Predictive value of single-nucleotide polymorphism signature for recurrence in localised renal cell carcinoma: a retrospective analysis and multicentre validation study

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Summary

Background Identification of high-risk localised renal cell carcinoma is key for the selection of patients for adjuvant treatment who are at truly higher risk of reccurrence. We developed a classifier based on single-nucleotide polymorphisms (SNPs) to improve the predictive accuracy for renal cell carcinoma recurrence and investigated whether intratumour heterogeneity affected the precision of the classifier.

Methods In this retrospective analysis and multicentre validation study, we used paraffin-embedded specimens from the training set of 227 patients from Sun Yat-sen University (Guangzhou, Guangdong, China) with localised clear cell renal cell carcinoma to examine 44 potential recurrence-associated SNPs, which were identified by exploratory bioinformatics analyses of a genome-wide association study from The Cancer Genome Atlas (TCGA) Kidney Renal Clear Cell Carcinoma (KIRC) dataset (n=114, 906 600 SNPs). We developed a six-SNP-based classifier by use of LASSO Cox regression, based on the association between SNP status and patients' recurrence-free survival. Intratumour heterogeneity was investigated from two other regions within the same tumours in the training set. The six-SNP-based classifier was validated in the internal testing set (n=226), the independent validation set (Chinese multicentre study; 428 patients treated between Jan 1, 2004 and Dec 31, 2012, at three hospitals in China), and TCGA set (441 retrospectively identified patients who underwent resection between 1998 and 2010 for localised clear cell renal cell carcinoma in the USA). The main outcome was recurrence-free survival; the secondary outcome was overall survival.

Findings Although intratumour heterogeneity was found in 48 (23%) of 206 cases in the internal testing set with complete SNP information, the predictive accuracy of the six-SNP-based classifier was similar in the three different regions of the training set (areas under the curve [AUC] at 5 years: 0·749 [95% CI 0·660–0·826] in region 1, 0·734 [0·651–0·814] in region 2, and 0·736 [0·649–0·824] in region 3). The six-SNP-based classifier precisely predicted recurrence-free survival of patients in three validation sets (hazard ratio [HR] 5·32 [95% CI 2·81–10·07] in the internal testing set, 5·39 [3·38–8·59] in the independent validation set, and 4·62 [2·48–8·61] in the TCGA set; all p<0·0001), independently of patient age or sex and tumour stage, grade, or necrosis. The classifier and the clinicopathological risk factors (tumour stage, grade, and necrosis) were combined to construct a nomogram, which had a predictive accuracy significantly higher than that of each variable alone (AUC at 5 years 0·811 [95% CI 0·756–0·861]).

Interpretation Our six-SNP-based classifier could be a practical and reliable predictor that can complement the existing staging system for prediction of localised renal cell carcinoma recurrence after surgery, which might enable physicians to make more informed treatment decisions about adjuvant therapy. Intratumour heterogeneity does not seem to hamper the accuracy of the six-SNP-based classifier as a reliable predictor of recurrence. The classifier has the potential to guide treatment decisions for patients at differing risks of recurrence.

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Introduction

Every year, approximately 300 000 people worldwide are diagnosed with renal cell carcinoma, resulting in around 129 000 deaths.[1,2] The major histological subtype is clear cell renal cell carcinoma, accounting for about 80% of all cases.[2] Approximately 30% of patients with localised clear cell renal cell carcinoma (stage I–III) will relapse after surgical excision.[3,4] A recent study reported that a subset of patients with more aggressive disease could benefit from adjuvant targeted therapy after surgery.[5] Therefore, accurate assessment of recurrence risk is the key to ascertaining which patients are at truly higher risk so that

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Research in context

Evidence before this study

We searched PubMed with the search terms "renal cell carcinoma", "molecular model", and "tumor recurrence" for articles published in English between Jan 1, 2009, and July 31, 2018. We identified several studies about gene-expression signatures and their association with tumour recurrence in patients with localised clear cell renal cell carcinoma. However, early studies with integrated models had several notable limitations. First, these studies had insufficient information (eg, risk score formulas or biomarker coefficients) about how to integrate multiple biomarkers into one model, which restricted widespread use of these models in the clinic. Second, gene-expression signatures developed by RT-PCR would be influenced by tissue-specific variability, and reference genes were needed to control for this tissue-specific variability. The risk score formulas and threshold values of these gene-expression signatures were not suitable for validation by other kinds of measurement data (eg, microarray data or RNA-sequencing data). Last, to the best of our knowledge, no previous study has systematically tested whether intratumour heterogeneity affects the precision of molecular models.

Added value of this study

In this retrospective analysis and multicentre validation study, we developed a six-SNP-based classifier with time-of-flight mass spectrometry to predict the recurrence risk of patients with localised clear cell renal cell carcinoma, and used an accuracy test to systematically assess the effect of intratumour heterogeneity on the precision of the classifier. The six-SNP-based classifier was able to classify patients with localised clear cell renal cell carcinoma as being at high or low risk of recurrence, irrespective of their tumour characteristics at presentation. Compared with previous gene-expression signatures, our six-SNP-based classifier incorporates fewer biomarkers and is not influenced by technical sources of variation; therefore, it is more feasible in clinical practice. To our knowledge, this classifier is the first to assess the effect of intratumour heterogeneity on a multi-biomarker model with an accuracy test through the whole cohort. We further developed and validated a nomogram comprising the six-SNP-based classifier, TNM stage, Fuhrman grade, and tumour necrosis status that predicted recurrence-free survival.

