An Optimal mRNA Marker for OSNA (One-step Nucleic Acid Amplification) Based Lymph Node Metastasis Detection in Colorectal Cancer Patients

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Received May 24, 2012; accepted December 6, 2012

Background: We previously reported that the one-step nucleic acid amplification assay is effective for lymph node metastasis detection in breast cancer patients. This paper describes the identification of CK19 mRNA as an optimal marker and its cut-off value for use in the detection of one-step nucleic acid amplification-based lymph node metastasis in colorectal cancer patients.

Methods: Candidate mRNA markers selected from the genome-wide expressed sequence tag database were evaluated by quantitative RT-PCR using a mixture of metastasis-positive and another mixture of metastasis-negative lymph nodes (n = 5 each), followed by quantitative RT-PCR using metastasis-positive and -negative lymph nodes (n = 10 each) from 20 patients. The one-step nucleic acid amplification assay for mRNA markers selected above was examined using 28 positive lymph nodes from 19 patients and 38 negative lymph nodes from the 11 pN0 patients.

Results: Quantitative RT-PCR analyses of the 98 mRNAs selected from the genome-wide expressed sequence tag database and the subsequent quantitative RT-PCR analyses of the nine mRNAs selected above indicated that CK19 and CEA mRNAs have the highest capability for distinguishing between positive and negative lymph nodes. CK19, CEA and CK20 mRNAs were evaluated by the one-step nucleic acid amplification assay. An area under a receiver-operating-characteristic curve for CK19 mRNA (0.999) was slightly larger than that for CEA mRNA (0.946; P = 0.062) and significantly larger than than for CK20 mRNA (0.875; P = 0.006).

Conclusion: We found that CK19 mRNA has the best diagnostic performance and its cut-off value for discriminating positive from negative lymph nodes can be set in the range of 75–500 copies/μl with 96.4% sensitivity and 100% specificity.

Key words: OSNA – colorectal cancer – lymph node metastasis – cytokeratin 19
INTRODUCTION

Stage IIIa colorectal cancer (CRC) patients treated with chemotherapy are reported to have significantly better prognosis than stage IIb CRC patients for whom chemotherapy is not recommended (1, 2). Several authors have suggested that this discrepancy may arise from a poor performance in the detection of lymph node (LN) metastasis with routine histopathologic examination (3–8).

Detection of LN metastasis in CRC patients is conventionally conducted by microscopic observation of tissue surfaces cut from a dissected LN, followed by H&E staining. This method frequently fails to detect micrometastases because of the localization of metastatic focus in LNs. Noura and Monden (8) reported that the detection rate of micrometastases using 1, 2 and 5 slices cut from an LN was 3.8, 6.3 and 11.8%, respectively. Although multi-section histopathology enables the detection of micrometastases, it is not a realistic solution for routine clinical use.

We recently reported that the one-step nucleic acid amplification assay (OSNA) with cytokeratin (CK) 19 mRNA (Fig. 1A), based on reverse transcription loop-mediated isothermal amplification (RT-LAMP) but not RT-PCR, is highly effective for intra-operative LN metastasis detection in breast (9–17) and gastric cancer patients (18). The method is characterized by the quantitative measurement of a target mRNA in a metastatic LN, brief reaction time and high specificity for the target mRNA using six primers, and is now being used clinically. Most recently, we have reported successful clinical studies for the detection of OSNA-based LN metastasis in CRC patients using CK19 mRNA as a molecular marker (19, 20). However, we have not yet reported the background for the identification of CK19 mRNA as an optimal marker.

In this paper we report an exhaustive search for an optimal mRNA marker through four screening phases from a genome-wide expressed sequence tag (EST) database. A cut-off value of CK19 mRNA for discriminating the positive from the negative LNs will also be discussed.

PATIENTS AND METHODS

LNs and Primary Tumors

LNs and primary tumors from the CRC patients were obtained from the Cancer Institute of the Japanese Foundation for Cancer Research and Osaka Police Hospital with the approval of their internal review boards. The samples were obtained from the patients who had not been treated with chemotherapy or radiation.

