nance vs 62% in those on MMC maintenance. A trend toward improvement in overall progression was also noted with BCG maintenance therapy.[4]

Malavaud performed a systematic review of the literature comparing conservative treatment and radical cystectomy, and concluded that immediate radical cystectomy is indicated in young patients with T1 tumors who have at least 1 additional factor associated with a poor prognosis such as multifocality, associated CIS, prostatic involvement or tumor located at a site difficult to resect.[40] Bianco et al performed a multivariate analysis to identify risk factors in patients undergoing cystectomy that influenced cancer specific survival, and found that those with concomitant CIS or those with persistent disease after an initial course of BCG had worse cancer specific survival.[41]

IBCG recommendations. Based on a review of current guidelines and evidence, for high risk disease the IBCG recommends BCG induction plus maintenance after complete TURBT (see figure). Immediate radical cystectomy should be considered for high grade, multiple T1 tumors; T1 tumors located at a site difficult to resect; residual T1 tumors on re-resection or high grade tumors with CIS.

Optimal BCG Induction and Maintenance Schedules

BCG instillations are classically given according to the empirical 6-weekly induction schedule of Morales et al introduced more than 30 years ago.[1,8,42] There is currently no agreement on the optimal BCG maintenance schedule. However, according to the FICBT and AUA,[4,5,8] the current optimal schedule is based on the Southwest Oncology Group regimen of 3 weekly instillations at 3 and 6 months after induction, and every 6 months thereafter for up to 3 years.[43] The EAU guidelines recommend at least 1 year of BCG maintenance therapy.[1]

Followup Schedule

The recommended followup schedules for low, intermediate and high risk disease vary among the 4 guidelines. However, all guidelines indicate the importance of regular followup cystoscopy. Although not evidence-based, the IBCG has proposed a schedule based on the EAU recommendations for followup with minor modifications.[1] For low risk disease surveillance cystoscopy at 3 months is recommended. If results are negative, the following cystoscopy is advised at 9 months and then yearly for a minimum of 5 years. No upper tract investigations are required. For high risk disease, cystoscopy and cytology at 3 months are recommended. If results are negative, the following cystoscopies and cytology assessments should be repeated every 3 months for 2 years, every 4 months in the third year, every 6 months thereafter until 5 years and annually thereafter. Annual upper urinary tract imaging should also be considered. For intermediate risk disease the followup schedule should be between that for low and high risk disease, and should be adapted according to individual patient factors.

Management of Recurrence and Treatment Failure

For the optimal management of NMIBC the IBCG emphasizes the importance of distinguishing recurrence from treatment failure and has proposed some definitions. Recurrence refers to the reappearance of disease (any grade, T category or CIS) after the completion of therapy. Failure of intravesical therapy occurs with any recurrence or progression during therapy.

Guideline comparison and supporting evidence. According to the EAU, patients with recurrence after intravesical chemotherapy may benefit from BCG instillations.[1] Patients with a high grade, nonmuscle invasive tumor at 3 months of BCG therapy can receive an additional BCG course as this has been associated with complete response in more than 50% of patients.[44] Although the EAU acknowledges that changing from BCG to chemotherapy can lead to further remission in select patients in whom BCG has failed, in most high risk patients with BCG failure immediate cystectomy is strongly advocated due to the high risk of progression to muscle invasive disease.[1]

The FICBT suggests that repeat BCG therapy may be appropriate for BCG resistant and BCG relapsing disease[8] as long as the recurrence is not T1 disease. Patients in whom induction BCG fails who experience recurrence of high grade disease at 6 months should be offered cystectomy (grade C). For patients with initial induction BCG therapy failure who are unfit, refuse cystectomy, or who have low or intermediate grade disease, an additional course of a BCG containing intravesical therapy is preferred (grade C). In the case of treatment failure before completion of maintenance BCG, cystectomy should be considered if high grade T1 or CIS is present (grade B). For high grade Ta recurrence the FICBT recommends repeat resection and continued BCG maintenance (grade B). If early failure occurs after the completion of maintenance BCG, cystectomy should be considered (grade B) for high grade NMIBC. However, if superficial recurrence occurs later, the FICBT recommends restarting BCG or other instillations as an alternative to cystectomy (grade B). Patients with recurrent T1 tumors should be considered for cystectomy if they have had 2 prior induction cycles of BCG (grade D).[8]

According to the NCCN a change in intravesical agent or cystectomy is recommended for patients

with CIS or Ta recurrence after intravesical therapy (no more than 2 consecutive cycles, category 2A). For high grade T1 recurrence, cystectomy is recommended (category 2A). Maintenance BCG therapy is optional in patients with recurrent or persistent disease showing complete response on followup cystoscopy regardless of whether 1 or 2 courses of induction therapy were administered.[3]

The AUA recommends repeat resection before additional intravesical therapy (standard) for patients with high grade Ta, T1 and/or CIS recurrence after intravesical therapy. Further intravesical therapy, particularly with BCG, may also be considered (an option) in these patients and cystectomy as a therapeutic alternative is recommended.[4,5] In fact, earlier cystectomy has been shown to improve the long-term survival of high risk patients in whom BCG therapy has failed.[45] A retrospective analysis of 90 patients with high risk NMIBC who ultimately underwent cystectomy demonstrated improved 15-year disease specific survival in those who underwent cystectomy within 2 years after initial BCG treatment.[45] Improved survival outcomes were also noted in patients who underwent cystectomy for recurrent disease compared to those treated for progression. Therefore, deferring cystectomy until progression to muscle invasive disease may negatively impact survival.

IBCG recommendations for treatment failure. The appropriate management strategies for cases of treatment failure depend on the type of failure (ie chemotherapy or BCG) as well as the risk level. For failure of chemotherapy in intermediate risk patients, TURBT plus BCG induction plus maintenance or additional intravesical chemotherapy is recommended. For high risk patients, TURBT plus BCG induction plus maintenance is recommended, or cystectomy can be considered. For BCG failure in intermediate risk patients, TURBT plus repeat BCG induction plus maintenance or radical cystectomy is recommended, whereas for high risk patients radical cystectomy is recommended.

IBCG recommendations for management of recurrence. The management of recurrence depends not only on previous and current levels of risk, but also on the previous treatment received. For recurrence in low risk patients the IBCG recommends treatment as for intermediate risk patients with TURBT plus intravesical chemotherapy or BCG induction plus maintenance. For recurrence in intermediate risk patients the risk category should be considered. If the patient is still classified as intermediate risk, TURBT plus repeat chemotherapy or BCG induction plus maintenance (depending on previous treatment) is recommended. If high risk, TURBT plus BCG induction plus maintenance or radical cystectomy is recommended (depending on previous treatment). For high grade recurrence in high risk patients, radical cystectomy is preferred, or TURBT plus additional intravesical instillations are recommended if cystectomy is not suitable for the patient.

CONCLUSIONS

Established areas of consensus among the 4 guidelines include the importance of TURBT in all patients with NMIBC and the benefit of adjuvant BCG for high risk disease. However, the guideline recommendations vary with regard to the definitions of low, intermediate and high risk disease as well as the appropriate treatment and followup of patients in each of these risk categories. Furthermore, there is currently no consensus on the definition and appropriate management strategies for primary intravesical treatment failure. To address these issues and provide urologists with more practical and unified guidance on the management of NMIBC, the IBCG has proposed the recommendations presented in this article.

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APPENDIX 1

Comparison of risk stratification definitions proposed by the EAU, FICBT, NCCN and AUA[1,3–5,7–9]

	Definitions		
	Low Risk	Intermediate Risk	High Risk
EAU	Low risk of tumor recurrence (EORTC recurrence score = 0) and progression (EORTC progression score = 0)	Intermediate (EORTC recurrence scores ranging from 1–9) or high (EORTC recurrence score ranging from 10–17) risk of recurrence and intermediate risk of progression (progression scores ranging from 2–6)	High risk of progression (EORTC progression scores ranging from 7–23)
	eg G1-2Ta	eg multifocal G2Ta, solitary G2T1	eg multifocal G2T1, G3Ta-T1, CIS
FICBT	Low grade Ta	Low grade Ta with high risk factors for recurrence or recurrent low grade Ta tumors	High grade Ta, all T1, CIS
NCCN	Low grade* Ta	High grade* Ta	All T1 (CIS listed separately)
AUA	Small volume, low grade Ta	Multifocal and/or large volume low grade Ta High risk of recurrence, low risk of progression	High grade Ta, all T1, CIS

Adapted from Persad et al.[10]

* Grading refers to the *World Health Organization International Histological Classification of Tumours,* 1973. The majority of grade 2 tumors are high grade. Some grade 2 tumors may be classified as low grade by some pathologists.

