Molecular Diagnosis of Sentinel Lymph Node Metastases in Cervical Cancer Using Squamous Cell Carcinoma Antigen

Song-Hua Yuan,1,3 Xue-Fang Liang,1 Wei-Hua Jia,2 Jian-Ling Huang,1 Mei Wei,3 Ling Deng,2 Li-Zhi Liang,3 Xiao-Yun Wang,1 and Yi-Xin Zeng2

Abstract

Purpose: To clarify the prognostic value of molecular diagnosis of SLN metastases in cervical cancer using SCCA.

Experimental Design: All SLNs and primary tumors, part of non-SLNs, were harvested from 36 patients with cervical cancer. Expression levels of SCCA, cytokeratin 19 (CK19), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in 178 samples (29 primary tumors, 5 histologic positive nodes, 60 histologic negative SLNs, 69 non-SLNs, and 15 normal nodes) were assessed by quantitative reverse transcription-PCR assay. The quantitative value of SCCA or CK19 mRNA was described as each value relative to GAPDH mRNA. The cutoff value was set at the upper limit of the quantitative value of nodes from noncancer patients, and those above this value constituted the molecular metastasis group.

Results: The SCCA mRNA expression values were more than 1 × 10^3 in 28 primary tumors and all histologic positive nodes, and its expression levels in SLNs were higher than in non-SLNs. SLNs from patients with adverse prognostic features had higher SCCA mRNA expression levels. Four histologic negative SLNs were diagnosed molecular metastases based on SCCA mRNA. Two cases with histologically uninvolved pelvic nodes recurred. Survival analysis indicates that molecular lymphatic metastasis based on elevated SCCA mRNA level is the best predictor of recurrence. However, CK19 is not a suitable marker due to its low specificity and relative higher baseline expression in normal nodes.

Conclusions: SCCA mRNA levels for molecular diagnosis of SLN metastases in cervical cancer more accurately identifies patients at risk for recurrence than the routine histology does.

One of the most important prognostic indicators of cervical cancer is the presence of pelvic lymph node metastases. The 5-year survival of cervical cancer patients with negative pelvic lymph node is 87% to 92%, which decreases to 42% to 57% when the pelvic lymph node is positive (1, 2). Therefore, the disease status of the pelvic lymph node is a critical variable to determine whether adjuvant radiotherapy is recommended. Despite favorable prognostic features, pelvic recurrence occurs in ~10% of patients with histologically negative lymph nodes (3). Only 50% of patients with recurrence have histologically positive lymph nodes (4). Histologically undetectable or dormant micrometastases in the lymphatic system probably account for recurrence of cervical cancer after variable disease-free intervals (5).

Histologic examination only samples a very small portion of each lymph node, and it has been calculated that a pathologist has only a 1% chance of detecting a micrometastatic focus within a three-tumor-cell diameter (6). Thus, there is a clear need for more sensitive detection of lymph node micrometastases, thereby allowing more individualized prognosis and treatment planning for cervical cancer patients. Immunohistochemical staining for tumor markers improves the sensitivity of micrometastasis detection and, when combined with serial sectioning to reduce the sampling error, results in upstaging of a significant number of patients. Some studies (7, 8) have shown that doing additional tissue sections and/or immunohistochemistry of lymph node increases metastases detection, whereas other studies (9–11), including our previous study (12), found that immunohistochemistry could not increase the detection rate of lymph node metastases in cervical cancer.

