Rapid and Sensitive Detection of Messenger RNA Expression for Molecular Differential Diagnosis of Renal Cell Carcinoma

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ABSTRACT

Purpose: **The aim of this study was to develop a practical technique to detect mRNA expression and to validate a panel of mRNA markers for molecular differential diagnosis of renal cell carcinoma (RCC).**

Experimental Design: **The renal cancer cell line SKRC-52 was used to set up the technique, which consisted of column extraction of RNA and one-step reverse transcription-PCR. We validated a panel of gene markers, including MN/CA9, cadherin-6, vimentin, mucin1, and parvalbumin, and studied 50 renal tumors (30 conventional, 9 papillary, and 5 chromophobe RCCs and 6 oncocytomas), 10 normal tissues, and 10 normal blood samples. We mimicked fine needle aspiration (FNA) biopsy in 10 kidneys with conventional RCC and applied this technique to 10 preoperative FNA samples from imaging-indeterminate renal tumors.**

Results: **The technique could detect as few as 10 SKRC-52 cells with MN/CA9 as mRNA marker and was less time consuming and labor intensive. MN/CA9 was a sensitive and rather specific gene marker for conventional RCC. Cadherin-6 gene expression was a sensitive marker for conventional and papillary RCC. Vimentin was highly specific for conventional RCC. Mucin1 mRNA was sensitive for papillary and chromophobe RCC and oncocytoma. Parvalbumin mRNA was a sensitive and highly specific marker for both chromophobe RCC and oncocytoma. Thus, these mRNA markers represent the biomarker genes for the subtypes of renal tumors. Finally, we successfully applied the technique to FNA specimens. Five preoperative FNA samples were MN/CA9 gene positive, suggesting a RCC, whereas the routine cytology was positive in only three cases.**

Conclusions: **A rapid and sensitive assay of mRNA markers was developed for molecular differential diagnosis of RCC. This molecular assay can be used as a powerful ancillary to surgical pathological diagnosis and cytological diagnosis of RCC.**

INTRODUCTION

The incidence of renal cell carcinoma (RCC) has increased steadily during recent years (1). Similar to other malignant cells, renal cancer cells contain abnormalities in the genetic code of their DNA. The first step in translation of the genetic code into function is the production of mRNA molecules. The mistake in the genetic code will result in an aberrantly expressed type of mRNA. Once a gene or DNA alteration occurs in the progenitor cells of a tumor, it marks all descendent cells for their entire life span; therefore, the detection of aberrantly expressed mRNA markers becomes an important target for molecular diagnosis of cancer and may predict the potential for metastasis.

Recent studies have demonstrated gene expression profiling in various cancers, including RCC (2–4). MN/CA9 and cadherin-6 are two new molecular markers for conventional RCC (5–8). MN/CA9, first detected on the cell surface of the cervical carcinoma cell line HeLa (9), belongs to the carbonic anhydrase family. MN/CA9 mRNA and protein are up-regulated in many cancers in response to hypoxic conditions. MN/CA9 was recently explored in RCC and considered a reliable biomarker (5, 6). Its encoded protein is recognized by two monoclonal antibodies, M75 and G250 (6, 10, 11). Cadherin-6 was recently found to play an important role in fetal kidney development (12). Cadherin-6 is specifically expressed in adult renal proximal tubules, whereas other organ tissues do not express it (8). Cadherin-6 was also found to have prognostic value in RCC (13). Vimentin was found to be a specific marker of conventional RCC in recent microarray studies (2, 3). Mucin1 is a transmembrane glycoprotein. Immunohistochemical study of RCC shows that most specimens stain strongly for mucin1 (14–17). Parvalbumin has been shown to be a super immunohistochemical marker for distal nephron (18, 19). This gene was recently found to be overexpressed in chromophobe RCC and oncocytoma (2). With the completion of the Human Genome Project, we can design specific primers to detect the specific gene transcripts of these markers by the sensitive technique reverse transcription-PCR (RT-PCR). Therefore, these new markers can be readily used for molecular diagnosis of RCC. However, the technical inconvenience is a major obstacle for direct clinical application. Such molecular assays require serious improvement before they can become clinical diagnostic tools.

