Speckle-type POZ protein acts as a predictive biomarker for mTOR target therapy in renal cell carcinoma

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Renal cell carcinoma (RCC) develops resistance to most of the conventional therapies. In recent years inhibitors are being used to interfere with growth of cancer cells at molecular level and are found to be the most effective treatment option. However, some patients have to deal with severe side effects. Till date there have been no specific predictive biomarkers available to predict the target response and patient’s stratification. The expression of speckle-type POZ protein (SPOP) as a predictive biomarker in the presence of mTOR target therapy. Tissue samples were collected after nephrectomies from patients with RCC. Further primary culture was established from tissues of low- and high-grade RCC. Working concentration of inhibitors (CCI-779 and SB203580) was selected by MTT assay on A-498 cell line. Western blot was performed to study the p38MAPK, mTOR and SPOP protein expression. Primary culture showed more than 70% positivity for pan-cytokeratin. The concentration of 20 \(\mu\)M of CCI-779 and 25 \(\mu\)M SB203580 caused 30% and 20% cell death respectively. Expression of SPOP protein was decreased in CCI-779-treated cells. The combined treatment of CCI-779 and SB203580 had more inhibitory effects on SPOP in all cells types. However, SB203580 had no effect on SPOP expression. The results underline that, SPOP could be used as a potential predictive biomarker to assess the response of therapy with mTOR inhibitor (CCI-779).

**Keywords:** Protein expression, predictive biomarker, renal cell carcinoma, target therapy.

Renal cell carcinoma (RCC) is the third most common type of urologic cancer. RCC is the most lethal, because of its diagnosis in late stage and resistance to the existing treatments. Although there are some newly developed effective therapies used to treat RCC, these show severe side effects. The area of oncology has now entered the era of personalized medicine and the selection of treatment for every cancer patient is becoming customized to avoid unnecessary side effects. This advancement reflects the molecular and genetic composition of the tumours and progress in biomarker technology, and according to the patient’s disease, it allows us to align the most appropriate treatment. The advances in our ability to identify predictive biomarkers, which can provide companion diagnostics for stratifying and subgrouping patients, represent the next leap forward in improving the quality of clinical care in oncology and are widely accepted. Recently, predictive biomarkers are gaining importance in advanced RCC as they are helpful in screening and prediction of the treatment; these can be assessed in serum and tissue.

Molecular mechanisms responsible for the proliferation of RCC have been identified, and molecular targeted therapy has also been developed. Temsirolimus (CCI-779), a major inhibitor of mammalian target of rapamycin (mTOR) has shown to prolong survival in first-line treatment of poor prognosis RCC of all histologies. However, there are many side effects such as gastrointestinal disorders, rash, fatigue, oedema and dyspnea as well as hematologic and metabolic abnormalities occurring in patients receiving temsirolimus. Hence it is important to give such therapy to those patients who are positive for mTOR to reduce unnecessary side effects.

Speckle-type POZ protein (SPOP) is a small protein known for its controversial role both in RCC and breast cancer as tumour inducer and tumour suppressor respectively. **Basically SPOP is a nuclear protein and is also known as E3 ubiquitin ligase adaptor identified as an auto-antigen in serum samples of patients with Scleroderma pigmentosum.** In our previous study we have found that SPOP is associated with grade and stage in RCC. mRNA expression in blood and tissue samples showed that patients with RCC had higher level of SPOP compared to normal samples.

Currently, for RCC, several studies have focused on the clinical validation of newly investigated markers, which enable to bridge molecular mechanisms for clinical diagnosis and therapeutics responsibly. Here, we study the effect of mTOR inhibitor (CCI-779) on SPOP in RCC cells. We also study the combined effect of CCI-779 and SB203580 (p38 MAPK inhibitor) on SPOP in RCC cells. A-498, a renal cancer cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India. Each tissue from low- and high-grade RCC was used for primary cell culture. Anti-pan-cytokeratin, mTOR, p-mTOR, p38 MAPK, p-p38 MAPK and act, secondary antibodies and protease phosphatase inhibitors cocktail were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti SPOP antibody was purchased from Abcam (Cambridge, England), mTOR inhibitor (CCI-779 or temsirolimus) and p38 MAPK inhibitor (SB203580) were purchased from Sigma Chemical Company (St...
Louis, MO, USA). BCA assay kit and ECL plus Western blotting substrate kit were purchased from Pierces (Thermo Scientific, USA). PVDF membrane was purchased from Santa Cruz (USA).