Implications of all the available evidence

Our six-SNP-based classifier appears to be a practical and reliable predictor that can complement the current staging system for prediction of localised renal cell carcinoma recurrence, which might enable physicians to make more informed treatment decisions about adjuvant therapy for their patients.

they can be considered for adjuvant treatment. TNM stage and pathological grade are commonly used to assess the risk of tumour recurrence in patients with localised clear cell renal cell carcinoma after surgery. However, patients with the same TNM stage and pathological grade can have diverse outcomes; therefore, predictive value needs to be added to the existing staging system, which could be achieved with validated biomarkers.

Intratumour heterogeneity can impair the precise molecular analysis of solid tumours, and clear cell renal cell carcinoma is a typical example of a tumour with substantial heterogeneity.[6–9] Several multigene classifiers have been developed to predict the risk of recurrence in patients with clear cell renal cell carcinoma;[10–13] however, only studies with small sample sizes have been done to assess intratumour heterogeneity and none has systematically tested whether or not intratumour heterogeneity affects the precision of prognostic multi-biomarker models. Additionally, no reliable prognostic biomarkers for clear cell renal cell carcinoma have been used routinely in clinical practice to date. Single-nucleotide polymorphisms (SNPs) are the most common type of genetic differences in humans. As genome-wide technologies continue to develop, our understanding of SNPs that are associated with disease outcomes, including cancer, continues to rapidly improve.[14–17]

We developed a multi-SNP-based classifier with time-of-flight mass spectrometry technology to predict the recurrence risk of patients with localised clear cell renal cell carcinoma, and used an accuracy test to assess the effect of intratumour heterogeneity on the precision of the biomarker. We also validated the predictive accuracy and reproducibility of the classifier in three different datasets with more than 1000 cases of localised clear cell renal cell carcinoma.

Methods

Study design and participants

In this retrospective analysis and multicentre validation study, we used formalin-fixed, paraffin-embedded (FFPE) tissue samples from 881 patients (aged 18–90 years) who underwent resection of a localised, sporadic, clear cell renal cell carcinoma. Patients with stage I–III clear cell renal cell carcinoma and with available clinicopathological characteristics and follow-up information were included. Exclusion criteria were synchronous or metachronous bilateral renal cell carcinoma or a history of inherited Von Hippel-Lindau disease, neoadjuvant therapy, or adjuvant therapy. For the training and internal testing sets, data were obtained from 453 patients treated between Jan 1, 2004, and Dec 31, 2012, at the First Affiliated Hospital and Cancer Center of Sun Yat-sen University, Guangzhou, Guangdong, China. We used computer-generated random numbers to assign 227 of these patients to the training set and 226 patients to the internal testing set. Intratumour heterogeneity was investigated from two other morphologically distinct regions within the

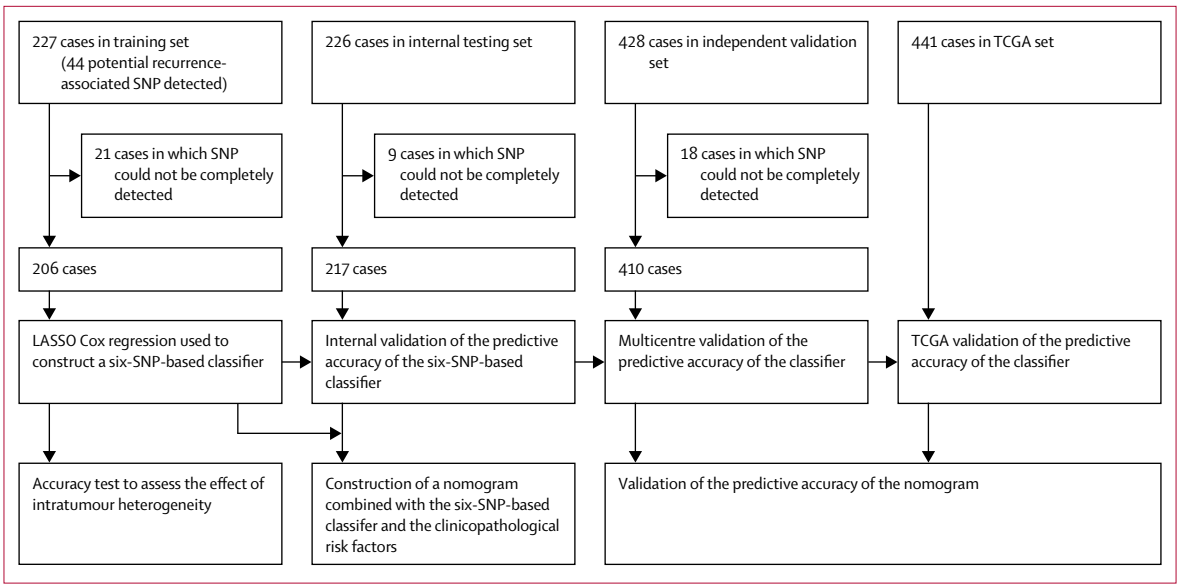

Figure 1: **Study design**
SNP=single-nucleotide polymorphism. TCGA=The Cancer Genome Atlas.

tumours in the training set (these two different regions were coded as region 2 and region 3). The independent validation set comprised 428 patients treated between Jan 1, 2004 and Dec 31, 2012, at three hospitals in China: Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai, Shandong, China (199 patients); Renji Hospital of Shanghai Jiao Tong University, Shanghai, China (113 patients); and Tongji Hospital of Huazhong University of Science and Technology, Wuhan, Hubei, China (116 patients). The TNM 2016 staging system was used to classify patients with clear cell renal cell carcinoma.[18] The histopathological grading system used in this study was based on the Fuhrman four-grade scale. A pathological re-review of all tumours was done by genitourinary pathologists at each institution where the surgery was done. The institutional review board at each participating institution approved the retrospective analysis of anonymous patient data.

