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Quantification of plasma cell-free DNA[1] in predicting therapeutic efficacy of sorafenib on metastatic clear cell renal cell carcinoma

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Abstract.
PURPOSE: The objective of this study is to determine whether or not plasma cfDNA levels can predict efficacy of sorafenib in patient with metastatic cRCC.
MATERIALS AND METHODS: Plasma cfDNA levels were quantified by quantitative real-time PCR at six different time-points (before treatment, 4 weeks, 8 weeks, 12 weeks, 16 weeks, and 24 weeks) in 18 metastatic cRCC patients receiving sorafenib, as assessed by CT examination according to RECIST 1.1.
RESULTS: A significantly lower plasma cfDNA level, measured from 8 weeks to 24 weeks, was found in patients with remission or stable disease than in those with progression. Higher levels in plasma cfDNA levels during the course of treatment indicated poor outcome. For predicting progression, a sensitivity of 66.7% was achieved at 100% specificity using cfDNA levels at 8 weeks.
CONCLUSIONS: Monitoring of plasma cfDNA levels during the course of sorafenib therapy could identify metastatic cRCC patients who are likely to exhibit a poor response at an early stage.

Keywords: Cell-free DNA, sorafenib, response, metastatic clear cell renal cell carcinoma

1. Introduction

Approximately 30% of renal cell carcinoma (RCC) patients present with distant metastasis at the time of diagnosis [1]. Moreover, one-third of patients who undergo resection of localised disease will develop distant metastasis [2]. As most patients with metastatic RCC respond poorly to chemotherapy, and its response to cytokine therapy including highdose interleukin-2 and/or interferon-alfa is less than 20% [3,4], the prognosis for patients with metastatic RCC is very poor, the 5-year overall survival rate is less than 10% and the median survival is only 13 months [5].

Sorafenib, an orally active multikinase inhibitor with effects on tumor-cell proliferation and tumor angiogenesis, was initially identified as a Raf kinase inhibitor. It also inhibits vascular endothelial growth factor receptors 1, 2, and 3; platelet- derived growth factor receptor β; FMS-like tyrosine kinase 3; c-Kit protein; and RET receptor tyrosine kinases [6,7]. The efficacy of sorafenib on metastatic RCC had been confirmed in both phase II and phase III trials, which had resulted in the approval of its use as a secondline treatment in metastatic RCC [8,9]. The efficacy of sorafenib had also been approved in most Asian countries/regions including China for metastatic RCC [10]. Sorafenib is expensive and is associated with certain adverse effects that impair quality of life. Thus, evaluation of efficacy is extremely attractive for patients with metastatic RCC which respond poorly to sorafenib.

In peripheral blood of cancer patients, previous studies indicate that circulating cell-free (cfDNA) is originated from tumors, through apoptosis, necrosis, or cell

[1]Plasma cell-free DNA is a useful marker for predicting therapeutic efficacy of sorafenib on metastatic clear cell renal cell carcinoma.

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lysis of tumor cells and circulating tumor cells [11]. It is noteworthy that the levels of circulating cfDNA associate with the progression of radiotherapy or cytotoxic therapy in some cancer patients [12,13]. Thus, we hypothesize that circulating cfDNA might be a novel biomarker for response to sorafenib therapy on metastatic clear cell renal cell carcinoma (cRCC).

The objective of our study is to determine whether or not plasma cfDNA levels can predict efficacy of sorafenib in patient with metastatic cRCC.

2. Materials and methods

2.1. Patient population

Eighteen non-selected patients with metastatic cRCC (between December 2009 and December 2011) were enrolled in the study. All patients had pathologically confirmed cRCC from their primary or metastatic site(s), and their initial tumors were staged according to the 2002 UICC TNM classification. Nuclear grading was based on the criteria of Fuhrman by the single pathologist. Prior immunotherapy was allowed and nephrectomy was not a requirement. Life expectancy should be minimum 3 months. Age < 18 or > 80 years, ECOG performance status > 1 were not compatible with eligibility. Adequate haematological, renal and hepatic function was required. Pretreatment evaluation also consisted of CT scan of the chest, CT scan or MRI of the abdomen and pelvis, and total body bone scan.

Informed consent was required and obtained from all patients before treatment. All patients received 400 mg of sorafenib (BAY 43-9006) orally twice daily, spaced 12 hours apart, on continuous basis. The treatment continued until disease progress or intolerance to the treatment occurred. Among 18 patients, 7 patients initiated their treatment with sorafenib only and 11 patients had received sorafenib after they had failed interleukin- and/or interferon-based therapy.

