Role of Growth Factor Receptor–Bound Protein 7 in Hepatocellular Carcinoma

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Abstract
The human growth factor receptor–bound protein 7 (Grb7) is an adaptor molecule and is related to cell invasion. In this present study, we investigated the clinical and biological significance of Grb7 expression in human hepatocellular carcinoma (HCC). We reviewed 64 consecutive patients who had undergone liver resection for HCC, and we investigated the correlation between Grb7 expression and clinical outcome. To analyze the biological behavior of Grb7 in vitro and in vivo, we established Grb7 stable knockdown HCC cells using RNA interference technology. The positive staining of Grb7 protein was correlated with portal venous invasion (P < 0.01), hepatic venous invasion (P < 0.01), and intrahepatic metastasis (P < 0.05). Positive expression of Grb7 was significantly correlated with focal adhesion kinase (FAK) protein levels in HCC (P < 0.01). The Grb7- and FAK-positive group showed a significantly poorer prognosis as compared with the Grb7- and FAK-negative group (P < 0.05). Grb7 knockdown HCC cells exhibited significantly lower levels of invasion potential (P < 0.05) and motility (P < 0.05) than the control cells in vitro; moreover, Grb7 knockdown HCC cells showed delayed onset of the tumors compared with the control cells in vivo. Grb7 expression can modulate the invasive phenotype of HCC. Grb7 plays an important role in HCC progression and is strongly associated with expression of FAK. Grb7 could be a therapeutic target in HCC. (Mol Cancer Res 2007;5(7):667–73)

Introduction
Hepatocellular carcinoma (HCC) is the most frequent epithelial cancer of the liver, the fifth most frequent cancer in the world (1). It causes significant mortality, especially in countries where there is a high prevalence of chronic hepatitis B virus and hepatitis C virus infection. Despite various forms of therapies, including hepatectomy, ablation, lipiodolization, transarterial embolization, and percutaneous ethanol injection therapy, many patients show disease recurrence and finally progress to the advanced stages with vascular invasion and multiple intrahepatic metastases. The prognosis for HCC is generally poor, the 5-year survival rate ranges ~50%, and the recurrent rate is ~70% (2-4).

The human growth factor receptor–bound protein 7 (Grb7) is the founding member of a family of adaptor molecules (5, 6) including Grb10 (7) and Grb14 (8). The family members have a highly conserved structure: the NH2-terminal region has proline-rich motifs and Ras association [Ral guanine nucleotide dissociation stimulator and afadin] domain; the central region has a pleckstrin homology domain; and the COOH-terminal region has an Src homology 2 domain. The Grb7 family molecules share sequence homology with the Caenorhabditis elegans Mig-10 gene, which is involved in the regulation of embryonic neural cell migration (9, 10). In addition, the Grb7 family plays important functions as downstream signaling molecules of tyrosine kinase receptors including epidermal growth factor receptor (5), ErbB2 (6), and EphB1 (11). In particular, Grb7 plays a role in cell migration by its interaction with focal adhesion kinase (FAK; refs. 12).

Grb7 has been shown to be amplified and overexpressed in breast cancer (13) and esophageal cancer (14). Grb7 and Grb7 variant, a novel variant of human Grb7 lacking the Src homology 2 domain, were associated with cell invasion and metastatic progression of esophageal cancer (15). In addition, Grb7/FAK complex formation and its correlation with increased invasion were also reported in esophageal carcinoma cells (16).

We previously reported that overexpression of FAK protein was significantly associated with portal venous invasion and poor prognosis in HCC (17). In the present study, expression of Grb7 in HCC tissue was immunohistochemically examined, and the relationships with regard to clinicopathologic features and FAK expressions were investigated. In addition, the aim of this study was also to investigate whether Grb7 is involved in the invasive potential of HCC and whether RNA interference (RNAi) of Grb7 protein could be a therapeutic tool in HCC.

Results
Protein Expression of Grb7 in HCC
The expression of Grb7 protein was immunohistochemically examined in 64 HCC patients (Fig. 1). Positive
expression of Grb7 was found at main lesion in 18 (28.1%) patients. Moreover, positive expression of Grb7 was found at portal venous invasive lesion in 11 of 20 (55%) patients. There was a significant association between positive expression of Grb7 in the main lesion and portal venous invasive lesion \((P < 0.05)\).