APPENDIX 2

Comparison of guideline recommendations for low, intermediate and high risk disease[1,3–5,7–9,32]

Guideline	Low Risk Disease	Intermediate Risk Disease	High Risk Disease
EAU	• TURBT • Single immediate post operative instillation of chemotherapy (grade A)	• TURBT • Single, immediate postoperative instillation of chemotherapy followed by: – Induction BCG plus maintenance (at least 1 year) (grade A), or – Maintenance intravesical chemotherapy (grade A) of 6–12 months (grade B)	• Repeat TURBT 2–6 weeks after initial resection (grade B) • Intravesical BCG induction plus maintenance for at least 1 year (grade A) • Immediate radical cystectomy for highest risk patients (grade C) — Multiple recurrent high grade tumors — High grade T1 tumors — High grade tumors with concomitant CIS CIS: • Intravesical BCG plus maintenance for at least 1 year (grade A) – Assess response at 3 months: ■ If no response: • Continue with 3 weekly boosters (grade B), or • Additional 6-week course of BCG (grade B), or • Cystectomy (grade B) – No complete response at 6 months: radical cystectomy (grade B)

(appendix continued)

APPENDIX 2 (continued)

Guideline	Low Risk Disease	Intermediate Risk Disease	High Risk Disease
FICBT	• TURBT • Single immediate postoperative instillation of chemotherapy (grade A)	Multiple low grade Ta • TURBT • Single immediate postoperative instillation of chemotherapy • Further adjuvant intravesical therapy: — First line: intravesical chemotherapy less than 6 months (grade B) — Second line: BCG (grade A)	High grade Ta • Second look TURBT and bladder mapping biopsies 2–4 weeks after initial resection (grade B) • If residual tumor is found: — Re-resection and 1 immediate instillation of chemotherapy — Followed 2–3 weeks later by 6-week BCG induction and 1–3 years of BCG maintenance (grade A)
		Recurrent low grade Ta • Office fulguration only in select patients with less than 5 small (less than 0.5 cm) low grade recurrent tumors and negative cytology (grade C) • Formal TURBT if clinical doubt that tumor is low grade, cytology positive, or change in tumor appearance has occurred (grade C) • Adjuvant intravesical therapy (see above)	T1 • Repeat TURBT (grade B) • Initial intravesical BCG for patients with completely resected primary and recurrent T1 tumors (based on a negative repeat resection) (grade C) CIS • Intravesical BCG for 6 weeks (grade A) • Maintenance BCG for 1 year or more (grade A)
NCCN	• TURBT • Observe (category 2A), or • Consider single immediate postoperative instillation of chemotherapy (category 2A), and/or • Induction intravesical chemotherapy (category 2A)	• TURBT • Repeat TURBT if incomplete resection or no muscle in specimen (category 2A) • Observe or • Intravesical therapy — BCG (preferred) or — MMC (category 2A)	All T1: Strongly advise re-resection or cystectomy for high grade *Residual disease:* • BCG preferred (category 1), or • Cystectomy (category 2A) *No residual disease:* • BCG preferred (category 1), or • MMC (category 2A) Any CIS/Tis • Complete resection followed by intravesical BCG (category 2A)
AUA	• TURBT • Single immediate postoperative instillation of chemotherapy (recommendation)	• TURBT • Intravesical BCG or MMC (recommendation) • Maintenance BCG or MMC (option)	• Repeat resection if lamina propria invasion without muscularis propria in specimen prior to intravesical therapy (standard) • Induction BCG followed by maintenance (recommendation) • Cystectomy (option)

Adapted from Persad et al.[10]

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The liposome-incorporating cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guéin can directly enhance the susceptibility of cancer cells to lymphokine-activated killer cells through up-regulation of natural-killer group 2, member D ligands

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OBJECTIVE

- To conduct a preclinical evaluation of the ability of natural killer cells to cytolyze bladder cancer cells that were modified to show enhanced expression of natural-killer group 2, member D (NKG2D) ligands by R8-liposome-bacillus Calmette-Guéin (BCG)-cell wall skeleton (CWS) treatment.

MATERIALS AND METHODS

- The T24 cells and RT-112 cells were co-cultured with R8-liposome-BCG-CWS and BCG for 2, 4, or 6 h, and then the surface expression of NKG2D ligands was analyzed using TaqMan real-time quantitative RT-PCR.
- Peripheral blood mononuclear cells were obtained with a conventional preparation kit, and then lymphokine-activated killer (LAK) cells were generated from these purified peripheral blood mononuclear cells via interleukin-2 stimulation.
- The anti-tumour effect of LAK cells against untreated and R8-liposome-BCG-CWS co-cultured with cells of the human bladder cancer cell lines T24 and RT-112 was analyzed using the cytotoxic WST-8 assay method at 4 h of culture at various effector/target (E : T) ratios.

RESULTS

- Major histocompatibility complex class I-related chain B (MICB) expression was increased ≈1.5-fold on T24 cells and RT-112 cells with BCG.
- UL-16-binding protein (ULBP) 1 expression was also increased ≈1.5-fold on T24 cells and RT-112 cells with BCG. R8-liposome-BCG-CWS increased the surface expression of MICB 2.2-fold on T24 cells but did not increase it significantly on RT-112 cells.
- ULBP1 expression was increased ≈2.2-fold on RT-112 cells, although no differences were observed between the expression of ULBP2 and 3 with R8-liposome-BCG-CWS.
- T24 cells that were co-cultured with R8-liposome-BCG-CWS showed an ≈1.3-fold increase in sensitivity to cytolysis by LAK cells at an E : T ratio of 4 and RT-112 cells showed an ≈1.4-fold increase at an E : T ratio of 2.

CONCLUSIONS

- In the present study, the induction of surface NKG2D ligands by R8-liposome-BCG-CWS rendered cancer cells more susceptible to cytolysis by LAK cells.
- T24 cells and RT-112 cells, even when cultured singly in the absence of immune cells, can directly respond to R8-liposome-BCG-CWS.
- The results obtained in the present study may therefore indicate a novel adoptive immunotherapy against bladder cancers.

KEYWORDS

liposome, BCG-CWS, bladder cancer, LAK cells

INTRODUCTION

Intravesical *Mycobacterium bovis* BCG therapy is effective against carcinoma *in situ* and as a prophylaxis against the recurrence of bladder cancer [1–5]. In addition to the direct anti-tumour effect, it is widely recognized that intravesical BCG therapy is more potent in preventing tumour recurrence than intravesical chemotherapy [6]. Although intravesical BCG therapy is effective, it is not free from serious side effects (e.g. high fever, granulomatous prostatitis, pneumonitis, hepatitis, and BCG sepsis) [7]. To avoid such unfavourable events, it is necessary to develop a more active and less toxic immunotherapeutic agent.

Although the BCG-cell wall skeleton (CWS) has long been investigated for this purpose, its clinical use is very limited because of difficulties relating to solubility and stability. To overcome these unfavourable physicochemical properties of the BCG-CWS preparation, we have applied octaarginine-modified liposomes (R8-liposomes) as a vector to transport BCG-CWS into the cytoplasm effectively. R8-liposomes were initially developed to transfer highly negatively charged DNA molecules into the cytoplasm by macropinocytosis [8–10]. R8-liposomes resemble an envelope-type virus and their surface are modified by anchored R8, a characteristic and efficient cell-penetrating peptide [9].

We have previously reported that R8-liposome-incorporating mycobacterial cell walls (R8-liposome-BCGCW) successfully attached to the surface of MBT-2 cells and were efficiently internalized into the cytoplasm within 1 h of co-incubation [11]. Internalized BCG-CW was then distributed to the lysosome of the MBT-2 cells. Furthermore, R8-liposome-BCGCW has been shown to completely inhibit the growth of MBT-2 tumours *in vivo* [11]. However, the mechanisms of the anti-tumour effect remain to be clarified, especially for the immune effector cells involved in R8-liposome-BCGCW-induced immunity.

In BCG immunotherapy, both the anti-tumour activity mediated by cytotoxic T lymphocytes (CTLs) and the anti-tumour activity mediated by an innate immune response in natural killer (NK) cells have a direct anti-tumour effect, as well as a prophylactic effect. The role of NK cells in this process was initially unclear [12], although more recent observations have shown that NK cells play a central role in the immune response that eradicates bladder cancer after intravesical instillation of BCG. In an *in vivo* mouse orthotopic bladder cancer model, Brandau *et al.* [13] noted that BCG-activated killer (BAK) cells are essential for a positive response to BCG. Furthermore, Suttmann *et al.* [14] reported the molecular mechanisms of BCG-immunotherapy involved in the process of cell-mediated cytotoxicity of both BAK cells and lymphokine-activated killer (LAK) cells against bladder cancer cells. NK cells were the major effector cell population of both BAK cells and LAK cells.

More recently, the natural killer group 2, member D (NKG2D) has been shown to be an important activating receptor present on the surface of NK cells. The NKG2D serves as a primary activation receptor, which is able to trigger cytotoxicity by itself. Previous studies have established that the expression of NKG2D ligands such as MHC class I-related chain A (MICA), MHC class I-related chain B (MICB) and a structurally distinct family of UL-16-binding protein (ULBP) proteins on tumours renders them susceptible to killing by NK cells [15–17]. However, the role of NKG2D and its ligands in BCG immunotherapy has not yet been investigated.

In the present study, we investigated whether BCG or R8-liposome-BCG-CWS treatment could induce the up-regulation of NKG2D ligands in human bladder cancer cell lines. In addition, we examined the susceptibility to LAK cells of cancer cells with or without R8-liposome-BCG-CWS treatment. The findings obtained show that the non-live bacterial agent, R8-liposome-BCG-CWS, can directly enhance the susceptibility of bladder cancer cells to LAK cells.