Real-time reverse transcription-PCR (RT-PCR) has been used to accurately measure levels of gene expression (13, 14) and has become widely used for detecting occult micrometastatic tumor cells in the resected lymph node specimens in various malignant diseases (15–19) as well as in cervical cancer (20). The advantages of this technique are the following: the capability to differentiate between baseline levels of gene...
expression in normal tissue versus altered levels in cancer cells; the benefit of a simple, rapid, and automated procedure; and the ability to analyze the entire specimen and decrease cells; the benefit of a simple, rapid, and automated procedure; and the ability to analyze the entire specimen and decrease... -TCCGTCTCAAAC T-ACAG

Annealing Temperature: 60°C

Table 1. Primer and probe sequences for RT-PCR amplification of CK19, SCCA, and GAPDH mRNA

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Amplification</th>
<th>Oligo</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK19 (NM002276)</td>
<td>Exons 2 and 3</td>
<td>Probe: 5’-TCCGTCTCAAC[T]TGTT-3’&lt;br&gt;Forward primer: 5’-AACTCCAGATTGCTCTGAGA-3’&lt;br&gt;Reverse primer: 5’-GCTCATGGCGAGCCGT-3’&lt;br&gt;Probe: 5’-ACAAGAATTTAGATGCCATC-3’&lt;br&gt;Forward primer: 5’-AGACAAAGCTCTTGGTAAATT-3’&lt;br&gt;Reverse primer: 5’-GCCAACAAGCTCTTGGAGA-3’&lt;br&gt;Probe: 5’-CCCACTACATCCTTTAATCC-3’&lt;br&gt;Reverse primer: 5’-CAGGCTGGAATCATATTGGAAC-3’</td>
<td>93 bp</td>
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<tr>
<td>SCCA (NM006919)</td>
<td>Exons 3 and 4</td>
<td>Probe: 5’-TCCGTCTCAAC[T]TGTT-3’&lt;br&gt;Forward primer: 5’-AACTCCAGATTGCTCTGAGA-3’&lt;br&gt;Reverse primer: 5’-GCTCATGGCGAGCCGT-3’&lt;br&gt;Probe: 5’-ACAAGAATTTAGATGCCATC-3’&lt;br&gt;Forward primer: 5’-AGACAAAGCTCTTGGTAAATT-3’&lt;br&gt;Reverse primer: 5’-GCCAACAAGCTCTTGGAGA-3’&lt;br&gt;Probe: 5’-CCCACTACATCCTTTAATCC-3’&lt;br&gt;Reverse primer: 5’-CAGGCTGGAATCATATTGGAAC-3’</td>
<td>90 bp</td>
</tr>
<tr>
<td>GAPDH (BC083511)</td>
<td>Exons 3 and 4</td>
<td>Probe: 5’-TCCGTCTCAAC[T]TGTT-3’&lt;br&gt;Forward primer: 5’-AACTCCAGATTGCTCTGAGA-3’&lt;br&gt;Reverse primer: 5’-GCTCATGGCGAGCCGT-3’&lt;br&gt;Probe: 5’-ACAAGAATTTAGATGCCATC-3’&lt;br&gt;Forward primer: 5’-AGACAAAGCTCTTGGTAAATT-3’&lt;br&gt;Reverse primer: 5’-GCCAACAAGCTCTTGGAGA-3’&lt;br&gt;Probe: 5’-CCCACTACATCCTTTAATCC-3’&lt;br&gt;Reverse primer: 5’-CAGGCTGGAATCATATTGGAAC-3’</td>
<td>66 bp</td>
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</table>

NOTE: Frame indicates the exon splice junction.
soaked in RNAlater-ICE and stored in the tumor bank of Cancer Center (tissues stayed overnight at 4°C, were taken out the next day, and stored at -80°C until the RNA extraction). The time elapsed between the removals of the tissue specimens and putting the tissue in RNAlater-ICE ranged 15 to 100 min. Among them, the timing for lymph node (ranging 60-100 min) was longer than for primary tumor (ranging 15-60 min) because bilateral pelvic lymphadenectomy was conducted followed by abdominal radical hysterectomy. The other half was sent for routine histopathology (one or two slides per node using H&E staining). Normal lymph nodes (n = 20) were obtained from seven patients who underwent a surgical procedure for benign tumors and lymph nodes using H&E staining. Normal lymph nodes (ranging 60-100 min) was longer than for primary tumor. For each patient. None of these patients had a history or clinical evidence of malignancy, and informed consent was obtained from each patient. The whole lymph node was soaked in RNAlater-ICE and sent to the tumor bank.