During treatment of sorafenib, all patients were evaluated weekly. Patients also had to be followed-up after the termination of their treatment, if intolerance to the treatment occurred. The adverse-effects secondary to the treatment were evaluated at each visit during and after the treatment, and were recorded according to the Common terminology criteria for adverse events v3.0 (CTCAE) of the National Cancer Institute.

CT examinations of cRCC primary ($n = 4$) or metastatic lesions ($n = 14$) were performed at baseline (2 days or less before treatment). RECIST measurements were performed by using CT at baseline, with follow-up scans obtained at 6-week intervals for the first 24 weeks (or until disease progression), and every 8 weeks thereafter. Responders were defined as those achieving complete response (CR), partial response (PR) and non-responders as those with stable disease (SD) or progressive disease (PD) after 8 weeks of treatment according to RECIST 1.1 (2009) [14]. For patients with unconfirmed response, a CT scan for confirmation was performed 4 weeks after the first recorded response. Lesions identified and measured were evaluated using the same machine and the same investigator.

10 healthy individuals were used as controls.

2.2. Analysis of plasma cfDNA levels

Peripheral venous blood (3 ml) was taken from all patients on six occasions: 1 days before treatment, and week (W) 4, 8, 12, 16 and 24 weeks after starting treatment, W0, W4, W8, W12, W16 and W24, respectively, peripheral venous blood was collected into EDTA-containing tubes and processed within 2 h of venipuncture. To ensure cell-free plasma collection, all EDTA–blood samples were centrifuged in 2 steps (1800 g for 10 min and then 12000 g for 10 min). The cell-free plasmas were stored at $-20°C$ until extraction.

Total DNA was isolated with the QIAamp DNA Blood Mini Kit (Qiagen) according to the Qiagen blood and body fluids protocol. Each column was loaded with 400 μL plasma and the extracted DNA was eluted with distilled water in a final volume of 50 μL. DNA was quantified using a Nanodrop spectrophotometer (Thermo scientific).

Quantitative real-time PCR was performed on an ABI Prism® 7900 HT. Each 10 μl reaction consisted of 1XSYBR® GreenER™ quantitative PCR SuperMix, 1 μl DNA sample, and 0.2 μmol/l forward and reverse primers (ACTB: forward GCTATCCCTGTACGCCTCTG; reverse AGGAAGGAAGGCTGGAAGAG; size of PCR product is 384bp) [15]. The thermal profile was a first denaturation step at 90°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed to confirm PCR product specificity. Both calibrators and samples were analyzed in triplicate. An external standard curve using serial dilutions (100 ng/ml, 10 ng/ml, 1 ng/ml, 0.1 ng/ml and 0.01 ng/ml) of genomic DNA from peripheral lymphocytes of a healthy individual. Serial dilutions of an external standard and water blanks were included in every run.

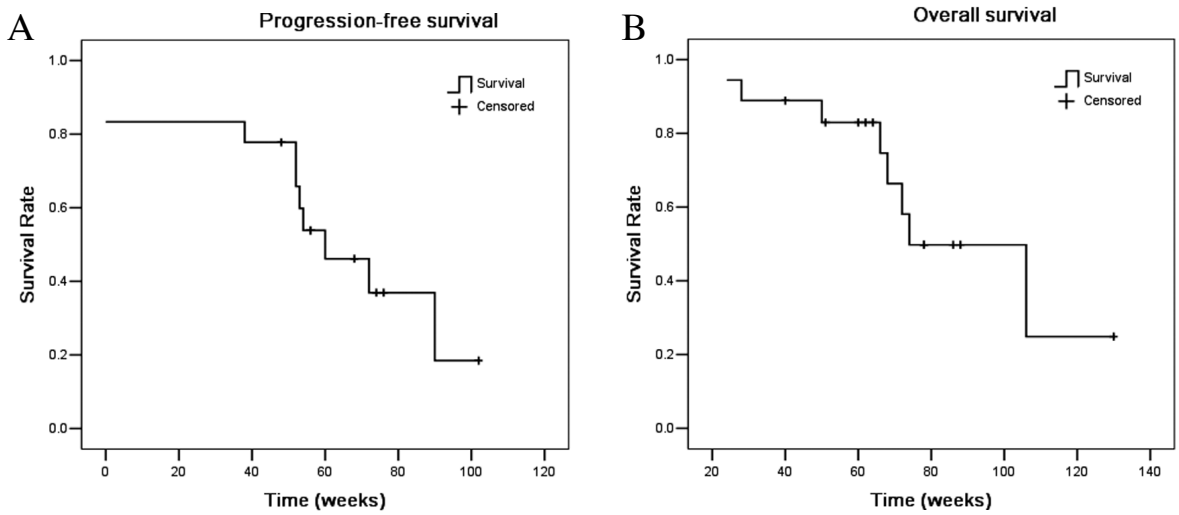

Fig. 1. Progression-free survival of patients (A). Overall survival of patients (B).

