Knockdown of the novel proteasome subunit Adrm1 located on the 20q13 amplicon inhibits colorectal cancer cell migration, survival and tumorigenicity

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Abstract. The novel proteasome subunit Adrm1 located on the 20q13 amplicon was differentially expressed in colorectal cancer by semiquantitative RT-PCR. Adrm1 mRNA was overexpressed in 46.2% (18/39) colorectal cancer tissues compared to their matched normal mucosa and significantly correlated with lymph node metastasis of colorectal cancer (P=0.037). Knockdown of Adrm1 by shRNA in human colon carcinoma RKO cells inhibited their anchorage-independent growth, cell migration as well as cell proliferation through inducing apoptosis and cell cycle arrest at the G1 phase. In addition, stable RNA interference of Adrm1 gene synergistic with 5-Fu treatment suppressed RKO cell growth in vitro. Collectively, these data suggested that Adrm1 is potentially oncogenic and may play an important role in colon tumorigenesis. Regiment with combined application of Adrm1 RNA interference and chemotherapy may emerge as a novel therapeutic strategy for Adrm1 overexpressed colorectal cancer.

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths throughout the world. Although advances in treatment have reduced patient morbidity, the survival rates of CRC have remained low. Molecular investigations have provided evidence that multiple genetic alterations are involved in colorectal tumorigenesis. A molecular genetic model of preferential sequence has been proposed (1). However, even with the present knowledge of the cellular and molecular mechanisms of CRC, no biological parameter can predict the behavior of cancers. Karyotypic/cytogenetic data regarding CRC have been accumulated over the last 10 years (2,3), and a gain of 20q, especially the 20q13 region, was frequently reported as a common genetic aberration (4-7). Several novel candidate oncogenes have been identified in that region, such as the novel zinc finger gene ZNF217 which is amplified in CRC (8). These data suggest that 20q13 harbors one or more oncogenes which are important for CRC development.

In this study, we found that the novel proteasome subunit Adrm1 located on the 20q13 amplicon was overexpressed in CRCs by semiquantitative RT-PCR. Knockdown of Adrm1 by shRNA in human colon carcinoma RKO cells inhibited their anchorage-independent growth, cell migration and proliferation. Our results suggested that Adrm1 is potentially oncogenic and may play an important role in colon tumorigenesis.

Materials and methods

Patients and tissue samples. A total of 39 frozen tumor and matched normal mucosa blocks (20 Dukes’ B, 18 Dukes’ C and 1 Dukes’ D) retrieved from patients with colorectal adenocarcinoma who underwent surgery from June 2006 to December 2006 at the Sir Run Run Shaw Hospital (Hangzhou, Zhejiang, P.R. China) were investigated in this study. There were 25 male and 14 female patients ranging in age from 40 to 87 years (61.8±11.2). Patients who received preoperative chemotherapy were excluded from this study.

Cell cultures. All human colorectal cancer cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma-Aldrich) under humidified conditions in 95% air and 5% CO2 at 37°C.

Semiquantitative RT-PCR. The extraction of total RNA from frozen sections of CRC specimens was carried out using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s specifications. First-strand complementary DNA (cDNA) synthesis was performed using a GeneAmp RNA PCR core kit (Perkin Elmer, Branchburg,
After blocking in TBS-T buffer with 5% dry milk PAGE and transferred electrophoretically to a nitrocellulose membrane. 532 micrograms of sample per well were loaded on a 12% SDS-PAGE. Total protein concentration was measured by using a BCA (bicinchoninic acid) assay (Pierce, Rockford, IL, USA). RKO cells were subjected to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, according to the manufacturer’s protocol (Sigma-Aldrich).