For establishment of primary culture cells from RCC tissue, single cell suspension was prepared according to a previously described protocol. All the procedures were carried out in sterile and pathogen-free conditions. Tissues were excised and diced into small pieces with the help of sterile and autoclaved scalpel. Thereafter, RCC tissues were placed in a flask containing medium with 20% FBS and kept in CO₂ incubator at 37°C for one day. The next day, the medium was changed and FBS was decreased up to 10%. The medium was changed at two days interval until the cells became confluent.

The A-498 cell lines and primary cultures were maintained at 37°C temperature and 5% CO₂ in RPMI-1640 (primary culture) and DMEM (A-498) medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml amphotericin (Life Technologies, USA). Trypsin-EDTA solution (0.25% trypsin, 0.30% EDTA in PBS solution) was added to detach the cells from the culture flask. The detached cells were collected and centrifuged at 200 g for 5 min. Pellet was washed with RPMI-1640 or DMEM medium and re-suspended in fresh medium with 10% FBS for experiments.

To characterize primary cell culture, anti-cytokeratin (1°Ab) was used as specific markers for epithelial tumour identification. Cells (~6 × 10⁵) were washed with PBS and centrifuged at 4000 rpm for 5 min. Thereafter, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min, washed twice with 1× PBS and centrifuged. Then they were kept overnight at 4°C in blocking solution (3% BSA), washed thrice with 1× PBS and centrifuged. Cells were incubated with primary antibody (1 : 200) for 2.30 h at room temperature followed by three washings with 1× PBS. They were then incubated with FITC-labelled secondary antibody (1 : 500) for 1 h at room temperature, washed once with PBS and finally suspended in 400 µl PBS for FACS analysis.

Cytokeratin positivity in cytoplasm was also studied by confocal microscopy using anti-pan-cytokeratin antibody. Cells were grown overnight on cover slip, washed with 1× PBS and fixed in 4% paraformaldehyde for 10 min. Cells were permeabilized with permeabilization buffer (0.2% Tween 20 + 0.1% sodium azide + 1% FBS in 1× PBS) for 30 min followed by two washings. Cover slip containing cells was placed on a glass slide and incubated with primary antibody (1 : 200) for 1 h at room temperature. Cells were rinsed with 1× PBS and incubated with FITC-labelled secondary antibody (1 : 250) for 30 min. Again, the cells were rinsed with PBS and observed under confocal microscope.

Effect of inhibitor on cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

mide (MTT) assay. Briefly, 3 × 10⁵ cells were seeded in 96-well plate and allowed to grow overnight. After 24 h of priming, cells were treated with different concentrations of inhibitor for 48 h. Four hours before the end of desired time interval, 20 µl of MTT solution (2.5 mg/ml) was added to each well. After 4 h, the resulting formazan crystals were dissolved in 40 µl lysis buffer (20% SDS dissolved in 50% each of DMF and ddH₂O). The developed colour was read at 570 nm on an ELISA reader and percentage of relative cell viability was calculated. Each experiment was performed in triplicate and repeated three times.

After treatment, cells were rinsed twice with ice-chilled 1× PBS under non-denaturing conditions. PBS was removed and cells were scraped-off and transferred to micro-centrifuge tubes. Cells were centrifuged and the supernatant was removed to collect cell pellet. The pellet was lysed in cell extraction buffer (prepared and added according to kit instructions) for 30 min on ice and vortexed for 30 min at 10 sec intervals. Cells were centrifuged at 13,000 rpm for 10 min at 4°C. Aliquots were made of clear lysate in clean microfuge tubes. The lysates were stored at −80°C for further use.

Protein content of cell lysate was estimated by BCA method (Pierces) kit. Bovine serum albumin (BSA) was used as a protein standard. Twenty-five microlitre of cell lysate was added to each well in microtitre plate. To