MATERIALS AND METHODS

PREPARATION OF R8-LIPOSOME-BCG-CWS

R8-liposome-BCG-CWS was prepared using a method described previously [18].

EXPANSION OF LAK CELLS

For the expansion of LAK cells [19,20], peripheral blood mononuclear cells (PBMCs) were prepared from 20 mL of heparinized peripheral blood with a conventional preparation kit (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). The cells were washed twice with calcium- and magnesium-free Dulbecco's PBS. The PBMCs (1×10^6 cells/mL, 1 mL/well) were then seeded into each well of 24-well tissue culture plates. AVM-V (Invitrogen, Tokyo, Japan) medium supplemented with 5% autologous plasma and interleukin-2 (IL-2) (200 U/mL) was used for the culture of lymphocytes. The LAK cell culture was continued with appropriate changes of the medium including IL-2 (at least half of the medium was changed every 2 days). After 2 weeks in culture, the number of lymphocytes was counted and the phenotypes of the lymphocytes were analyzed by flow cytometry. For flow cytometry, lymphocytes were stained with the monoclonal antibodies: fluorescein isothiocyanate-labelled anti-CD3 (UCHT1, IgG1) and phycoerythrin-labelled anti-CD56 (MOC-1, IgG1; Dako, Kyoto, Japan) and fluorescein isothiocyanate-labelled anti-CD16 (3G8, IgG1) and phycoerythrin-labelled anti-CD56 (V NK75, IgG1; BD Pharmingen, San Diego, CA, USA). Isotype-matched control monoclonal antibodies were used as negative controls. Cells were stained with these monoclonal antibodies for 30 min at 4 °C. After washing, the cells were immediately analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

CYTOTOXICITY ASSAY

The non-radioisotopic tetrazolium salt, WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, a monosodium salt, was used to mesure the cytotoxic activity of lymphocytes against anchorage-dependent target tumour cells, as described previously [21]. This assay is compatible with the standard ^{51}Cr-release assay. Briefly, 1×10^4 target cells in 100 μL of culture medium were seeded in each well of a 96-well plate and pre-cultured overnight. After washing the culture plates with PBS, the cultured lymphocytes suspended in 100 μL of medium were added as effector cells to each well at the indicated effector/target (E : T) ratio. The cells were co-cultured for 4 h and then washed once gently with the appropriate amount of PBS. Adherent target cells were stained with WST-8 solution (10% in RPMI medium; 100 μL/well) for 90 min at room temperature. The optical density at 570 nm was determined for each well. The percentage of surviving target cells was expressed as: $(B - C)/A \times 100$, where A is the absorbance of control target cells pre-cultured on a separate plate just before adding the effector cells, B is the absorbance of target cells remaining after the effector cells were added and C is the absorbance of effector cells only. Each value represents the mean of triplicate expreriments.

BLADDER CANCER CELL LINES AND RNA EXTRACTION

The human TCC cell lines used in the present study were RT-112 and T24. These TCC cell lines display phenotypes associated with well-differentiated and poorly differentiated TCC cells. Although RT-112 cells originate from carcinomas invading bladder muscle, they show a superficial pattern *in vitro* and are widely used as a model of superficial bladder cancer [22]. T24 cells are widely used as

models of carcinomas invading bladder muscle [23,24]. RT-112 cells and T24 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and 1% L-glutamine in an incubator at 37 °C under a 5% CO_2 atmosphere. Upon reaching 80% confluence (5–10 × 10^6 cells), these cells were washed twice with PBS, and then cultured for 24 h before re-inoculation in serum-free RPMI-1640 medium containing R8-liposome-BCG-CWS (0.1 mg/mL) alone.

The medium was exchanged with RPMI after 1 h, and RNA was extracted 0, 1, 3 or 5 h later. Briefly, cells were washed with PBS and lysed with 1 mL of TRIzol reagent (a phenol and guanidine isocyanate solution; Gibco BRL, Grand Island, NY, USA). Chloroform (200 μL) was added, and the mixture was centrifuged at 4 °C and 12 000***g*** for 15 min. The liquid phase was precipitated with isopropanol. The RNA pellets were dissolved in Tris EDTA (TE) buffer.

BCG

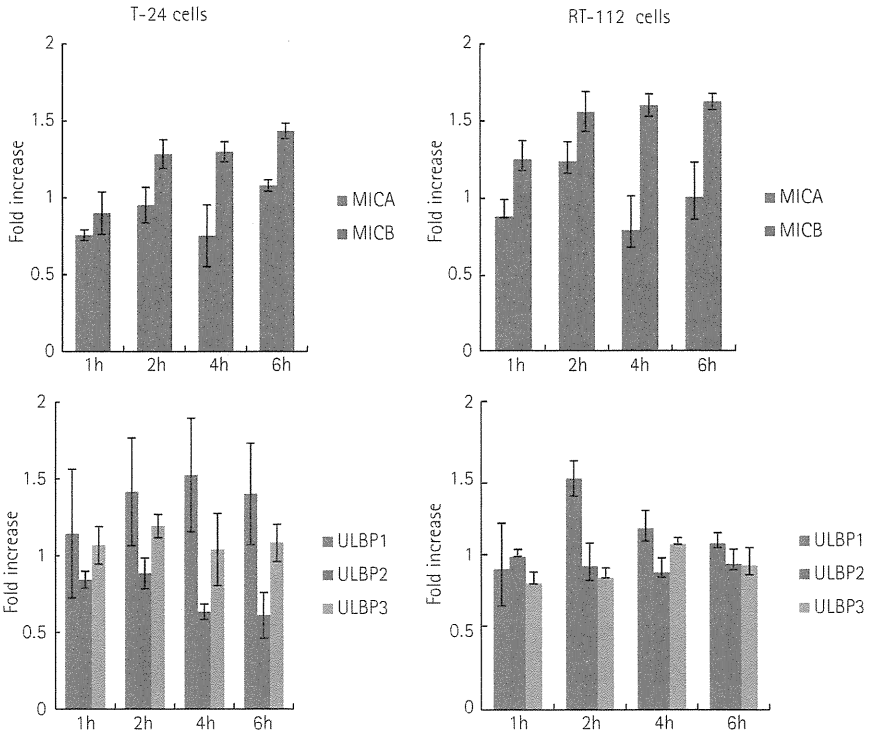
Lyophilized preparations of BCG, strain Tokyo 172 (Japan BCG Laboratory, Tokyo, Japan), which contained 22.9 × 10^6 colony-forming units per 0.5 mg wet weight were used.

T24 cells and RT-112 cells were incubated overnight in RPMI-1640 medium in a culture flask. The culture medium was then replaced with 1 mL of RPMI-1640 containing 0.1 mg (wet weight) of BCG. After various periods of incubation with BCG matching those of R8-liposome-BCG-CWS, the flask was thoroughly washed with PBS to remove the extracellular BCG and the medium was replaced with the same medium containing 100 μg/mL streptomycin to inhibit the extracellular growth of the remaining BCG.

TAQMAN REAL-TIME QUANTITATIVE RT-PCR

A sample comprising 1 μg of RNA extracted from the primary tumour and normal renal cell lines was reverse-transcribed using random hexamers in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Real-time quantitative RT-PCR was performed using 5 μL of diluted cDNA (1 μL in 20 μL of water) in a final volume of 25 μL in accordance with the manufacturer's instructions (Applied Biosystems). PCR primers and probes for MICA, MICB, ULBP1, ULBP2 and ULBP3 gene targets were designed by Applied Biosystems and used in accordance with the manufacturer's instructions. The amount of sample RNA was normalized by amplification of an endogenous RNA control (18S). The relative quantification of the transcripts was derived using a TaqMan Gene Expression assay and an ABI PRISM 7900HT (Applied Biosystems). The relative expression of each gene was calculated using the formula $2^{-\Delta\Delta Ct}$, according to the method described by Livak and Schmittgen [25], where ΔCt represents the difference between the cycle threshold of the amplification curve of the target gene and that of the endogenous control gene.

FIG. 1. MHC class I-related chain (MIC) B gene expression is activated with live BCG on T24 cells and RT-112 cells at 6 h of incubation. UL-16-binding protein (ULBP) 1 gene expression is also up-regulated.



RESULTS

PHENOTYPES OF LAK CELLS

LAK cells were prepared from normal human peripheral blood and incubated *in vitro* in the presence of IL-2. Flow cytometry measuring CD3, CD4, CD8, CD16 and CD56 was used to assess the composition of lymphocytes in samples from peripheral blood and NK products. Lymphocytes other than $CD16^+CD56^+$ cells consisted mainly of $CD3^+CD56^-$ cells, which correspond to the phenotype of LAK cells or non-specific T cells. Flow cytometry was used to classify the NK cells into two subpopulations, $CD3^-CD56^+$ and $CD16^+CD56^+$ cells, making up 49% and 52%, respectively, of total LAK cells. The subsets of $CD4^+$ and $CD8^+$ cells, which corresponded to helper T cells and CTLs, accounted for 13% and 34%, respectively.