### Relationship between Immunohistochemical Results and Clinicopathologic Features

Table 1 shows a comparison of the clinicopathologic factors between the Grb7-positive group and the Grb7-negative group in HCC. Protein expression of Grb7 was significantly correlated with portal venous invasion \((P < 0.01)\), hepatic venous invasion \((P < 0.01)\), and intrahepatic metastasis \((P < 0.05)\).

### Grb7 Expression Correlates with FAK Expression in HCC

We previously reported that the overexpression of FAK protein was significantly associated with portal venous invasion and poor prognosis in 64 HCC samples (17). The relationship between protein expression of Grb7 and FAK was examined in 64 same samples of HCC. We refer to our previous data about FAK expression in 64 HCC samples (17). Thirteen of 18 cases with positive expression of Grb7 showed positive expression of FAK, and 41 of 46 cases with negative expression of Grb7 showed negative expression of FAK (Table 2). This indicated that protein expression of Grb7 was significantly correlated with FAK protein levels in HCC \((P < 0.01)\).

### Relationship between Immunohistochemical Results and Survival

With regard to prognosis, the Grb7-positive group showed a tendency to poor prognosis as compared with the Grb7-negative group \((P = 0.07)\). The survival curves of the positive versus negative expressers of FAK and Grb7 showed a significant separation \((P = 0.03, \text{Fig. 2})\). We also carried out a multivariate analysis of survival using the Cox proportional hazards regression model including serum albumin level, Edmondson-Steiner grade, histologic differentiation, tumor-node-metastasis classification, portal venous invasion, intrahepatic metastasis, and FAK/Grb7 expression, which were found to be prognostic factors on univariate analysis. This analysis showed that FAK/Grb7 expression was not an independent factor.

### Grb7 Expression in HCC Cell Lines

Western blot analysis was done to determine whether HCC cell lines express Grb7 protein. Protein expression of Grb7 was detected in all three HCC cell lines by Western blot analysis (Fig. 3). Hep3B expressed higher levels of Grb7 protein compared with Huh7 and PLC/PRF/5. Therefore, we used the Hep3B cell line for the subsequent experiments.

### Suppression of Grb7 Protein in Hep3B Cells by RNAi Technology

To address if Grb7 could serve as a therapeutic target for invasive HCC, we used small interfering RNA (siRNA) oligonucleotides to suppress Grb7 expression in HCC cells. We designed siRNA oligonucleotides targeting Grb7 mRNA. The siRNA oligonucleotides were transfected into a Grb7-overexpressing HCC cell line, Hep3B. The protein lysates were harvested 24, 48, and 72 h after transfection and analyzed by Western blot. We found that Grb7 expression levels were suppressed in Hep3B cell lines by at least 72 h (Fig. 4A). These data indicated that siRNA could effectively suppress Grb7 overexpression.

To investigate the functional role of Grb7 protein in HCC cells, we generated Grb7 stable knockdown Hep3B cells using siRNA expression plasmid. As seen in Fig. 4B, Western blot analysis of whole-cell lysates showed that transfection of the

### Table 1. Grb7 Protein Expression and Clinicopathologic Factors in HCC

<table>
<thead>
<tr>
<th>Factors</th>
<th>Grb7 Positive ((n = 18))</th>
<th>Grb7 Negative ((n = 46))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (cm)</td>
<td>4.9 ± 1.2</td>
<td>3.6 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>I and II</td>
<td>12</td>
<td>37</td>
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</tr>
<tr>
<td>III and IV</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Well</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Moderately</td>
<td>11</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>7</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Capsule formation, n (%)</td>
<td>17 (94)</td>
<td>35 (76)</td>
<td>NS</td>
</tr>
<tr>
<td>Portal venous invasion, n (%)</td>
<td>14 (78)</td>
<td>12 (23)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hepatic venous invasion, n (%)</td>
<td>5 (28)</td>
<td>1 (2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Intrahepatic metastasis, n (%)</td>
<td>8 (44)</td>
<td>7 (15)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

