Cadherin-6 Gene Expression in Conventional Renal Cell Carcinoma: A Useful Marker to Detect Circulating Tumor Cells

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Abstract. Background: Dissemination of cancer cells into the circulation is an essential step in the development of a metastasis. Detection of circulating cancer cells may improve the monitoring methods for cancer patients. However, the detection of circulating renal cancer cells is mainly hampered by the lack of markers available for renal cell carcinoma (RCC). In this study, we evaluated cadherin-6 mRNA as a new molecular marker for the detection of circulating renal cancer cells. Materials and Methods: Forty-six blood samples of conventional RCCs were included. A standard protocol of RT-PCR, assisted by computer densitometric analysis to establish a cut-off, was performed to examine cadherin-6 mRNA expression by using specific primers. A renal cancer cell line, SKRC-59 and forty tumor biopsies from conventional RCCs were used as positive controls. Twenty-five blood samples from non-RCC patients were also analyzed. Results: Cadherin-6 mRNA could be detected in 38/40 (95%) conventional RCC specimens. Cadherin-6 mRNA was positive in 21/46 (45.7%) blood samples of RCC patients, while no positivity was found in non-RCC blood samples. Among the localized RCCs, 14/35 (40.0%) blood samples were positive while 7/11 (63.6%) were positive among the blood samples from metastatic RCCs. Conclusion: Our data indicate that cadherin-6 gene is frequently expressed in conventional RCCs. Cadherin-6 is a useful molecular marker to detect the circulating cancer cells disseminated from conventional RCC.

The incidence of renal cell carcinoma (RCC) has increased steadily during recent years. Although radiological imaging has improved the diagnosis of RCC, nearly one-third of patients present metastatic disease at the time of diagnosis. Besides, as many as 40% of patients with local tumors will ultimately relapse with metastatic disease after surgery (1). These clinical facts indicate that the cancer cells have disseminated at the time of surgery. One of the major difficulties in monitoring RCC patients is that RCC lacks either tissue-specific or efficient serum markers. The establishment of new monitoring methods is urgently needed.

With the advent of RT-PCR technology, many researchers have resumed their interest in detecting circulating cancer cells (2, 3). Theoretically, the primary tumors shed tumor cells into the blood flow before these cells can establish a metastasis. Therefore, this renewed interest aims at detecting micrometastasis at an earlier stage before it can be found by imaging, thus allowing monitoring of postoperative cancer patients in an individual style. Such molecular detection in many solid tumors has been reported (4-8). Some have been suggested to be sensitive and specific. Unfortunately, molecular detection in RCC is hampered by the lack of an efficient marker. We previously demonstrated that conventional RCC expresses common epithelial markers such as epithelial specific antigens and cytokeratins (9). However, these markers have not been proved powerful for targeting circulating cancer cells.

Cadherin is a family of transmembrane glycoproteins. The family of cadherins are best characterized as adhesion molecules. E-cadherin is known to be the most important adhesion molecule in epithelial tissues (10). The loss of E-cadherin expression has been studied in relation to tumor invasiveness among many epithelial tumors (10). However, in conventional RCC, cadherin-6 but not E-cadherin has proved to be a major cell adhesion molecule (11-13). Cadherin-6 was originally isolated from a hepatocellular carcinoma cell line. Cadherin-6 is found to relate to fetal kidney development and is considered to be a quite specific tissue marker to renal proximal tubules where conventional RCC originates (14-16), although cadherin-6 can be found in other tissues such as in the nervous system (17).
Immunostaining of cadherin-6 was also found to have prognostic value in negative E-cadherin RCC (12). This recent evidence suggests that cadherin-6 may be a candidate RT-PCR marker for the detection of circulating renal cancer cells. Therefore, we studied the cadherin-6 gene expression in RCC tumor specimens and further tested the possibility of using this gene marker for the detection of circulating renal cancer cells.

Materials and Methods

Blood samples and tumor tissues. Forty-six blood samples of conventional RCC patients were included. About 8 ml blood was drawn into a EDTA-tube from the peripheral vein before operation. The blood samples were subjected to a Ficoll to isolate the targeted cells. The cells were stored at -80°C until use. Twenty-five blood samples from non-RCC patients were also analyzed. Forty tumor biopsies from conventional RCCs and 10 normal renal tissues adjacent to the tumor were used as positive controls. Approximately 0.5 cm³ of tumor tissue was immediately obtained after the removal of the diseased kidney. Tumor tissues were snap-frozen in liquid nitrogen and then stored at -80°C until the extraction.

All these tumors were pathologically proved to be conventional (clear cell) RCCs. They were staged according to the 1997 TNM classification and graded according to Fuhrman criteria (18, 19).

This research protocol was approved by a local research committee and informed consent was obtained from the patients who participated in this protocol.