2.3. Statistical analyses

Levels of plasma cfDNA were expressed as Mean ± SD (range). The data between different groups were compared with Mann-Whitney U test. The cfDNA levels in different response groups were compared by analysis of variance with Bonferronis correction. Progression-free survival (PFS) time was measured from the date of the initiation of sorafenib treatment until documented radiologically confirmed disease progression or death of patient. The duration of overall survival (OS) was calculated from the date of the initiation of sorafenib treatment until death or until the date of the last follow-up visit for patients still alive. Both PFS and OS duration were calculated by the Kaplan-Meier method. In order to identify the diagnostic biomarker for therapeutic efficacy, receiver operating characteristic (ROC) curves and the corresponding areas under the curve (AUC) were calculated. In addition, sensitivity and cut-off values for PD were calculated at 100% specificity (with 95%CI)). In order to test the association of W0-W24 with the overall survival of the patients, Kaplan–Meier curves and log-rank analyses were established using their median values as cut-off points. A P-value of < 0.05 was considered significant. Statistical analyses were performed with the SPSS software (version 13.0; SPSS).

3. Results

10 healthy individuals (6 males and 4 females) had a median age of 62.0 years (range 51–70). 18 patients treated with sorafenib included in this study (13 males and 5 females) had a median age of 63.6 years (range 42–75) at the start of treatment. There was no statistical difference in age between the patients and controls ($P = 0.026$).

The median follow-up time of patients was 68 weeks (range 13–178). The clinical response of patients was assessed using RECIST criteria 1.1 (2009). Radiologically confirmed CR, PR, SD (of more than 6 months), and PD were observed in 0/18 (0%), 4/18 (22.2%), 11/18 (61.1%), and 3/18 (16.7%) patients. The 1-year estimated PFS and OS were 59.5% and 69.9% (Fig. 1). The median PFS was 60 weeks (95% CI 38–81), and the median OS was not reached at the time of this analysis. At the time of this analysis, 8 patients (44.4%) had deceased. No statistical differences were observed in OS or PFS for patients received sorafenib as their first-line treatment or after cytokine therapy.

The pretreatment level of plasma cfDNA (W0) in patients with metastatic cRCC (4.771 ± 0.404 ng·ml^{-1}, range 3.357–5.239 ng·ml^{-1}) was significantly higher than that in healthy individuals (0.622 ± 0.288 ng·ml^{-1}, range 0.208–1.022 ng·ml^{-1}) ($P < 0.001$). No association was found between the W0 and age ($P = 0.829$), gender ($P = 0.443$), ECOG-PS ($P = 0.052$), or sites of metastatic diseases ($P = 0.350$). The significant associations were found between the W0 and TNM stage ($P = 0.047$), Fuhrman grade ($P = 0.035$) or number of metastatic foci ($P = 0.029$) (Table 1). The plasma cfDNA level of W4, W8, W12, W16 and W24 were also quantified during the course of sorafenib therapy, a decrease in cfDNA levels was observed in patients

Table 1
The plasma cfDNA levels before treatment of sorafenib in patients with metastatic cRCC and those in healthy individuals

	No. of patients	Plasma cfDNA (Mean ± SD, ng·ml^{-1})	P-value
Metastatic cRCCs	18	4.771 ± 0.404 (range 3.357–5.239)	
Age (years)			0.829
⩽ 65	8	4.827 ± 0.120	
> 65	10	4.726 ± 0.144	
Gender			0.443
Male	13	4.817 ± 0.104	
Female	5	4.652 ± 0.217	
ECOG-PS			0.052
0	8	4.582 ± 0.194	
1	10	4.922 ± 0.097	
TNM Stage			0.047
T1	4	4.519 ± 0.214	
T2	4	4.463 ± 0.249	
T3	10	4.995 ± 0.057	
Fuhrman Grade			0.035
G2	6	4.399 ± 0.168	
G3	8	4.889 ± 0.091	
G4	4	5.092 ± 0.066	
Sites of metastatic diseases			0.350
Lung	9	4.889 ± 0.119	
Liver	5	4.811 ± 0.178	
Bone	2	4.759 ± 0.166	
Other	2	4.550 ± 0.224	
Number of metastatic foci			0.029
⩽ 3	5	4.583 ± 0.142	
> 3	13	4.845 ± 0.126	
Healthy controls	10	0.622 ± 0.288 (range 0.208–1.022)	
Age (years)			0.841
⩽ 65	5	0.665 ± 0.162	
> 65	5	0.579 ± 0.102	
Gender			0.762
Male	6	0.661 ± 0.137	
Female	4	0.564 ± 0.116	