EFFECTS OF R8-LIPOSOME-BCG-CWS ON THE EXPRESSION OF NKG2D LIGANDS OF BLADDER CANCER CELL LINES

To investigate the activation of bladder cancer cells by live BCG, we cultured T24 cells and RT-112 cells with BCG containing RPMI medium for 1 h. Then the RPMI medium was exchanged with streptomycin, and T24 cells and RT-112 cells were harvested after 0, 1, 3 and 5 h of culture and total cellular RNA was isolated. We then performed a TaqMan (Applied Biosystems) real-time quantitative RT-PCR, and the results obtained showed that both cell types had increased levels of MICB and ULBP1 transcripts (Fig. 1).

Additional quantitative analyses for the genes for ULBP1, ULBP2, ULBP3, MICA and MICB

FIG. 2. MHC class I-related chain (MIC) B gene expression is activated with R8-liposome-BCG-cell wall skeleton (CWS) on T24 cells at 4 h of incubation. UL-16-binding protein (ULBP) 3 gene expression is also up-regulated. ULBP1 gene expression is activated by R8-liposome-BCG-CWS treatment on RT-112 cells at 2 h of incubation. MICB gene expression is not up-regulated on RT-112 as it is on T24 cells.

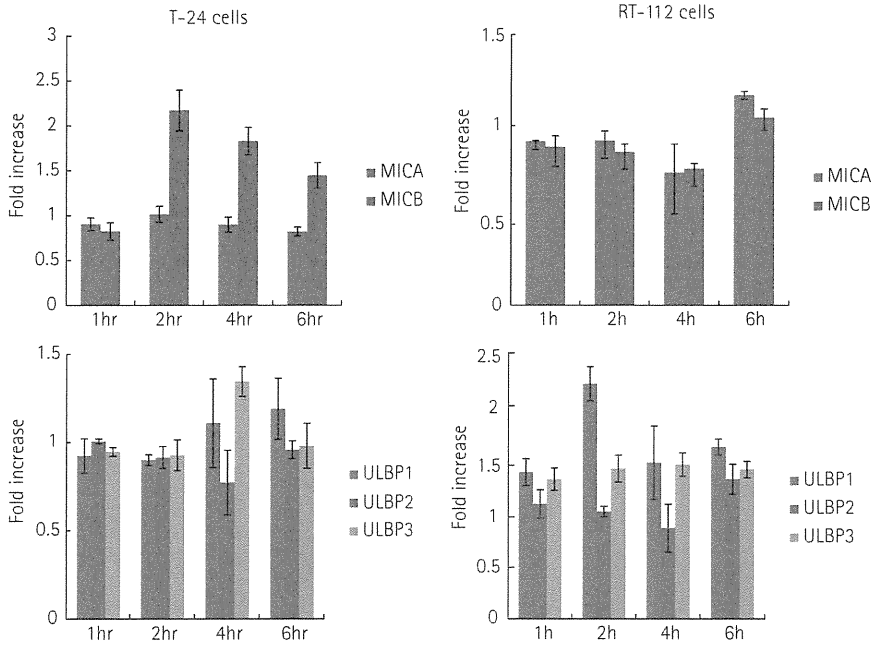
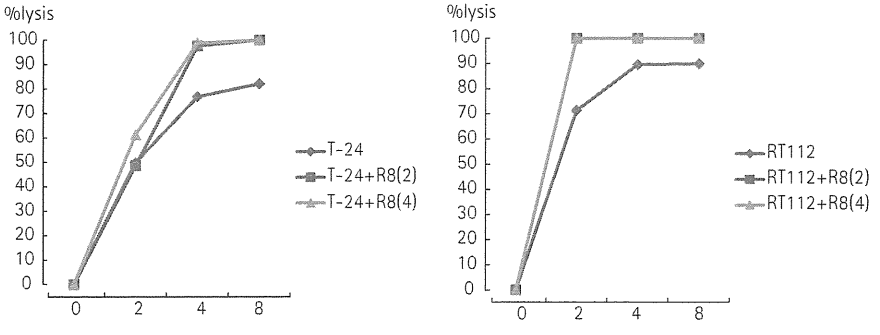


FIG. 3. Cytotoxic activity of the lymphokine-activated killer (LAK) cells against T24, RT-112 and R8-liposome-BCG-cell wall skeleton (CWS) co-cultured cells assayed by WST-8. T24 cells and RT-112 cells with R8-liposome-BCG-RWS were more susceptible than utreated cells to the LAK cells. R8(2): co-incubation with R8-liposome-BCG-RWS for 2 h. R8(4): co-incubation with R8-liposome-BCG-RWS for 4 h.



were performed on the T24 cells and RT-112 cells that received R8-liposome-BCG-CWS treatment, and the results obtained showed that expression of the gene for MICB was up-regulated in T24 cells and ULBP1 gene expression was up-regulated in RT-112 cells (Fig. 2).

MICB expression was increased ≈ 2.2-fold on T24 cells with R8-liposome-BCG-CWS treatment but only ≈ 1.4-fold expression was observed on cells treated with BCG.

Similarly, ULBP1 expression was also increased ≈2.2-fold on RT-112 cells with R8-liposome-BCG-CWS treatment, although only ≈1.5-fold expression was shown by BCG treatment. These results suggest that the NKG2D ligands may be more strongly expressed by R8-liposome-BCG-CWS treatment than by BCG treatment.

NKG2D ligands such as the MICA, MICB, ULBP1, ULBP2 and ULBP3 proteins may be induced by cellular stress, and most of them are frequently expressed on tumour cells [26,27]. These results indicate that NKG2D provides a first-line surveillance against bladder cancer.

SUSCEPTIBILITY OF R8-LIPOSOME-BCG-CWS-TREATED BLADDER CANCER CELLS TO LAK CELLS

Cytotoxic assays using LAK cells incubated with T24 cells with R8-liposome-BCG-CWS treatment showed more effective killing than LAK cells incubated with untreated T24 cells. At T24 cells, cytotoxicity was almost 100% with R8-liposome-BCG-CWS at an E : T ratio of 4. However, the cytotoxicity was only 77% when R8-liposome-BCG-CWS treatment was not used (Fig. 3). RT-112 cells that exhibit a superficial pattern *in vitro* when treated with R8-liposome BCG-CWS also were more susceptible to the LAK cells. The cytotoxicity was 100% with R8-liposome-BCG-CWS treatment at an E : T ratio of 2 but was only 71% in the absence of R8-liposome-BCG-CWS treatment (Fig. 3).

DISCUSSION

We previously reported that R8-liposome-BCG-CW completely inhibited the growth of MBT-2 tumours in C3H/HeN mice, whereas BCG-CW alone did not. Animals vaccinated with a mixture of MBT-2 cells and R8-liposome-BCG-CW showed a significant inhibition of the growth of R8-liposome-BCG-CW pretreated MBT-2 cells [11]. This suggests that, under conditions of immune tolerance from the host, bladder cancer cells usually can be recognized by the presence of BCG-related molecules in cancer cells.

Both the antigen-specific activity of CTLs and innate immune activity of NK cells are considered to be involved in BCG-induced anti-tumour immunity. Recent studies have reported that NK cells are essential for effective BCG immunotherapy. In addition, there is increasing evidence to support a significant role of NKG2D and its ligands in NK cell cytotoxicity. For example, human macrophages infected with either influenza or Sendai virus are known to have up-regulated MICB expression, which stimulates NKG2D-dependent interferon-γ release by NK cells [28].

The present study aimed to investigate the immune mechanism activated by a non-live bacterial agent, R8-liposome-BCG-CWS, using human bladder cancer cell lines and allogenic LAK cells. The results obtained clearly showed that the R8-liposome-BCG-CWS can directly enhance the susceptibility of bladder cancer cells to LAK cells, possibly through the up-regulation of NKG2D ligands on cancer cells. In the present study, we decided to use LAK cells rather than BAK cells as NK cell dominant effector cells in an attempt to establish a well known, non-live bacterial model. Generally, LAK cells represent a composite of $CD3^-$ NK cells and $CD3^+$ T cells, and have the capacity to kill a variety of tumour cells and MHC class I-negative target cells. However, most importantly, activated NK cells, but not T cells, play a major role in LAK cell activity [29,30].

One of the cell lines used in the present study, T24, which is a well known line of human bladder cancer [31], expresses a markedly lower level of MHC class I molecules compared to normal cells. Hence, the T24 cells may be regulated by cells in a class I MHC molecule-unrelated manner, rather than by the class I MHC molecule-restricted CTLs.

In the present study, the induction of the surface NKG2D ligands MICB by R8-liposome-BCG-CWS treatment rendered T24 cells more susceptible to LAK cells. Higuch *et al.* [32] reported that cytotoxicity against T24 cells by live BCG-treated PBMCs containing mostly activated NKT cells, as well as some γδT and NK cells, was markedly inhibited by an anti-MICA/MICA specific antibody [32]. Therefore, MICA/MICB molecules on cancer cells appear to be possible tumour cell ligands for BCG-activated innate immune cell recognition. MICB expression was increased on the T24 cells when they were cultured with R8-liposome-BCG-CWS, and ULBP1 expression was also increased on the RT-112 cells in the present study. MICB and ULBP1 expression were also increased on T24 and RT-112 co-cultured with live BCG. The reasons for the different expression of MICB and ULBP1 on these cells treated with R8-liposome-BCG-CWS remain to be clarified.