Carcinoma cell line and culture. A carcinoma cell line (a kind gift from Dr. Oosterwijk) , SKRC-59, derived from RCC was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. The confluent cells were harvested, washed with Hanks and stored at -80°C until use. SKRC-59 was used as a positive control because it is known to be positive for the cadherin-6 gene (13).

Extraction of RNA and RT-PCR. Frozen tissues were pulverized under liquid nitrogen. The frozen powder was suspended in 2 ml RNABe solution (Eurobio, Les Ulis, France) for extraction. The total RNA was extracted by using the standard chloroform-isopropanol-ethanol technique. Cells in culture were dispatched by Hanks-EDTA, centrifuged and washed two times with Hanks’ solution. The cultured cells were suspended in RNABe as tumor tissue. The RNA was obtained after isopropanol and ethanol precipitation. The RNA pellet was resuspended in 20-50 µl of distilled water according to the amount of RNA extracted. RNA was quantified by using UV spectrophotometry at 260 nm. RNA specimens were stored at -80°C until RT-PCR. One µg of RNA was used to synthesize cDNA by employing a first-strand cDNA synthesis kit (Invitrogen™, Life Technologies, Paisley, UK). The cDNA samples were stored at -20°C.

We designed specific primers for the PCR amplification of cadherin-6. The primer for β-actin was also used to check the quality of extraction of RNA and RT-PCR. The primers for cadherin-6 (509 base pairs) were as follows: sense primer 5'-ATT CAG CCA CGG TTA GAA TTG-3'; antisense primer 5'-TGA GGC ACT GCC TGC TTC A-3', and for β-actin (507 base pairs): sense primer 5'-TAC CAC TGG CAT CGT GAT GGA CT-3', and antisense primer 5'-TGC TTC TGC ATC CTG TCG GCA AT-3'. These primers were designed to span the splice junction so that genomic DNA contamination could be monitored. The PCR reaction mixture contained 4 µl of cDNA. PCR was performed with denaturing temperature at 94°C for 1 min, annealing temperature at 57°C (cadherin-6) or 60°C (β-actin) for 1 min and extension at 72°C for 1 min. Thirty-five cycles were performed. For each PCR, SKRC-59 was used as a positive control and reaction without template RNA was used as a negative control.

Analysis of PCR product. PCR products were separated by electrophoresis on a 1.5% agarose gel. DNA fragments were visualized and photographed under UV light with ethidium bromide staining. The expected band for cadherin-6 and β-actin was identified by a co-migration of a DNA marker ladder electrophoresed in an adjacent lane. In addition, the fidelity of the PCR amplification was confirmed by the sequencing of representative PCR products.

To make a quantitative analysis of the PCR product, we adopted a densitometry-assisted method (20, 21). Briefly, a serial dilution with 0.0, 1.0, 2.0, 4.0 and 8.0 µl of SKRC-59 cDNA was made and spiked with normal blood cDNA. The same protocol of PCR and electrophoresis was performed. The levels of cadherin-6 gene expression were estimated by computer-assisted densitometry. We established the linear range of the densitometric quantification as reflected by the correlation of signal intensity and the corresponding cDNA. In our study, we used the intensity level corresponding to the linear range.

Statistics. The χ² test was performed for comparisons between group frequencies. P<0.05 was considered as significant.

Results

Forty-six blood samples from conventional RCC were obtained. There were 35 localized tumors (N0M0) and 11 metastatic tumors. The tumor stage for localized tumors was determined according to the new TNM classification and graded according to Fuhrman criteria (18, 19). The possibility of using this gene marker for the detection of circulating renal cancer cells.

Table I. Cadherin-6 mRNA expression rate in the peripheral blood.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total number</th>
<th>Positive number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT1 - PT2</td>
<td>20</td>
<td>7 (35.0%)</td>
</tr>
<tr>
<td>PT3</td>
<td>15</td>
<td>7 (46.7%)</td>
</tr>
<tr>
<td>RCCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I - II</td>
<td>21</td>
<td>7 (33.3%)</td>
</tr>
<tr>
<td>(N0M0)</td>
<td>14</td>
<td>7 (50.0%)</td>
</tr>
<tr>
<td>Metastatic RCCs (N+ or M+)</td>
<td>11</td>
<td>7 (63.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>21 (45.7%)</td>
</tr>
<tr>
<td>Controls</td>
<td>25</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

was: 18 pT1, 2 pT2 and 15 pT3. The Fuhrman grade for localized tumors was: 6 grade I, 15 grade II, 10 grade III and 4 grade IV.

The cadherin-6 mRNA expression rate in the peripheral blood samples is shown in Table I. Cadherin-6 mRNA could be detected in 38/40 (95%) clear cell RCC specimens and in 9/10 (90%) normal renal tissue samples. In total blood samples from conventional RCC, cadherin-6 mRNA was positive in 21/46 (45.7%). The positive examples are demonstrated in Figure 1. In contrast, no positive was found in non-RCC blood samples. The difference in percentages between the control and RCC patients was statistically significant (p<0.01).