Table 2
Distribution of plasma cfDNA levels in various patient response groups for monitoring response to sorafenib

	Plasma DNA (Mean ± SD, ng·ml^{-1})			P-value					
	PR	SD	PD	Overall	PR vs. SD	PR vs. PD	SD vs. PD	PR+SD vs. PD	PR vs. SD+PD
W0	4.596 ± 0.144	4.814 ± 0.143	4.846 ± 0.129	0.176	0.104	0.098	0.292	0.271	0.073
W4	4.442 ± 0.279	4.839 ± 0.141	4.860 ± 0.189	0.083	0.091	0.062	0.523	0.125	0.040
W8	3.665 ± 0.362	4.869 ± 0.148	4.946 ± 0.083	0.019	0.015	0.007	0.047	0.021	0.016
W12	3.181 ± 0.239	5.005 ± 0.146	5.362 ± 0.384	0.007	0.009	0.003	0.039	0.013	0.009
W16	2.776 ± 0.259	5.117 ± 0.156	5.688 ± 0.330	0.004	0.005	< 0.001	0.036	0.009	0.004
W24	2.551 ± 0.288	5.268 ± 0.057	6.069 ± 0.261	< 0.001	< 0.001	< 0.001	0.007	0.002	< 0.001

with PR, whereas the levels increased in patients with SD or PD (Fig. 2).

In order to assess the efficacy of plasma cfDNA levels in predicting remission, stable disease and progression, W0, W4, W8, W12, W16 and W24 were compared in different response groups (PR, SD and PD). W0 and W4 were lower in group PR compared with group SD and PD, however, the differences between these groups did not reach statistical significance ($P = 0.104$, $P = 0.098$; $P = 0.091$, $P = 0.062$). Overall, among all three groups, the significant differences in levels of plasma cfDNA were observed from 8 weeks after treatment of sorafenib (W8 $P = 0.019$, W12 $P = 0.007$, W16 $P = 0.004$, W24 $P < 0.001$). When compared among any two groups, significantly lower W8, W12, W16 and W24 were observed in patients with PR or SD as compared to patients with PD (Table 2). W0 could not predict remission, stable disease and progres-

