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Original article

Suppression of GLTSCR2 expression in renal cell carcinomas

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ABSTRACT

Nucleolar protein PICT-1/GLTSCR2 (GLTSCR2) has both tumor suppressive and oncogenic activities, depending on the types of cancer tissue and its expression level. The role of GLTSCR2 in renal cell carcinoma (RCC) has not yet been addressed. The aims of this study were to evaluate GLTSCR2 expression in RCC tissue and to determine pathological significance of GLTSCR2 in terms of tumor grade. RCC and adjacent normal tissue from 84 different patients was retrieved from nephrectomy specimens. The expression level of GLTSCR2 in RCC tissues was determined via immunohistochemical staining and invasion was determined using transwell chambers with Matrigel-coated membranes. The expression of GLTSCR2 was suppressed in about 80% of the carcinoma specimens compared to noncancerous renal tissue and inversely correlated with Fuhrman nuclear grade ($r = -0.40$, $p < 0.05$). Knockdown of GLTSCR2 expression increased the invasiveness of SNU267 RCC cells. The expression of GLTSCR2 was suppressed in RCCs and its downregulation accentuated the malignant phenotype.

1. Introduction

Protein interacting with PTEN carboxyl terminus 1/glioblastoma tumor suppressive candidate region gene 2 (GLTSCR2) is a nuclear/nucleolar protein with both tumor suppressive and oncogenic activity [1,2]. As a tumor suppressor, GLTSCR2 upregulates the expression of p53 by inhibiting its mouse double minute 2 homolog (MDM2)-mediated degradation and, consequently, delays cell cycle progression [3]. It is downregulated in brain tumors including glioblastomas and neuroblastomas, breast cancers, and ovarian cancers [4–7], and its expression reciprocally correlates with the degree of differentiation in prostatic adenocarcinomas [8]. As an oncoprotein, high expression of GLTSCR2 results in rapid proliferation of tumor cells, and expression of its transcript is increased in oesophageal and colon cancers [1]. The divergent roles of GLTSCR2 in tumor progression may reflect the type of cancer, the intracellular signalling pathways determined by the genetic status of the cancer, and the expression levels of GLTSCR2's binding partners as well as its own expression level.

The aim of this study was to elucidate the role of GLTSCR2 in RCC. In this study, we immunohistochemically determined the expression levels of GLTSCR2 in RCC tissue from 84 different cases and the pathological significance of GLTSCR2 expression.

2. Materials and methods

2.1. Cell culture, tissue samples and pathological analysis

SNU267 RCC clear cell-type cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum. Surgically resected RCC tissue from 84 different cases and accompanying clinical data were retrieved from the pathology laboratories at our institution in accordance with an institutional review board-approved protocol. Tissue slides were prepared using conventional methods. Histopathological analyses and Fuhrman nuclear grading of routine tissue slides stained with hematoxylin and eosin were performed by two pathologists.

2.2. Immunohistochemical staining and evaluation

Immunohistochemical staining was performed using the EnVision-HRP kit (Dako, Carpinteria, CA, USA) and visualized via chromogen 3-3′ diaminobenzidine (5 min incubation), followed by

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counterstaining with hematoxylin. Rabbit anti-GLTSCR2 antibody (dilution 1:100) was used as described in our previous study.[9] GLTSCR2 expression was evaluated in RCC and non-cancerous renal tissue within the same specimen according to a semiquantitative scoring system. Intensity was scored as follows: 0, negative; 1, weak expression; 2, moderate expression; and 3, strong expression. Scores were based on the maximum intensity of positive cells. The percentage of positive cells was scored as follows: 0, negative; 1, < 25%; 2, 26–50%; 3, 51–75%; and 4, > 76%. The final grade was the sum of the intensity and percentage scores; Grade 1 refers to a score of 0–1, Grade 2, to a score of 2–3, Grade 3 to a score of 4–5, and Grade 4 to a score of 6–7.

2.3. Adenovirus construction and infection

Adenovirus expressing GLTSCR2 was constructed as described previously [3]. Infection was carried out by incubating SNU267 cells with adenovirus for 12 h in serum-free medium, and cells were harvested for western blotting 48 h after infection.

2.4. Knockdown of GLTSCR2 and invasion assay

To deplete GLTSCR2, SNU267 cells were transiently transfected with small interfering RNA (siRNA) (Qiagen, CA, USA) to GLTSCR2; control cells received scrambled siRNA. siRNA transfection was performed using oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Invasion assays were carried out using a cell invasion kit (Chemicon, MA, USA) according to the manufacturer's protocol. Briefly, 1×10^4 cells were plated on Matrigel-coated membranes in transwell invasion chambers and incubated at 37 °C for 24 h. Non-invading cells were removed by wiping the upper side of the membrane. Invading cells (i.e., cells that penetrated the membrane) were fixed with methanol and stained with hematoxylin. Three independent invasion assays were performed in triplicate. On average, cells in six random fields were counted using a light microscope.