**Figure 1.** Characterization of primary culture. *a,* Representative FACS cytogram of primary culture cells. FACS histogram overlay showing the characterization of primary culture cells from patient’s samples using pan-cytokeratin antibody. Overlay 1 represents unstained cells, overlay 2 indicates isotype staining and overlay 3 pan-cytokeratin. *b,* Confocal microscopy shows pan-cytokeratin positive primary culture cells at 60× magnification.
determine the expression of various proteins, whole cell lysates were prepared after inhibitor treatment. The standard protocol was followed for Western blotting. Samples were prepared by using a method described by Laemmeli. Prior to loading of the sample on the gel, it was incubated in a boiling water bath for 5 min. The discontinuous gel system had resolving gel in 1.5M Tris-HCl (pH 8.8) and stacking gel in 0.5M Tris-Cl (pH 6.5) respectively. It was run in Laemmeli buffer at a constant voltage of 80 V till the completion of electrophoresis. The gel was stained in staining solution or transferred to PVDF membrane. The membrane was rinsed in phosphate-buffered saline containing 0.1% Tween 20 (PBST) and blocked in 5% BSA overnight at 4°C with constant shaking. The membrane was washed and then incubated for 2–3 h or overnight with primary antibody (SPOP, mTOR, p-mTOR, p38 MAPK, p-p38 MAPK and actin) diluted 1:1000 with 3% BSA in PBS at 4°C, followed by three washes for 5 min each. Affinity purified HRP conjugated secondary antibody was added at a dilution of 1:3000 with 3% BSA in PBS and incubated for 1–2 h at 4°C. The reaction was developed using enhanced chemiluminescence. Band expression was detected on ChemiDoc™ XRS+ by Bio-red. Intensity of protein expression was measured using ImageJ software.

Results were expressed as mean ± SD and multiple variables were analysed by one-way ANOVA. \( P < 0.05 \) was considered statistically significant.

Primary culture of RCC was established from tumour of nine RCC cases. The purity of RCC primary culture at fourth passage was measured by flow-cytometry using anti-pan-cytokeratin antibody (specific markers for epithelial tumour identification). Primary cultured cells with >70% positivity for pan-cytokeratin were used for the experiment (Figure 1). For further validation, the
expression of pan-cytokeratin was also checked under confocal microscope (Olympus 1X81). We observed that with increase in the number of passages, the percentage of cells with pan-cytokeratin expression was found to increase in RCC cells from primary culture.

Inhibitory concentration (IC) was taken for mTOR inhibitor (CCI-779) and p38-MAPK inhibitor (SB203580) as optimum dose selection. The dose was selected on the basis of cell viability by MTT assay in A-498 cell line. A dose of 20 μM of CCI-779 for 48 h was selected (Figure 2). Dexamethasone (25 μM) was taken as positive control for cell death and DMSO as solvent control. We found that cell viability significantly decreased with SB203580 in a time- and dose-dependent manner (Figure 3). Also, 25 μM of SB203580 was selected as optimum concentration at 48 h.

SB203580 (p38-MAPK inhibitor) was used to check the inhibitory effect on SPOP expression. For this, primary cells from low-grade, high-grade and A-498 cells were treated with SB203580 (25 μM) for 48 h in complete medium and whole cell lysates were subjected to Western blot analysis. To verify the efficacy of SB203580 on p38 inhibition, activated form of p38, i.e. phosphorylated-p38 (p-p38) was also checked. It was found that p-p38 expression levels decreased from (control = 0.593 to treated = 0.379) in the presence of SB203580 in all cell types, indicating that SB203580 is effectively working on p38 expression (Figure 4). The basal level of SPOP was found to be higher in high-grade compared to low-grade RCC cells. However, we did not find significant changes in SPOP expression in the presence of p38 inhibitor (control = 1.06, treated = 0.982) in both low- and high-grade RCC cells as well as in A-498 cells.

Further, to see whether inhibition of mTOR alone or in combination with p38 inhibitor has any effect on SPOP expression in RCC, primary cultured cells and A-498 cells were treated with either SB203580 (25 μM) or CCI-779 (20 μM) alone or a combination of both for 48 h in complete medium. The efficacy of mTOR inhibitors was measured by significantly decreased expression (control = 0.70, treated = 0.41, P ≤ 0.05) of p-mTOR (activated mTOR) (Figure 5). We found that SB203580 and CCI-779 reduced the levels of p-p38 and p-mTOR respectively. However, treatment with CCI-779 significantly decreased (control = 1.32, treated = 0.82, P ≤ 0.05) the levels of SPOP in primary cells derived from high-grade, low-grade and A-498 cells at 48 h of treatment (Figure 6). Combined treatment with SB203580 and CCI-779 also significantly decreased (control = 1.32, treated = 0.704, P ≤ 0.005) the expression of SPOP in primary cells as well as in A-498 cells. HepG2 cells were used as positive control for SPOP. These cells reported to have high basal level of SPOP expression. For mTOR and p38 expression, rotenone (mETC complex I inhibitor known for induction of oxidative stress which leads to p38 activation) was used as positive control to validate their expression in RCC cell line11.

