Expression of potential molecular markers in renal cell carcinoma: impact on clinicopathological outcomes in patients undergoing radical nephrectomy

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OBJECTIVES
To evaluate the expression levels of several potential molecular markers in renal cell carcinoma (RCC), to clarify the significance of these markers as prognostic predictors in patients undergoing radical nephrectomy (RN).

PATIENTS AND METHODS
The study included 153 patients with clinically organ-confined RCC undergoing RN. Expression levels of 12 proteins, including Aurora-A, Bcl-2, Bcl-xL, clusterin, heat-shock protein 27 (HSP27), HSP70, HSP90, Ki-67, matrix metalloproteinase (MMP)-2 and -9, p53 and vascular endothelial growth factor, in RN specimens obtained from these 153 patients were measured by immunohistochemical staining.

RESULTS
Of the 12 markers, clusterin, HSP27, Ki-67, MMP-2 and -9 expression were significantly associated with several conventional prognostic factors. Univariate analysis also identified these five markers as significant predictors of disease recurrence, while mode of presentation, pathological stage, grade and microvascular invasion were also significant. Of these significant factors, Ki-67 expression, pathological stage and microvascular invasion appeared to be independently related to disease recurrence. Furthermore, there were significant differences in recurrence-free survival according to positive numbers of these three independent factors, i.e. disease recurred in four of 56 patients who were negative for risk factors (7%), 17 of 71 positive for one risk factor (24%), and 20 of 26 positive for two or three risk factors (77%).

CONCLUSIONS
Combined evaluation of Ki-67 expression, pathological stage and microvascular invasion would be particularly useful for further refinement of the system for predicting the outcome after RN for patients with RCC.

KEYWORDS
renal cell carcinoma, molecular marker, radical nephrectomy, prognosis

INTRODUCTION
Radical nephrectomy (RN) has been regarded as an effective therapy for patients with clinically localized RCC, but 20–30% of these patients have a local and/or distant disease recurrence [1]. Thus intensive efforts have been made to identify variables that precisely predict the outcome of RN, and which are of great utility in planning both postoperative therapy and the follow-up schedule in an individual patient.

To date, various factors, including pathological stage, tumour grade, histological subtype, microvascular invasion (MVI) and inflammatory response, have been shown to be significantly associated with treatment failure after RN [2,3]. Furthermore, several investigators have shown the usefulness of nomograms in calculating the probability of disease recurrence after surgical resection of localized RCC [4,5]. However, RCC has been shown to be characterized by unique biological features and a heterogeneous genetic background. Indeed, RCCs with similar clinicopathological factors can show different biological behaviour and result in different clinical outcomes [1]. These findings suggest the limitations for predicting outcomes after RN in patients with localized RCC using conventional variables alone.

In an attempt to provide more reliable prognostic information in patients with RCC, several studies examined the value of a wide variety of molecular markers, and some of these molecules were shown to be significantly related to treatment failure [2,3,6–10]. For example, Kim et al. [6] reported that carbonic anhydrase-9, PTEN, vimentin and p53 could be used as independent predictors of disease-specific survival in patients with metastatic RCC who had RN before immunotherapy. However, there have been a few studies evaluating the prognostic significance of multiple molecular markers in localized rather than metastatic RCC. Accordingly, in the present study, we evaluated the expression levels of 12 potential molecular markers, including Aurora-A, Bcl-2, Bcl-xL, clusterin, heat-shock protein 27 (HSP27), HSP70, HSP90, Ki-67, matrix metalloproteinase (MMP)-2 and -9, p53 and vascular endothelial growth factor (VEGF), in RN specimens with immunohistochemical staining, and analysed these outcomes.
MOLECULAR MARKERS IN RCC AND OUTCOMES AFTER RADICAL NEPHRECTOMY

The characteristics of 153 patients with RCC who had RN

| Variable                      | Median (range) or n (%) |
|-------------------------------|-------------------------|
| Age, years                    | 60.5 (30–87)            |
| Male/female                   | 93 (60.8)/60 (39.2)     |
| Mode of presentation          |                         |
| Incidental                    | 109 (71.2)              |
| Symptomatic                   | 44 (28.8)               |
| Pathological stage            |                         |
| pT1                           | 101 (66.0)              |
| pT2                           | 29 (18.9)               |
| pT3                           | 20 (13.1)               |
| pT4                           | 3 (2.0)                 |
| Grade                         |                         |
| 1                             | 80 (52.3)               |
| 2                             | 67 (43.8)               |
| 3                             | 6 (3.9)                 |
| MVI                           |                         |
| Negative                      | 88 (57.5)               |
| Positive                      | 65 (42.5)               |
| Histological subtype          |                         |
| Clear cell                    | 132 (86.3)              |
| Not clear cell                | 21 (13.7)               |

FIG. 1. RFS of 153 patients with clinically localized RCC after RN.

