Expression of RhoA and RhoC in colorectal carcinoma and its relations with clinicopathological parameters

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Abstract

Background: Ras homologous (Rho) family GTPases play a pivotal role in the regulation of numerous cellular functions associated with malignant transformation and metastasis. To evaluate the role of these GTPases in colorectal cancer, the mRNA expression levels in matched sets of tumor and non-tumor tissues from surgical specimens were analyzed. The relationship between the mRNA levels in tumor tissues to the clinicopathological features was also assessed.

Methods: A total of 68 patients with colorectal carcinoma were recruited and the levels of RhoA and RhoC mRNA transcripts in cancer, paratumoral and normal tissues were characterized by quantitative real-time polymerase chain reaction (QRT-PCR). Their correlation to clinical histopathological parameters was analyzed.

Results: The levels of RhoA and RhoC mRNA transcripts in carcinoma tissues were significantly higher than those in the matched paratumor and normal tissues from the same patient (p < 0.05). The expression levels of both genes were significantly correlated with metastasis of cancer cells to lymph nodes and liver (p < 0.05). The levels of RhoA expression were significantly correlated with the histopathological degree of cancer, while the expression of RhoC was correlated with the extent of local invasion to intestine.

Conclusions: This is the first study with QRT-PCR to examine the expressions of RhoA and RhoC genes in colorectal carcinoma of Chinese patients. The significantly up-regulated RhoA and RhoC expressions suggest that they may contribute to the initiation, development, invasion and metastasis of colorectal carcinoma in Chinese patients. Clin Chem Lab Med 2009;47:811–7.

Keywords: colorectal carcinoma; gene expression; real-time polymerase chain reaction (RT-PCR); RhoA; RhoC.

Introduction

Colorectal carcinoma is one of the most common malignancies with an increasing annual incidence (1). Colorectal carcinoma is usually accompanied by local invasion and distant metastasis, which are the main causative factors for cancer-related death (2). The initiation, development, local invasion and distal metastasis are closely regulated by multiple genes, whose expression are determined by internal or external factors. Therefore, elucidation of these factors and the pattern of their expression may help to understand the progression of colorectal carcinoma, and help predict the clinical outcome of patients with colorectal carcinoma.

The Ras homologous (Rho) sub-family of low-molecular-weight GTP-binding proteins contains Rho (e.g., RhoA, B, C), Rac and Cdc42 proteins (3). These molecules are involved in the regulation of a variety of cellular processes, such as the organization of the microfilament network, cell–cell contact and malignant transformation (4). Furthermore, they play important roles in intracellular signal transduction, regulate cell proliferation, apoptosis, adhesion and motility (5). Abnormal expression and activation of both RhoA and RhoC have been demonstrated to correlate with the development and metastasis of malignant carcinoma (3). Increased expression of RhoC has been found to correlate with poor outcome in Caucasians with colorectal carcinoma, and may also be used as a prognostic marker in these patients (6). Increased expression of RhoA has been observed in Asians with colorectal carcinoma (7). However, little is known about whether the expression of those oncogenes is altered in Chinese patients with colorectal carcinoma, and how this expression is correlated with the clinicopathological characteristics of Chinese patients with colorectal carcinoma.

The quantitative real-time polymerase chain reaction (QRT-PCR) is a new fluorescence-based technology that can be used to amplify and simultaneously quantify targeted DNA molecules. Together with reverse transcription, this technology can be employed to quantitatively measure mRNA transcripts of one gene at a particular time in certain cells.
or tissues. In this study, we employed the QRT-PCR to examine the expressions of RhoA and RhoC genes in colorectal carcinoma tissue from Chinese patients, and analyze the relationship between its expression and various clinicopathological parameters. Our data demonstrated high levels of RhoA and RhoC mRNA transcripts in colorectal carcinoma from Chinese patients, which may contribute to the initiation, progression and metastasis of colorectal carcinoma.

Materials and methods

Subjects and sample collection

A total of 68 patients with colorectal carcinoma who underwent surgery in the Department of General Surgery, the Affiliated Hospital of Qingdao University Medical College between January 2007 and April 2008, were recruited for this study after informed consent was obtained. There were 38 males and 30 females, with an average age of 61.5 years (range 36–80 years). For the 68 cases, there were 31 with colon carcinoma and 37 with rectal carcinoma. Diagnoses were confirmed by pathological examinations. Prior to surgery, none of the patients received chemotherapy or radiotherapy. At surgery, tumor tissue, paratumor tissue (3–5 cm from the border of the tumor) and normal tissue (>5 cm from the border of the tumor) were resected. Based on the histopathological classifications, six out of 68 cases were highly differentiated, 45 moderately differentiated and 17 poorly differentiated. Four out of 68 cases were from the border of the tumor. Based on the TNM staging for colorectal carcinoma (UICC, 2006), there were six cases at T1, 18 at T2, 35 at T3 and nine at T4. Twenty-four patients had metastasis to lymph nodes, and 11 to the liver, including two with concomitant metastasis to the liver and lung. The tissues were immediately frozen in liquid nitrogen and stored at -70°C until use.