**Cell transfection and stable colony selection.** For transfection, RKO cells were plated in 24-well plates at a density of 1x10^5 cells per well and allowed to grow overnight to 90-95% confluency. The next day, the cells were transfected with the mixture of 0.8 μg DNA (Adrm1 shRNA plasmid or negative control plasmid) and 2 μl lipofectamine 2000 (Invitrogen) in 100 μl serum-free medium according to the manufacturer’s instructions. To produce stably transfected cells, after transfection with the shRNA expression construct, 1500 μg/ml of hygromycin (Roche, Germany) was added at 48 h to the medium (DMEM +15% FBS). The cells were left in selective medium for two weeks after which they were trypsinized and recultured in selective medium for propagation.

**Western blotting.** Cell extracts were prepared with lysis buffer. Total protein concentration was measured by using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Ten micrograms of sample per well were loaded on a 12% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. After blocking in TBS-T buffer with 5% dry milk for 1 h at room temperature, the membranes were incubated sequentially with primary antibody overnight at 4°C and HRP-conjugated secondary antibody (1:1000, Santa Cruz, CA, USA) for 2 h at room temperature in TBS-T milk. Primary antibodies were used as follows: anti-Adrm1 mouse monoclonal antibody (3C6, 1:1000, Abnova, Taiwan); anti-caspase-3 rabbit monoclonal antibody (sc-7148, 1:1000, Santa Cruz Biotechnologies, CA); anti-cleaved caspase-8 mouse monoclonal antibody (1C12, 1:1000, Cell Signaling, USA) and anti-GAPDH mouse monoclonal antibody (1:1000, Santa Cruz Biotechnologies). Detection was carried out using the ECL kit (Amersham Biosciences, Piscataway, NJ, USA) and the blots were developed using a Fujifilm Las-4000 imaging system.

**MTT assay.** RKO cells were subjected to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, according to the manufacturer’s protocol (Sigma-Aldrich).

**Annexin V-FITC/PI double staining.** To measure the numbers and the ratio of apoptotic cells, the Annexin V-FITC/PI apoptosis detection kit (Beyotime, Shanghai, P.R. China) was utilized. The cells were seeded at 1x10^5/well into 6-well plates, harvested two days later by trypsinization, washed twice with cold PBS, and then performed according to the manufacturer’s instructions.

**Cell cycle analysis.** Cell cycle analysis was performed with PI. Cells were collected in 5 ml PBS and spun for 5 min at 200 x g. Cells were resuspended in 0.5 ml PBS and further fixed in ice-cold 70% ethanol for at least 2 h. Fixed cells were next centrifuged at 200 x g for 5 min and the pellet was resuspended in 1 ml of freshly prepared PI staining solution (20 μg/ml PI/0.1% Triton/0.2 mg DNase-free RNase A). Samples were kept for 30 min at room temperature and maintained at 4°C overnight in the dark.

**Soft agar assay.** For the soft agar colony formation assay, ~500 cells were suspended in medium containing 0.3% low melt agarose, seeded into a six-well plate that was overlaid to avoid off-target match. The pSilencer™ siRNA expression cassette (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). To produce stably transfected cells, after transfection with the shRNA expression construct, 1500 μg/ml of hygromycin (Roche, Germany) was added at 48 h to the medium (DMEM +15% FBS). The cells were left in selective medium for two weeks after which they were trypsinized and recultured in selective medium for propagation.

**Construction of Adrm1 shRNA.** The target sequence against human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs). The most effective SEC targeting human Adrm1 cDNA (NM_0070002) was designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The pH-100 shRNA expression vector kit (Ambion, Austin, TX, USA) was used according to the manufacturer’s protocol to produce shRNA expression cassettes (SECs).
Statistical analysis. Statistical analysis was performed in SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). The two-tailed Chi-square and Fisher’s exact test were used to analyze the association of the Adrm1 mRNA expression with the different clinicopathological parameters. P<0.05 was considered statistically significant.

Results

Identification of Adrm1 mRNA differentially expressed in colorectal cancer versus matched normal mucosa. To search for the Adrm1 mRNA expression status in colorectal cancer, semiquantitative RT-PCR was performed in 11 colorectal cancer cell lines and 39 CRC cases (RNA quality of these cases was excellent and the amount of cDNA was normalized). Adrm1 mRNA was expressed in all the cell lines (Fig. 1A) and overexpressed in 46.2% (18/39) colorectal cancer tissues compared to their matched normal mucosa (Fig. 1B).