Median (range) duration of follow-up after RN of this series was 69 (39–117) months. Generally, all patients were followed by laboratory and radiological evaluations, and a physical examination, every 6 months to monitor recurrence and metastasis. In the absence of a relapse of RCC by 5 years after surgery, the interval between re-examinations was increased. Informed consent for this study was obtained from all of these patients, and the study design was approved by the Research Ethics Committee of our institution.

The RN specimens were stained immunohistochemically as previously described [11]. Briefly, sections from formaldehyde-fixed, paraffin-embedded tissue from the 153 specimens were deparaffinized by xylene and rehydrated in decreasing concentrations of ethanol. After blocking endogenous peroxidase with 3% hydrogen peroxidase in methanol, sections were boiled on 0.01 M citrate buffer for 20 min and incubated with 5% normal blocking serum in Tris-buffered saline for 20 min. The sections were then incubated with the following antibodies: anti-human Aurora-A rabbit polyclonal antibody (Abcam, Cambridge, UK), anti-human Bcl-2 mouse monoclonal antibody (Dako, Carpinteria, CA, USA), anti-human Bcl-xl mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human clusterin rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human HSP27 mouse monoclonal antibodies (Novocastra Laboratories, Newcastle, UK), anti-human HSP70 mouse monoclonal antibodies (Novocastra), anti-human Ki-67 mouse monoclonal antibody (Novocastra), anti-human Ki-67 mouse monoclonal antibody (Dako), anti-human MMP-2 mouse monoclonal antibody (Daichi Fine Chemical, Takaoka, Japan), anti-human MMP-9 mouse monoclonal antibody (Daichi Fine Chemical), anti-human p53 mouse monoclonal antibody (Novocastra) and anti-human VEGF rabbit polyclonal antibody (Santa Cruz). The sections were then incubated with biotinylated goat antimouse or rabbit IgG (Vector Laboratories, Burlingame, CA, USA). After incubation in an avidin–biotin peroxidase complex for 30 min, the samples were exposed to diaminobenzidine tetrahydrochloride solution and counterstained with haematoxylin.

Staining results were interpreted by two independent observers who were unaware of the clinicalopathological data. For Ki-67 and p53 analyses, only nuclear staining was considered, and strong expression of Ki-67 and p53 was defined as the proportion of positively stained tumour cells >5% and >20%, respectively, as previously reported [12]. The highest intensity of immunohistochemical staining for the remaining 10 proteins was visually scored from several fields of each section and classified as negative, weak, moderate and strong. According to the previous study [13], either moderate or strong staining intensity in >10% of tumour cells was considered as strong expression.

The chi-squared test was used to analyse the association between several clinical-pathological factors and expression levels of molecular markers. The recurrence-free survival (RFS) rates were calculated by the Kaplan–Meier method, and differences were determined using the log-rank test. The prognostic significance of certain factors was assessed by the Cox proportional hazards regression model. In all tests, P < 0.05 was considered to indicate significance.

RESULTS

The characteristics of the 153 patients included in the study are shown in Table 1. The association of the clinical-pathological factors and expression levels of the 12 potential molecular markers in RN specimens was then analysed. As shown in Table 2, clusterin, HSP27, Ki-67, MMP-2 and MMP-9 expression were significantly associated with several conventional prognostic variables, whereas expression levels of the remaining seven proteins showed significant correlation with either no or limited prognostic variables.