Primer design and synthesis

The specific primers used for amplifying the targeted gene fragments with QRT-PCR were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA), based on the sequences of these genes, and analyzed using the basic local alignment search tool (BLAST) to determine their specificity. The primers for the human RhoA gene were: sense 5'-CGGGAGCTAGCCAAGATGAA-3', antisense 5'-CTCATCTCCGAGGGATC-3', fluorescent probe 5'-FAM-AGAGATATGGCAAACAGGATTGGCG-TAMRA-3', and the amplicon size was 158 base pairs (bp). The primers for the human RhoC gene were: sense 5'-CCTCATGTGCTTCTCAT-3', antisense 5'-CTCCTGACAGGGCTCAGTA-3', fluorescent probe 5'-FAM-AGAGATATGGCAAACAGGATTGGCG-TAMRA-3', and the amplicon size was 158 base pairs (bp). The primers for the human GAPDH gene were: sense 5'-CTTAGCTGACCCAAAAGCTGCCCATT-3', antisense 5'-GGTTTTGCTTCACTCCAT-3', and the amplicon size was 168 bp. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control with the specific primers: sense 5'-CTTAGCACCCTCTGAGCIAGG-3', antisense 5'-GGTTTTGCTTCACTCCAT-3', fluorescent probe 5'-FAM-CATGCGATCAGTCCAGAGAGCCAG-3', and the amplicon size was 150 bp. The primers and fluorescent probes were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

RNA and cDNA preparation and QRT-PCR

Total RNA was extracted from tissue samples using Trizol (Invitrogen, Gaithersburg, MD, USA) and reverse transcribed into cDNA using the PrimeScript RT-PCR kit (TaKaRa Bio Inc, Shiga, Japan) according to the manufacturer's instruc-

Results

Primer specificity

To determine the levels of RhoA and RhoC mRNA transcripts, total RNA was extracted from individual samples and the purity of these RNA samples determined by the ratio of optical density (OD260/OD280). The ratio was between 1.8 and 2.0, suggesting high purity of RNA. Following reverse transcription into cDNA, the target gene fragments of RhoA, RhoC and GAPDH were amplified by PCR (Figure 1). As expected, individual DNA products were observed with RhoA (158 bp), RhoC (136 bp) and GAPDH (150 bp), respectively (Figure 1), suggesting high-specificity of the primers. Further sequence analysis revealed that the PCR products were RhoA, RhoC and GAPDH DNA fragments (Figure 2). These preliminary results provided a reasonable basis for quantitative characterization of RhoA, RhoC and GAPDH mRNA transcripts in the tissue samples.

Amplification efficiency

Amplification curves of serially diluted cDNA samples exhibited a standard S shape, suggesting a good amplification efficiency and a linear relationship. For
the RhoA and RhoC genes, the log value of each cDNA dilution was plotted vs. ΔCT, showing a slope of 0.0797 and 0.0864, respectively (Figures 3 and 4). This indicated that both target genes had amplification efficiencies similar to the internal control genes (GAPDH), justifying application of the $2^{-\Delta\Delta CT}$ method for relative quantification (9).

**High levels of RhoA and RhoC mRNA transcripts in colorectal carcinoma tissues**

We first examined the expression of RhoA, RhoC and GAPDH in tissue samples obtained from 68 patients with colorectal carcinoma (Figure 5). Data analysis showed the values of RhoA and RhoC mRNA in tumor samples to be significantly higher compared with that found in paratumor or normal tissue (p < 0.05). The relative levels of RhoA and RhoC mRNA in tumor samples compared with normal samples were 4.53 (3.81–5.39) and 3.83 (3.11–4.73), respectively. These results demonstrated significantly higher expression of RhoA and RhoC mRNA in tumor samples (p < 0.05). However, there was no significant difference between paratumor and normal tissue samples (p > 0.05) (Table 1). The high levels of RhoA and RhoC mRNA transcripts shown in colorectal carcinoma tissues suggest that both RhoA and RhoC expression may contribute to the tumorigenesis of colorectal carcinoma in Chinese patients.

