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Expression of ROR1 in Patients with Renal Cancer-A Potential Diagnostic Marker

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ABSTRACT

Background: The ectopic expression of receptor tyrosine kinase Ror1 has been reported in patients with hematological malignancies such as chronic lymphocytic leukemia and acute lymphoblastic leukemia. Here we report, for the first time, expression of *ROR1* gene in both tumor tissues and peripheral blood mononuclear cells (PBMC) from patients with renal cancer (RC). **Methods:** In the current study, the expression of *ROR1* gene was semi-quantitatively measured in PBMC and tumor tissues from 16 RC patients as well as PBMC from 22 healthy individuals relative to the expression of the housekeeping gene phosphoglucomutase 1 by RT-PCR. **Results:** Our results showed that *ROR1* was expressed at gene level in 81.3% of renal tumor tissues (13 out of 16) whereas it was expressed in 94% of PBMC from RC patients (15 out of 16). A weak expression of *ROR1* was observed in PBMC of 4 out of 22 healthy individual. A significant expression of *ROR1* was observed in PBMC from RC patients when compared to that in PBMC from normal healthy individuals (P<0.001). The expression of *ROR1* in PBMC may reflect a shedding of tumor cells into blood stream. **Conclusion:** We conclude that detection of a high level of *ROR1* expression in blood cells might assist in early detection of renal malignancies, providing taking into consideration the clinical symptoms of the disease. *Iran. Biomed. J. 14 (3): 77-82, 2010*

Keywords: *ROR1*, Ectopic expression, Renal cancer

INTRODUCTION

Early detection of cancer cells significantly increases the possibility of successful treatment of patients with cancer. There are several molecular markers for diagnosis and prognosis of patients with renal cancer (RC) which includes Von Hippel-Lindau, Hypoxia-inducible factor 1 alpha, and carbonic anhydrase 9 [1-3]. However, these molecular markers are still insufficient as prediction tools in treatment and prognosis of the RC patients and have not been established as reliable tools in clinical practice. In this regard, the identification of more reliable tumor markers capable of characterizing cancer cells using more accessible patients' specimens such as peripheral blood or urine would be of great assist in managing the patients.

Recently, we and others have reported expression of receptor tyrosine kinase (RTK) Ror1 at gene and protein levels in patients with chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) [4-8]. Human Ror 1 (hRor1) has a role in cancer and also it has been identified as a potent survival kinase in HeLa cervical carcinoma cells [9]. Ror proteins are type-1 transmembrane RTK and like other RTK, they are predominantly located in the plasma membrane [10, 11]. hROR1 gene is

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located on chromosome 1p31-p32 [12, 13] and consists of 937 amino acids (914 after cleavage of the signal peptide) [14]. The expression of Ror1 is restricted to heart, kidney, brain, skeletal muscle and lung in embryonic stages [15].Also it is expressed in the heart, and prostate [16, 17]. Normal adult tissues do not express cell surface Ror1 molecule [7]. Our preliminary RT-PCR results in a panel of solid tumor cell lines showed that *ROR1* expression is not restricted to B cell malignancies and it is also expressed in some other solid tumors including renal cell carcinoma cell lines (Unpublished data). This finding directed us to investigate the expression of *ROR1* in tumor tissues as well as peripheral blood mononuclear cells (PBMC) from RC patients.

MATERIALS AND METHODS

Patients. Sixteen patients with renal malignancies were selected from five hospitals in Tehran (Iran). The study was approved by the Ethical Committee of Avicenna Research Institute and an informed consent was obtained from patients and healthy volunteers. The patients (n = 16) are composed of 3 women and 13 men with the age of 56.9 ± 10.4 years from whom 16 fresh tumor in addition to their peripheral blood samples were collected. This group included 8 patients with renal cell carcinoma, 5 with transitional cell carcinoma, and 3 with papillary cell carcinoma. Data about the stages of tumors and the age groups of the patients with RC are summarized in Table 1. The control group included blood samples from 22 normal individuals (11 women and 11 men, aged 56.3 ± 16.2) with no background of cancer.

Isolation of peripheral blood mononuclear cells. PBMC were isolated using Ficoll (Amersham Pharmacia Biotech, Chicago, IL, USA) density-gradient centrifuge [18]. Then, PBMC were washed twice with PBS.