During the observation period of the study, 41 of the 153 patients (26.8%) developed disease recurrence, and the 1-, 3- and 5-year RFS rates were 94.8%, 79.0% and 74.2%, respectively (Fig. 1). By univariate analysis using the Cox proportional hazards regression model, expression levels of clusterin, HSP27, Ki-67, MMP-2 and MMP-9 were identified as significant factors associated with RFS, while mode of presentation, pathological stage, grade and MVI were also significant among several clinical-pathological factors examined (Table 3). Furthermore, multivariate analysis of nine significant factors determined by the univariate analysis was used to evaluate the predictive value for disease recurrence, and
### TABLE 2 Correlation of expression levels of molecular markers in RN specimens with several clinicopathological factors

| Factor                        | Marker                                      | Total patients | Age, years | Gender | Mode of presentation | Pathological stage | Grade | MVI | Histological subtype |
|-------------------------------|---------------------------------------------|----------------|------------|--------|----------------------|-------------------|-------|-----|----------------------|
|                               | Aurora-A | Bcl-2 | Bcl-xL | Clusterin | HSP27 | HSP70 | HSP90 | Ki-67 | MMP-2 | MMP-9 | VEGF | p53 |
| Age, years                    |          |       |        |          |       |       |       |       |       |       |      |     |
| <60                           | 21       | 57    | 56     | 42       | 37    | 55    | 53    | 42    | 39    | 36    | 59   | 26  |
| ≥60                           | 20       | 56    | 54     | 38       | 33    | 53    | 50    | 46    | 43    | 41    | 58   | 25  |
| P                             | 0.82     | 0.75  | 0.62   | 0.46     | 0.52  | 0.63  | 0.53  | 0.58  | 0.57  | 0.53  | 0.74 | 0.82 |
| Gender                        |          |       |        |          |       |       |       |       |       |       |      |     |
| Male                          | 28       | 67    | 64     | 48       | 42    | 64    | 61    | 50    | 47    | 44    | 70   | 32  |
| Female                        | 13       | 46    | 46     | 32       | 28    | 44    | 42    | 38    | 35    | 33    | 47   | 19  |
| P                             | 0.25     | 0.96  | 0.29   | 0.84     | 0.86  | 0.55  | 0.57  | 0.24  | 0.35  | 0.35  | 0.66 | 0.73 |
| Mode of presentation          |          |       |        |          |       |       |       |       |       |       |      |     |
| Incidental                    | 28       | 85    | 76     | 65       | 56    | 75    | 74    | 75    | 70    | 62    | 87   | 34  |
| Symptomatic                   | 13       | 28    | 34     | 15       | 14    | 33    | 29    | 13    | 12    | 15    | 30   | 17  |
| P                             | 0.63     | 0.068 | 0.35   | 0.004    | 0.028 | 0.45  | 0.81  | <0.001<0.001<0.001<0.001<0.001 | 0.12 | 0.38 |
| Pathological stage            |          |       |        |          |       |       |       |       |       |       |      |     |
| pT1                           | 31       | 72    | 71     | 61       | 54    | 72    | 69    | 69    | 66    | 61    | 75   | 35  |
| ≥ pT2                         | 10       | 41    | 39     | 19       | 16    | 36    | 34    | 19    | 16    | 16    | 42   | 16  |
| P                             | 0.13     | 0.31  | 0.54   | 0.005    | 0.008 | 0.79  | 0.71  | <0.001<0.001<0.001<0.001<0.001 | 0.37 | 0.63 |
| Grade                         |          |       |        |          |       |       |       |       |       |       |      |     |
| 1                             | 27       | 60    | 55     | 50       | 42    | 53    | 51    | 55    | 52    | 48    | 62   | 32  |
| 2 or 3                        | 14       | 53    | 55     | 30       | 27    | 55    | 52    | 33    | 30    | 29    | 55   | 19  |
| P                             | 0.042    | 0.74  | 0.36   | 0.008    | 0.038 | 0.22  | 0.97  | 0.003 | 0.003 | 0.012 | 0.75 | 0.067 |
| MVI                            |          |       |        |          |       |       |       |       |       |       |      |     |
| −ve                           | 23       | 64    | 63     | 55       | 47    | 62    | 63    | 67    | 63    | 59    | 67   | 28  |
| +ve                           | 18       | 49    | 47     | 25       | 23    | 46    | 40    | 21    | 19    | 18    | 50   | 23  |
| P                             | 0.83     | 0.71  | 0.92   | 0.003    | 0.027 | 0.97  | 0.19  | <0.001<0.001<0.001<0.001<0.001 | 0.91 | 0.64 |
| Histological subtype          |          |       |        |          |       |       |       |       |       |       |      |     |
| Clear cell                    | 36       | 96    | 96     | 70       | 63    | 93    | 89    | 76    | 70    | 66    | 100  | 45  |
| Not clear cell                | 5        | 17    | 14     | 10       | 7     | 15    | 14    | 12    | 12    | 11    | 17   | 6   |
| P                             | 0.74     | 0.43  | 0.57   | 0.64     | 0.22  | 0.93  | 0.95  | 0.97  | 0.73  | 0.84  | 0.60 | 0.62 |