**Correlation of RhoA and RhoC gene expressions to clinical histopathological features of colorectal carcinoma**

We analyzed the relationship between the levels of RhoA and RhoC gene expression. The levels of RhoA mRNA transcripts were correlated with that of RhoC mRNA expression in the colorectal tumor samples ($r = 0.847$, $p < 0.01$). Further analysis of the relationship between the levels of RhoA or RhoC expression and various clinical histopathological features revealed that the expression of both genes was significantly correlated with the presence of metastasis to lymph nodes or liver (p < 0.05), but was independent of patient’s age and gender. In addition, the level of
RhoA expression was correlated with histopathological degree of cancer, while RhoC was correlated with the extent of local invasion to the intestine (p < 0.05) (Table 2).

Discussion

The Rho family of GTPase functions as a molecular switch within cells. In response to intra- and extracellular signals, it cycles between an active GTP-bound form and an inactive GDP-bound form. The Rho family not only regulates the organization of the actin filament system but also modulates cell motility, proliferation, apoptosis, cell cycle progression, and invasion and metastasis of malignant tumor cells (10). Small GTPase of the Rho family plays an important role in eukaryotic signal transduction, which primarily affects the organization of the cytoskeleton involved with movement. Rho-associated protein kinase (ROCK) is a key downstream effector of Rho proteins (11, 12). Recent data have implicated that the Rho/ROCK pathway plays an important role in the invasion and metastasis of cancer cells (13–15).

RhoA has been shown to regulate the activities of multiple transcription factors. The majority of these factors have been implicated in cancer progression (16) by modulating cancer cell adhesion, contraction, movement, release of cellular adhesion, degradation of extra-cellular matrix, and invasion into blood or lymph vessels (17–19). In gastric cancer, high levels of RhoA expression were observed in tumors at more advanced stages, histologically diffuse, aggressive metastasis into lymph nodes and poorer survival (20). RhoA itself also has transformation activity, often interacting with Ras gene mutations, especially K-ras, which is a common event in colorectal carcinoma, to promote cellular transformation (21).

RhoC also contributes to tumor development, especially invasion and metastasis of cancer cells. Its functions are likely mediated by the following mechanisms (22, 23): 1) disrupting cellular polarity, 2) depriving cells of adherent junctions, 3) promoting cellular motility and remodeling extra-cellular matrix to enhance the local invasiveness of cancer cells, 4) up-regulating the expression of factors involved in angiogenesis to promote the intravasation of tumor cells into blood vessels. RhoC has been considered a marker for early metastasis of cancer (24). Clark and colleagues identified high RhoC gene expression in highly metastatic melanoma cells by microarray. They were also the first to demonstrate the causative role of RhoC expression in tumor cell invasion, proposing the involvement of RhoC in tumor invasion and metastasis (25). In prostate cancer, over-expression of RhoC enhances cellular invasion, and its
expression level correlates directly with the invasiveness of prostate cancer cells (26). In liver carcinoma, retroviral transduction of RhoC siRNA inhibits cellular motility (27).

Fluorescence-based QRT-PCR is a fast and accurate technique, and can be used for quantifying the levels of mRNA transcripts. It has been widely used in different fields of molecular biology. In this study, we characterized the relative levels of RhoA and RhoC mRNA transcripts in tissues by QRT-PCR. In theory, relative quantification requires comparison of the quantity of mRNA between samples derived from the same number of cells. This is very difficult in practice given the difficulties in obtaining the same number of cells from different samples. In addition, there is variability in RNA extraction and PCR amplification. We standardized the mRNA levels of our target genes against GAPDH, which is an internal housekeeping gene. Then we compared mRNA levels between different samples, improving the validity of the result (28, 29).

Table 1 Relative expression of RhoA and RhoC genes in different tissues.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Sample number (n)</th>
<th>RhoA</th>
<th>Relative to normal(a)</th>
<th>RhoC</th>
<th>Relative to normal(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta CT)</td>
<td></td>
<td></td>
<td>(\Delta CT)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>68</td>
<td>0 ± 0.2014</td>
<td>1 (0.87–1.15)</td>
<td>0 ± 0.2512</td>
<td>1 (0.84–1.19)</td>
</tr>
<tr>
<td>Paratumor</td>
<td>68</td>
<td>−0.1551 ± 0.1711</td>
<td>1.11 (0.98–1.25)</td>
<td>−0.0955 ± 0.2437</td>
<td>1.07 (0.90–1.27)</td>
</tr>
<tr>
<td>Tumor</td>
<td>68</td>
<td>−2.1799 ± 0.2509</td>
<td>4.53 (3.81–5.39)</td>
<td>−1.9387 ± 0.3019</td>
<td>3.83 (3.11–4.73)</td>
</tr>
</tbody>
</table>

\(a\)Data are expressed as the mean 2\(^{-\Delta CT}\) (range).