For The Cancer Genome Atlas (TCGA) set, clinical data comprised 441 retrospectively identified patients who underwent resection between 1998 and 2010 from 13 medical centres in the USA for localised clear cell renal cell carcinoma. Clinical data and SNP data (the Affymetrix SNP 6.0 array) were downloaded from the Genomic Data Commons Data Portal and are in whole or in part based on data generated by the TCGA Research Network.

Procedures

For each sample of FFPE tumour tissue, subsequent sections were stained with haematoxylin and eosin for histological confirmation of the presence (>80%) of tumour cells. Four 15 μm tissue sections from each sample were used to obtain sufficient DNA. DNA was extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen; Hilden, Germany) following the manufacturer's instructions.

44 candidate SNPs, which are potentially related to renal cell carcinoma recurrence, were identified by exploratory bioinformatics analyses of a genome-wide association study from TCGA Kidney Renal Clear Cell Carcinoma (KIRC) dataset (n=114). The initial 906 600 SNPs were reduced to 438 662 by removing the following SNPs: those in the sex chromosomes, those with low minor allele frequency, and those with a high number of missing genotypes. Associations exceeding the genome-wide significance threshold ($p<10^{-4}$) were seen at 44 SNPs; details are described in the appendix (pp 2, 11). We examined these 44 candidate SNPs using time-of-flight mass spectrometry (MassARRAY system, Sequenom; San Diego, CA, USA) to analyse extracted DNA from the 227 FFPE samples in the training set (appendix pp 2–4). 19 of the 44 SNPs that had a call rate of less than 99% were excluded. The LASSO Cox regression model[19–21] was used to select the most useful prognostic markers among the rest of the 25 SNPs in 206 of 227 cases with complete SNP information and to construct an SNP-based classifier for prediction of recurrence-free survival (appendix pp 2, 5, 12). The SNP-based classifier comprised six of the 25 SNPs in the regions of the following genes: rs4718593 (in *LINC01372*), rs7934644 (in *OR8D2*), rs17050001 (in *SRGAP3*), rs9618567 (in *C22orf39*), rs7739947 (in *MARCKS*), and rs4479520 (in *HIRA*). To test whether intratumour heterogeneity affected the prediction accuracy of the six-SNP-based classifier, the classifier was assayed in the other two regions (region 2 and region 3) in the training set. To

See **Online** for appendix

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For the TCGA Research Network see https://cancergenome.nih.gov/

	Training set (n=206)	Internal testing set (n=217)	Independent validation set (n=410)	TCGA set (n=441)	Total (n=1274)
Sex					
Women	63 (31%)	72 (33%)	113 (28%)	164 (37%)	412 (32%)
Men	143 (69%)	145 (67%)	297 (72%)	277 (63%)	862 (68%)
Age, years					
<60	146 (71%)	143 (66%)	220 (54%)	204 (46%)	713 (56%)
≥60	60 (29%)	74 (34%)	190 (46%)	237 (54%)	561 (44%)
Pathological stage					
I	117 (57%)	122 (56%)	243 (59%)	262 (59%)	744 (58%)
II	52 (25%)	60 (28%)	102 (25%)	57 (13%)	271 (21%)
III	37 (18%)	35 (16%)	65 (16%)	122 (28%)	259 (20%)
Fuhrman grade					
G1	19 (9%)	25 (12%)	53 (13%)	14 (3%)	111 (9%)
G2	106 (52%)	110 (51%)	201 (49%)	212 (48%)	629 (49%)
G3	68 (33%)	66 (30%)	131 (32%)	169 (38%)	434 (34%)
G4	13 (6%)	16 (7%)	25 (6%)	38 (9%)	92 (7%)
Data not available	..	..	..	8 (2%)	8 (1%)
Tumour necrosis					
Absent	157 (76%)	164 (76%)	301 (73%)	236 (54%)	858 (67%)
Present	49 (24%)	53 (24%)	109 (27%)	174 (39%)	385 (30%)
Data not available	..	..	..	31 (7%)	31 (2%)
Median follow-up, months (IQR)	69 (54–93)	70 (52–91)	76 (62–98)	44 (22–66)	65 (43–87)
Recurrence-free survival (95% CI)					
3 years	83·0% (78·0–88·3)	80·5% (75·4–86·0)	84·3% (80·8–88·0)	85·7% (82·2–89·5)	83·8% (81·7–85·9)
5 years	76·6% (70·9–82·8)	74·9% (69·1–81·1)	76·2% (72·1–80·6)	80·6% (76·1–85·4)	77·1% (74·7–79·7)
7 years	73·2% (66·7–80·4)	70·1% (63·6–77·4)	70·5% (65·8–75·5)	75·0% (69·0–81·4)	72·0% (69·0–75·0)
Overall survival (95% CI)					
3 years	95·6% (92·9–98·5)	94·0% (90·8–97·2)	94·8% (92·7–97·0)	85·0% (81·4–88·7)	91·7% (90·2–93·3)
5 years	88·1% (83·6–92·8)	87·8% (83·4–92·4)	88·3% (85·1–91·5)	72·3% (67·3–77·6)	83·6% (81·4–85·8)
7 years	75·9% (69·0–83·6)	76·1% (69·5–83·2)	72·1% (67·2–77·4)	62·1% (55·6–69·3)	70·4% (67·3–73·7)

Data are n (%), unless otherwise specified. TCGA=The Cancer Genome Atlas. SNP=single-nucleotide polymorphism.