In the present study, we developed a rapid and sensitive molecular method to detect the mRNA markers for the purpose of molecular diagnosis of RCC. Our efforts could facilitate clinical applications.

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MATERIALS AND METHODS

Renal Carcinoma Cell Lines and Normal Renal and Tumor Tissues. Two renal carcinoma cell lines, SKRC-52 and SKRC-59, were a gift from Dr. E. Oosterwijk (University Medical Center Nijmegen, Nijmegen, The Netherlands). SKRC-52 was used as a positive control for MN/CA9 and SKRC-59 as a positive control for cadherin-6, vimentin, mucin1, and parvalbumin. Both cell lines were cultured in RPMI 1640 tissue culture solution supplemented with antibiotics (1%), glutamine (1%), and calf serum (5%).

This study included 50 renal tumors (30 conventional, 9 papillary, 5 chromophobe RCCs, and 6 oncocytomas), 10 normal renal tissue samples, and 10 normal blood samples. The RCCs were staged according to 1997 TNM classification and graded according to the Fuhrman criteria (20, 21). The renal tumor and adjacent normal tissue were obtained immediately after the kidney was removed. The renal tissues were placed in RPMI 1640 tissue culture solution for transportation and were processed within 2 h. In addition, another tumor tissue sample and adjacent normal tissue sample were snap-frozen in liquid nitrogen and stored in −80°C for future use. Special attention was paid to keep the tissues sterile.

Management of Cell Lines and Renal Tissues. The cell lines were harvested at confluence with Hanks-EDTA and washed twice with Hanks solution. The cells were counted, and 1×10^6 cells were used for RNA extraction.

Renal tissues were dissociated mechanically as described previously (22, 23). Briefly, a small tissue piece of ~0.3 cm^3 plus 1.5 ml of Hanks solution were placed in a Medicon chamber (Dako A/S, Copenhagen, Denmark). The Medicon was inserted in a Medimachine and run for ~1 min. The cell suspension was removed by syringe aspiration and centrifuged for 10 min at 300 × *g* at 4°C. The cell pellet was washed once with Hanks solution.

Dilution of Renal Carcinoma Cell Line. In our previous experiment, we found that SKRC-52 was strongly MN/CA9 positive (22). We therefore chose SKRC-52 to test the sensitivity of our technique. SKRC-52 cells were harvested as mentioned above. The cells were counted and diluted with Hanks solution, and dilution aliquots containing 10, 100, and 1000 SKRC-52 cells were obtained.

Fine Needle Aspiration (FNA) Biopsy. To mimic the FNA biopsy, we first experimented with FNA aspiration in 10 kidneys with conventional RCC. When the kidney was removed, we performed FNA aspiration with a 21-gauge needle. The aspirated cells were placed in a tube containing 2 ml of RPMI 1640 and used for experiments within 2 h. The needle was washed several times with RPMI 1640 in the tube. The FNA specimen was centrifuged for 10 min at 300 × *g* at 4°C and washed once with Hanks solution. The cells prepared in this way were ready for RNA extraction.

Ten patients with a kidney tumor had a preoperative FNA biopsy under ultrasound guidance for routine cytological examination. These were atypical cystic tumors or suspected oncocytomas. The malignant or benign nature of these tumors could not be established by imaging techniques such as ultrasound, computed tomography, and magnetic resonance imaging. An extra FNA biopsy was taken for RNA extraction and RT-PCR analysis. The cells were processed as in the experiment of FNA biopsy. This research protocol was approved by a local research committee, and informed consent was obtained from each patient.

Column Extraction of Total RNA. Total RNA was extracted by use of a RNeasy mini kit (Qiagen S.A.). Briefly, 350 μl of CTL solution was used to lyse cells. The lysed cells were homogenized by use of a springing column, and then 350 μl of 70% ethanol were added to the lysate. The solution was then washed with RW buffer and REP buffer. The RNA was eluted in 30 μl of RNase-free water and then quantified by UV spectrophotometry at 260 nm. The RNA specimens were stored in −80°C until RT-PCR. For the SKRC-52 dilution test, we did not measure the RNA quantity because its yield was very low. In this case, we used the maximum volume for RT-PCR.