**RNA isolation and cDNA synthesis.** Total RNA was isolated from primary tumors and lymph nodes with Trizol reagent (Invitrogen). Each specimen was removed from -80°C storage and immediately grinded into powder in liquid nitrogen, not allowing the tissue to thaw. Total RNA was isolated following the manufacturer’s instructions. The RNA pellet was dissolved in 30 µL of diethylpyrocarbonate-treated distilled water. RNA was quantified using spectrophotometry (DU-640, Beckman) and RNA quality was checked by visualization of 18S and 28S rRNA on an agarose gel after ethidium bromide staining. Total RNA (8 µL) and 2.5 µL of oligo(dT) (TaKaRa) were incubated for 10 min at 75°C, cooled on ice for at least 1 min, and incubated with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) in a reaction volume of 20 µL for 90 min at 37°C. Reverse transcriptase was then inactivated by heating at 95°C for 1 min. The resulting cDNA was stored at -20°C until further use.

**Real-time RT-PCR.** Real-time RT-PCR was done using the Taqman MGB method (24). The primers and probe were designed using Primer Express software (version 2.0; PE Applied Biosystems). To avoid detection of genomic DNA, amplification was done on spliced regions of the genes (Table 1). Amplification and detection of CK19 (Genbank accession number M33101) and CK19b pseudogenes (Genbank accession number U85961) is impossible because both primers and probe contain mismatches (Table 2). To confirm amplification specificity of CK19, conventional RT-PCR product (amplified by using the above-mentioned primers) was subjected to sequencing (Supplementary Fig. S1).

An external standard curve was generated by dilution of the target conventional RT-PCR product (cDNA obtained from the primary tumor), which was purified by agarose gel electrophoresis (25). The absolute concentration of the external standard was measured according to Eq. A.

\[
\text{Copies} = \frac{\text{OD}_{260} \times \text{dilution factor} \times 10^{-6} \times 6.02 \times 10^2}{1 \text{ mL} \times 324.5 \times 2 \times \text{number of bases}}
\]

Reactions were recorded and analyzed with the ABI PRISM 7900HT Sequence Detection System. Each PCR amplification was done with a 15 µL final reaction mixture consisting of 7.5 µL of 2× Premix Ex Taq (DRR039, TaKaRa), 0.15 µL of 20 µmol/L forward primer, 0.15 µL of 20 µmol/L reverse primer, 0.2 µL of 20 µmol/L Taqman MGB probe, 1.2 µL CDNA, and 0.3 µL ROX reference dye (50×). The thermal cycling conditions comprised an initial step at 95°C for 10 s followed by 40 cycles with each cycle at 95°C for 5 s and 60°C for 35 s. Two nontemplate controls were included in each amplification run and all measurements were done in duplicate under blind conditions by a single technician. Both the precise amount and quality of mRNA in each sample are extremely difficult to assess, so transcripts of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were quantified as an internal reference for the quantitative PCR assay and each sample was normalized based on its GAPDH content. The quantitative value of SCCA or CK19 mRNA was described as each value relative to GAPDH mRNA, and relative signal intensity (RSI; value of 100,000 × SCCA/GAPDH and 100,000 × CK19/GAPDH) was measured for quantification (19).

**Data collection.** Data from the quantitative RT-PCR analysis were collected and graphed in an Excel file (Microsoft Corp.).