Table 1: **Baseline characteristics of patients by the six-SNP-based classifier assessment set**

estimate the reproducibility and validity of this classifier, we assayed the classifier in the internal testing set and the independent validation set. 217 of 226 cases in the internal testing set and 410 of 428 cases in the independent validation set were successfully detected. We tested the predictive accuracy of this classifier in these two sets (the internal testing set and the independent validation set) and in the TCGA set.

The study design is shown in figure 1. Clinical features of patients in the training, internal testing, independent validation, and TCGA sets are described in table 1.

Outcomes

The main outcome was recurrence-free survival, defined as the time from surgery to first renal cell carcinoma recurrence (local or distant metastases identified by imaging, biopsy, or physical examination). The secondary outcome was overall survival, defined as the time from surgery to death from any cause.

Statistical analysis

Biomarker profiling studies often use high-throughput assays to differentially screen biomarker profiles in small subgroups of patients, and then validate the findings by use of low-throughput methods in larger populations.[20,22] LASSO has been extended and broadly applied to the Cox proportional hazard regression model for survival analysis with small sample sizes and high-dimensional predictors.[19–21] In the training set, in which the sample size to SNP variables ratio was less than 10:1, the LASSO Cox regression model was used to construct an SNP-based classifier. The Kaplan-Meier method was used to analyse the correlation between variables and recurrence-free survival and overall survival, and the log-rank test was used to compare survival curves. The Cox regression model was used for multivariate survival analysis, and Cox regression coefficients were used to generate a nomogram. Calibration curves were used to assess whether actual outcomes approximately predicted

outcomes for the nomogram. Time-dependent receiver operating characteristic (ROC) curves and areas under the curves (AUCs) at 5 years were generated to assess prognostic accuracy.[23] Statistical tests were done with R software (version 3.5.0). Statistical significance was set at p values less than 0·05.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. J-HL had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

The distribution of the six-SNP status of 206 patients in the training set is shown in the appendix (p 13). Using the LASSO Cox regression models, we calculated a risk score for each patient based on the six-SNP status:

$$\begin{aligned}\text{risk score} = {} & (0{\cdot}1186 \times \text{rs4479520}) - (0{\cdot}0074 \times \text{rs4718593}) \\ & + (0{\cdot}0072 \times \text{rs9618567}) + (0{\cdot}0633 \times \text{rs7934644}) \\ & - (0{\cdot}2123 \times \text{rs7739947}) - (0{\cdot}1466 \times \text{rs17050001}) \\ & - 0{\cdot}1650\end{aligned}$$

The risk scores of the 206 patients in the training set ranged from –0·4914 to 0·2222 (appendix p 13). When we assessed the distribution of risk scores for the six-SNP-based classifier and recurrence status in the training set, patients with lower risk scores generally had lower recurrence than did patients with higher risk scores. We used a time-dependent ROC curve to describe the predictive value of the classifier and the six SNPs. The AUC at 5 years of the classifier was 0·749 (95% CI 0·660–0·826), and the AUC at 5 years of the six SNPs ranged from 0·558 to 0·631. The predictive accuracy of the classifier was significantly higher than that of any single SNP alone (appendix p 13).

Patients in the training set were divided into high-risk (n=103) and low-risk (n=103) groups, with the median risk score (of 0) as the cutoff. Compared with patients in the low-risk group, patients in the high-risk group had shorter recurrence-free survival (hazard ratio [HR] 6·78 [95% CI 3·17–14·49], p<0·0001; appendix p 13). Patients in the high-risk group also had shorter overall survival than did those in the low-risk group (HR 5·31 [95% CI 2·66–10·61]; p<0·0001; appendix p 16).

To ascertain whether or not intratumour heterogeneity affected the risk score based on the six-SNP-based classifier, the six-SNP status was measured in two other regions within the same tumours in the training set (region 2 and region 3). The distribution of the six-SNP status of 206 patients in the region 2 and region 3 groups is shown in appendix (p 13). Compared with the region 1 group, 48 (23%) of 206 cases had a different SNP status and different risk scores between different regions in the same tumour, which suggests that these cases were affected by intratumour heterogeneity. When patients were classified into high-risk and low-risk groups with the cutoff, 14 (7%) of 206 cases received a different risk assessment. The predictive accuracy of the classifier was tested in the region 2 and region 3 groups, as shown in the appendix (p 13). The AUCs at 5 years were 0·734 (95% CI 0·651–0·814) in the region 2 group and 0·736 (0·649–0·824) in the region 3 group, which was similar to the predictive accuracy of the region 1 group of the training set. Patients in the high-risk groups had shorter recurrence-free survival than patients in the low-risk groups in the region 2 and region 3 groups, as was the case in the region 1 group (HR 5·55 [95% CI 2·69–11·48] in region 2 and 5·93 [2·87–12·25] in region 3; appendix p 13). Therefore, intratumour heterogeneity did not seem to hamper the reliability of our classifier as a predictive model.