Half of an LN was snap-frozen in liquid nitrogen and stored at −80°C until use. The frozen LNs used throughout this study were obtained from 40 patients with 26 well-differentiated tubular adenocarcinoma, 6 moderately differentiated tubular adenocarcinoma, 1 poorly differentiated adenocarcinoma and 7 adenocarcinoma of unknown differentiation. LN blocks (b in Fig. 1A) obtained from the pathologically negative LNs of pN0 patients and those from the pathologically positive LNs were used as metastasis-negative and -positive LNs, respectively.

Paraffin-embedded primary tumors were obtained from 187 patients with 177 adenocarcinomas (72 well-differentiated tubular adenocarcinoma, 96 moderately differentiated tubular adenocarcinoma, 96 moderately differentiated tubular...
adenocarcinoma and 9 poorly differentiated adenocarcinoma), 9 mucinous carcinomas and 1 signet ring cell carcinoma.

**PREPARATION OF LN LYSE AND RNA SOLUTION**

The study protocol is shown in Fig. 1A. Histopathologic examination with H&E was carried out on the center of a whole LN (Fig. 1A, i). The central portion (block b, 2–3 mm of thickness) of one half of a frozen LN was cut out. From this block, two slices (Fig. 1A, ii and iii) were cut and subjected to histopathologic examination with immunohistochemistry using antibody against CK (anti-keratin AE1/AE3; Dako, Glostrup, Denmark). Metastasis-positive LNs were defined as being positive at least at one of the three levels (Fig. 1A, i, ii and iii).

Blocks (Fig. 1A, a and c) were used for the preparation of an LN lysate for use in OSNA or quantitative RT-PCR (QRT-PCR). The blocks (<600 mg) were homogenized for 90 s in 4 ml of a lysis buffer (Lynorhag) (Sysmex, Kobe, Japan) on ice using a Physcotron Warring blender with an NS-4 shaft (MicroTec Nichion, Tokyo, Japan). The homogenate was centrifuged at 10 000 g for 1 min at room temperature and the supernatant was used as the LN lysate. RNA was purified from the LN lysate using the RNeasy mini kit (QIAGEN, Hamburg, Germany) according to the manufacturer’s instructions. The purified RNA solution was quantified by UV spectrophotometry at 260 and 280 nm. The RNA quality was confirmed by identifying 18s and 28s rRNA bands on a 1% agarose gel stained with ethidium bromide.

**SELECTION OF mRNA MARKERS AND QRT-PCR**

An optimal marker for the OSNA assay was selected in four phases. In the first screening phase, we used data for 220 196 EST tags from 978 colon cancer libraries and 219 955 EST tags from 128 LN libraries using the genome-wide EST database of the Cancer Genome Anatomy Project (CGAP, http://cgap.nci.nih.gov/Info/CGAPDownload) in November 2004 (21). We compared the expression frequency of each gene in the colon cancer libraries with its expression in the LN libraries. The expression frequency of each gene was obtained from the total tag number for each gene out of the total tag number for each library.

In the second screening phase, the expression of each mRNA markers in metastasis-positive and -negative LNs were investigated by QRT-PCR. A mixture of five metastasis-positive LNs and another mixture of five metastasis-negative LNs were used as positive and negative LN samples, respectively. QRT-PCR was carried out with an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). The purified RNA (2 μl) was subjected to QRT-PCR with QuantiTect SYBR Green (QIAGEN) according to the manufacturer’s instructions. Primers were designed using ABI Primer Express Version 2.0 (Applied Biosystems). Forward and reverse primers were prepared for 98 mRNAs and β-actin, which was used as a control (Supplementary Table S1). The detailed QRT-PCR procedure was described in our previous report (9). The expression level of each marker was evaluated based on a ΔΔCt [Δ (threshold cycle, ΔCt = Ct (for each mRNA) − Ct (for β-actin)]. The difference between the expressions of each marker in the metastatic-positive and -negative LNs was evaluated based on a ΔΔΔCt [ΔΔCt = ΔCt (for negative LNs) − ΔCt (for positive LNs)] (22).

The reactions were performed in duplicate. Each marker was evaluated based on its ΔΔCt value indicating the difference between ΔCt values for positive and negative LNs.