Our preliminary study revealed that SPOP and mTOR mRNA levels increase in blood and tissue samples of RCC. Hence, in the present study, we evaluated the status of mTOR and SPOP in primary cells cultured from RCC tumour. The primary cell cultures from tumour preserve in vivo properties that could potentially tailor medical therapy allowing pharmacological evaluation of sensitivity/toxicity and therapeutic responses that could predict in vivo responses in the same patient to a specific drug12. In this study, primary culture cells of low-grade and high-grade RCC were established, and the explants or mechanical method was used in preference to enzymatic method, because of the lengthy procedure of the latter in sequential enzymatic treatments, which results in low
yield. Only those cultures which show more than 70% of pan-cytokeratin at fourth passage (for confirming RCC cells) were used in the present study. The epithelial nature of these cells was further confirmed by confocal microscopy.

Our *ex vivo* data on primary culture showed that at basal level the SPOP expression was higher in high-grade RCC compared to low-grade RCC, correlating with our RT-PCR results in tumour tissue, wherein SPOP expression was found to increase in a grade-dependent manner in RCC.

Previously Shor and coworkers have reported that a concentration of mTOR inhibitor (CCI-779) at 20 µM can be considered as a clinically relevant dose. We evaluated the effect of p38 and mTOR inhibition on SPOP expression in order to use this knowledge to monitor the response of mTOR inhibitor and design treatment strategies for RCC.

We observed that CCI-779 alone and in combination with SB203580 had inhibitory action on SPOP protein in primary culture and A-498 cells. This may be due to the effect of mTOR inhibitor through HIF-1α, as it has been observed that SPOP expression is regulated by HIF-1α. HIF-1α has been shown to play an important role in renal tumorigenesis and found to be constitutively active in cRCC. Flaherty *et al.* have shown that CCI-779 prevents angiogenesis by inhibiting HIF-1α in RCC; therefore, inhibition of SPOP expression in RCC cells on treatment with CCI-779 may be used to monitor the effect of mTOR inhibitor, which warrants further clinical study.

In the last decade, a greater understanding of the molecular mechanism of mTOR involved in the pathogenesis of RCC has identified specific targets for therapeutic intervention, and allosteric inhibitors of mTOR have demonstrated promising activity in the treatment of advanced RCC. Our results showed that CCI-779 alone and in combination with SB203580 had inhibitory effect on SPOP protein in primary culture and A-498 cells, which might be owing to the effect of mTOR inhibitor through HIF-1α, as it has been observed that SPOP expression is regulated by HIF-1α. HIF-1α has been shown to play an important role in renal tumourigenesis and found to be constitutively active in cRCC.

Bunce *et al.* have shown that SPOP ubiquitination activity can be reduced through p38-MAPK inhibition by SB203580 in HEK293 cells. So far, direct inhibitor of SPOP is not available; therefore we used p38-MAPK inhibitor, SB203580. The sub-toxic dose of SB203580 was selected by MTT assay on A-498 RCC cell line in a time- and dose-dependent manner. We selected an optimum
concentration of 25 μM for further experiment. At this concentration of SB203580, expression of p-p38 was markedly reduced, confirming its activity. However, we found that SB203580 had no effect on SPOP expression. Bunce et al. have further shown that the stimulation of the p38-MAPK pathway enhances the ubiquitin ligase activity of Cul3-SPOP via PI3K activation in HEK293 and HeLa cells. Our result was surprising, and in contrast to previous findings, that inhibition of p38-MAPK with SB203580 had no effect on SPOP expression in primary cells as well as in A-498 cells. This discrepancy may relate to either difference in the cell lines or inhibition of SPOP ubiquitination activity alone by SB203580. Besides, the discrepancy also may be due to absence of SPOP in the downstream of p38 signaling axis in RCC cells. It is interesting to know that SB203580 does not affect the expression of SPOP in RCC cell line. Increased p38-MAPK and SPOP expression in primary culture indicates their role in tumour progression. These findings were found consistent with earlier results, wherein p-p38 expression was shown to be high in high grade of RCC. High expression of SPOP in metastatic RCC compared to normal kidney tissue can be used for diagnosis of primary site by immunohistochemistry/RT-PCR of metastatic lesion.

In the present study, both SPOP and mTOR expression were higher in high-grade RCC compared to low-grade RCC. The data presented here not only support the conclusion that SPOP can serve as predictive biomarker in RCC cases, but also demonstrate that SPOP expression in RCC patients can help in stratification, which ultimately helps in personalized medicine decision. The results also show that combination of p38 MAPK and mTOR inhibitors can be used in RCC treatment, as SPOP is found to increase with severity of disease. The expression of SPOP and mTOR should be further studied with a large number of patients to establish SPOP as a predictive biomarker for cancer therapy.

Conflict of interest: The authors declare that they have no competing interests.


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