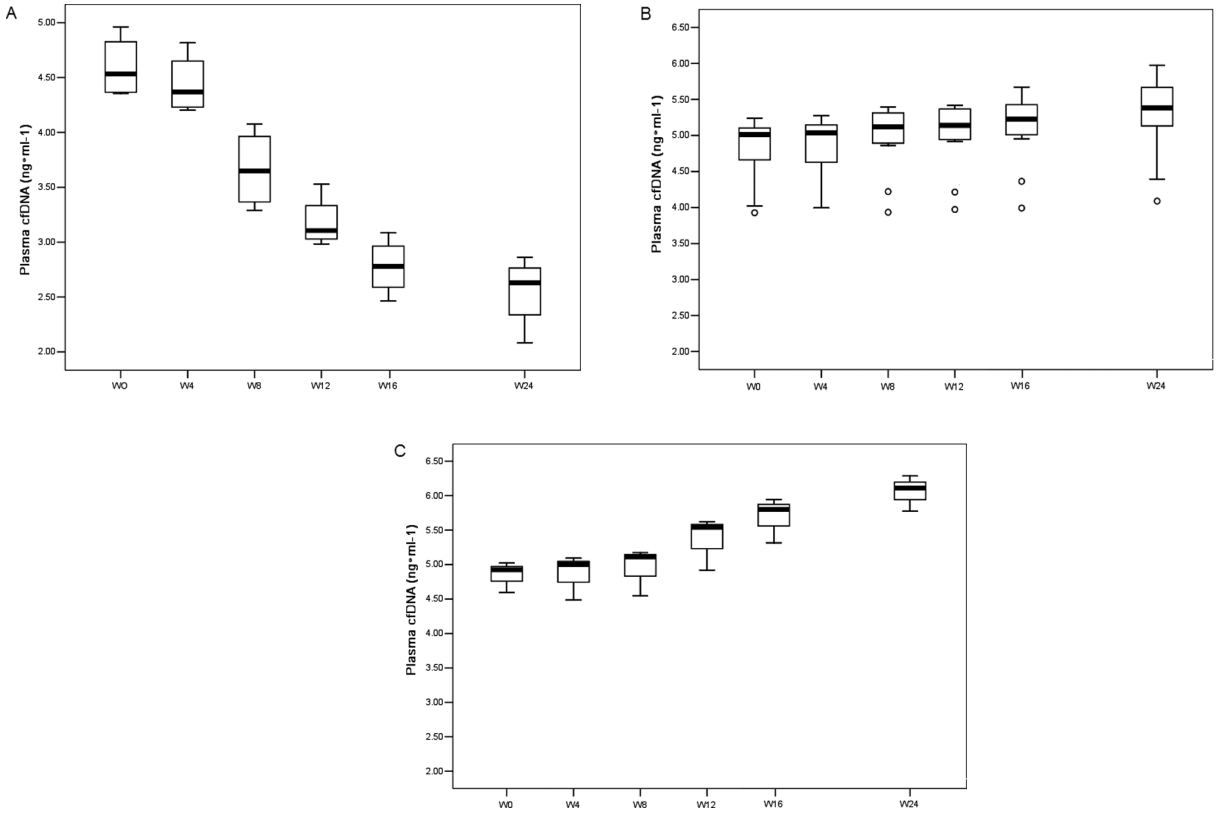

Fig. 2. Distribution of plasma cfDNA levels in patients with Remission (A), Stable disease (B) and Progression (C) during the course of sorafenib therapy.

sion ($P = 0.271$, $P = 0.073$). Although W4 could not predict remission, stable disease ($P = 0.125$), it was able to predict progression ($P = 0.040$). Further, W8, W12, W16 and W24 were able to predict remission, stable disease and progression (Table 2).

In order to test the potential of plasma cfDNA levels as a biomarker for the prediction of progression, ROC curves were plotted for W0, W4, W8, W12, W16 and W24. Using W0, progression could be predicted with a sensitivity of 26.8% at absolute (100%) specificity (AUC 0.511; cut-off value 4.915 ng·ml^{-1}). Using W4, progression could be predicted with a sensitivity of 44.6% at absolute (100%) specificity (AUC 0.578; cut-off value 4.942 ng·ml^{-1}). Using W8, progression could be predicted with a sensitivity of 66.7% at absolute (100%) specificity (AUC 0.800; cut-off value 5.019 ng·ml^{-1}). Further, W12, W16 and W24 were able to predict progression with a sensitivity of 71.2% (AUC 0.822; cut-off value 5.48 ng·ml^{-1}), 76.3% (AUC 0.889; cut-off value 5.738 ng·ml^{-1}) and 80.8% (AUC 0.956; cut-off value 6.048 ng·ml^{-1}), respectively, at 100% specificity.

With the median cfDNA level as the cut-off value, patients were divided into high (above median) and low (below or equal to median) groups. No association was observed between W0, W4, W8 and survival. However, W12, W16 and W24 were significantly related to survival (Table 3).

4. Discussion

The evaluation of response to sorafenib is performed with RECIST in routine clinical care and in clinical trials. With the use of RECIST, patients are said to respond, stabilize, or progress, depending on treatment-induced changes in tumor size measured on CT or MRI. However, cancers treated with sorafenib often show little change in size during early stage of therapy despite substantial clinical benefit. Radiologic criteria that additionally take into account functional changes in the enhancement of lesions on contrast material–enhanced CT scans or dynamic contrast-enhanced MRI have helped to identify patients who went on to show

Table 3
Association between plasma cfDNA levels and survival in metastatic clear cell renal cell carcinoma

Cut-off value ($ng \cdot ml^{-1}$)	Survival weeks (Median, 95% confidence interval)	P-value
W0		0.471
$\leqslant 4.929$	106.0 (47.1–164.9)	
> 4.929	82.0 (63.9–80.1)	
W4		0.212
$\leqslant 4.883$	107.0 (48.3–166.5)	
> 4.883	82.0 (62.5–80.1)	
W8		0.117
$\leqslant 4.925$	108.0 (50.3–168.2)	
> 4.925	82.0 (60.9–80.1)	
W12		0.047
$\leqslant 4.976$	109.0 (52.1–167.9)	
> 4.976	74.0 (56.7–79.6)	
W16		0.018
$\leqslant 5.185$	109.0 (48.7–168.9)	
> 5.185	68.0 (43.8–92.1)	
W24		0.018
$\leqslant 5.226$	109.0 (48.7–168.9)	
> 5.226	68.0 (43.8–92.1)	

clinical benefit as early as 9 weeks into treatment [16–18].