In the third screening phase, the expression of the mRNA markers selected above was evaluated by QRT-PCR using 10 positive and 10 negative LNs from 20 patients. The reaction condition was same as mentioned above. The performance of each marker was evaluated based on a ΔΔΔCt value, indicating the difference between the mean ΔCt value for positive LNs and the lowest ΔCt value for negative LNs.

In the fourth screening phase, the mRNA marker expression was examined with the OSNA assay using 28 positive LNs from 19 patients and 38 negative LNs from 11 pN0 patients.

**OSNA**

The OSNA included solubilization of an LN followed by gene amplification using RT-LAMP (23). Figure 1B shows a schematic diagram of OSNA. The LN lysate obtained from the homogenization of LN blocks described in the above section was diluted 10 times with Lynorhag and then a 2 μl of the diluted solution was subjected to RT-LAMP assay. The RT-LAMP reaction was carried out at 65°C on RD-100i (Sysmex) and finished within 16 min. The reaction mixture in the RT-LAMP reaction yields a white turbidity with the passage of time (24). Mori et al. (24) reported that pyrophosphate, which is produced in the course of the RT-LAMP reaction, binds to a magnesium ion to result in magnesium pyrophosphate. The amount of magnesium pyrophosphate increases with the passage of the reaction. Magnesium pyrophosphate has a low solubility in the aqueous solution, and precipitates when its concentration reaches saturation. The amplification of an mRNA marker was automatically monitored at 6-s intervals on RD-100i by measuring the turbidity of the reaction mixture. An mRNA copy number in the reaction mixture was calculated based on the threshold time defined as the time at which the turbidity exceeded 0.1.

Figure 1C shows the time courses of RT-LAMP reactions using CK19 mRNA solutions of known concentration. An inverse correlation between the threshold time in the RT-LAMP step and the CK19 mRNA concentration was observed in a range of CK19 mRNA concentrations of 2.5 × 107 to 2.5 × 102 copy/μl (9).

Six different primers were used for the RT-LAMP reaction based on the impressive studies reported by Notomi et al. (23, 25). A set of two inner and two outer primers as well as
an additional set of two primers including the forward and backward loop primers was designed specifically to recognize the eight distinct regions on a target gene. The primers for CK19 mRNA were designed and prepared as described in our previous paper (9). Primers for CEA and CK20 were designed by PrimerExplorer V4 (Fujitsu System Solutions, Tokyo, Japan): CK20 FA, 5’-CTGCTGAGACTCTAGAGCTGACTTGGAGATCAGCTT-3’; CK20 RA, 5’-CTGCTGAGACTCTAGAGCTGACTTGGAGATCAGCTT-3’; CK20 F3, 5’-CAATTTGGTGTTTG-3’; CEA FA, 5’-CAATTTGGTGTTTG-3’; CEA RA, 5’-CAATTTGGTGTTTG-3’; CEA F3, 5’-CAATTTGGTGTTTG-3’; CEA LPF, 5’-CAATTTGGTGTTTG-3’; CEA LPR, 5’-CAATTTGGTGTTTG-3’; CEA F3, 5’-CAATTTGGTGTTTG-3’; CEA LPF, 5’-CAATTTGGTGTTTG-3’. To avoid the amplification of genomic DNA, the primers were designed to amplify the exon junction regions in each gene.

ROC Curve Analysis and a Cut-off Value

The area under the ROC (receiver operating characteristic) curve (AUC) for CK19, CEA and CK20 mRNAs was obtained based on the data acquired in the fourth screening phase. The statistical analysis was carried out by the Wilcoxon test using the StatMate IV software (ATMS, Tokyo, Japan). Based on the ROC curve for CK19 mRNA, we set a cut-off value for discriminating the metastasis-positive from the metastasis-negative LNs at a copy number at which the sensitivity reaches a maximum when the specificity is set at 100%.

Immunohistochemical Staining with Anti-CK19 Antibody

Paraffin-embedded primary tumors were sectioned at a 4-μm thickness. After deparaffinization and rehydration, a section was trypsinized for 20 min at 37°C with 0.1% standard calcium chloride solution (pH 8.5) and then treated for 20 min with 10% normal rabbit serum. The section was incubated at 4°C in mouse monoclonal antibody CK19 antibody (RCK108, Dako, Glostrup, Denmark), and stained with streptavidin–biotin immunoperoxidase (iVIEW DAB Detection Kit, Ventana, Arizona) according to the manufacturer’s instructions.