To investigate whether combining the risk score in the three regions would increase the predictive accuracy of the six-SNP-based classifier, the average risk score and the maximum risk score in the three regions were used to assess the classifier. When assessing the average risk score of the classifier, the AUC at 5 years was 0·742 (95% CI 0·661–0·823) and was therefore similar to that of the region 1, region 2, and region 3 groups (appendix p 14). When assessing the maximum risk score of the classifier in the three regions, the predictive accuracy of the classifier slightly increased (AUC at 5 years 0·757 [95% CI 0·676–0·835], appendix p 14).

To estimate the reproducibility and validity of this classifier, we tested the classifier in 217 cases of the internal testing set, in 410 cases of the independent validation set, and in 441 cases of the TCGA set. The distribution of the six-SNP status in the three sets is shown in the appendix (p 15). The risk score for each patient in the sets was calculated with the same formula as that used in the training set. The classifier achieved similar and stable predictive accuracy in the internal testing set (AUC at 5 years 0·739 [95% CI 0·666–0·813]), independent validation set (0·719 [0·659–0·780]), and TCGA set (0·746 [0·670–0·821]; appendix p 15). The sensitivity, specificity, and positive and negative predictive value of the six-SNP-based classifier are described in the appendix (p 6). Patients in these three sets (the internal testing set, the independent validation set, and TCGA set) were classified into high-risk and low-risk groups, with the same cutoff as that used in the training set. Patients in the high-risk groups had shorter recurrence-free survival (HRs ranging from 4·62 to 5·39; appendix p 15) and shorter overall survival (HR 1·93–3·83; appendix p 16) than did patients in the low-risk groups in all three sets.

After adjusting for clinical variables (age, sex, TNM stage, Fuhrman grade, and tumour necrosis status; table 2; appendix p 7) by multivariate Cox regression analysis, the six-SNP-based classifier remained an independent prognostic factor for predicting both

	Training set		Internal testing set		Independent validation set		TCGA set	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Sex (men *vs* women)	1·50 (0·79–2·88)	0·22	1·28 (0·72–2·27)	0·41	0·74 (0·50–1·10)	0·14	1·68 (1·00–2·81)	0·049
Age (≥60 years *vs* <60 years)	1·74 (0·99–3·06)	0·053	1·84 (1·09–3·10)	0·022	2·05 (1·40–3·00)	0·00021	1·38 (0·86–2·20)	0·18
Stage (III *vs* II *vs* I)	1·79 (1·29–2·49)	0·00053	1·81 (1·31–2·49)	0·00029	1·96 (1·57–2·45)	<0·0001	2·11 (1·63–2·73)	<0·0001
Fuhrman grade (G3 or 4 *vs* G1 or 2)	1·81 (1·04–3·14)	0·035	2·53 (1·50–4·28)	0·00052	2·32 (1·59–3·37)	<0·0001	2·17 (1·33–3·55)	0·0020
Tumour necrosis (present *vs* absent)	2·29 (1·30–4·02)	0·0041	2·00 (1·16–3·44)	0·013	3·82 (2·63–5·53)	<0·0001	3·34 (2·00–5·56)	<0·0001
Six-SNP-based classifier (high risk *vs* low risk)	6·78 (3·17–14·49)	<0·0001	5·32 (2·81–10·07)	<0·0001	5·39 (3·38–8·59)	<0·0001	4·62 (2·48–8·61)	<0·0001

TCGA=The Cancer Genome Atlas. HR=hazard ratio. SNP=single-nucleotide polymorphism.

Table 2: **Univariate association of the six-SNP-based classifier with recurrence-free survival in the four sets**

	Training set		Internal testing set		Independent validation set		TCGA set	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Sex (men *vs* women)	1·49 (0·77–2·89)	0·23	1·84 (1·01–3·33)	0·046	0·99 (0·66–1·49)	0·97	1·54 (0·89–2·66)	0·13
Age (≥60 years *vs* <60 years)	1·37 (0·76–2·48)	0·30	1·90 (1·11–3·24)	0·019	2·05 (1·40–3·01)	0·00022	1·11 (0·67–1·85)	0·68
Stage (III *vs* II *vs* I)	1·47 (1·03–2·11)	0·036	1·90 (1·33–2·69)	0·00036	1·67 (1·32–2·12)	<0·0001	1·81 (1·36–2·42)	<0·0001
Fuhrman grade (G3 or 4 *vs* G1 or 2)	1·06 (0·57–1·99)	0·85	1·25 (0·70–2·23)	0·45	1·91 (1·28–2·85)	0·0016	1·07 (0·63–1·83)	0·81
Tumour necrosis (present *vs* absent)	1·82 (0·98–3·40)	0·058	1·50 (0·84–2·69)	0·17	2·11 (1·41–3·15)	0·00028	2·07 (1·19–3·60)	0·0098
Six-SNP-based classifier (high *vs* low risk)	6·07 (2·81–13·12)	<0·0001	6·66 (3·40–13·03)	<0·0001	4·71 (2·89–7·66)	<0·0001	4·12 (2·12–8·00)	<0·0001

TCGA=The Cancer Genome Atlas. HR=hazard ratio. SNP=single-nucleotide polymorphism.