Accordingly, studies on molecular markers in evaluation of metastatic RCC treatment are relevant. Klatte's study demonstrates for the first time that increased 20S proteasome levels are associated with cRCC, advanced disease, and poor prognosis. Furthermore, in patients responding to sunitinib, 20S proteasome levels were lower than in patients with stable disease and progressive disease [19]. Kortsaris's group reports the value of plasma NT-pro-BNP measurement for predicting response to sunitinib treatment in patients with metastatic RCC [20]. Baseline serum levels of MMP-9 and TNF-a are also shown as the predictive markers of sunitinib activity in patients with metastatic RCC [21].

Circulating cfDNA, a biomarker that is easily accessible and preferably non-invasive, has been shown to have diagnostic and prognostic potential in RCC [22]. In patients with RCC, Hauser's group demonstrates that levels of circulating cfDNA are increased and predominantly originates from tumors [23]. As necrosis is the predominant type of cell death in tumor tissue, longer DNA fragments representing non-apoptotic DNA may be a suitable marker in cancer. The level of plasma cfDNA had been detected in this study. We observed that plasma cfDNA levels in patients with metastatic cRCC were significantly higher than those in healthy controls. Furthermore, there were positive associations among plasma cfDNA levels, TNM stage, Fuhrman grade, and number of metastatic foci in patients with metastatic cRCC before treatment.

The previous studies indicate that circulating cfDNA may play a potential role in monitoring the efficacy of cancer therapies. Cheng's group demonstrates that the total plasma cfDNA levels in cancer patients show dynamic changes associated with the progression of radiation therapy [12]. Öfner's group demonstrates that circulating DNA in plasma of rectal cancer patients undergoing preoperative chemoradiation might serve as a surrogate marker to discriminate between responders and nonresponders [24]. Kumar's group demonstrates that monitoring of plasma DNA levels during the course of chemotherapy could identify patients who are likely to exhibit an insufficient therapeutic response and disease progression at an early stage [25].

In our study, the trend in plasma cfDNA level analysed at six different time-points (W0-W24) in metastatic cRCC patients receiving sorafenib was correlated with response to therapy. During the course of treatment, a decrease in plasma cfDNA levels was observed in patients with PR, whereas the levels increased in patients with SD or PD. Compared with patients with PR or SD, patients with PD showed significantly higher W8-W24. Compared with patients with SD or PD, patients with PR showed significantly lower W4-W24.

For predicting progression (PD versus PR+SD), it was observed that W0 and W4 were not able to predict PD, however, W8-W24 predicted progression with the greater sensitivity at 100% specificity. For predicting remission (PR versus SD+PD), it was observed that, except for W0, W4-W24 were able to predict remission. These findings suggest that monitoring the levels of plasma cfDNA during the course of sorafenib therapy may help in predicting response to therapy.

The associations between plasma cfDNA measured at different time-points and survival time were also analyzed in an effort to analyze its utility as a prognostic marker for survival in patients with metastatic cRCC. The associations were observed between W12, W16, W24 and survival. The patients with lower plasma cfDNA levels survived longer.

With the advance in laboratory techniques, it has now become easy to isolate circulating cfDNA from plasma/serum. Furthermore, it has now become easy to quantify circulating cfDNA by quantitative real-time PCR. Comparing to more expensive imaging tools, cost-effectiveness of circulating cfDNA analysis is a clinical and well established routine analysis, and plasma samples are easily managed.

Small sample size was a limitation of the present study; however, we feel that the results are promising enough to encourage further research. The multicen-

tric and large-scale prospective validation studies are required in order to confirm our current findings. Additionally, other blood-based biomarkers, such as VEGF, FGF-2, MMP-9, were not been studied in this study. These circulating angiogenic factors which can be used in combination with cfDNA, may improve the diagnostic power of current imaging tools for indicating early sorafenib efficacy on metastatic cRCC.

5. Conclusions

In conclusion, monitoring of plasma cfDNA levels during the course of sorafenib therapy could identify metastatic cRCC patients who are likely to exhibit a poor response at an early stage. This may help in individualising treatment, and could lead to better management of metastatic cRCC.

Acknowledgements

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