MICA and MICB expression is restricted or absent on normal tissues but is induced in response to various stresses and pathological conditions, including epithelial-derived tumours [33,34]. Additionally, the MICA/MICB protein has been detected in intestinal epithelial cells infected with *Mycobacterium* tuberculosis or *Escherichia coli* [35], and MICA has been shown on the surface of cytomegalovirus-infected fibroblasts [36,37]. ULBP ligands are expressed at the mRNA level in many tissues and cell lines, including the lung, heart, liver, testis, brain and colon, but cell surface expression by normal cells has not been detected [26,38]. These findings suggest that transcriptional regulation of individual NKG2D ligands may differ substantially between normal tissues and tumours.

Unfortunately, little is known about the regulation and expression of these molecules, except that they all share the common property of being inducible by cellular distress. Nevertheless, the available data clearly suggest that they are differentially expressed in normal tissues and in tumours from different origins. MICA and MICB proteins are frequently overexpressed in epithelial tumours of multiple origins but are less frequently expressed in haematopoietical malignancies [33,39–41]. This variable pattern of expression of NKG2D in cancer could be part of the immunoediting process [42], although it is more likely related to the fact that the expression of NKG2D ligands is controlled by different activation pathways. Up-regulation of MICA expression is considered to be mediated by the heat shock elements identified in the promoters of genes for MIC [43]. By contrast, ULBP family members lack these motifs [43]. Taken together, this suggest that NKG2D ligand diversity not only reflects the redundant expression of molecules with the same function, but also may indicate that these ligands have evolved to be differentially regulated in diverse physiological or pathological situations.

In conclusion, the results obtained in the present study show that R8-liposome-BCG-CWS up-regulated the expression of the ligand of NKG2D on bladder cancer cells. Accordingly, LAK cells recognized the bladder cancer cells and induced cytolysis of the cells. Therefore, LAK cells are crucial for the BCG-induced control of bladder tumour growth. In the future, the development of this non-live bacterial agent may provide a more active and less toxic tool as a substitute for live BCG in immunotherapy against bladder cancer.

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CONFLICT OF INTEREST

None declared.

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Abbreviations: **BAK**, BCG-activated killer; **CTL**, cytotoxic T lymphocyte; **CWS**, cell wall skeleton; **E : T**, effector/target; **IL-2**, interleukin-2; **LAK**, lymphokine-activated killer; **MICA**, MHC class I-related chain A; **MICB**, MHC class I-related chain B; **NK**, natural killer; **NKG2D**, natural-killer group 2, member D; **PBMC**, peripheral blood mononuclear cell; **ULBP**, UL-16-binding protein.

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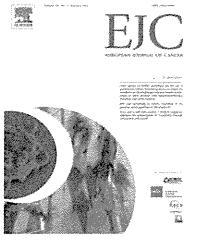

Key predictive factors of axitinib (AG-013736)-induced proteinuria and efficacy: A phase II study in Japanese patients with cytokine-refractory metastatic renal cell Carcinoma

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ABSTRACT

Background: Axitinib (AG-013736) is an oral, selective and potent inhibitor of vascular endothelial growth factor receptors (VEGFR)-1, 2 and 3. This phase II study investigated axitinib efficacy, safety and biomarkers in Japanese patients with cytokine-refractory metastatic renal cell carcinoma (mRCC).

Patients and methods: In an open-label, multicentre study, 64 patients received an axitinib starting dose of 5 mg twice daily.

Results: Objective response rate (ORR) was 50.0% and median progression-free survival (PFS) was 11.0 months per independent review committee. Common treatment-related adverse events were hypertension (84%; 70% grade ⩾3), hand–foot syndrome (75%; 22% grade ⩾3) and diarrhoea (64%; 5% grade ⩾3). Eighteen patients (28%) developed proteinuria ⩾2 g/24 h and required dose reduction or treatment interruption/discontinuation. Proteinuria was a major cause for treatment discontinuation. Baseline urine protein levels were associated with development of proteinuria ⩾2 g/24 h (hazard ratio [HR] = 5.457, P = 0.0035 in patients with baseline proteinuria ⩾1+ versus <1+). Baseline urine protein levels correlated more strongly with axitinib-related proteinuria than other baseline renal function test values or blood pressure. Patients with greater decreases in soluble VEGFR-2 concentrations had significantly higher ORR and longer PFS than those with smaller

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decreases (ORR: 64.5% versus 37.5%, P = 0.045; median PFS: 12.9 months versus 9.2 months, HR = 0.42, P = 0.01).

Conclusions: Axitinib showed significant antitumour activity and was well tolerated in Japanese mRCC patients. Baseline proteinuria and soluble VEGFR-2 levels may be key indicators of axitinib-induced proteinuria and efficacy, respectively.

1. Introduction

Renal cell carcinoma (RCC) is the most common form of kidney cancer,[1] accounting for 2–3% of adult malignancies worldwide[2] and increasing at a rate of 2–3% per decade.[1] Approximately 30% of RCC patients have metastatic disease (mRCC) at the time of diagnosis.[1,3–5] Although the overall incidence of RCC is lower in Asian countries than in North America and Europe, it is particularly high among Japanese males.[5] In 2008, the incidence rate (per 100,000 persons at risk) for kidney cancer was 17.7, 13.6 and 2.0 in North America, Europe and Asia, respectively; the rate among Japanese males was 16.2.[6] Surgical removal of primary tumours is widely recommended,[2] but systemic therapy is needed for mRCC patients. Furthermore, mRCC is resistant to traditional cancer treatments, e.g. chemotherapy and radiation. Until recently, cytokine treatment with interferon-α (IFN-α) and/or interleukin-2 (IL-2) was the standard of care for mRCC.[4,7] However, these therapies offer modest clinical benefit.[4]

Rising rates of RCC, prevalence of metastatic cases and limited efficacy of available therapies highlight the need for novel treatment options. Pathways leading to angiogenesis, which is essential for tumour progression and metastasis, are key targets of current research. Most clear-cell RCC, which accounts for 75% of mRCC cases,[8] exhibit loss of function of the von Hippel Lindau gene.[9,10] This leads to acceleration of the vascular endothelial growth factor (VEGF) pathway and promotion of angiogenesis.[9]

Axitinib, an oral, selective and potent inhibitor of vascular endothelial growth factor (VEGF) receptors (VEGFR)-1, 2 and 3,[11] demonstrated clinical efficacy in phase II studies of various tumour types.[12–17] Single-agent axitinib is active and well tolerated as second-line treatment for mRCC.[12,15] Objective response rates (ORR) for axitinib were 44.2% and 22.6% in phase II studies of patients with cytokine-refractory[12] and sorafenib-refractory mRCC,[15] respectively, conducted in Western countries.

The present phase II study investigated the efficacy, safety and biomarkers of axitinib in Japanese patients with cytokine-refractory mRCC. This was the first study to investigate the activity and safety of axitinib for mRCC in Asian patients.

2. Patients and methods

2.1. Study design and end-points

This was an open-label, multicentre phase II study. The primary end-point was ORR. Secondary end-points included progression-free survival (PFS), duration of response (DR), safety and changes in the plasma concentrations of potential biomarkers (soluble VEGFR [sVEGFR]-1, 2, 3; VEGF and soluble stem cell factor receptor [sKIT]). Exploratory analyses evaluated the relationship between changes in plasma concentration profiles of biomarkers and efficacy end-points. Potential predictive factors of axitinib-induced proteinuria were investigated as post hoc exploratory analyses.

This study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation guidelines on Good Clinical Practice, the study protocol and applicable local regulatory requirements and laws. All participants provided informed consent and agreed to comply with the study protocol, which was approved by an institutional review board at each site. The trial is registered on ClinicalTrials.gov (NCT00569946).

2.2. Patient population

Patients aged ⩾20 years had histologically confirmed mRCC with clear-cell component, ⩾1 target lesion defined by Response Evaluation Criteria in Solid Tumours (RECIST, version 1.0),[18] prior nephrectomy, and were refractory to first-line cytokine therapy (IFN-α and/or IL-2). Patients were required to have Eastern Cooperative Oncology Group performance status of 0 or 1; adequate bone marrow, hepatic and renal function; baseline proteinuria <2+ by urine dipstick or <2 g/24 h urine collection; and blood pressure (BP) ⩽140/90 mmHg (antihypertensive medications were permitted). Patients with clinically evident gastrointestinal disorders potentially affecting ingestion or absorption were excluded. Other exclusion criteria were active seizure disorders; evidence of brain metastases, spinal cord compression or carcinomatous meningitis; myocardial infarction, severe or unstable angina, coronary or peripheral artery bypass graft, symptomatic congestive heart failure or cerebrovascular accident ⩽12 months prior to study registration.

2.3. Study treatments

Axitinib was orally administered with food at a starting dose of 5 mg twice daily (BID) continuously. The axitinib dose could be increased to 7 mg BID and then to a maximum of 10 mg BID in patients with no grade >2 treatment-related adverse events (AEs) and with ⩽150/90 mmHg for ⩾2 weeks without the use of antihypertensive medication. The axitinib dose was reduced to 3 mg BID and then to 2 mg BID in patients who developed grade 3 treatment-related non-haematologic AEs and patients with two readings of systolic BP >150 mmHg or diastolic BP >100 mmHg who were receiving maximal antihypertensive therapy. The axitinib dose was interrupted in patients with grade 4 treatment-related AEs, two readings of

systolic BP >160 mmHg or diastolic BP >105 mmHg, or ⩾2 g protein/24 h and resumed at one lower dose level when AEs improved to grade ⩽2, BP was <150/100 mmHg or <2 g protein/24 h was present.