Table 3: **Multivariate Cox regression analysis of the six-SNP-based classifier with recurrence-free survival in the four sets**

recurrence-free survival (table 3) and overall survival (appendix p 8) in the training, internal testing, independent validation, and TCGA sets. When stratified by clinical factors (age, sex, TNM stage, Fuhrman grade, and tumour necrosis status), the six-SNP-based classifier was still a clinically and statistically significant prognostic model for prediction of recurrence-free survival (figure 2; appendix pp 9, 17, 18) and overall survival (appendix pp 19, 20). Indeed, the recurrence risk of the subgroup of the classifier-defined high risk in Fuhrman grade 1 or 2 was higher than the classifier-defined low risk in grade 3 or 4 (appendix p 17), and the recurrence risk of the subgroup of the classifier-defined high risk in stage I or II disease was higher than the classifier-defined low risk in stage III disease (appendix p 18). Thus, our findings suggest that the six-SNP-based classifier can provide predictive value that complements clinical prognostic features.

We constructed a nomogram that combined the six-SNP-based classifier with the clinicopathological risk factors (TNM stage, Fuhrman grade, and tumour necrosis status) to ascertain how the six-SNP-based classifier added prognostic value to the clinicopathological staging system and to provide clinicians with a quantitative method to predict the probability of recurrence in a patient with clear cell renal cell carcinoma. The nomogram was developed with data from 423 cases in the training and internal testing sets (developing set, appendix p 21), and the calibration curve of the nomogram showed good agreement between prediction and observation (appendix p 21), although the level of agreement did decrease in all three sets at higher survival probabilities. The AUC at 5 years of this nomogram in the developing set was 0·811 (95% CI 0·756–0·861), and the AUCs at 5 years of the classifier or clinicopathological risk factors ranged from 0·605 to 0·743 in the developing set. The predictive accuracy of the nomogram was significantly higher than that of the classifier or clinicopathological risk factors alone in the developing set (figure 3). 410 cases in the independent validation set and 441 in the TCGA set were used to validate the predictive accuracy of the nomogram; the AUC at 5 years was 0·776 (95% CI 0·717–0·835) in the independent validation set and 0·808 (0·727–0·889) in the TCGA set, and both AUCs were also better at predicting recurrence probabilities than the classifier or clinicopathological risk factors alone (figure 3). Furthermore, the addition of the classifier to the clinical model combining TNM stage, grade, and necrosis significantly improved the accuracy of predicting tumour recurrence (figure 3; appendix p 10). The predictive

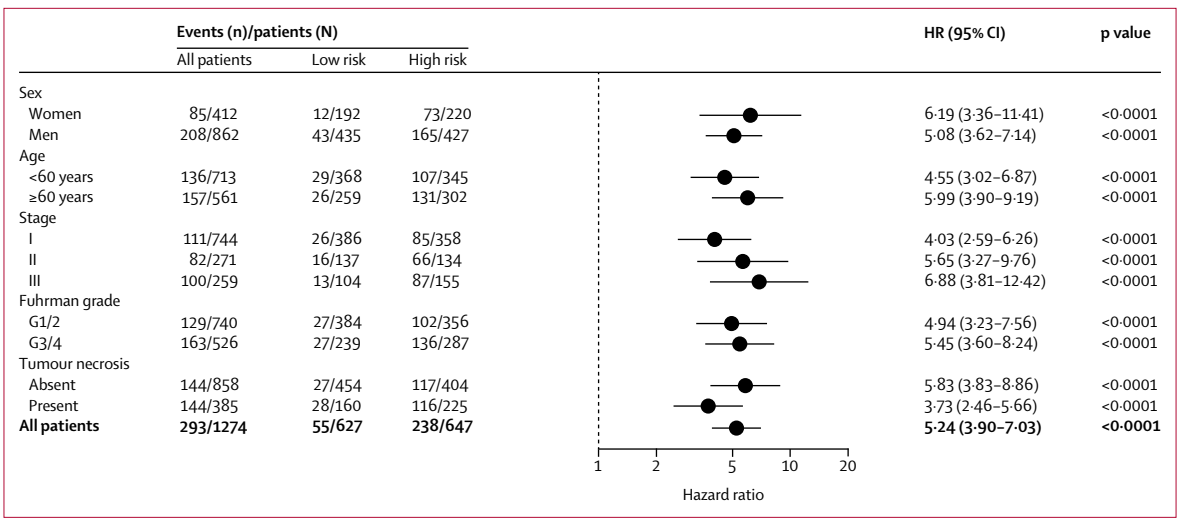

Figure 2: **Hazard ratio of tumour recurrence for all 1274 patients with localised clear cell renal cell carcinoma according to the six-SNP-based classifier in different subgroups stratified by clinical parameters**
SNP=single-nucleotide polymorphism.