One-Step RT-PCR and Analysis of PCR Products. One-step RT-PCR was performed according to the manufacturer's specifications (Invitrogen) with 200 ng of RNA. The RT-PCR reaction mixture contained 25 μl of 2× reaction buffer, 1 μl of sense primer (10 μM), 1 μl of antisense primer (10 μM), 1 μl of RT/PlatinuM Tag mixture, 200 ng of RNA, and distilled water to a final volume of 50 μl. The following RT-PCR conditions were used: 1 cycle of 50°C for 30 min followed by 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min.

We designed six specific primers: β-actin, MN/CA9, cadherin-6, vimentin, mucin1, and parvalbumin. The primers were as follows:

For β-actin (507 bp): sense primer, 5′-TAC CAC TGG CAT CGT GAT GGA CT-3′; antisense primer, 5′-TCC TTC TGC ATC CTG TCG GCA AT-3′;

For MN/CA9 (386 bp): sense primer, 5′-GGA CAA AGA AGG GGA TGA CC-3′; antisense primer, 5′-AAA GGC GGT GCT GAG GTG AA-3′;

For cadherin-6 (509 bp): sense primer, 5′-ATT CAG CCA CGG TTA GAA TTg-3′; antisense primer, 5′-TGA GCC ACT GGC TGC TTC A-3′;

For vimentin (643 bp): sense primer, 5′- TAT GCC ACG CGC TCC TCT G-3′; antisense primer 5′-GCT GAC GTA CGT CAC GCA G-3′;

For mucin1 (562 bp): sense primer, 5′-ACC ACT CTG ATA CTC CTA CC-3′, antisense primer, 5′-GCA CTG ACA GAC AGC CAA GG-3′;

For parvalbumin (329 bp): sense primer, 5′-GAT GAC AGA CTT GCT GAA CGC-3′; antisense primer, 5′-CTT AGC TTT CAG CCA CCA GAG-3′.

These primers were designed to span the splice junction so that genomic DNA contamination could be monitored (24).

For each RT-PCR, SKRC-52 was used as a positive control for MN/CA9 and SKRC-59 for cadherin-6, vimentin, mucin1, and parvalbumin. Reactions without template RNA were used as a negative control. The RT-PCR for β-actin was used to check the quality of the RNA extraction and RT-PCR.

We analyzed 15 μl of PCR products by electrophoresis on a 1.5% agarose gel. DNA fragments were visualized and photographed under UV light after ethidium bromide staining. The expected band for each marker was identified by comigration of a DNA marker ladder electrophoresed in an adjacent lane. In addition, the amplified fragments in a representative case for each marker were sequenced to confirm the fidelity of PCR amplification.

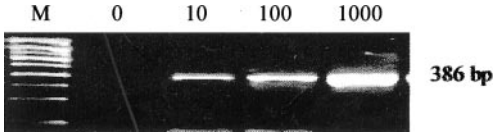

Fig. 1 Sensitivity of the assay. *M*, molecular marker. SKRC-52 cells were diluted to test the sensitivity of MN/CA9 (386 bp). The assay could detect as few as 10 SKRC-52 cells.

RESULTS

Set-up of Assay. As expected, MN/CA9 was always detected in SKRC-52 cells, and cadherin-6 was always detected in SKRC-59 cells. In our dilution test, we found that a signal could be detected when 10 SKRC-52 cells were analyzed (Fig. 1).

Analysis of Renal Tissues. The TNM classification of RCC was as follows: 13 T1a, 10 T1b, 2 T2, 10 T3a, 8 T3b, and 1 T3c. There were 4 N+ and 4 M+ tumors. The RCC grade was as follows: 6 grade I, 21 grade II, 12 grade III, and 5 grade IV.