2.4. Assessments

Tumours were radiologically assessed prior to initiation of axitinib therapy and every 8 weeks thereafter according to RECIST, version 1.0. Tumour responses were also assessed by an independent review committee (IRC). AEs were evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.[19] Urinalysis was conducted at screening and at each clinic visit; for urinary protein ⩾2+ by semiquantitative dipstick analysis, a 24-h urine collection was performed. Thyroid function tests were conducted at screening; cycle 1 days 1, 8, 15 and 22; cycles 2 and 3 day 1; day 1 of odd-numbered cycles thereafter; and at end of treatment. Blood samples for evaluation of the changes in plasma concentrations of sVEGFR-1, 2 and 3; VEGF; and sKIT were collected pre-dosing, day 1 of cycles 2–7, and at end of treatment. Plasma concentrations of proteins were measured by an enzyme-linked immunosorbent assay (Alta Analytical Laboratory; El Dorado Hills, CA, USA).

2.5. Statistical analysis

Sixty-three patients were required to test the null hypothesis that the true ORR was ⩽10% versus the alternative hypothesis that the true ORR was ⩾25% with a one-sided alpha level of 5% and 90% power. All patients receiving at least one dose of axitinib were included in the safety and efficacy analyses. This analysis was conducted 1 year after all patients (excluding those who discontinued treatment) initiated axitinib.

3. Results

3.1. Patient characteristics and treatment exposure

Sixty-four patients were enrolled (Table 1). Median duration of treatment with axitinib was 326 days (range, 13–696) with a mean daily dose of 7.1 mg (range, 1.6–16.4). Axitinib dosing was titrated >5 mg BID in five patients (8%), and reduced to <5 mg BID in 42 patients (66%). In all, 37 patients discontinued the study, 13 due to treatment-related AEs and 24 due to disease progression. As of the analysis cut-off date, 27 patients (42%) were still receiving axitinib.

3.2. Efficacy

A summary of axitinib efficacy is shown in Table 2. Thirty-two (50.0%; 95% confidence interval [CI], 37.2–62.8) and 35 (54.7%; 95% CI, 41.7–67.2) patients achieved an objective response according to IRC and investigator assessments, respectively. Median PFS was 11.0 months (95% CI, 9.2–12.0) (Fig. 1A) and 12.0 months (95% CI, 9.2–14.8) according to IRC and investigator assessments, respectively. Tumour size decreased by ⩾30% in 37 patients (58%) according to IRC assessment (Fig. 1B). Median DR ($n = 32$) was 11.5 months (95% CI, 8.3 – not estimable) by IRC assessment.

Table 1 – Baseline patient characteristics.

	N = 64
Male/female, n (%)	44 (69)/20 (31)
Age, median (range) (year)	63 (34–80)
ECOG PS 0/1, n (%)	57 (89)/7 (11)
Primary histology, n (%)	
Clear cell	62 (97)
Papillary carcinoma	1 (2)
Spindle cell	1 (2)
Prior adjuvant therapy, n (%)	
Yes	10 (16)
No	54 (84)
MSKCC risk criteria[a]*, n (%)*	
Favourable	10 (16)
Intermediate	47 (77)
Poor	4 (7)

ECOG PS, Eastern Cooperative Oncology Group performance status and MSKCC, Memorial Sloan-Kettering Cancer Center.
[a] Unknown for three patients.

Table 2 – Summary of efficacy.

	IRC assessment N = 64	Investigator assessment N = 64
Best response by RECIST, n (%)		
Partial response (PR)	32 (50.0)	35 (54.7)
Stable disease[a] (SD)	29 (45.3)	26 (40.6)
Progressive disease	1 (1.6)	1 (1.6)
Indeterminate[b]	2 (3.1)	2 (3.1)
Objective response rate (PR)	32 (50.0)	35 (54.7)
95% CI	37.2–62.8	41.7–67.2
Median PFS, months	11.0	12.0
95% CI	9.2–12.0	9.2–14.8

CI, confidence interval; IRC, independent review committee; PFS, progression-free survival and RECIST, Response Evaluation Criteria in Solid Tumours.
[a] Stable disease ⩾8 weeks.
[b] No tumour assessment after dosing due to adverse event-related discontinuation.

3.3. Safety

The most frequently reported treatment-related non-haematologic AEs were hypertension, hand–foot syndrome, diarrhoea and dysphonia (Table 3). The most common grade ⩾3 treatment-related non-haematologic AEs were hypertension and hand–foot syndrome. Few patients experienced treatment-related haematologic AEs (Table 3). The treatment-related laboratory abnormality most frequently reported as an AE was proteinuria (Table 3). Treatment-related AEs leading to study discontinuation were proteinuria ($n = 7$; 11%), anxiety, decreased weight, polycythemia, subarachnoid haemorrhage, malaise and thyrotoxicosis ($n = 1$ each; 2%).

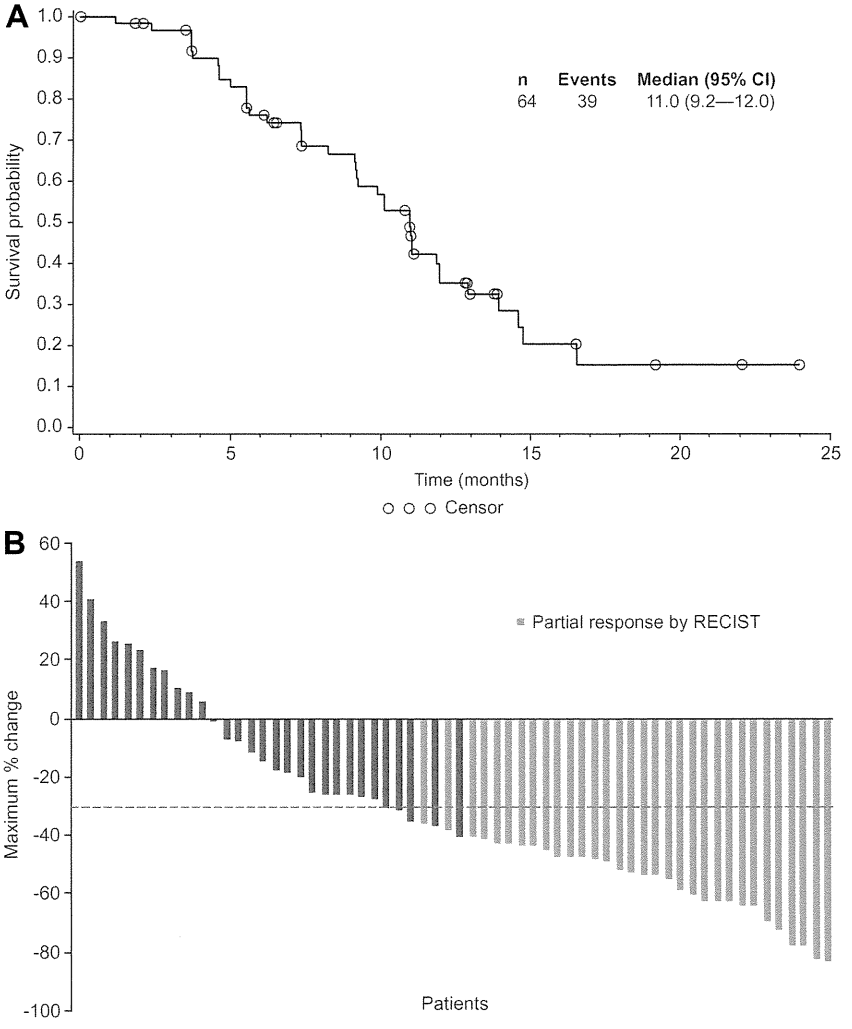

Fig. 1 – Efficacy. (A) Kaplan–Meier plot of progression-free survival (IRC assessment) and (B) maximum percentage change in target lesion size (IRC assessment). Bars represent individual patients. IRC, independent review committee.

3.4. *Proteinuria*

Eighteen patients (28%) developed proteinuria ⩾2 g/24 h, requiring dose reduction or treatment interruption/discontinuation. Median proteinuria prior to dose interruption was 2.8 g/24 h (range, 2.1–10.8). Proteinuria ⩾2 g/24 h resolved to <2 g/24 h within 3–47 days (median, 7 days) of treatment interruption. Median proteinuria was 1.0 g/24 h (range, 0.5–1.9) when axitinib treatment was resumed at a lower dose level. Seven patients discontinued treatment because proteinuria ⩾2 g/24 h was still observed at the lowest available dose (2 mg BID). Baseline urine protein levels correlated more strongly with axitinib-related proteinuria ⩾2 g than other baseline renal function-related test values or BP (Table 4). The hazard ratio [HR] for the development of axitinib-related proteinuria ⩾2 g was 5.457 (95% CI, 1.749–17.029; P = 0.0035) in patients with baseline proteinuria ⩾1+ compared with those with baseline proteinuria <1+. Patients with baseline proteinuria ⩾2+ had a higher risk of shorter treatment duration due to axitinib-induced proteinuria (Table 5). Time to treatment discontinuation due to proteinuria ranged from 44 to 118 days compared with 158–344 days in patients with baseline proteinuria ⩾2+ and <2+, respectively. Higher levels of baseline urine protein were associated with axitinib-induced proteinuria ⩾2 g (Table 6).