Values are expressed as the number of patients with strong expression of each molecule.

### TABLE 3 Univariate and multivariate analyses of association between several variables with RFS in 153 patients with RCC who had RN

| Variable                        | N patients (n with disease recurrence) | P Univariate | Multivariate |
|---------------------------------|----------------------------------------|--------------|--------------|
| Age, years (<60 vs ≥60)         | 76 (19) vs 77 (22)                     | 0.680        | –            |
| Gender (male vs female)         | 93 (27) vs 60 (14)                     | 0.420        | –            |
| Mode of presentation (incidental vs symptomatic) | 109 (21) vs 44 (20) | 0.036 | 0.230 |
| Pathological stage (pT1 vs ≥pT2) | 101 (19) vs 52 (22) | 0.002 | 0.042 |
| Grade (1 vs 2 or 3)             | 80 (14) vs 73 (27)                     | 0.018        | 0.330        |
| MVI (negative vs positive)      | 88 (12) vs 65 (29)                     | <0.001       | 0.018        |
| Histological subtype (clear cell vs not) | 132 (36) vs 21 (5) | 0.630 | – |
| Weak expression vs strong expression for: | | |
| Aurora-A                       | 41 (12) vs 112 (29)                    | 0.140        | –            |
| Bcl-2                          | 113 (30) vs 40 (11)                    | 0.330        | –            |
| Bcl-xL                         | 110 (31) vs 43 (10)                    | 0.480        | –            |
| Clusterin                      | 80 (14) vs 73 (27)                     | 0.030        | 0.180        |
| HSP27                          | 70 (12) vs 83 (29)                     | 0.037        | 0.370        |
| HSP70                          | 108 (29) vs 45 (12)                    | 0.260        | –            |
| HSP90                          | 103 (27) vs 50 (14)                    | 0.550        | –            |
| Ki-67                          | 88 (17) vs 65 (24)                     | <0.001       | 0.037        |
| MMP-2                          | 82 (17) vs 71 (24)                     | 0.019        | 0.089        |
| MMP-9                          | 77 (14) vs 76 (27)                     | 0.029        | 0.360        |
| p53                            | 117 (31) vs 36 (10)                    | 0.310        | –            |
| VEGF                           | 51 (17) vs 102 (24)                    | 0.280        | –            |
showed that Ki-67 expression level, pathological stage and MVI were independently associated with disease recurrence irrespective of other factors included (Table 3). RFS curves according to Ki-67 expression level, pathological stage and MVI are shown in Fig. 2. There were significant differences in RFS among these three groups.

To more precisely characterize the prognostic features after RN we categorized patients according to positive numbers of three independent risk factors for disease recurrence, including Ki-67 expression level, pathological stage and MVI. Disease recurrence occurred in four of 56 patients who had no risk factor (71%), 17 of 71 positive for one risk factor (24%), and 20 of 26 positive for two or three risk factors (77%). As shown in Fig. 3, there were significant differences in RFS among these three groups.

**DISCUSSION**

A precise prediction of postoperative prognosis in patients undergoing surgical resection for malignant tumours, using a worldwide consensus, would be important for physicians to make decisions about the follow-up schedule and additional treatment, which is particularly true in patients with RCC because of its highly resistant phenotype to conventional non-surgical treatments [1]. Historically, staging according to the TNM classification system has been the standard for stratifying patients into risk groups for prognosis; however, recent studies indicate that stage alone is not reliable for predicting disease recurrence in patients with localized RCC [2,3,14]. Consequently, several molecular markers, in addition to conventional variables, have been investigated as prognostic indicators in RCC. Although some of these systems were of some use in predicting postoperative clinical outcomes in patients with RCC [2,3,6–10], there have been no systems widely introduced into clinical practice, due to several limitations. In the present study therefore we analysed the values of conventional clinicopathological prognostic factors and several potential molecular markers for predicting the clinical outcome in 153 patients undergoing RN for clinically localized RCC.