**Statistical analysis.** Statistical analysis was done using Statistical Package for the Social Sciences software (SPSS for windows, version 11.0). The Mann-Whitney U test was used to compare the differences of RSIs among the following groups: primary tumor, histologic positive lymph nodes, histologic negative SLNs, non-SLNs, and normal control lymph nodes. The same test was also used to compare the differences of RSIs in SLNs among different tumor grades, FIGO stages, and tumor sizes. The primary end point was disease recurrence measured from the time of surgery to the time of diagnosed recurrence or last date of follow-up. The cutoff value was set at the upper limit of the quantitative value of lymph nodes from noncancer patients, and those above this cutoff value constituted the molecular metastasis group. Kaplan-Meier disease-free survival curves were plotted for predictors, including FIGO stage, grade, histologic lymph node metastasis, tumor size, and the molecular lymph node metastases based on SCCA and CK19 mRNA, respectively, in each patient. The analysis of disease-free survival was conducted by log-rank tests. P value of <0.05 was considered statistically significant.

### Results

**Demographic and clinical characteristics.** One hundred and seventy-eight samples from 36 patients were analyzable, including 29 primary tumors, 65 SLNs (5 of them were positive by H&E staining), 69 non-SLNs, and 15 normal lymph nodes. The median of patients’ age was 41 years old (range, 28-57 years old). Histotypes were squamous cell carcinoma (31 cases), adenosquamous carcinoma (3 cases), and adeno-carcinoma (2 cases). FIGO stage was IB1 (22 cases), IB2 (11 cases), and IIA (3 cases). Tumor grades were low grade in 3 cases, moderate grade in 11 cases, and high grade in 22 cases. Fifty-nine samples, including 7 primary tumors, 20 SLNs (2 of them were positive by H&E staining), 27 non-SLNs, and 5 control lymph nodes, were excluded from the analysis.

### Table 2. Amplicon generated by amplification of CK19 mRNA

| CK19 | AACTCCAGAGTTGTCTCTGAGA-34 bp-AACCCAGTTGTCTCTGAGA | CAGGCTCTGCCGCTGAGCC |
| CK19b | AACTCCAGAGTTGTCTCTGAGA-34 bp-AACCCAGTTGTCTCTGAGA | CAGGCTCTGCCGCTGAGCC |

**NOTE:** Frames identify location of upstream and downstream primers. The sequence recognized by the labeled probe is shaded. Sequences of pseudogenes CK19a (GI 186694) and CK19b (UBS5961) are also shown. Underlines indicate the mismatches compared with CK19 mRNA.
because the quantities of GAPDH mRNA were less than 10,000 copies/µl and they were considered to contain inadequate RNA. Preoperative brachytherapy and/or chemotherapy had been carried out on 11 patients due to bulky tumors. Seven cases have histologic positive pelvic lymph node. The clinical, demographic, and histopathologic characteristics of the population are reported in Table 3.

**Quantitative analysis of SCCA expression.** The RSIs were plotted and analyzed for five sources, including primary tumors, lymph nodes that were histologically positive, histologically negative SLNs, non-SLNs, and benign lymph nodes from patients free of cancer (Fig. 1). The RSI of SCCA mRNA expression in 28 of all 29 primary tumors (26 cases of squamous cell carcinoma and 3 cases of adenosquamous cell carcinoma) ranged from $4.7 \times 10^3$ to $9.8 \times 10^6$, although the RSI of the rest one (histotype was squamous cell carcinoma) was only 21. The RSI of SCCA mRNA in five histologic positive lymph nodes ranged from $1.8 \times 10^4$ to $1.7 \times 10^5$, and no difference existed between those of primary tumors and positive lymph nodes ($P > 0.50$). The expression levels in SLNs were significantly higher than those in non-SLNs ($P = 0.01$). No difference existed between those of non-SLNs and benign lymph nodes ($P > 0.05$). All of the histologically negative lymph nodes had lower SCCA expression levels than those of the 28 primary tumors or 5 positive lymph nodes.

SCCA expression levels for 65 SLNs from 31 patients were analyzed according to clinicopathologic prognostic features, such as FIGO stage, tumor size, and tumor grade. We found that patients with tumor size $\geq 4$ cm had higher RSIs of SCCA mRNA than those with tumor size $<4$ cm ($P < 0.03$; Fig. 2). SCCA expression levels were significantly higher in the SLNs from patients with tumor of FIGO stage IB$_2$/IIA than in those with FIGO stage IB$_1$ ($P < 0.02$; Fig. 2). However, there is no significant difference between those of grade 1 to 2 and grade 3 ($P > 0.10$; data not shown).