The correlations between the clinicopathological parameters of patients with colorectal cancer and the Adrm1 mRNA overexpression are summarized in Table I. The Adrm1 mRNA overexpression was significantly correlated with
the G1 phase, in other words, suppress RKO cell proliferation due to cell cycle arrest at 58.80±1.10% (Table II).

Adrm1 knockdown increased the spontaneous apoptosis of RKO cells. We utilized TUNEL staining to evaluate DNA damage of the cells. As shown in Fig. 2A, significantly larger number of TUNEL-positive nuclei were observed in Adrm1 shRNA transfected cells compared with those of parental and scrambled shRNA transfected cells. Quantification of TUNEL-positive nuclei revealed that the fraction of TUNEL-positive cells increased by 10-fold with Adrm1 shRNA transfected compared with untransfected and scrambled shRNA transfected.

Then, we examined the effects of Adrm1 knockdown on caspases by Western blotting. Fig. 2B demonstrated that Adrm1 knockdown induced cleavage (activation) of caspase 8 which produce p43/41 while procaspase-3 was markedly attenuated. Caspase-3 activation plays a key role in initiation of cellular events during the early apoptotic process. It suggested most cells were in the late apoptotic stage. Together, these experiments indicated that Adrm1 knockdown increased the spontaneous apoptotic responses in RKO cells.

Adrm1 knockdown suppressed the migration of RKO cells. Comparison of the spindle morphology of parental and scrambled shRNA transfected RKO cells, the Adrm1 shRNA transfected cells grown at both low and high density were characterized by the deficiency of lamellipodial extensions and displayed rounded morphology resulting in complete detachment from tissue culture dishes (Fig. 3A). The effect of Adrm1 knockdown on colony-forming efficiency in soft agar is shown in Fig. 1D. The Adrm1 mRNA overexpression had no significant correlation with patient gender, age, tumor location, and differentiation.

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Adrm1 mRNA expression</th>
<th>n=39</th>
<th>Low (n=21)</th>
<th>High (n=18)</th>
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<td>25</td>
<td>13 12</td>
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<tr>
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<td>8 9</td>
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<tr>
<td></td>
<td>&gt;61.8</td>
<td>22</td>
<td>13 9</td>
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<tr>
<td>Location</td>
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<td>7 10</td>
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<tr>
<td></td>
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<td>22</td>
<td>14 8</td>
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</tr>
<tr>
<td>Differentiation</td>
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<td>13 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>11</td>
<td>6 5</td>
<td></td>
<td></td>
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<tr>
<td>Dukes’ stage</td>
<td>A+B</td>
<td>20</td>
<td>14 6</td>
<td></td>
<td>0.037*</td>
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<tr>
<td></td>
<td>C+D</td>
<td>19</td>
<td>7 12</td>
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<tr>
<td>Lymph node metastasis</td>
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<td>7 12</td>
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<tr>
<td></td>
<td>Negative</td>
<td>20</td>
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*Statistically significant (P<0.05).

A Adrm1 knockdown inhibited the proliferation of RKO cells. To reduce Adrm1 expression, we developed an Adrm1 shRNA plasmid that caused significant reduction of Adrm1 expression without any detectable change in GAPDH expression (Fig. 1C). The effect of Adrm1 knockdown on colony-forming efficiency in soft agar is shown in Fig. 1D. We further examined cell migration of the Adrm1 shRNA transfected, but otherwise an decrease of S/G2 phase cells from 62.68±1.70% to 58.80±1.10% (Table II). Adrm1 knockdown appeared to suppress RKO cell proliferation due to cell cycle arrest at the G1 phase, in other words, Adrm1 may promote RKO cell proliferation by accelerating cell cycling.