**Abbreviation:** NS, not significant \((P > 0.05)\).
Low Invasion and Motility Potential of Grb7 Knockdown Hep3B Cells

We examined the invasion and motility potential of the Grb7 knockdown cells using an in vitro Matrigel invasion assay. Grb7 knockdown Hep3B cells exhibited significantly less invasive potential than control cells (Fig. 5A; P < 0.05). To further investigate the motility potential of Grb7 knockdown cells, we did an in vitro wound healing assay (Fig. 5B). There was significantly lower motility with Grb7 knockdown cells as compared with control cells (Fig. 5C; P < 0.05). These results indicate that Hep3B cells have low invasive potential and motility due to suppression of the Grb7 protein. The proliferation of Grb7 knockdown Hep3B cells was not statistically significantly different from that of control cells (Fig. 5D).

Suppression of Grb7 Protein Delayed the Onset of the Tumors in Nude Mice

Next, we sought to determine whether RNAi of Grb7 could serve as a therapeutic agent against HCC tumor formation in nude mice. Grb7 knockdown cells and control plasmid–transfected cells were injected into nude mice, and the growth of tumors was measured weekly. As shown in Fig. 6, tumor size and the growth curves of the tumors from Grb7 knockdown cells and control cells were similar after 8 weeks, and the growth rate of Grb7 knockdown cells was very similar to that of control cells. Moreover, the mean of the tumor doubling times in control cells (15.6 ± 2.7 days) was not significantly modified by Grb7 knockdown (14.7 ± 1.2 days; Fig. 6, inset). These data showed that Grb7 inhibition delayed the onset of the tumors but had no effect on the growth rates of the tumors.

Discussion

In this report, we showed that Grb7 protein was overexpressed in main lesion and portal venous invasion lesion in HCC. Grb7 overexpression was significantly correlated with portal venous invasion, hepatic venous invasion, and intrahepatic metastasis. Similar studies have been done on a few other carcinomas. For example, it was shown that the Grb7 protein was overexpressed in 14 of 31 (45%) cases as compared with the adjacent normal mucosa, and this finding was significantly correlated with the presence of lymph node metastases in esophageal carcinoma (16). It was observed that DNA and messenger RNA levels of Grb7 were elevated in 32% of Barrett's adenocarcinomas (18). Our present study made it clear that Grb7 strongly reflects the biological aggressiveness of HCC and plays an important role in its progression; moreover, this is the first clinical study on the expression of Grb7 protein in HCC.

We also showed that Grb7 expression was significantly correlated with FAK protein levels. FAK is a cytoplasmic nonreceptor tyrosine kinase and one of the major mediators of signal transduction (proliferation, migration, cytoskeleton, and antiapoptosis; ref. 19). We previously showed that the overexpression of FAK protein was significantly associated with portal venous invasion (17). It was reported that Grb7 protein interacted with and was phosphorylated by FAK under the regulation of matrix-integrin signaling in esophageal carcinoma cells (16). Using immunoblotting and immunoprecipitation methods, we observe a direct interaction of FAK protein with Grb7 protein in HCC cells (data not shown). Together with our study, it is suggested that FAK/Grb7 formation might be one of the important roles of tumor invasion in HCC. Additional studies will be required to clarify the interaction and phosphorylation of FAK/Grb7 protein in relation to cell invasion in HCC cells.

The survival curves of the positive versus negative expression of Grb7 showed no significant separation. The FAK- and Grb7-positive expression group showed a significantly poorer prognosis than the negative expression group. Unexpectedly, FAK- and Grb7-positive expression was not an independent factor. We previously showed that the overexpression of FAK protein was an independent factor in determining the prognosis of patients in multivariate analysis (17). FAK has been implicated in the regulation of cell proliferation, migration, cytoskeleton, and apoptosis (19). Grb7 is one of the downstream signaling molecules of FAK in cell migration (12). It might be that FAK expression, rather than Grb7 expression, was an adequate independent prognostic factor in HCC.