3.5. *Thyroid function*

Fifty-six patients (88%) experienced increases and/or transient decreases in thyroid-stimulating hormone (TSH) levels beyond the normal range during axitinib treatment (Fig. 2A and B). Twenty (31%) and 14 patients (22%) had transient increases in levels of free triiodothyronine (T3) and free thyroxine (T4), respectively (Fig. 2C and D, respectively). Thirty-two patients (50%) received thyroid hormone replacement therapy during the study.

3.6. *Changes in soluble plasma protein concentrations and correlation with clinical activity*

Overall, levels of VEGF increased while levels of sVEGFR-1, 2 and 3 decreased during therapy (Fig. 3A and B). Small de-

Table 3 – Safety findings.

	All grades *n* (%)	Grades 3/4[a] *n* (%)
Treatment-related non-haematologic AEs[b]		
Hypertension	54 (84)	45 (70)
Hand–foot syndrome	48 (75)	14 (22)
Diarrhoea	41 (64)	3 (5)
Dysphonia	34 (53)	0 (0)
Fatigue	31 (48)	3 (5)
Hypothyroidism	31 (48)	0 (0)
Decreased appetite	23 (36)	3 (5)
Weight decreased	19 (30)	2 (3)
Nausea	16 (25)	0 (0)
Headache	15 (23)	0 (0)
Stomatitis	15 (23)	0 (0)
Epistaxis	14 (22)	0 (0)
Rash	13 (20)	0 (0)
Arthralgia	12 (19)	2 (3)
Dysgeusia	12 (19)	0 (0)
Vomiting	10 (16)	0 (0)
Malaise	8 (13)	4 (6)
Abdominal pain	8 (13)	0 (0)
Chest pain	7 (11)	0 (0)
Constipation	7 (11)	0 (0)
Cough	7 (11)	0 (0)
Treatment-related haematologic laboratory AEs[c]		
Thrombocytopenia	7 (11)	1 (2)
Neutropenia	4 (6)	1 (2)
Haemoglobin decreased	3 (5)	0 (0)
Anaemia	2 (3)	1 (2)
Lymphopaenia	2 (3)	1 (2)
Polycythaemia	2 (3)	0 (0)
Leucopoenia	2 (3)	0 (0)
Treatment-related laboratory test AEs[b]		
Proteinuria[d]	37 (58)	6 (9)
TSH increased	20 (31)	0 (0)
ALT increased	15 (23)	2 (3)
AST increased	15 (23)	1 (2)
ALP increased	11 (17)	0 (0)
LDH increased	8 (13)	0 (0)

AE, adverse event; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase and TSH, thyroid-stimulating hormone.

[a] No grade 5 AEs (deaths) were reported.

[b] Reported in ⩾10% of patients.

[c] Reported in ⩾3% of patients.

[d] Includes proteinuria, protein urine, and protein urine present.

creases in sKIT plasma concentrations were observed. Patients with a percent change in sVEGFR-2 levels <median (greater reduction) demonstrated significantly higher ORR compared with those with a percent change ⩾median (smaller reduction) (64.5% [95% CI, 45.4–80.8] versus 37.5% [95% CI, 21.1–56.3]; $P = 0.045$) (Fig. 3C). Similarly, patients with a greater reduction in sVEGFR-2 levels demonstrated significantly longer median PFS compared with those with a smaller reduction (12.9 months [95% CI, 9.3–16.6] versus 9.2 months [95% CI, 7.4–11.0]; HR 0.42 [95% CI, 0.21–0.83], $P = 0.01$) (Fig. 3D).

4. Discussion

In this study, axitinib was well tolerated and demonstrated significant clinical activity as second-line treatment for Japanese patients with cytokine-refractory mRCC. Moreover, tumour shrinkage was observed in the majority of patients. This clinical benefit is encouraging and warrants further investigation of axitinib in this population. Final analysis of efficacy data from this study, including overall survival, is awaited.

Common treatment-related AEs were hypertension, hand–foot syndrome, diarrhoea, and dysphonia, toxicities frequently associated with VEGF pathway inhibitors.[12,13,15,16,20–23] Other than hypertension, most AEs were grade ⩽2. Hypertension was the most commonly reported AE (84% of patients) and was manageable with antihypertensive medication, which was administered to 94% of patients. The incidence of proteinuria and hand–foot syndrome was higher in this study than in the Western study of axitinib for cytokine-refractory mRCC (58% versus 8%, and 75% versus 8%, respectively).[12] In contrast,

Table 4 – Proteinuria ⩾2 g/24 h and baseline renal function-related test values or blood pressure.

Baseline laboratory tests	HR (95% CI)[a]	P value[b]
Urine protein	5.457 (1.749–17.029)[c]	0.0035
Creatinine	1.446 (1.010–2.069)	0.0439
eGFR	0.647 (0.423–0.987)	0.0435
eCcr	0.781 (0.599–1.020)	0.0693
BUN	1.368 (0.809–2.314)	0.2425
Albumin	0.830 (0.649–1.062)	0.1378
Systolic BP	1.593 (0.915–2.774)	0.0998
Diastolic BP	0.822 (0.631–1.071)	0.1470

BP, blood pressure; BUN, blood urea nitrogen; CI, confidence interval; eCcr, estimated creatinine clearance; eGFR, estimated glomerular filtration rate and HR, hazard ratio.

[a] Crude HR for one-level increment by Cox proportional hazard model. One increment: creatinine 0.2, eGFR 10, eCcr 10, BUN 10, albumin 0.2, systolic BP 10, diastolic BP 5.

[b] Wald-type test.

[c] HR for urine protein ⩾1+ versus <1+ (<1+ is reference).

Table 5 – Characteristics of patients discontinuing due to proteinuria.

Age (year)	Gender	Time to discontinuation due to proteinuria (days)	Baseline urine protein (mg/24 h)	Axitinib dose level	Urine protein/24 h (g)	Creatinine (mg/dL)	Best response by IRC
61	F	44	2+ (935)	5 mg BID	10.8	1.0	Indeterminate[a]
				2 mg BID[b]	4.8	1.0	
54	F	113	2+ (460)	5 mg BID	4.5	1.56	SD
				3 mg BID[b]	3.4	1.58	
				2 mg BID[b]	2.3	1.58	
72	M	118	2+ (920)	5 mg BID	2.7	1.49	SD
				3 mg BID[b]	3.0	1.47	
				2 mg BID[b]	2.9	1.55	
70	M	158	–	3 mg BID[c]	3.3	1.02	SD
				2 mg BID[b]	4.3	1.18	
79	M	173	±	5 mg BID	2.7	1.67	SD
				3 mg BID[b]	1.8	1.56	
				2 mg BID[b]	2.1	1.68	
74	M	225	–	5 mg BID	2.2	1.1	PR
				3 mg BID[b]	3.0	1.2	
				2 mg BID[b]	2.3	1.3	
65	M	344	–	3 mg BID[c]	2.7	0.85	PR
				2 mg BID[b]	2.6	0.92	

Shaded area: baseline urine protein of 2+.

BID, twice daily; F, female; IRC, independent review committee; M, male; PR, partial response and SD, stable disease.

[a] No tumour assessment after dosing due to discontinuation from study.

[b] The dose was reduced due to proteinuria.

[c] The dose was reduced from 5 mg BID to 3 mg BID due to hypertension prior to development of proteinuria ⩾2 g/24 h.

Table 6 – Proteinuria ⩾2 g/24 h and baseline urine protein.

Baseline urine protein (*n*)	Proteinuria <2 g during treatment, *n* (%)	Proteinuria ⩾2 g during treatment, *n* (%)
– (51)	40 (78)	11 (22)
± (7)	4 (57)	3 (43)
1+ (2)	1 (50)	1 (50)
2+ (4)	1 (25)	3 (75)
P value[a]	0.013	

[a] Cochran-Armitage exact test.