Based on the highest SCCA level from normal lymph nodes, the cutoff level was set as RSI > 352 for elevated SCCA mRNA expression in micrometastases. Four histologic negative lymph

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<th>Stage</th>
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<td>III</td>
<td>$&lt;4$ cm</td>
<td>—</td>
<td>-</td>
<td>-</td>
<td>—</td>
<td>N</td>
</tr>
<tr>
<td>36</td>
<td>38</td>
<td>Squamous</td>
<td>IB$_2$</td>
<td>II</td>
<td>$\geq 4$ cm</td>
<td>—</td>
<td>-</td>
<td>+</td>
<td>—</td>
<td>Y</td>
</tr>
</tbody>
</table>

NOTE: Cases 1 to 5: only primary tumor could be analyzed. Cases 6 to 12: only lymph nodes could be analyzed. Cases 13 to 36: both primary tumor and lymph nodes could be analyzed.

Abbreviation: LNM, lymph node metastases.

*Patient whose SLNs and non-SLNs were studied.

Table 3. The clinical, demographic, histopathologic, and molecular pathologic characteristics of the 36 patients
Molecular Diagnosis of Lymph Node Metastases

Fig. 1. RSI of mRNA levels of two genes in different groups. RSI of SCCA (A) and CK19 (B) mRNA levels were plotted for the following five groups: primary tumor (T), histologic positive lymph nodes (P+LN), histologic negative SLNs (NSLN), non-SLNs (NSLN), and normal control lymph nodes (CLN). The median of each group was labeled by a transverse line (all RSI equaled zero was set as 0.01).

Discussion

Recently, many studies have used quantitative real-time RT-PCR to detect lymphatic micrometastasis and identified the presence of molecular metastasis as an independent factor...
to predict recurrence (21, 26–31). However, similar studies in cervical cancer were rare. To date, only one prospective study designed to assess the value of molecular detection of lymphatic micrometastasis in cervical cancer has been reported by Van Trappen et al. (20)—that the level of CK19 expression in pelvic lymph nodes was related to clinicopathologic features and prognosis. CK19 is a commonly used marker in many studies on micrometastasis, detected by either conventional or real-time RT-PCR (16, 20, 26, 32–35). However, the results were not consistent with each other. Noguchi et al. (32) and Van Trappen et al. (20) reported no expression of CK19 in control lymph nodes, whereas studies by Bostick et al. (33) and Min et al. (34) showed that this marker is expressed in normal lymph nodes and therefore not suitable for molecular detection of micrometastatic breast cancer. In a study by Gillanders et al. (16), CK19 was found with relatively higher baseline level of expression in normal lymph nodes than the other five markers (CEA, mam, mamB, PIP, and PDEF). This inconsistency may be due to simultaneous amplification of two CK19 pseudogenes or the qualitative detection by conventional RT-PCR. In this study, we used a fully quantitative real-time RT-PCR and avoided simultaneous amplification and detection of CK19a and CK19b pseudogenes. In addition, the amplification specificity of CK19 was confirmed by sequencing the conventional RT-PCR product. Therefore, our results showed that CK19 is not a suitable marker for molecular diagnosis of lymphatic metastasis in cervical cancer due to its low specificity and high baseline expression level in normal lymph nodes.