The expression rates of the mRNA markers are shown in Table 1. Conventional RCC frequently expressed MN/CA9 (97%), cadherin-6 (93%), and mucin1 (57%). Papillary RCC was positive for MN/CA9 (56%), cadherin-6 (89%), and mucin1 (89%). Chromophobe RCC expressed mainly mucin1 (80%) and parvalbumin (100%). Oncocytomas were frequently positive for cadherin-6 (67%), mucin1 (100%), and parvalbumin (83%). Normal renal tissues expressed cadherin-6 (90%) and parvalbumin (50%), but were negative for MN/CA9 and vimentin. Normal blood samples were negative for MN/CA9.

The sensitivity, specificity, positive predictive value and negative predictive value of each marker for each subtype of renal tumors were calculated and are shown in Fig. 2. In our study, the sensitivity measures the probability that the marker in a subtype is positive (number positive in the subtype/number of tumors in the subtype), and the specificity measures the probability that the marker in other subtypes is negative (number negative in other subtypes/number of tumor in other subtypes). The positive predictive value assesses the reliability of a positive result for the marker (number of positives in the subtype/total number of positives), and the negative predictive value assesses the reliability of a negative result for the marker (number of negatives in the subtype/total number of negatives). MN/CA9 and vimentin were highly specific for the conventional RCC. Parvalbumin was highly specific for both chromophobe carcinoma and oncocytoma. The typical mRNA expression of a conventional RCC was differentiated from that of an oncocytoma (Fig. 3). In our study, cadherin-6 and mucin1 were not specific markers for subtype differentiation.

Analysis of FNA Biopsy. In the experiment, the 10 FNA specimens from conventional RCCs demonstrated MN/CA9 and cadherin-6 gene expression (Fig. 4). A typical MN/CA9 mRNA expression pattern in a preoperative FNA sample is shown in Fig. 5. The results of the preoperative FNA biopsies are shown in Table 2. Five of the preoperative FNA biopsies were MN/CA9 gene positive, suggesting a RCC. Cytology was positive in three cases, and one false positive was found (case 8). One patient with a cystic conventional RCC was not detected by either the cytological examination or the molecular assay (case 6).

DISCUSSION

The utilization of gene markers could result in more accurate and sensitive diagnoses and classifications of cancer. With rapid advances in molecular biology, genetic diagnosis of RCC may be realized by detection of mRNA markers. To obtain this objective, two fundamental requirements must be fulfilled: the assay protocol must be practical and the mRNA markers must be efficient.

Although many results in basic research have been successful, translation into the clinical laboratory use is not necessarily easy. The potential use of molecular assays requires a technique to be sensitive, specific, simple to manipulate, and easy to standardize. Here we explored the possibility of genetic diagnosis of RCC. RT-PCR is an efficient technique of gene diagnosis, but this technique has rarely been used in clinical samples for cancer diagnosis, which suggests the technical difficulty of direct application. We intended to overcome this difficulty by combining rapid column extraction of RNA with one-step RT-PCR. Boylan *et al.* (25) found that column extraction of RNA was more sensitive than the commonly used chloroform–ethanol method. We also found that the RNA yield in column extraction was higher than that in chloroform–ethanol extraction (data not shown). This column extraction of RNA was recently used to extract RNA from microdissection specimens in which the number of cells was limited (25, 26) and needs only 20 min. In our study, we also found that the extraction could be effective when as few as 10 SKRC-52 cells were used. One-step RT-PCR limits the chances of possible contamination and makes the process more easily standardized when a certain quantity of RNA is used for analysis. The combination of column extraction of RNA and one-step RT-PCR significantly reduced the labor and time required for the analysis. This allows more rapid and efficient detection of gene expression in cancer tissues. The whole process can be finished within several hours,