The recent progress of RNAi technology has shown the potential to overcome those limitations. siRNA is a double-stranded RNA that is more resistant to nuclease degradation...
reported that the pleckstrin homology domain of Grb7 adaptor proteins, and cytoskeletal proteins (21). It was also mice including protein kinases, phospholipases, GTPases, amino acids that has been reported in a variety of signaling interesting. The NH2-terminal region of Grb7 has proline-rich domain. Pleckstrin homology domain is a module of lesions, Ras association domain, and pleckstrin homology 3 domain of various proteins (23). However, no interacting proteins have yet been identified in the proline-rich lesions of Grb7. Ras association domain is mostly RasGTP effectors and includes Ral guanine nucleotide dissociation stimulator, afadin, and c-Raf (24). This suggests the possibility that Grb7 might serve as an effector and regulate the Ras signaling pathway. Our preliminary data show that the activation of p42/p44 mitogen-activated protein kinase in Grb7 knockdown Hep3B cells is down-regulated as compared with control cells (data not shown). Further studies will be necessary to identify the downstream pathway of Grb7 binding to clarify its precise molecular role in HCC progression.

As molecular targets are identified, novel agents that are specific for such targets can be designed that might improve the treatment of HCC. FAK and Grb7 are possible molecular targets in HCC. FAK signals to many pathways. Thus, we think that more side effects might occur with FAK as a molecular target. In this study, Grb7-positive HCC was more strongly correlated with vascular invasion rather than FAK-positive HCC. Therefore, we think that we would be able to control HCC progression more specifically if Grb7 was chosen as a molecular target. Grb7 would be a novel molecular target in HCC with vascular invasion.

In conclusion, high expression of Grb7 protein is associated with clinical progression of HCC, and Grb7 expression is significantly correlated with FAK expression in HCC. Furthermore, we clarified that Grb7 knockdown using RNAi technology could modulate the malignant phenotype of HCC more, we clarified that Grb7 knockdown using RNAi technology to consider its therapeutic tool in the future. We made an attempt to modulate Grb7 protein expression using RNAi technology to consider its therapeutic tool in the future. We suppressed Grb7 protein using siRNA oligonucleotides and siRNA stable expression technique in Hep3B cells, characterized by high expression of Grb7 protein. The production of Grb7 protein was greatly reduced in Grb7 siRNA oligonucleotide-transfected cells and pSilencer 3.1-H1 hygro/Grb7 RNAi plasmid–transfected cells compared with control cells. Furthermore, Grb7 knockdown HCC cells exhibited low invasion and motility compared with control cells in vitro. The proliferation of Grb7 knockdown Hep3B cells was not statistically significantly different from that of control cells. Grb7 inhibition delayed the onset of the tumors but had no effect on the growth rates of the tumors in vivo. In this clinical study, protein expression of Grb7 was not significantly correlated with tumor size. It was reported that the Grb7 peptide inhibitor significantly attenuated cell migration in vitro and peritoneal metastasis in a mouse model of pancreatic cancer cells, but the proliferation of pancreatic cells treated with the Grb7 peptide inhibitor was not significantly different from that of untreated cells (20). These data suggested that Grb7 protein played an important role in cell invasion of HCC cells, but might not contribute to cell proliferation. Taken together, these findings indicated that HCC progressiveness was deteriorated by Grb7 RNAi.

The actual downstream pathway of Grb7 in HCC cells is interesting. The NH2-terminal region of Grb7 has proline-rich lesions, Ras association domain, and pleckstrin homology domain, and the COOH-terminal region has an Src homology 2 domain. Pleckstrin homology domain is a module of ~100 amino acids that has been reported in a variety of signaling molecules including protein kinases, phospholipases, GTPases, adaptor proteins, and cytoskeletal proteins (21). It was also reported that the pleckstrin homology domain of Grb7 interacted with phosphoinositides and this interaction mediated signal transduction pathways in cell migration (22). Several minimal consensus repeats, PXXP, are located within the proline-rich lesions of Grb7. Theses consensus sequences can modulate protein-protein interactions by binding to the Src homology 3 domain of various proteins.