Adrm1 knockdown increased the sensitivity to 5-fluorouracil (5-Fu) in RKO cells. Since 5-Fu is a widely used chemotherapeutic agent during the past two decades in treatment of CRC, we explored the sensitivity of Adrm1 knockdown RKO cells to 5-Fu treatment. A dose-response curve for 5-Fu
revealed that IC50 on RKO cells was 12.5 μM for 48 h (data not shown). Then we measured the effect of Adrm1 knockdown on RKO cell growth using the MTT assay. The Adrm1 shRNA transfected cells displayed greatly reduced cell numbers (Fig. 3C), indicating apoptotic cell death (Fig. 3D), upon treatment with 5-Fu.

Discussion

In this study, Adrm1 located on 20q13 amplicon was found to be overexpressed in 46.2% (18/39) colorectal cancer tissues compared to their matched normal mucosa. This is consistent with previous studies. Simins et al have identified Adrm1 as a novel cell adhesion-promoting receptor that was up-regulated in metastatic cancer cells. Northern blot analysis of human breast cancer cell lines revealed 3- to 5-fold elevated Adrm1 mRNA levels in metastatic as compared to non-metastatic cells (9). Moreover, Pilarsky et al identified Adrm1 as commonly overexpressed gene in solid tumors compared to normal tissues by comparison of microarray data and also validated its differential expression by hybridization of a gene-specific probe (10). Recently, Fejzo et al also identified Adrm1 as amplification target after comprehensive analysis of 20q13 genes in ovarian cancer. Overexpression of Adrm1 correlated significantly with shorter time to recurrence and overall survival of ovarian cancer (11).

Adhesion-regulating molecule 1 (Adrm1), also known as GP110, hRpn13 or MGC29536, is located on the 20q13 amplicon. It is suggested that the chromosome segment 20q13 may harbor one or more oncogenes that are important for colorectal cancer development. Adrm1 was described initially as an interferon-inducible membrane glycoprotein with a possible role in cancer (12). Database search analysis suggested that Adrm1 was a human ortholog of Rpn13, a subunit of budding yeast proteasomes (13). Four independent research groups have demonstrated that Adrm1 was a novel component of 19S regulatory complex of 26S proteasome (14-17), directly associated with the deubiquitinating enzyme UCH37 and anchored this enzyme to the proteasome.

The 26S proteasome plays a central role in cell cycle progression, transcription factor activation, apoptosis, and other cellular events (18). Inhibiting proteasome activity leads to cell cycle apoptosis and the deregulation of the proteasome pathway was often associated with cancer-related processes such as oncogenic transformation, tumor progression, escape from immune surveillance and drug resistance (19). More interesting, recently we also found the proteasome subunit PSMA7 located on the 20q13 amplicon was over-expressed and associated with liver metastasis in colorectal cancer (20). The available information suggests that involvement in a malignant phenotype is conceivable for the proteasome subunit Adrm1 (9,10).
In the present study, we found Adrm1 overexpression was significantly correlated with lymph node metastasis of colorectal cancer (P=0.037). Knockdown of Adrm1 by shRNA inhibited not only anchorage-independent growth but also the migration of RKO cells as well as their proliferation through inducing apoptosis and cell cycle arrest at the G1 phase in vitro. This property may involve interactions of Adrm1 with Uch37 and proteasomes, although Adrm1 may have other functions that are yet to be discovered. The molecular mechanisms by which Adrm1 plays a role in colorectal cancer progression will be investigated in detail in a future study.

In summary, we reported the first evidence that the novel proteasome subunit Adrm1 located on the 20q13 amplicon is an important mediator of migration, metastasis in human colorectal cancer cells and also modulates in vitro colorectal cancer cell growth. Additionally, our data showed that Adrm1 RNA interference combined with 5-Fu treatment efficiently suppressed colorectal cancer cell growth in vitro. Regimen with combined application of Adrm1 RNAi and chemotherapy may emerge as a novel therapeutic strategy for Adrm1 overexpressed colorectal cancer.

Acknowledgements

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References