Table 1 mRNA expression for the different subtypes

	No. (%) of samples positive for expression of				
	MN/CA9	Cadherin-6	Vimentin	Mucin 1	Parvalbumin
RCC					
Conventional	29/30 (97%)	28/30 (93%)	18/30 (60%)	17/30 (57%)	3/30 (10%)
Papillary	5/9 (56%)	8/9 (89%)	2/9 (22%)	8/9 (89%)	1/8 (13%)
Chromophobe	0/5 (0%)	1/5 (20%)	1/5 (20%)	4/5 (80%)	5/5 (100%)
Oncocytoma	0/6 (0%)	4/6 (67%)	1/6 (17%)	6/6 (100%)	5/6 (83%)
Normal tissue	0/10 (0%)	9/10 (90%)	0/10 (0%)	3/10 (30%)	5/10 (50%)
Normal blood	0/10 (0%)	1/10 (10%)	Not tested	Not tested	Not tested

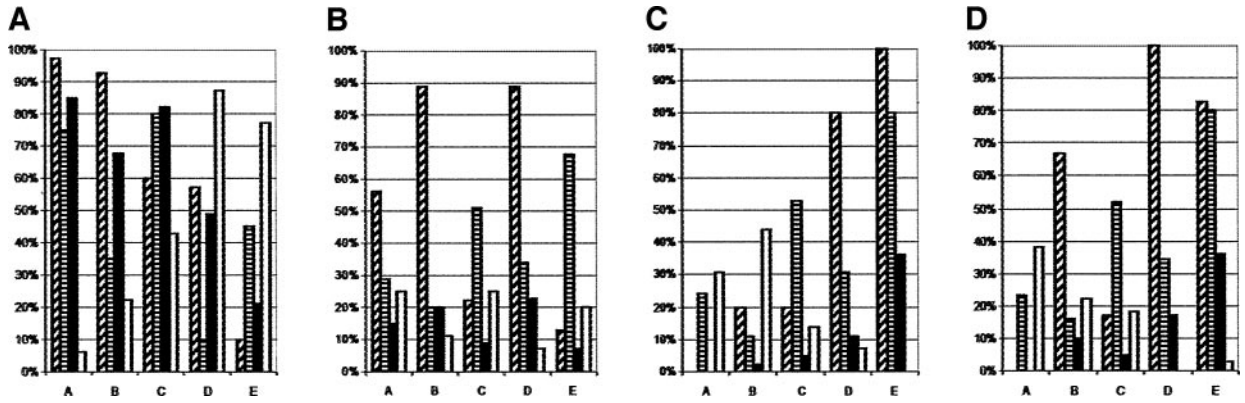

Fig. 2 Sensitivity (▨), specificity (▤), positive predictive value (■), and negative predictive value (□) of each mRNA marker for each renal cell carcinoma (RCC) subtype. *A*, conventional RCC; *B*, papillary carcinoma; *C*, chromophobe carcinoma; *D*, oncocytoma. *Column sets A, B, C, D,* and *E* represent the mRNA markers MN/CA9, cadherin-6, vimentin, mucin1, and parvalbumin, respectively.

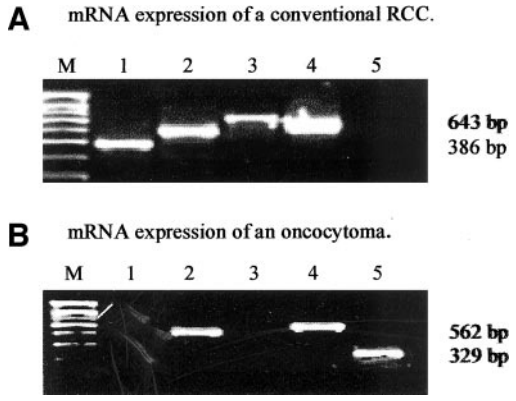

Fig. 3 mRNA expressions of a conventional renal cell carcinoma (RCC) *versus* an oncocytoma. *A*, conventional RCC; *B*, oncocytoma. The conventional RCC was positive for MN/CA9 (*Lane 1*; 386 bp) and vimentin (*Lane 3*; 643 bp) mRNA, whereas the oncocytoma was negative for these two markers. The conventional RCC was negative for parvalbumin (*Lane 5*; 329 bp), whereas the oncocytoma showed positive expression. Both the conventional RCC and oncocytoma expressed cadherin-6 (*Lane 2*; 509 bp) and mucin1 (*Lane 4*; 562 bp) mRNA. *Lane M*, molecular marker.

which is comparable to the time needed for routine pathological examination or immunohistochemical staining.