**Materials and Methods**

**Clinical Samples**

We reviewed 64 consecutive patients who had undergone initial liver resection for HCC without preoperative treatment at the Department of Surgery and Science, Kyushu University Hospital, between January 1991 and February 1999. The ages

**FIGURE 4.** Western blot analysis of Grb7 and \(\beta\)-actin in Hep3B cells using RNAi technology. A, Transfection with siRNA oligonucleotides targeting Grb7 mRNA or control. Each cell lysate was prepared at 24, 48, and 72 h after transfection. Grb7 expression levels were suppressed in Hep3B cell lines by at least 72 h. B, Transfection with pSilencer 3.1-H1 hygro/Grb7 RNAi plasmid or control. In lysates of two transfectants, Grb7 stable knockdown was observed.
of the patients ranged from 42 to 78 years, with an average age of 61.2 years. Clinicopathologic variables were defined according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer of the Liver Cancer Study Group of Japan (25). None of the patients had previously received any other therapy such as ablation, transarterial embolization, and percutaneous ethanol injection therapy. Patients who underwent noncurative surgery or had an inadequate follow-up were excluded from this surgery. After surgical resection, HCC specimens from each patient were fixed in 10% buffered formalin, embedded in paraffin, stained with H&E, and examined microscopically.

**Immunohistochemistry**

Immunohistochemical observations were done on adjacent deparaffinized sections using the peroxidase-labeled streptavidin-biotin technique with the Histofine SAB-PO kit (Nichirei). The primary antibody used in this study was Grb7 protein (rabbit polyclonal antibody; Santa Cruz Biotechnology). Three-micrometer-thick histologic sections were cut and removed from paraffin with xylene and rehydrated in descending dilutions of ethanol. The endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 30 min. To retrieve the antigen, pretreatment with citrate buffer (0.01 mol/L citric acid, pH 6.0) for 20 min at 99°C in a microwave oven was done. After sections were exposed to 10% nonimmunized goat serum in PBS for 10 min, they were incubated overnight at 4°C with primary antibodies diluted 1:250. The sections were subsequently incubated with a second-stage biotinylated antibody for 20 min, followed by incubation with horseradish peroxidase–labeled streptavidin for 20 min at room temperature. After rinsing in PBS, the reaction products were visualized by immersing the section in
diaminobenzidine tetrahydrochloride as a chromogen. No significant staining was observed in the negative controls, which were prepared using rabbit immunoglobulin at the same concentration. Finally, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

The Grb7 expression of the tumor was evaluated independently by two pathologists (T.M. and S.A.) according to the proportion of positively stained cells. When the stained cells accounted for >20% of the total, the tumor was designated as high expression.

Cell Culture

Three established human HCC cell lines, Huh7, Hep3B, and PLC/PRF/5 (Riken Cell Bank), were used in this study. Cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 500 units/mL penicillin, and 500 μg/mL streptomycin (Life Technologies). All cells were maintained at 37°C in 5% CO₂.

Western Blot Analysis

Growing cells were harvested by scraping on ice into a lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 0.5 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, and 2 μg/mL leupeptin. Lysates were cleared by centrifugation (15,000 rpm) at 4°C for 10 min. The supernatant protein concentration was determined using a Bradford Protein Assay Kit (Bio-Rad). Normalized lysates were boiled in sample buffer, run on a 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Invitrogen). Membranes were blocked for 1 h in TBS containing 5% skim milk and 0.05% Tween 20. The membrane was probed with Grb7 antibody (1:1,000 dilution; rabbit polyclonal antibody; Santa Cruz Biotechnology) and β-actin (1:2,500 dilution; mouse monoclonal antibody; clone AC-15, Sigma-Aldrich) for 2.5 h. The membrane was then washed twice with TBS containing 0.05% Tween 20 for 10 min and then incubated with TBS containing 1:5,000 diluted peroxidase-conjugated second anti-bodies (Amersham Pharmacia Biotech) for 1 h. After being washed thrice, the membrane was developed by an enhanced chemiluminescence system (Amersham Pharmacia Biotech) and analyzed with the use of LAS-3000 (Fujifilm).

Transfection with siRNA Oligonucleotides

siRNAs were synthesized and high-performance purified (Qiagen-Xeragon). siRNA oligonucleotides were as follows: Grb7 sense, CGAGUCCACAGUGUAAGUG-dTdT; Grb7 antisense, AACGUACACGGUGAGCUG-dTdT; control sense, UUCUCGGAACUGUGUCAG-dTdT; and control antisense, ACGUGACAGCGUUCGGAGA-dTdT. Hep3B cells (1 × 10⁵ per well) were transfected with siRNA oligonucleotides in six-well plates using LipofectAMINE 2000 (Invitrogen) following the manufacturer’s protocol.