RNA extraction and cDNA synthesis. Total cellular RNA was extracted from tumor tissues and PBMC with RNA-Bee reagent (BioSite, Täby, Sweden) according to the manufacturer's instructions. Reverse transcription was carried out in 20 µl reaction containing 5 µg total RNA, 1 U murine leukemia virus (M-Mulv) reverse transcriptase, 1 µl 20 pmol/µl random hexamer (N6), 1 U RNase inhibitor, 1 mM each of dNTP, and 4 µl 5× buffer (Invitrogen, Carlsbad, CA, USA). The tubes were incubated at 42°C for 45 min and then at 90°C for 5 min to terminate the reaction. The reaction products were used as template for PCR.

Table 1. *ROR1* expression profile and phenotypic characterization of 16 RC patients.

No.	IC	Age	Sex	Stage	RCC Phenotype	*ROR1* (RT-PCR)
1	74	49	M	T3, N1	Papillary renal cell carcinoma	+
2	33	54	M	T2	Transitional cell carcinoma of renal pelvis	+
3	18	50	M	T1	Transitional cell carcinoma of renal pelvis	+
4	66	31	M	T2, N1	Renal cell carcinoma (sarcomatoid features)	+
5	7	46	M	T2	Transitional cell carcinoma of renal pelvis	+
6	10	82	M	T2, N1	Renal cell carcinoma (clear cell type)	+
7	12	61	F	T1	Papillary renal cell carcinoma	+
8	17	71	M	T3	Papillary Transitional cell carcinoma of renal pelvis	+
9	57	57	M	T2	Renal cell carcinoma (chromophobe cell type)	+
10	9	49	F	T1	Transitional cell carcinoma of renal pelvis	+
11	55	62	M	T2	Renal cell carcinoma (clear cell type)	+
12	84	53	M	T1	Renal cell carcinoma (clear cell type)	+
13	14	56	M	T2	Transitional cell carcinoma of renal pelvis	-
14	90	50	M	T2	Renal cell carcinoma (clear cell type)	-
15	5	66	M	T2	Renal cell carcinoma (clear and granular cell type)	-
16	31	67	F	T2	Renal cell carcinoma (clear and granular cell type)	+

IC, internal code; M, male; F, female; T, tumor; N, nodes; T1, the tumor is no more than 7 cm across and is completely inside the kidney; T2, the tumor is more than 7 cm across, but is still completely inside the kidney; T3, the cancer has spread through the kidney capsule, to a major vein, the adrenal gland or other tissues immediately surrounding the kidney; N1, cancer spread to one nearby lymph node only.

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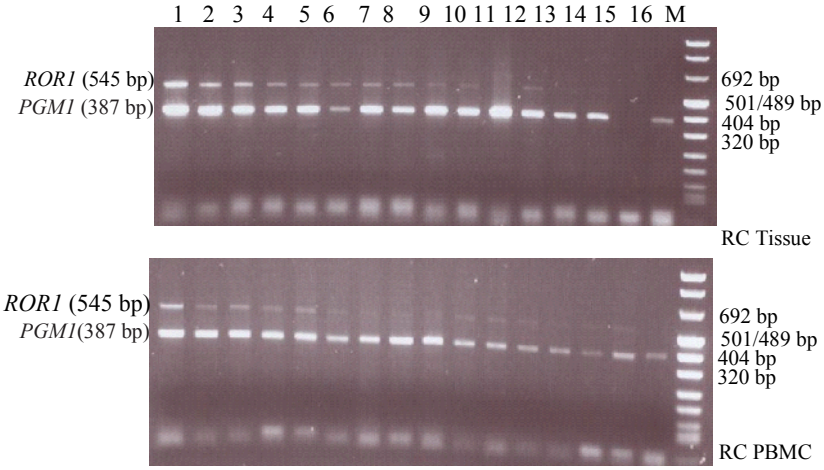

Fig. 1. RT-PCR of *ROR1* and *PGM1* genes. *ROR1* and *PGM1* PCR products related to each sample were electrophoresed together in equal volumes in the same wells of 1.5% ethidium bromide. (Upper panel) lanes 1 to 16: tissues of patient with RC. (Lower panel) lanes 1 to 16: PBMC from renal cancer patients. M, molecular weight marker VIII Roche (Roche Diagnostic, Mannheim, Germany).

Reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was performed with Ror1 primers [6] and phosphoglucomutase 1 (PGM1) primers (TCCGACTGAGCGGCACTGGGAGTGC as sense and GCCCGCAGGTCCTCTTTCCCTCA CA as anti-sense) [19]. PCR cycling parameters for the ROR1 primers were at 95°C for 2 min followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C. PCR cycling parameters for the PGM1 primers sets were 2 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 67°C, and 30 s at 72°C. PCR was carried out in a 25 µl reaction containing 1 µl of RT product, 1 µl of forward and reverse primers (10 Pmol/µl), 1 µl dNTP (10 mM), 1 mM $MgCl_2$, 2.5 µl 10× buffer, and 1U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplicon sizes of *ROR1* and PGM1 PCR products were 545 and 387 bp, respectively. *ROR1* and *PGM1* PCR products related to each sample were electrophoresed together in equal volumes in the same wells of 1.5% ethidium bromide-stained agarose gels and visualized under UV light. The intensity of bands was analyzed with Labworks 4.0 software (UVP, USA) and the expression of *ROR1* gene was measured semi-quantitatively relative to the expression of *PGM1*, in each sample.

Statistical analysis. Statistical analysis of the data was performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL) version 11.2. The differences between values of parameters in the independent and dependent groups were determined using Mann-Whitney U test and Wilcoxon signed-rank test, respectively. In addition, Fisher's exact and Chi square test were used for assessment of differences between qualitative variables. *P* value <0.05 was assigned to define the significance levels.

RESULTS

The representative RT-PCR amplification of *ROR1* in RC PBMC and tissue samples are shown in Figure 1. In this study, PBMC of 22 healthy individuals, with no history of cancer diseases, were evaluated for relative expression of *ROR1* gene transcript. Among these, 18 individuals did not show any *ROR1* expression whereas 4 exhibited low levels of *ROR1* expression (Data not shown). Based on these data, an arbitrary cut off level of 4.2 (mean + 2 SD) was defined. Accordingly, only 2 of the normal donors had *ROR1* expression above the cut off level. *ROR1* was expressed in 13 out of 16 RC tissue samples about cut off level, whereas its expression was detected in 15 out of 16 RC PBMC (Figs. 2 and 3).

When *ROR1* gene expression was measured in PBMC of RC patients, there was a significantly higher expression of *ROR1* in PBMC of RC patients when compared to normal controls (P<0.001). In addition, the *ROR1* expression levels in tumoral tissues related to RC patients were significantly higher than that in normal PBMC (P<0.001). In Figures 2 and 3, we show relative expression of *ROR1* in tissue and PBMC of RC patients, respectively. No significant difference was observed between *ROR1* expression in tumoral tissues and their PBMC counterparts (P>0.05, Fig. 4).

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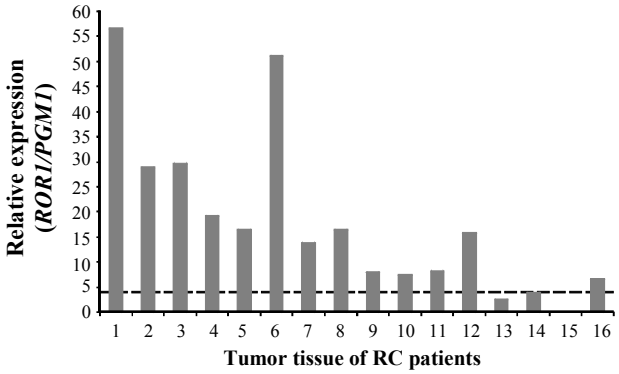

Fig. 2. Relative expression of *ROR1* in 16 RC tissue samples. Dotted line shows the cut off level (4.2). The numbering corresponds to patients numbering in Table 1.

There was no significant difference between various stages of RC for *ROR1* expression in both blood and tissue. *ROR1* gene expression in different age groups showed that the age had no effect on *ROR1* expression in peripheral blood and tumoral tissues of patients with RC.