New classification of histological subtypes has recently been introduced for epithelial renal tumors according to specific chromosome alterations: conventional (clear cell) carcinoma, papillary carcinoma, chromophobe carcinoma, and benign oncocytoma (27–29). Differential diagnosis of each subtype has important clinical significance because the clinical course of each subtype is different. At present, the standard diagnosis of RCC is by postoperative morphological study under microscope. However, the cytological features of different subtypes may be very similar because granular or eosinophilic cells may occur in conventional carcinomas, papillary, chromophobes, and oncocytomas (28, 30). There has been considerable interest in the application of an adjunctive technique, such as immunohistochemistry, to this differential diagnosis.

Recently, chromosome markers have been found in RCC. Researchers have developed fluorescence *in situ* hybridization or comparative genomic hybridization techniques to differentially diagnose RCC by detecting chromosome aberrations (31, 32). Because the gene expression pattern is correlated with a specific morphology (33), we explored the possibility of using mRNA markers as a new way to differentiate the RCC subtypes. We studied a panel of mRNA markers based on recent findings for gene expression of RCC (2, 3, 5, 6, 16). RCC is mainly composed of conventional RCC (70–80%), which is thought to originate from the proximal tubule cells (28). To date, MN/CA9 has been the most significant molecular marker for conventional RCC. We previously found that the percentage of renal cancer cells positive for this marker was higher than for other epithelial markers, except for the cytokeratins (23). MN/CA9 and cadherin-6 have a high expression rate in conventional RCC. The highly frequent expression rates indicate their usefulness, *e.g.*, in detection of circulating renal cancer cells (7, 34). Normal renal tissues expressed cadherin-6 but not MN/CA9,

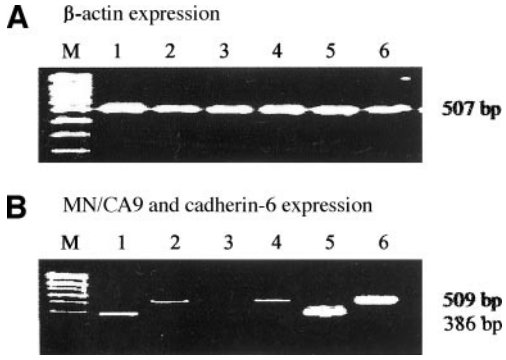

Fig. 4 MN/CA9 and cadherin-6 expression in an experimental fine needle aspirate (FNA). *A*, *Lanes 1–6*, β-actin (507 bp) expression, used to check the quality of extraction. *B*, MN/CA9 (386 bp) and cadherin-6 (509 bp) expression. *Lane 1*, MN/CA9 in tumor tissue; *Lane 2*, cadherin-6 in tumor tissue; *Lane 3*, MN/CA9 in normal tissue; *Lane 4*, cadherin-6 in normal tissue; *Lane 5*, MN/CA9 in an experimental FNA; *Lane 6*, cadherin-6 in an experimental FNA. *Lane M*, molecular marker.

which is in agreement with previous reports (5–8, 22). In our study, we showed that renal oncocytomas were MN/CA9 negative (5, 6, 22) but that they could express cadherin-6.

Vimentin may be another specific molecular marker for conventional RCC. Our results supported that it can be detected in the majority of conventional RCCs. However, we also found one positive among the oncocytomas and one positive among the chromophobe RCCs, which agrees with the report by Moch *et al.* (3). We found that mucin1 mRNA was widely present in RCCs. The detection of vimentin and mucin1 mRNA markers may be useful because recent studies have shown that the expression of vimentin or mucin1 in RCC provides prognostic value (2, 15).