RESULTS

QRT-PCR was used to evaluate a large number of mRNAs in the second and third screening phases because the primers can be prepared in a short period of time; OSNA requires considerable time and effort for the preparation of an optimal set of six primers. In the fourth screening phase, candidate markers were finally evaluated by OSNA to determine the best marker for clinical use.

A Δ Ct value was used as an indicator of the expression level for an mRNA marker; the smaller the Δ Ct value, the higher the expression. A ΔΔ Ct value was used as an indicator of the difference between the expression levels for an mRNA marker in metastasis-positive and -negative LNs.

Selection of Optimal mRNA Markers and Setting of a Cut-off Value

In the first screening phase, candidate 98 mRNA markers were selected from genome-wide EST database by comparing an expression frequency in colon cancer with that in LN libraries for each gene (Supplementary Table S1). In the second screening phase, the 98 candidates were evaluated by QRT-PCR using metastasis-positive and -negative LNs. The results are summarized as ΔΔ Ct values (Supplementary Table S1), which were obtained from the differences between Δ Ct values for positive and negative LNs. We selected nine markers with ΔΔ Ct > 12 including mRNAs of carcinoembryonic antigen (CEA), CK19, CK20, mucin (MUC) 2, MUC11, MUC12 and MUC13, FXYD domain-containing ion transport regulator 3 (FXYD3) and cystic fibrosis transmembrane conductance regulator (CFTR).

In the third screening phase, candidate markers were selected according to the following two criteria: the first was Δ Ct > 9, which indicates a difference between the lowest Δ Ct value for negative LNs and the mean Δ Ct value for positive LNs; the second was the mean Δ Ct for positive LNs < 5. Of the 9 markers, CK19, CEA, FXYD3 and CK20 mRNAs met the first criterion, among which CK19 and CEA mRNAs met the second criterion (Fig. 2, Table 1). CK19 and CEA mRNAs indicating a high capability for distinguishing between the positive and negative LNs were
further evaluated for use in the OSNA assay. In addition to the above two markers, CK20 mRNA was selected because it has been well documented as a marker for LN metastasis detection in CRC patients (26–31).

In the fourth screening phase, CK19, CEA and CK20 mRNAs were evaluated by the OSNA assay. The results are provided in Fig. 3. CK19 mRNA was expressed in all histopathologically positive LNs. On the other hand, the expressions of CEA and CK20 mRNAs were not observed for three and six histopathologically positive LNs, respectively, as indicated by arrows in Fig. 3. On the other hand, the expression level of each mRNA was not different in most of the histopathologically negative LNs.

It is well known that the ROC curve analysis for a diagnostic marker is a precise and valid measure for diagnostic performance (32). We examined the ROC curve analyses for CK19, CEA and CK20 mRNAs to compare their performances (Fig. 4). The AUC for CK19 mRNA (0.999) was slightly larger than that for CEA mRNA (0.946) \((P = 0.062)\) and significantly larger than for CK20 mRNA (0.875) \((P = 0.006)\). It should also be noted that the AUC for CEA mRNA was significantly larger than that for CK20 mRNA \((P = 0.008)\). The maximum sensitivities for CK19, CEA and CK20 mRNAs were 96.4%, 89.2% and 75.0%, respectively, when the specificity of each gene was set at 100%.

The OSNA assay with CEA and CK20 mRNAs showed very low levels of expression for several positive LNs (Fig. 3). Only CK19 mRNA was expressed at higher levels than the others for all positive LNs and showed a significantly acceptable difference in the expression between positive and negative LNs.

The above ROC curve analysis indicates that a cut-off value of CK19 mRNA for discriminating metastasis-positive and -negative LNs can be set in the range of 75–500 copies/μl with 96.4% sensitivity and 100% specificity.