There were limitations to this study; a sample size of 153 patients in such a comparatively common disease like RCC is not large enough. In addition, although the study included 12 proteins as potential molecular markers for RCC, these 12 had not been selected based on scientifically objective criteria. Therefore, there might be other molecules more closely associated with the prognostic outcome in RCC than these 12 molecules. However, most previous studies focused on the significance of either one or a few molecular markers in each series [2,3,6–10], and some of them used tissue microarrays, which do not always provide representative information in an individual case [6,7]. Collectively, these findings suggest the advantages of the present study, simultaneously investigating 12 molecular markers using immunohistochemical staining of the RCC specimen from each patient.

Expression of the 12 molecular markers investigated in this study was detected in most of RCC tissues. However, there were varied patterns in the relation between the expression level of each molecule and the conventional prognostic variables, i.e. expression levels of clusterin, HSP27, Ki-67, MMP-2 and MMP-9 had a significant effect on these variables, whereas expression levels of the remaining seven proteins showed a significant association with either no or limited prognostic factors. However, the significance of these seven proteins in RCC progression cannot be completely denied based on the present findings. For example, Aurora-A has been shown to be widely expressed in various types of malignant disease, irrespective of the degree of disease progression, and thus regarded as being involved in the initiation rather than the progression of malignant diseases [15]. There was strong expression of Aurora-A protein in

**FIG. 2.** RFS of patients with clinically localized RCC after RN, according to expression level of Ki-67 (A), pathological stage (B) and MVI (C).

**FIG. 3.** RFS of patients with clinically localized RCC after RN according to the number of independent risk factors for disease recurrence, including strong Ki-67 expression, advanced pathological stage (i.e. ≥pT2) and MVI.
73.2% of RCC specimens evaluated in the present series. Accordingly, it would be necessary to interpret the findings of immunohistochemical staining considering the functional characteristics of evaluated molecular markers.

The effect of conventional prognostic variables and potential molecular markers on RFS after RN were subsequently compared. Univariate analysis showed that mode of presentation, pathological stage, grade and MVI, expression levels of clusterin, HSP27, Ki-67, MMP-2 and -9 were significantly associated with disease recurrence; however, only the expression level of Ki-67, pathological stage and MVI were independent prognostic predictors by multivariate analysis. Pathological stage and MVI are well established prognostic factors after RN [2,5,16], and the usefulness of the Ki-67 expression level as a prognostic marker has been also confirmed in previous studies [6–9]. Therefore, it is interesting to evaluate the ability to predict disease recurrence after RN on combining these three independent risk factors, as using an approach similar to that in the present study, Dall’Oglio et al. [17] reported that risk assessment and stratification based on MVI, tumour size and Fuhrman grade in RN specimens might allow better individualization of follow-up and treatment schedules for patients with RCC. In the present study, when the 153 patients were classified into the three groups of no risk factors, positive for one and positive for two or three, there were significant differences in RFS among these groups. These findings suggest that consideration of the three main risk factors identified by multivariate analysis (i.e. Ki-67 expression level, pathological stage and MVI) might contribute to developing a novel system that can more precisely predict disease recurrence after RN.

In the present series, we investigated the significance of several conventional prognostic variables and expression levels of several potential molecular markers in RN specimens simultaneously, as predictors of disease recurrence for clinically localized RCC, and expression levels of Ki-67, pathological stage and MVI were identified as independent prognostic predictors after RN. Moreover, it has been suggested that the utility of the combined use of these three factors could contribute to further refinement of this predictive system. However, a prospective study investigating additional potential molecular markers involved in the progression of RCC will be necessary before a definitive conclusion can be drawn.

CONFLICT OF INTEREST

None declared.

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Abbreviations: RN, radical nephrectomy; HSP, heat-shock protein; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; MVI, microvascular invasion; RFS, recurrence-free survival.