2.5. Statistical analysis

Statistical analysis was carried out using SPSS software, version 13.0 (SPSS, Chicago, IL, USA). Data were analysed using Fisher's exact test or the Pearson chi-square test. Correlations between GLTSCR2 expression and Fuhrman nuclear grade were determined using Spearman's rank correlation coefficient. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Clinicopathologic findings of RCCs

The clinicopathologic findings are listed in Table 1. Selected eighty four cases were composed of 56 men and 28 women (men to women ratio, 2:1), with a mean age of 59 years. Histologically, 75 (89%) cases were clear cell RCC (CRCC), 4 (5%) papillary RCC (PRCC), and 5 (6%) chromophobe RCC (CPRCC). Fuhrman nuclear grading system was as follows: 12 (14%) were grade I, 37 (44%) were grade II, 30 (36%) were grade III, and 5 cases (6%) were grade IV.

3.2. Immunohistochemical findings of GLTSCR2 in noncancerous renal tissues

To investigate GLTSCR2 expression in noncancerous renal tissues, we performed immunohistochemical staining on surgically resected kidney tissues from 84 different cases. In renal tubular cells, GLTSCR2 immunostaining reactivity varied from grade 1 to 4, but over 85% of the cases were categorized to grade 3 and 4 (Fig. 1A). Although immunoreactivity was different between the selected cases, there was no significant difference according to the areas in staining intensity. In addition, difference in staining intensity between proximal tubular, distal tubular, and collecting duct epithelial cells within the same case was negligible. Individual tubular cells revealed coarse GLTSCR2 immunoreactivity within the nucleus with stronger nucleolar staining (Fig. 1B). In the glomerulus, about sixty percents of mesangial cells were positively stained but intensity was below than tubular cells. Glomerular capillary endothelial cells were weakly stained. In contrast, strong immunoreactivity was detected in parietal epithelial cells (Fig. 1C).

Table 1
Clinicopathologic characteristics of the renal cell carcinomas.

Clinicopathologic variable	Number of cases (%)	
Tumor size	Less than 7 cm	68 (81)
	More than 7 cm	16 (19)
Fuhrman nuclear grade	Grade I	12 (14)
	Grade II	37 (44)
	Grade III	30 (36)
	Grade IV	5 (06)
TNM Stage	Stage I	62 (73)
	Stage II	14 (17)
	Stage III	5 (06)
	Stage IV	3 (04)
Histologic types	CRCC	75 (89)
	PRCC	4 (05)
	CPRCC	5 (06)

CRCC, clear cell type renal cell carcinoma; PRCC, papillary renal cell carcinoma; CPRCC, chromophobe renal cell carcinoma

3.3. Immunohistochemical findings of GLTSCR2 in RCCs

In parallel with noncancerous renal tubular cells, GLTSCR2 was immunohistochemically stained in the nucleus. GLTSCR2 immunoreactivities varied from grade 1 to grade 4, but about 78% of RCCs were categorized to grade 1 and grade 2 (Fig. 2A). There were no significant differences in GLTSCR2 immunoreactivity between histological tumor types (data not shown). In comparison with immunohistochemical findings in noncancerous renal tissues, we found that GLTSCR2 expression was significantly downregulated in RCCs (Fisher's exact test, $p < 0.05$) (Table 2). In addition, GLTSCR2 expression in RCC tissue was significantly downregulated compared with matched normal tissue in 81% of the selected cases (Fisher's exact test, $p < 0.05$) (Fig. 2B). Next, we investigated the relation between GLTSCR2 immunoreactivity and pathologic parameters including tumor size, TNM stage, and nuclear grade. Among them, GLTSCR2 immunoreactivity was reciprocally correlated with nuclear grade (Spearman's correlation coefficient; $r \equiv -0.40$, $p < 0.05$) (Table 3).

3.4. Knockdown of GLTSCR2 in renal carcinoma cells is associated with an invasive phenotype

Because nuclear grade is associated with tumor aggressiveness in RCC [9], we asked whether suppression of GLTSCR2 expression in RCC cells affected their invasive potential. SNU267 clear cell-type renal carcinoma cells were transfected with siRNA to GLTSCR2 (siGLT) or scrambled siRNA (siSCR). Three days after transfection, GLTSCR2 expression was reduced more than 80% in siGLT-transfected cells compared with siSCR-transfected cells (Fig. 3A). Invasion assays using transwell chambers with Matrigel-coated membranes were performed. siGLT increased the number of invading cells, whereas siSCR did not (Fig. 3B). Controlled adenovirus-mediated expression of GLTSCR2 (Ad-GLT) in cells expressing siGLT reduced the number of invading cells almost to