCTR1,

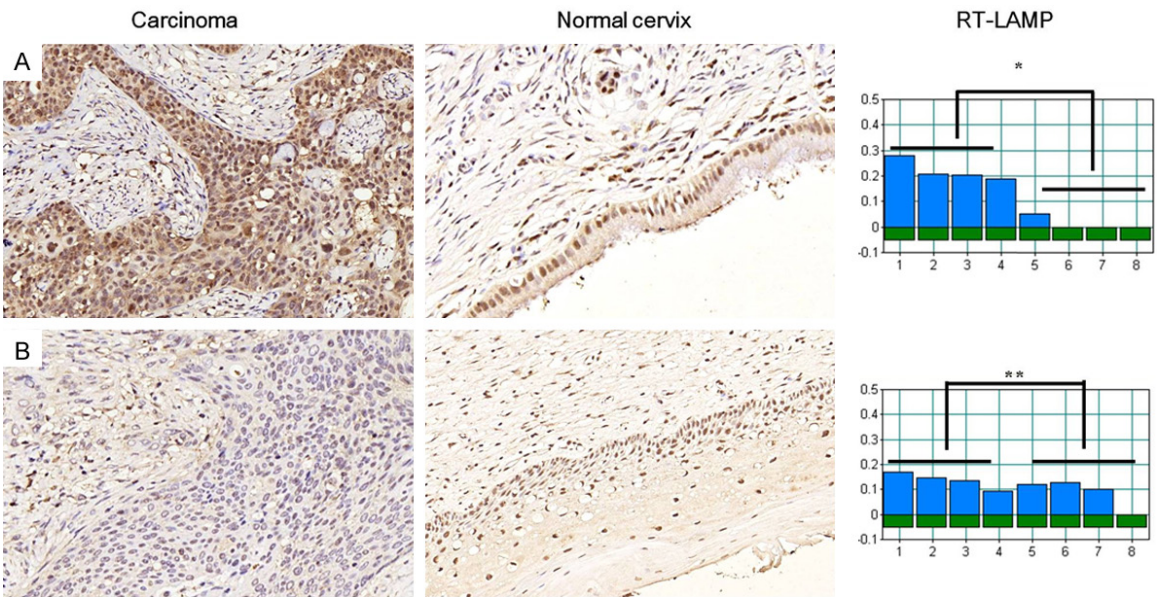

Figure 2. Representative hCTR1 over-expressed (A) cervical squamous cell carcinoma and low-expressed carcinoma (B) cases analyzed by immunohistochemical staining and RT-LAMP. In RT-LAMP reaction, carcinoma RNA was loaded into lane 1 to 4 as duplicates, while adjacent normal tissue RNA was loaded into lane 5 to 8 as duplicates. *, $P<0.05$; **, $P>0.05$.

Table 2. Positive distribution of IHC and RT-LAMP diagnosis of cervical carcinoma patients

Stages	Number of patients	Positivity of IHC analysis	Positivity of RT-LAMP analysis	LCT detection of cancer recurrence after 6 months
IA	2	1	1	0
IB	13	7	8	0
IIA	8	3	3	0
IIB	0	0	0	0
Total cases	23	11	12	0

was reported to be the major transporter of platinum drug and played an essential role in platinum chemotherapy and resistance [12, 13]. Some clinical researches revealed that hCTR1 was associated with good response of platinum-based chemotherapy and indicated as individual prognostic factor for progression-free survival and overall survival in ovarian and NSCLC patients [9, 14]. These results suggest that hCtr1 expression may also have prognostic value for predicting the treatment efficacy in cervical cancer chemotherapy using cisplatin.

Although feasibility studies with IHC or quantitative RT-PCR have shown considerable sensitivity and specificity in certain gene detection, these procedures require considerable time and are hardly acceptable during surgeries. Recently, Notomi and coworkers reported that a novel DNA/RNA amplification technique, LAMP [11]. RT-LAMP reaction has been shown to be feasible with crude samples without purification process of RNA in some virus and cancer biomarker assays [15-19]. In present study, we applied this technique for diagnosis of platinum drug sensitivity of cervical cancer targeting hCTR1 mRNA.

Total time required to obtain results for existence of hCTR1 are one of the most important issues in this study. All of the sections which contain comparable expression of hCTR1 showed positivity within 45 min of RT-LAMP reaction. About 30 min of RNA extraction need to precede the reaction, thus, a total of 75 min are required. This time could be tolerable to most of surgeons, taking duration required for routine histopathological frozen section analysis with IHC staining in our hospital into account.

Our present study revealed that the limit to detect hCTR1 mRNA was approximate 100 cell copies and 100% of hCTR1 positive tumor could be revealed by this new technique. False positivity of 8.3% (1 out of 12 patients) was observed in this analysis, however, it could

hardly account for the real false positive rate because our sample amount was limited. Although contamination, some sorts of instability of the reaction, or migration of cells of epithelial origin could affect the false positive rate, it requires further analysis. Furthermore, with the limitation of sample amount and follow-up period, no recurrence and according platinum drug resistance was observed in current study, more patients and long-term follow-up would be involved to summarize the correlation between hCTR1 expression and survival time/recurrence rate of cervical patients.

Conclusions

Although couples of issues remain to be further studied, the RT-LAMP assay represents a potential alternative for the molecular diagnosis and routine screening of hCTR1 positive cervical carcinomas. It could also be useful in monitoring the efficacy of individualized platinum chemotherapy programs.

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Disclosure of conflict of interest

None.

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