IMMUNOHISTOCHEMICAL ANALYSIS OF PRIMARY TUMORS

The low expression of CK19 mRNA in a metastatic LN might possibly cause a false-negative in the OSNA assay. CK19 expression in a primary tumor could become an indicator of CK19 mRNA expression in a positive LN. We examined the expression of CK19 in 187 primary tumors from CRC patients, finding a frequency of 98.4% (Table 2). The result suggests that CK19 mRNA would be expressed at a high frequency in positive LNs.

DISCUSSION

A marker group including mRNAs of matrix metalloproteinase 7, caudal type homeobox 1, CK20, MUC 1, CEA, guanylyl cyclase C, tumor-associated calcium signal transducer 1 and Villin 1 as well as a combination of these markers to compensate for the poor performance of a single mRNA marker was selected. An optimal mRNA marker for OSNA assay in CRC
(26–31, 33–36), has been selected empirically and examined for QRT-PCR-based LN metastasis detection in CRC patients. However, the QRT-PCR assay with these markers has not been applied for routine clinical use. Thus, our study began with an exhaustive search for an optimal marker via the first through fourth screening phases from the genome-wide EST database.

Of the nine markers selected in the second screening phase, five (CEA, CK19, FXYD3, CK20 and MUC13 mRNAs) showed relatively good separation in the expression between positive and negative LNs (Fig. 2, Table 1). The performance of the markers other than CK19, CEA and CK20 mRNAs for LN metastatic detection in CRC patients is not well known (26–31, 33–36). FXYD3 and MUC13 mRNAs have been reported to be good prognostic markers for CRC patients. FXYD3 is a cell membrane protein that may regulate the function of ion pumps and ion channels, and its strong expression has been reported to predict worse survival in CRC patients (37). MUC13 is a cell surface membrane-anchored mucin expressed abundantly in all CRCs, with the highest expression in more poorly differentiated tumors (38).

In the fourth screening phase, the ROC curve analyses showed that CK19 and CEA mRNAs had significantly better diagnostic performance than CK20 mRNA. Further analysis of the data (shown in Fig. 3) indicated that the OSNA with CK19 mRNA would make it easier to detect a metastatic focus in an LN than using CEA mRNA because OSNA could detect CK19 mRNA (100 copy/μl) more reproducibly than CEA mRNA (1 copy/μl). Thus, CK19 mRNA has a great advantage over CEA and CK20 mRNAs for use in OSNA.

False positives resulting from the amplification of CK19 pseudogenes are well known (39). Several reports have shown that RT-PCR with CK19 mRNA causes false-positive results for LN metastasis detection in CRC patients, presumably because of the amplification of pseudogenes (31, 39). To eliminate this kind of false positives, we designed six primers that can amplify only CK19 mRNA but not the pseudogenes, and used these primers for the OSNA assay.

The low expression of CK19 mRNA in a positive LN would cause a false negative for the OSNA assay. In our experiment using 187 primary tumors, we observed a high expression frequency (98.4%) for CK19 (Table 2). Other authors have reported that all primary tumors they examined (85 of 85) were positively stained with anti-CK19 antibody. These results suggest that CK19 mRNA is expressed at a high frequency in positive LNs from a broad range of patients. Interestingly, Tsuda et al. reported that CK19 is highly expressed in positive LNs from breast cancer patients even when its expression is not observed in primary tumors (unpublished data; presented by Tsuda et al. at the Ninth Annual Meeting of the Japanese Society for Sentinel Node Navigation Surgery, Tokyo, 2007).

Based on the results obtained in this study, we and other authors have conducted multicenter clinical studies in Japan (18) and Europe (40), obtaining promising results of 94.9–95.2% sensitivity and 97.7–97.9% specificity.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

Acknowledgements

We thank the staff of the clinical and pathology laboratories at each facility for their support. We also would like to thank Yoshihito Yamamoto, Yasumasa Akai and Masahiro Nishida for supporting the construction of the OSNA system.

Funding

This work was supported by research and development fund of Sysmex corporation.

Conflict of interest statement

Prof. N. Matsura has been a scientific advisory board member of SYSMEX Corporation. M. Daito, K. Hiyama, J. Ding, K. Nakabayashi and Y. Otomo are employees of Sysmex Corporation.

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