Among the blood samples of localized RCC, 14/35 (40.0%) were cadherin-6 mRNA-positive. In this group, the difference in percentages between the low stages (pT1 and pT2) and high stages (pT3) was not statistically significant (p>0.05). Also, the difference in percentages between low grades (I and II) and high grades (III and IV) was not statistically significant (p>0.05).

Among 11 blood samples from metastasized patients (M+ or N+), 7 (63.6%) were cadherin-6 mRNA-positive.

Discussion

Recent studies have demonstrated that cadherin-6 gene is important for fetal kidney development (15, 16, 22). During embryogenesis, the newly formed epithelium of the renal vesicle expresses E-cadherin near the ureteric bud tips and cadherin-6 more distally. In the S-shaped bodies, the cadherin expression patterns reflect the developmental fate of each region. The proximal tubule progenitors express cadherin-6, while the distal tubules express E-cadherin. Moreover, cadherin-6 has been recently found to be mainly expressed in renal proximal tubules. The conventional or clear cell RCC is generally regarded to originate from proximal tubule cells. In the present study, we found that 95% of conventional RCC expressed cadherin-6 mRNA. This result indicates that the cadherin-6 gene is one of the most frequently expressed genes in conventional RCC.

Detection of circulating cancer cells has been recently revived due to the RT-PCR technique. The detection of circulating cancer cells may provide a new way to diagnose or monitor patients after operation (23). Although some technical problems have to be overcome before direct clinical application, it has become an active area of cancer research for solid tumors. Conventional RCC can spread by a hematogenous mechanism. Therefore, a conventional RCC patient may be a good subject to detect cancer cells in blood. The detection of circulating renal cancer cells may be a useful surrogate serum marker for conventional RCC. However, its detection is largely hampered by the lack of markers. As a result, very limited data concerning circulating renal cancer cells are available. We and others have reported that G250/MN/CA9 may be a useful marker for the detection of circulating renal cancer cells (24, 25). However, the use of this tumor marker to target the circulating renal cancer cells needs an enhanced detection (24). Our personal data also suggested that a nested or enhanced RT-PCR was needed (data not shown). In fact, the selected mRNA marker plays an important role in the sensitivity of detecting circulating cancer cells. Another commonly used strategy is to choose the tissue markers. For example, PSA is used for targeting circulating prostate cancer cells, matrix metalloproteinase-7 for gastric cancer cells, mammaglobin for breast cancer cells and thyroglobulin for thyroid cancer cells (3-7). In this sense, cadherin-6 may be a candidate for tissue marker of renal epithelial cells of the proximal tube. The high frequency of cadherin-6 gene expression in conventional RCC attracts us to utilize it for the detection of circulating renal cancer cells.

Several laboratories have begun the molecular detection of circulating renal cancer cells (25-27). By using an enhanced RT-PCR assay for MN/CA9, McKiernan et al. found 18/37 (49%) of peripheral blood samples of conventional RCCs were positive (24). Ashida et al.
established an RT-PCR assay to use von Hippel-Lindau (VHL) gene mutation to detect the circulating renal cancer cells in the peripheral blood (26). They could detect cancer cells in the peripheral blood in 2/17 (11.8%) RCC patients before surgery. It should be pointed out that MN/CA9 gene expression was detected in almost all conventional RCCs, while mutations of the VHL tumor suppressor gene were detected in up to 60% of sporadic conventional RCCs. Here, we evaluated cadherin-6 mRNA as a marker to detect circulating renal cancer cells. By using cadherin-6 mRNA as a marker, we found that a total of 45.7% blood samples of conventional RCC patients showed positive, which was similar to the report by McKiernan et al. (24). In our study, 40.0% of the patients with localized RCC resulted positive for cadherin-6 mRNA. This is not surprising because some tumors can discharge tumor cells into blood at an early stage. How and when the tumor discharges its tumor cells into the blood is a very complex biological process. However, they do provide a possible new way to diagnose or monitor conventional RCC patients. Furthermore, the present study demonstrated that the majority of metastatic patients were cadherin-6 mRNA-positive. Very recently, Shimazui et al. also proved, by using a nested RT-PCR, that cadherin-6 mRNA was present in the blood of RCC patients (28). Taking these results together, we believe that cadherin-6 is a useful marker for the detection of circulating tumor cells. The next step is to improve the sensitivity and specificity of the detection by the new technique in order to monitor the micrometastasis of conventional RCC patients.

In conclusion, the cadherin-6 gene is frequently expressed in conventional RCCs. Cadherin-6 is a useful molecular marker to detect renal cancer cells disseminated into the circulation.

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References


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