DISCUSSION

Expression of Ror1 in two hematological malignancies of ALL and CLL has been reported previously. Here, we report the expression of *ROR1* (at gene level) in a solid tumor. Apart from the role of this RTK in pathogenesis of cancer, its detection in the peripheral blood cells is an important discovery which may pave the way for solving problems in early cancer detection. Our results clearly show that *ROR1* could be used as a tumor marker for diagnosis and detection of RC patients which of course needs further technical optimization. Among 16 RC patients' PBMC studied here, only one of them did not show expression of *ROR1* gene above the cut off level, indicating 95% detection rates. To our best knowledge, no single marker so far has yielded such a high percentage of positive cases in a given solid malignancy. We assume that the bias in expression of *ROR1* in tissues and PBMC from RC patients might be due to sample preparation either during the surgery or RNA preparation.

No significant difference was found in expression of *ROR1* with regard to the different stages of the disease indicating a constitutive expression of *ROR1*. This constitutive expression has previously been observed in a B cell-derived malignancy like CLL [6] and may imply that *ROR1* has also playing the same role in the pathogenesis of RC.

The *ROR1* gene is predominantly expressed during embryogenesis and principally is involved in development of different tissues. Interestingly, no cell surface expression of *ROR1* has been reported in normal adult tissues yet [7]; therefore, making it an ideal kidney tumor marker.

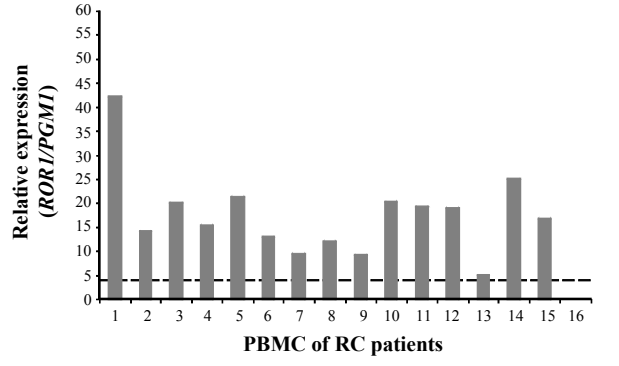

Fig. 3. Relative expression of *ROR1* in PBMC of 16 renal cancer (RC) patients. Dotted line shows the cut off level (4.2). The numbering corresponds to patients numbering in Table 1.

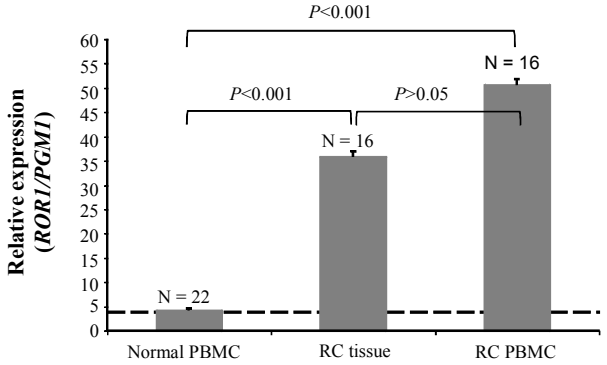

Fig. 4. Comparison of relative expression of *ROR1* in healthy PBMC (n = 22), renal cancer (RC) patients´ PBMC (n = 16), and renal cancer (RC) patients´ tissues (n = 16). Dotted line shows the cut off level (4.2). Significant values are bolded.

Although we have previously shown that Swedish normal healthy PBMC do not express *ROR1* transcript [6], a very low level expression of *ROR1* was observed in PBMC from 4 healthy individuals used in this study with no history of cancer. This low expression of *ROR1* gene might be due to either spontaneous stimulation of PBMC during Ficoll separation or it may reflect a genetic background of Iranian population as observed in our previous study [4]. As the ultimate goal is to establish a reliable RT-PCR based technique for detection of cancer cells using peripheral blood cells, considering such a low level of expression is essential for accurate

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diagnosis. Furthermore, it is obvious that real-time quantitative PCR would increase the accuracy of the assay. Further studies including a large number of samples in each group using real-time quantitative PCR as well as detection at protein level is warranted. Moreover, it would be interesting to investigate the expression of *ROR1* in the urine from patients with RC where the shedding of cancer cells is more probable.

To our knowledge, this is the first report describing expression of *ROR1* gene in a solid tumor as well as its detection in PBMC from these patients. Altogether, our results suggest that high levels of *ROR1* expression in blood cells might be used as a potential marker for detection and perhaps for monitoring RC patients following any treatment.

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