In this study, we clearly showed that SCCA is certainly a better marker than CK19 for molecular diagnosis of lymphatic metastasis in squamous cell and adenosquamous cervical cancer. SCCA is a member of the ovalbumin family of serine proteinase inhibitors, which can be detected in normal cervical squamous epithelium, columnar epithelium, squamous cell carcinoma, and most of adenocarcinoma but barely expressed in normal lymph nodes and endometrium (36–38). Stenman et al. (22) first detected SCCA-expressing tumor cells in peripheral blood by RT-PCR in cervical cancer and found that it can be used for staging and evaluation of prognosis in epidermoid carcinoma of the uterine cervix. Onishi et al. (19) conducted a study on oral cancer and found that genetic diagnosis by real-time quantitative PCR based on SCCA mRNA expression was more practical for detecting occult tumor cells in cervical lymph nodes than CK13. In a more recent study on molecular staging of cervical lymph nodes in squamous cell carcinoma of the head and neck, SCCA was one of the best markers in the 40 markers analyzed, which provided obvious classification of positive and benign lymph nodes (39).

However, case 3’s poorly differentiated primary squamous cell carcinoma tumor in this study showed a low SCCA mRNA level, and the tumor cells were highly malignant clinically. The serum SCCA level was slightly elevated before treatment and descended to normal after one cycle of neoadjuvant chemotherapy, and then it kept in normal value even after recurrence. However, the CEA level was highly elevated both preoperatively and after recurrence. Therefore, this disagreed with the pathology of squamous cell carcinoma (three senior pathologists read this slide and gave the same conclusion). These indicate that the heterogeneity does exist among the same histotype tumor cells, and the pathology judged by eye had some limits. All other primary tumors, including squamous cell and adenosquamous carcinoma, were positive. Unfortunately, we could not get the SCCA mRNA level in adenocarcinoma because two primary tumors with adenocarcinoma were excluded from the analysis due to inadequate RNA. Case 23, with SLN expression of SCCA mRNA lower than the cutoff value, developed distant metastasis 27 months later. It is possible that sampling error existed because the lymph node specimens were divided at the time of collection, with half of the specimen sent for routine pathology and half for real-time RT-PCR. Thus, there is the potential that the portion of the lymph node with micrometastatic squamous cell carcinoma was sent for routine pathology but not real-time RT-PCR.

SLN identification is currently thought feasible in the early-stage cervical cancer (23). The main significance was that the absence of metastatic disease with a SLN should enable a reduction of the extension of pelvic lymphadenectomy and a
possible morbidity-sparing effect. Moreover, such a strategy permits a thorough analysis of only a few lymph nodes in order to enhance the sensitivity and the specificity of the diagnosis of lymph node invasion. The feasibility of this encouraging change in surgical approaches mainly depends on a rapid and accurate SLN analysis in a single operating room. In the treatment of breast cancer and melanoma, in which SLN biopsy is commonly used, the sensitivity of intraoperative frozen section analysis ranges only from 38% to 74% (40–43). GeneXpert, a new biotechnology developed by Cepheid for molecular diagnostic testing, is capable of doing RNA isolation from lyzed tissue, reverse transcription, and quantitative PCR all in 30 min, and it gives the potential application of an intraoperative SLN analysis. When Ferris et al. (39) applied this rapid, multiplex QRT-PCR technique to SLNs from patients with squamous cell carcinoma of the head and neck, they showed that this technology has the potential to accurately stage the neck and allow the surgeon to provide optimal treatment in a single procedure. Histologic examination only samples a very small portion of each lymph node even using the serial section that already increases the cost and labor. However, with this molecular method we used, several sentinel nodes can be mixed and grinded together to form a “sample pool.” Only a little bit of tissue from this fully mixed sample pool is needed for next step examination. It certainly counteracts the increased labor and costs caused by this new technology while at the same time improves the molecular diagnosis results.

It would be of great value when combining an appropriate molecular marker with such a rapid, accurate intraoperative SLN analysis in cervical cancer because it allows the surgeon to determine the necessary extent of resection during the surgery and a more individualized treatment planning could be designed for these patients. In this study, we have verified that progression of the molecular metastasis in squamous cell and adenocarcinoma cervical cancer based on quantification of SCCA mRNA is more accurate than that based on histopathologic analysis. A new study will be conducted soon on the molecular metastasis diagnosis based on SCCA-mRNA combined with the GeneXpert.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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