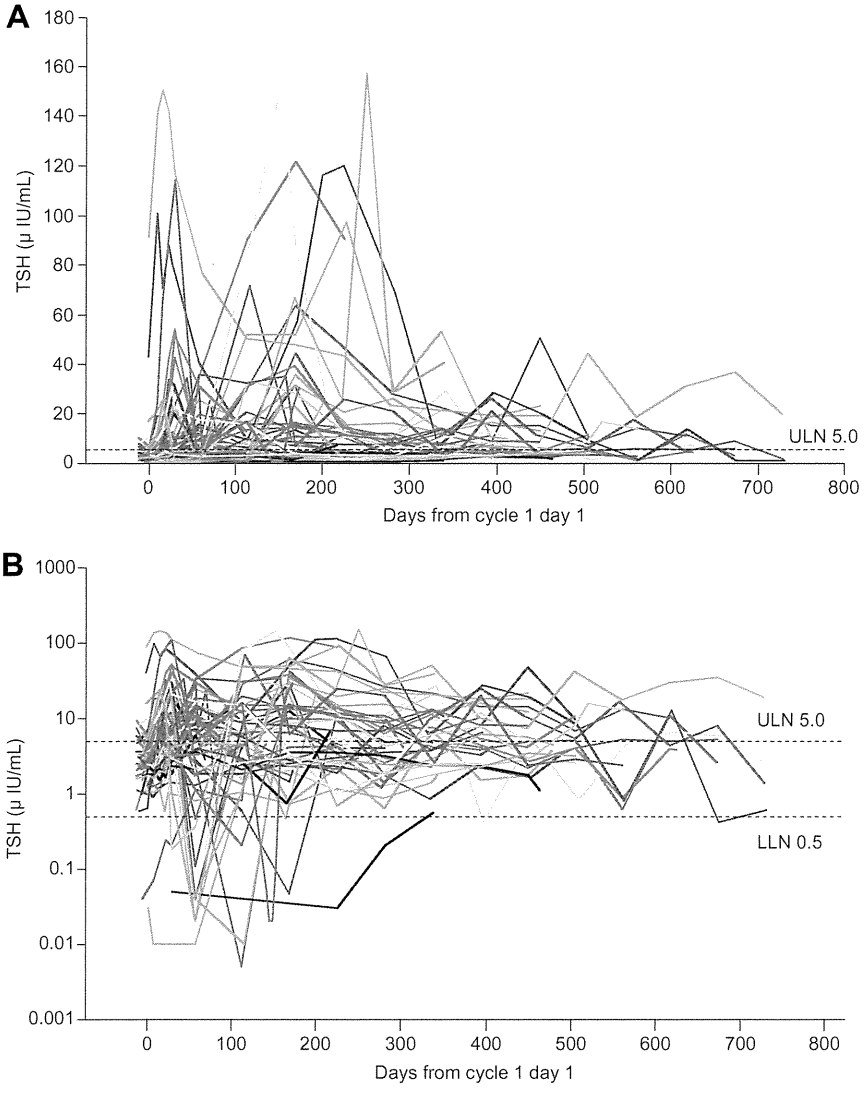

Fig. 2 – Thyroid function test levels over time. (A) Thyroid-stimulating hormone absolute values, and (B) log scale, (C) free triiodothyronine (absolute values) and (D) free thyroxine (absolute values). Lines represent individual patients. LLN, lower limit of normal range; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine and ULN, upper limit of normal range.

the incidence of dry skin was higher in the Western study (33% versus 5%).[12] Axitinib dose reductions were required in more Japanese patients (66%) than Western patients (29%) with cytokine-refractory mRCC.[12] It is unclear why the incidence of proteinuria and hand–foot syndrome was higher in Japanese patients compared with Western patients. Higher rates of AEs, including hand–foot syndrome and haematologic abnormalities, were also reported in Asian patients with RCC treated with sunitinib[24–27] or sorafenib[28] compared with Western patients.

Proteinuria is a widely reported side effect of antiangiogenic therapies.[29] In this study, proteinuria occurred in 58% of patients, with 28% developing proteinuria ⩾2 g/24 h and requiring dose interruption or reduction. All patients developing proteinuria ⩾2 g/24 h restarted axitinib at a lower dose level after proteinuria was resolved <2 g/24 h. However, 11% of patients were ultimately discontinued due to proteinuria ⩾2 g/24 h with the lowest available dose (2 mg BID). These results underscore the importance of monitoring patients receiving angiogenesis inhibitors, including axitinib, for proteinuria. Control of proteinuria during antiangiogenic therapy may require dose reductions or treatment discontinuation, and patients should be referred to nephrologists when appropriate.[29] An understanding of the predictive factors leading to proteinuria in patients receiving angiogenesis inhibitors will be important for managing this AE. Here, higher baseline protein levels correlated with the development of axitinib-induced proteinuria and were associated with early discontinuation due to proteinuria. Thus, evaluation of urine protein levels at baseline may be important for selecting patients for axitinib therapy. In this study, the axitinib dose was interrupted/reduced or discontinued according to predefined dose-modification criteria for proteinuria, which has been used across axitinib clinical studies. In clinical practice, periodic interruption of axitinib treatment when indicated may help limit proteinuria to a low grade; axitinib treatment may be resumed,

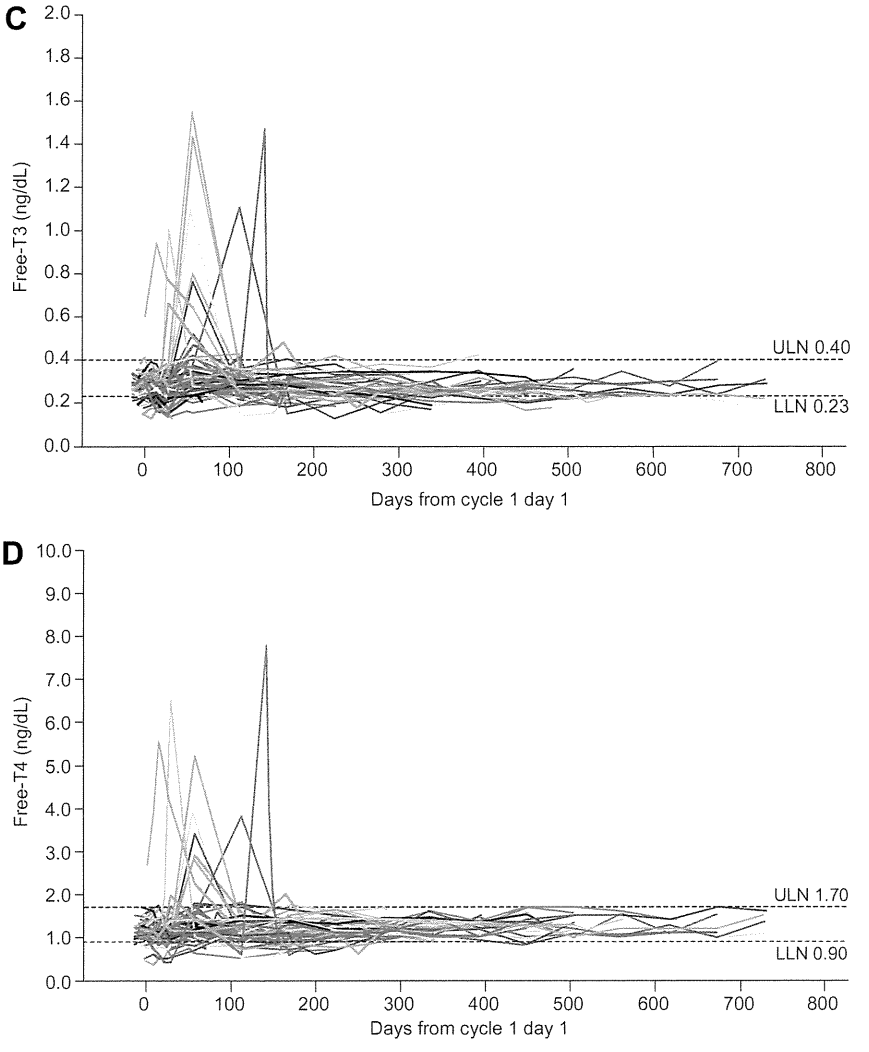

Fig 2. *(continued)*

especially when patients experience disease progression while off treatment. Proteinuria frequently correlates with the development of hypertension in patients receiving antiangiogenic therapies;[29] however, no clear correlation between development of proteinuria and change in BP during axitinib treatment was observed here. This may be related to multiple mechanisms of VEGFR inhibition leading to hypertension. In addition, 28 patients (44%) received antihypertensive medication prior to axitinib treatment initiation, and the majority of patients ($n = 54$; 84%) received antihypertensive medication during the first 28 days of axitinib treatment to manage BP. This might have obscured the potential to assess the correlation between development of proteinuria and elevated BP.

Abnormalities in TSH levels were frequently reported (88% of patients). Inhibitors of VEGFR may impair thyroid function by blocking VEGF binding to normal thyroid cells and impeding thyroid blood flow.[30,31] Based on data from this and prior studies, monitoring of thyroid function is recommended during axitinib treatment. Hypothyroidism and associated symptoms may be managed by proactive administration of thyroid hormone replacement therapy.[30–34]

Agents targeting the VEGF pathway have changed the treatment paradigm for mRCC, and ongoing research is aimed at identifying biomarkers that predict their clinical benefit. Numerous candidate biomarkers have been evaluated, but a validated predictive biomarker has not yet been identified.[35] Prior studies of VEGF and sVEGFRs as biomarkers of response to angiogenesis inhibitors have yielded inconsistent results, illustrating the need for dynamic evaluation of these proteins during treatment.[35] In this study, greater reductions in sVEGFR-2 plasma concentrations during therapy were associated with higher ORR and longer PFS. Thus, sVEGFR-2 may be a useful pharmacodynamic marker of clinical outcome with axitinib.

Although there was no comparator in this study, axitinib demonstrated significant efficacy for mRCC in Japanese patients and was well tolerated in this population. Although the incidence of some AEs (e.g. proteinuria and hand–foot syndrome) was higher in Japanese compared to Western mRCC patients receiving axitinib, careful monitoring and management may help to control these toxicities if they arise during therapy. Moreover, preliminary findings suggest that baseline proteinuria and sVEGFR-2 levels may be key indicators for