Table 2 Correlation between RhoA and RhoC \(\Delta CT\) values and clinical histopathological parameters.

<table>
<thead>
<tr>
<th>Pathological features</th>
<th>Case number</th>
<th>(\Delta CT) (RhoA)</th>
<th>p-Value</th>
<th>(\Delta CT) (RhoC)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>−2.255 ± 0.3621</td>
<td>&gt;0.05</td>
<td>−1.9767 ± 0.2611</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>−2.105 ± 0.2766</td>
<td></td>
<td>−1.9007 ± 0.3562</td>
<td></td>
</tr>
<tr>
<td>Site of tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>31</td>
<td>−2.1675 ± 0.2577</td>
<td>&gt;0.05</td>
<td>−1.8614 ± 0.2763</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Rectum</td>
<td>37</td>
<td>−2.1923 ± 0.2132</td>
<td>&gt;0.05</td>
<td>−2.0160 ± 0.3289</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Histological subtypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High and moderate differentiation</td>
<td>51</td>
<td>−2.0875 ± 0.1734</td>
<td>&lt;0.05</td>
<td>−1.9266 ± 0.3326</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Low differentiation</td>
<td>17</td>
<td>−2.3602 ± 0.2877</td>
<td></td>
<td>−1.9913 ± 0.3125</td>
<td></td>
</tr>
<tr>
<td>Extent of intestinal invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T_1 + T_2)</td>
<td>24</td>
<td>−2.1405 ± 0.2847</td>
<td>&gt;0.05</td>
<td>−1.7005 ± 0.2566</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(T_3 + T_4)</td>
<td>44</td>
<td>−2.2036 ± 0.1964</td>
<td></td>
<td>−2.0673 ± 0.2137</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
<td>−2.3503 ± 0.1926</td>
<td>&lt;0.05</td>
<td>−2.1503 ± 0.1344</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>−2.0732 ± 0.2533</td>
<td></td>
<td>−1.8362 ± 0.2581</td>
<td></td>
</tr>
<tr>
<td>Liver metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11</td>
<td>−2.4106 ± 0.0810</td>
<td>&lt;0.05</td>
<td>−2.2662 ± 0.1050</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Negative</td>
<td>55</td>
<td>−2.1422 ± 0.2198</td>
<td></td>
<td>−1.9405 ± 0.1911</td>
<td></td>
</tr>
</tbody>
</table>

In gastric cancer, the adenovirus-mediated siRNA against RhoA and RhoC down-regulates the expression of both genes and inhibits the proliferation and invasiveness of cancer cells (30). Our study also identified positive correlation between mRNA levels of these two genes (\(r = 0.847, p < 0.01\)). This suggests cooperativity between these two genes in the initiation and progression of cancer, especially invasion and metastasis of colorectal carcinoma.

To our knowledge, this is the first study on the levels of RhoA and RhoC mRNA transcripts in tissue samples from Chinese patients with colorectal carcinoma by QRT-PCR; similar studies have been performed with other human tumors. Our results are consistent with a previous study done by Faried et al. that used essentially the same method in patients with squamous cell carcinoma of the esophagus (31). For quantitative methods, our TaqMan probes are more precise than SYBR Green chemistry dyes used by others. Another study used QRT-PCR and Western blotting on tissue from 64 patients with liver cancer.
and found that the levels of RhoA mRNA transcripts were correlated with protein levels, and higher expression was correlated with tumor stage and metastasis (32). In ovarian cancer, levels of RhoA and RhoC mRNA and protein expression are up-regulated in cancer tissue, as determined by QRT-PCR and Western blot analysis (33). Shimada et al. examined mRNA and protein levels of RhoC in 49 non-small cell lung carcinoma. They found that mRNA levels were correlated with protein levels, with both significantly higher in tumor tissue, as compared with non-tumor tissue (34).

We believe that levels of RhoA and RhoC mRNA transcripts in colorectal carcinoma are correlated with protein expression. Further study on RhoA and RhoC expression in colorectal carcinoma may provide more insight into the mechanisms underlying the action of RhoA and RhoC in the development and metastasis of colorectal carcinoma. Therefore, RhoA and RhoC expression may be new markers of invasiveness of colorectal carcinoma, and used as potential targets for treatment.

Acknowledgements

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