As expected, parvalbumin mRNA could be detected in all chromophobe RCCs and in the majority of oncocytomas, both of which originate from the intercalated cells of distal tubules (28). This result supported that parvalbumin mRNA is a promising gene marker for chromophobe RCC and oncocytoma (2). Thus, the mRNA markers that we studied represent novel candidate tumor biomarker genes for RCC and its histological subtypes. In particular, the detection of three genes, such as MN/CA9, vimentin, and parvalbumin, indicates that tumors that originate from the proximal tubule cells (conventional and papillary RCCs) can be distinguished from tumors that originate from distal tubule cells (chromophobe RCC and oncocytoma). Our technique can be easily applied to surgical pathological samples to measure specific gene expression when morphological evaluation is difficult.

Biological samples such as peripheral blood, urine, stool, cavity effusion, aspiration biopsies, and tumor tissue can be easily obtainable for molecular assays (35–37). As a result of the frequent use of imaging, the incidental discovery of small asymptomatic renal tumors has been increasing. The major problem is that a diagnosis of malignant or benign cannot be established by imaging characterizations in some small tumors, *i.e.*, atypical cystic tumor or oncocytomas. Some conventional RCCs are cystic tumors. In this case, a FNA biopsy is the only way to establish a diagnosis of cancer. The FNA biopsy is frequently used for the diagnosis of cancer in breast, liver, thyroid, and other cancers, but a renal FNA biopsy is one of the most difficult tasks for cytopathologists for two reasons: the lack of cells and the cytological similarity of different subtypes, as discussed above. Because it requires only a few cells, mRNA analysis is easier than that of DNA because the copy number of each mRNA is usually larger than that of the corresponding DNA. Using the cells remaining in an aspiration needle, Takano *et al.* (38) found that RT-PCR could efficiently preoperatively diagnose medullary thyroid carcinoma. In the present study, we applied our technique to renal FNA biopsies to differentiate renal tumors from imaging-indeterminate cases. MN/CA9 is an ideal candidate gene marker because it is not expressed in normal or hematological cells or benign oncocytomas. In our experiment, we demonstrated that our technique could be readily used in renal FNA biopsy specimens to detect renal cancer cells. We have examined 10 imaging-indeterminate tumors and found 5 MN/CA9 gene-positive FNA biopsy samples, suggesting a RCC, whereas cytology examination was positive in only 3 cases. In addition, cytology examination showed a false positive (Table 2, *case 8*), which suggests that an accurate diagnosis requires a more objective method than cytological examination. Postoperative pathology confirmed these results, which demonstrated that RT-PCR could detect renal cancer cells not detected by cytological examination. Our initial analysis may suggest that the use of mRNA markers could improve the cytology-based diagnosis. Although the number of our FNA series was limited, we showed that the clinical application of the molecular marker MN/CA9 for differential diagnosis of RCC may help to solve a major problem in preoperative diagnosis. We are enlarging our series to detect renal cancer cells in FNA specimens by mRNA analysis for imaging-indeterminate cases. In addition, we believe that our technique can be readily applied to FNA biopsy samples of other types of cancer.

Table 2 Analysis of preoperative FNA biopsy

Case	Cytology	MN/CA9	Postoperative pathology
1	Negative	Positive	Conventional RCC
2	Positive	Positive	Conventional RCC
3	Negative	Positive	Conventional RCC
4	Negative	Negative	Benign cyst
5	Negative	Negative	Oncocytoma
6	Negative	Negative	Conventional RCC
7	Negative	Negative	Benign cyst
8	Positive	Negative	Benign cystic adenoma
9	Positive	Positive	Conventional RCC
10	Positive	Positive	Conventional RCC

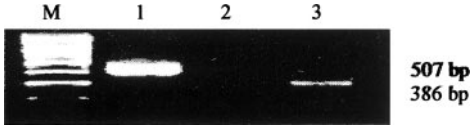

Fig. 5 MN/CA9 mRNA expression in a preoperative fine needle aspirate (FNA) sample. β-actin (*Lane 1*; 507 bp) was used to evaluate the quality of RNA extraction. *Lane 2*, negative β-actin control. *Lane 3*, sample positive for MN/CA9 mRNA (386 bp).

In conclusion, we have developed a rapid and sensitive mRNA expression assay for differential molecular diagnosis of RCC that can be used as a powerful ancillary to the diagnosis of RCC based on pathological and cytological analysis.

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