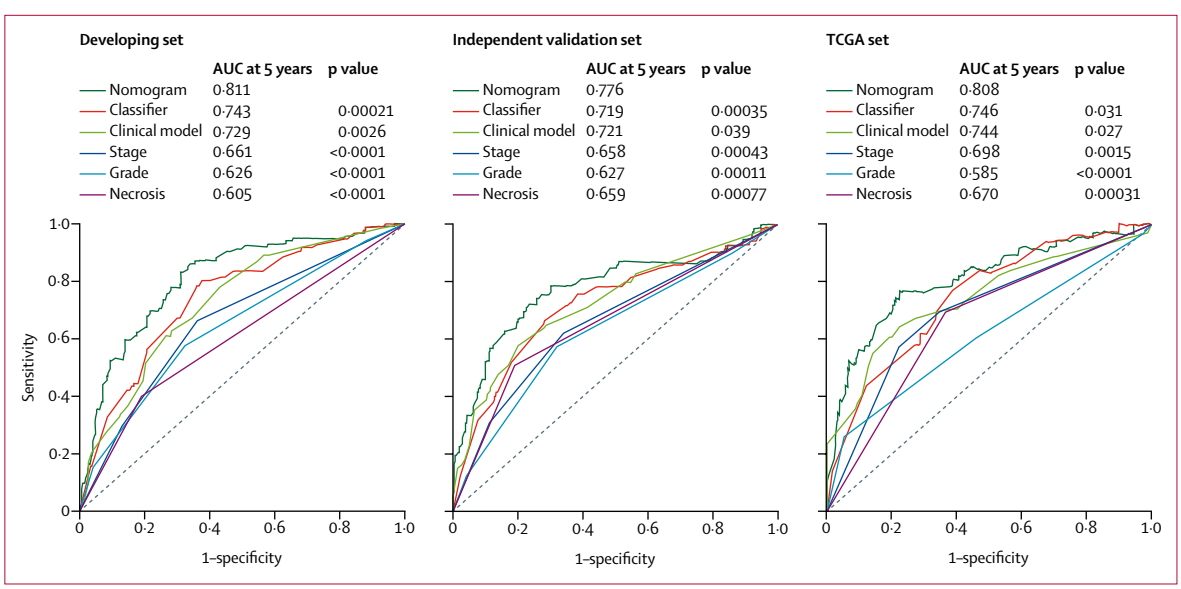

Figure 3: **Validation of a nomogram that combines the six-SNP-based classifier and tumour stage, grade, and necrosis to predict recurrence-free survival**
Time-dependent ROC curves and AUCs at 5 years were used to assess the prognostic accuracy of the nomogram, compared with the six-SNP-based classifier and tumour stage, grade, and necrosis. p values show the AUC at 5 years for the nomogram versus the AUC at 5 years for the classifier or other variables alone. The nomogram itself and its calibration are in the appendix (p 21). AUC=area under the curve. ROC=receiver operating characteristic. SNP=single-nucleotide polymorphism. TCGA=The Cancer Genome Atlas.

accuracy of combining the classifier and the Leibovich Score[24] was also significantly higher than the predictive accuracy of the classifier or the Leibovich Score alone; details are described in the appendix (p 22).

Discussion

In this retrospective analysis and multicentre validation study, we developed a six-SNP-based classifier to complement the existing staging system for prediction of localised renal cell carcinoma recurrence, which can enable physicians to make more informed treatment decisions about adjuvant therapy. Clinical trials of adjuvant targeted therapy in patients with localised renal cell carcinoma define stage III as high risk; however, our results show that the recurrence risk of the subgroup of the classifier-defined high risk in stage I or II disease was higher than the classifier-defined low risk in stage III (appendix p 18). Our results also show that the recurrence risk of the subgroup of the classifier-defined high risk in Fuhrman grade 1 or 2 was higher than the classifier-defined low risk in grade 3 or 4 (appendix p 17). The addition of the classifier to the nomogram combining

TNM stage, grade, and necrosis can significantly improve the predictive accuracy of tumour recurrence. The coefficient for the SNP classifier in the nomogram was higher than the coefficients for clinical factors of stage, Fuhrman grade, and necrosis (appendix p 21). This means that the SNP classifier has a higher point-increase for a one-unit increase than the clinical factors of stage, Fuhrman grade, and necrosis. Although the SNP classifier provides a wide range of points in the dataset, the SNP classifier varies by less than one unit across the four sets.

Several FFPE-based mRNA signatures, such as ClearCode34, the 16-gene assay, and the cell cycle proliferation score, have been developed to increase the predictive accuracy of recurrence in localised clear cell renal cell carcinoma.[10–13] These mRNA expression-based models incorporated 16–46 biomarkers detected by real-time PCR. Clinical translation of the multi-biomarker predictive models requires an understanding of factors that affect the precision and accuracy of high-throughput-based assays. Chief among these factors is the variability of biomarker measurements, which can be divided into technical (intrinsic to the platform) and pre-analytical (intrinsic to the sample) sources of variation.

mRNA expression would be influenced by tissue-specific variability with the RT-PCR assay. Several reference genes were introduced to control for tissue-specific variability, and mRNA expression was assessed by relative quantitation by normalising to reference genes.[10] The risk score formulas and threshold values of these mRNA signatures, which were developed by RT-PCR, are not suitable for validation by other types of measurement data (such as microarray data or RNA-sequencing data).[7] In this study, we developed a six-SNP-based classifier by use of time-of-flight mass spectrometry to predict recurrence of localised clear cell renal cell carcinoma after surgery. Each of the included SNPs has only three allelic variants represented as a rank variable. The composition of these SNPs is not influenced by tissue type and does not require adjustment on the basis of any other biomarker. The risk score formulas and threshold value of our six-SNP-based signature are suitable for validation with other tumour analysis approaches, including SNP microarray and DNA sequencing. Therefore, our six-SNP-based classifier is not influenced by the technical sources of variation with different platforms in different centres.