Establishment of Stable Grb7 Knockdown Hep3B Cells

It was reported that hairpin siRNA could efficiently induce RNAi of the target gene (26). To establish stable expression of siRNA in mammalian cells, we used the siRNA expression vector pSilencer 3.1-H1 hygro (Ambion, Inc.). To generate the pSilencer 3.1-H1 hygro/Grb7 RNAi plasmid, hairpin Grb7 siRNA oligonucleotides (sense, 5'-GATCCCGCGAGTCAACGCACGTTACGTTGGAGAC-TCTGTTTTTGGAA-3'; antisense, 5'-AGCTTTTCCAAGAACACAGTCACTGACCTGGCTCGCC-3') were inserted into the BamHI and HindIII sites of the pSilencer 3.1-H1 hygro. The plasmid was transfected into Hep3B cells by lipofection method as previously described (27), using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Stably transfected colonies showing suppression of Grb7 protein were selected after hygromycin (200 μg/mL) treatment and used for the subsequent experiments. The pSilencer hygro negative control RNAi plasmid (Ambion)–transfected cells were used for the control.

In vitro Cell Invasion Assay

According to previously described methods (17, 28), the invasive potential of Grb7 knockdown cells was determined by a Matrigel invasion assay using polycarbonate membranes (8.0 μm pore size) in the upper chamber of 24-well Transwell culture chambers coated with Matrigel (Becton Dickinson). Cells (1.0 × 10⁵ per well) were placed in the upper chambers, and the lower chambers were filled with 750-μL DMEM with 20% fetal bovine serum as a chemoattractant. After 36 h of incubation at 37°C, the membranes were stained with May-Grunwald and Giemsa solutions. The invasive cells that had migrated through the membrane to the lower surface were counted in three different fields under a light microscope at ×200. Each experiment was done in triplicate wells and repeated thrice.

In vitro Wound Healing Assay

Grb7 knockdown cells and control cells were used in the wound healing assay to examine for the alterations of cell motility and migration (29). Cells were initially seeded uniformly onto 60-mm culture plates with an artificial wound carefully created at 0 h, using sterile pipette tips to scratch on the

FIGURE 6. Murine xenograft model for tumor formation effect of Grb7. Grb7 knockdown cells (●) and control cells (○) at 2.5 × 10⁵ in 0.25-mL PBS were injected s.c. in the right flank of six mice in each group. Tumor formation was scored weekly. Points, mean volume (n = 5); bars, SD. Inset, corresponding logarithmic regression trend lines.
subconfluent cell monolayer. The wound monolayers were then incubated in DMEM containing 10% fetal bovine serum for 24 h and photographed under a phase-contrast microscope at ×200. The experiments were repeated in triplicate wells at least thrice. In vitro Cell Proliferation Assay

Grb7 knockdown cells and control cells were plated at a density of 1.0 × 10⁵ per well in three 6-cm plates and were harvested and counted on days 1, 3, and 7. The medium was changed every 72 h. This experiment was repeated thrice.

Murine Xenograft Model

Four-week-old female nude (nu/nu) mice were purchased from Charles River Laboratories and were maintained under specific pathogen-free conditions in the animal facility at the Kyushu University Medical School. These experiments were approved by the Kyushu University Institutional Animal Care and Use Committee and conformed to all the guidelines outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy for Sciences and published by the NIH. Grb7 knockdown cells and control cells were injected s.c. in the right flank with 2.5 × 10⁶ cells in 0.25-mL PBS. Each experiment was repeated at least twice. The tumor volumes were calculated according to the following formula: 

\[ V = \frac{A \times B^2}{2} \] (cm³), where A was the largest diameter (cm) and B is the smallest diameter (cm).

Statistical Analysis

Associations between Grb7 expression and clinicopathologic variables were analyzed by Student’s t test or Fisher’s exact test. Survival curves were drawn according to the Kaplan-Meier method. The Cox proportional hazards model with a stepwise method. The Cox proportional hazards model with a stepwise procedure was used in multivariate analysis of survival data. The variables such as the data using cell lines were compared by Mann-Whitney’s U test. All statistical differences were deemed significant at \( P < 0.05 \).

References

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