When solid tumour tissue samples are used to detect the expression of biomarkers, we have to consider whether intratumour heterogeneity would affect the predictive accuracy of these biomarkers, because biomarker expression can vary across different tumour regions.[6–9] Some previous studies have assessed the influence of intratumour heterogeneity in the multi-biomarker predictive model of clear cell renal cell carcinoma. Rini and colleagues[10] developed a 16-gene assay for use with FFPE tumour tissue samples to predict recurrence in localised clear cell renal cell carcinoma. To address concerns about intratumour heterogeneity, they focused on samples from eight patients for whom multiple regional tumour samples were available, and ascertained that, for one (12·5%) of the patients, the 16-gene profile assigned different risk ranks on the basis of intratumour heterogeneity. Similarly, Brooks and colleagues[13] used their 34-gene classifier (ClearCode34) to assign clear cell renal cell carcinoma tumours to subtypes (high risk and low risk) and found that two (20%) of ten patients with clear cell renal cell carcinoma would have received differential classification based on intratumour heterogeneity. In previous studies, small sample sizes were used to assess intratumour heterogeneity and indicated that intratumour heterogeneity would affect the predictive accuracy of multi-biomarker models, but no studies have systematically investigated whether or not this is the case. In our study, we used an accuracy test to investigate how intratumour heterogeneity influences the precision of biomarkers. We assayed the six SNPs in three different regions of tumour samples with the whole training set. Although 48 (23%) of 206 cases had different risk scores in different regions, the predictive accuracy of the classifier in all three regions was similar (AUCs at 5 years were 0·749, 0·734, and 0·736). Intratumour heterogeneity did not seem to hamper the accuracy of our classifier as a reliable predictive model. To our knowledge, our study is the first to show the effect of intratumour heterogeneity on multi-biomarker model precision with an accuracy test done in the whole cohort.

The six SNPs were in the regions of the genes *LINC01372* (7q11·22), *OR8D2* (11q24·2), *SRGAP3* (3p25·3), *C22orf39* (22q11·21), *MARCKS* (6q21), and *HIRA* (22q11·21). According to the information about chromosomal deletions and amplifications in the TCGA dataset,[25] the six SNPs are not located in the deletion frequency or amplification frequency chromosome bands. The HIRA protein is required for deposition of histone H3·3 at genes and enhancers[26] and for recruitment and function of polycomb complexes at gene promoters and dynamic restoration of chromatin after DNA damage repair.[27] Knockout of *HIRA* in mice substantially enhanced oncogene-induced hyperplastic cell proliferation.[28] srGAP3, a member of the Slit–Robo subfamily of Rho GTPase-activating proteins, controls actin and microtubule dynamics through negative regulation of Rac. It has been also reported to be a tumour suppressor in mammary epithelial cells.[29] Myristoylated alanine-rich C-kinase substrate (MARCKS), a substrate for protein kinase C, is localised in the plasma membrane and is an actin filament cross-linking protein.[30] Many studies have shown the implication of MARCKS in cancer aggressiveness, particularly the metastatic process and therapeutic resistance.[31–33] OR8D2 has been reported to be a predictor of recurrence risk and prognosis for patients with colon cancer.[34] C22orf39 is downregulated by MYC induction in porcine liver cancer stem cells and is related

to the carcinogenesis of hepatocellular carcinoma.[35] To our knowledge, no studies have been done to investigate the function of the *LINC01372* gene.

The generalisability of our retrospective study is limited because it only included patients from China and the USA. In the future, new systemic treatment options might lead to smaller differences in overall survival between high-risk and low-risk patients. Furthermore, our multivariable models did not include microvessel invasion or a sarcomatoid carcinoma component, because this information was not provided in the TCGA dataset and was not collected consistently in our study. Our results need to be further validated in prospective studies with larger cohorts and in other populations. Additionally, we selected the cutoff of the classifier risk score on the basis of results from the Asian population. When using this cutoff in non-Asian populations, the positive predictive value of the classifier was lower than in the Asian population (appendix p 6). This observation suggests that, in future studies, it might be reasonable to choose a more suitable cutoff of the classifier risk score for non-Asian populations.

In summary, our six-SNP-based classifier seems to be a practical and reliable prognostic tool for localised clear cell renal cell carcinoma. It can provide prognostic value to complement the existing staging system for predicting recurrence after surgery, which can enable more informed treatment decisions about adjuvant systemic therapy. The predictive accuracy of our six-SNP-based classifier seems to be stable and is not influenced by intratumour heterogeneity sources of variation. The classifier has the potential to guide treatment decisions for patients at differing risks of recurrence.

Contributors

J-HL designed the study. Z-HF, H-WZ, Z-HC, QW, HH, JZ, Y-ZX, B Li, J-TW, WX, JL, Z-LZ, H-HY, Y-HP, LS, Z-LG, Y-RH, F-JZ, S-GW, Z-PL, and WC obtained and assembled data. J-HW, YC, B Liao, G-MQ, G-PW, CL, QL, C-XL, P-XL, W-FC, DX, and J-HL analysed and interpreted the data. J-HW and J-HL wrote the report, which was edited by all authors, who have approved the final version.

Declaration of interests

We declare no competing interests.

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