INTRODUCTION

RCC accounts for ≈3% of adult cancers and constitutes 80–90% of all primary malignant renal tumours [1]. To date, tumour stage and nuclear grade were considered the most important prognostic variables for patients with RCC [2,3] but in many cases they are insufficient to predict the clinical behaviour of these tumours.

Tumour cell proliferation and angiogenesis are necessary for tumour growth and metastasis, in part resulting in the colonization of malignant cells by vessels generated from host tissues [4,5]. The first step in the distant metastasis of a tumour is venous invasion by cancer cells from the primary tumour [6–8]. CD44 is a transmembrane glycoprotein involved in cell-cell and cell-matrix interactions; it represents a large family of cell-adhesion molecules, which differ mainly in primary structure, with a predominant form (CD44H, standard or haematopoietic form) and several variant isoforms. The standard form CD44H is the main receptor for hyaluronate. CD44H and variants have been investigated in various tumours [9–13]. This molecule, which allows tumour cells to bind to high endothelial venules, may be important in tumour dissemination [5,14].

Vascular endothelial growth factor (VEGF) is an angiogenic factor that may be involved in tumour growth and metastasis. Although the physiological relevance of VEGF overexpression in tumoral angiogenesis is well accepted, its potential prognostic value is debated [4,15,16].

In this study, we retrospectively evaluated the prognostic value of Ki-67 labelling index (LI), CD44H LI, VEGF expression level and microvessel invasion (MVI) in RCCs, and correlated them with the usual prognostic variables (tumour size, stage and nuclear grade) and cancer-specific survival.

OBJECTIVE

To determine if use of cell proliferation, cell adhesion, level of angiogenesis-related factors and presence of microscopic vascular invasion (MVI) could better predict the biological behaviour of renal cell carcinoma (RCC), which has a widely variable clinical outcome despite the use of conventional prognostic factors (staging and grading).

MATERIALS AND METHODS

The expression of Ki-67, CD44H and vascular endothelial growth factor (VEGF) were assessed immunohistochemically in formalin-fixed, paraffin-embedded tissues from 48 RCCs, using a Ki-67 labelling index (LI), CD44H LI and level of VEGF expression, respectively. In addition all the pathological slides were reviewed retrospectively for the presence and absence of MVI. The prognostic value of all the variables assessed was then evaluated, and correlated with the usual prognostic variables and cancer-specific survival.

RESULTS

Univariate analysis of cancer-specific survival showed that tumour stage (P < 0.001), tumour size (P = 0.005), metastasis, MVI, Ki-67 LI, CD44H LI and VEGF expression (all P < 0.001) were predictors of tumour-related death. There was a statistical correlation between CD44H LI and each of Ki-67 LI (r = 0.61), expression level of VEGF (r = 0.72) and presence of MVI (r = 0.71). Independent predictors of cancer-specific survival in a multivariate analysis were: in all patients with RCC, the MVI (P = 0.003) and VEGF expression (P = 0.01); in those with no metastases, MVI (P = 0.01); in patients with no MVI, VEGF (P = 0.04); and in patients with MVI, Ki-67 LI (P = 0.003). No independent predictor was identified in patient with metastases.

CONCLUSION

This study suggests that cell proliferation, cell adhesion, the level of VEGF expression and the presence of MVI represent a complex tumour-host interaction that may favour the progression of RCC. Cell proliferation, CD44H and VEGF expression appear to be powerful markers for identifying patients with an adverse prognosis.

KEYWORDS

renal cell carcinoma, Ki-67, CD44, VEGF, microvessel invasion, prognosis
Tumour stage was defined according to the International Union Against Cancer and the American Joint Committee on Cancer 1997 TNM classification [18].

Paraffin-embedded 5-μm sections were stained immunohistochemically for Ki-67, CD44H and VEGF, with microwave pretreatment and using the avidin-biotin-peroxidase (ABP) method. For each case of RCC a representative slide of the tumour with the highest nuclear grade and the corresponding paraffin block were selected. After microwave pretreatment in citrate buffer (0.01 mol/L, pH 6), tissue sections were incubated for 10 min with one of the following primary antibodies: anti-Ki-67 mouse monoclonal antibody (proliferation marker; ready solution for use, Ab-2 Clone M867, NeoMarkers, Fremont, USA), anti CD44/HCAM mouse monoclonal antibody (ready solution for use, Std. Ab-4 Clone 156–3C11, NeoMarkers), and anti–VEGF, 1–26, polyclonal antibody (1 : 200 dilution, M 134 002FD, TakaRa, Otsu, Shiga, Japan). The primary antibodies were followed by biotinylated secondary antibody and peroxidase-labelled streptavidin detection system. This horseradish peroxidase detection system (Laboratory Vision Corporation, Fremont, USA) was then used with diaminobenzidine as the chromogen (Sigma Chemical Company, Poole, England). Positive and negative controls were run in parallel with each batch, and appropriate results obtained.

Immunostaining was independently evaluated by two pathologists and any differences resolved in a common reading. The immunohistochemical studies were scored as follows: The Ki-67 LI and CD44H LI were expressed as percentages of the Ki-67- and CD44H-positive cells by counting at least 1000 tumour cells at ×400. For VEGF, staining was determined semiquantiatively according to a three-grade scale; 0, no staining of tumour cells; 1+, membranous stain with no cytoplasmic immunostaining or with light cytoplasmic staining of some tumour cells (<50%); 2+, diffuse and strong membranous and cytoplasmic staining of some tumour cells (<50%), and most tumour cells (>50%) [15].

To detect MVI all of the pathological slides were reviewed retrospectively. MVI was defined as tumour that locally infiltrates through the complete wall of the vessel, including the endothelium, giving rise to a free tumour extension into the lumen [11,13]. MVI was considered present when tumour was seen in a vessel, i.e. at least one or more endothelial cells or the tunica media of the vessel were recognized to surround a neoplastic cell group. In selected cases elastin stains, and Factor VII (ready solution for use, Clone B1–3CS, DAKO A/S, Denmark) and CD34 (1 : 100 dilution, C8,2002, Quartett, Berlin, Germany) immunostains were used to identify vascular wall and endothelial cells, respectively.

The results were analysed statistically using the chi-square test for categorical variables. Correlations were analysed using the Spearman rank correlation test. Cases were categorized into two groups with either a low (<20%) or high (≥20%) CD44H LI and low (<15%) or high (≥15%) Ki-67 LI, respectively. The threshold of Ki-67 LI and CD44H LI was selected according to the receiver operating characteristic curves. Survival time was evaluated using the Kaplan–Meier method, and the difference between groups tested using the log-rank test. Cox regression analysis was used for multivariate statistical analysis. P < 0.05 was considered to indicate statistical significance in all tests.

RESULTS

The median (50, range) tumour diameter was 8 (4, 2–25) cm; the Fuhrman grade was I in nine, II in 11, III in 13, and IV in 14 tumours. The tumour stage was pT1 in 17, pT2 in 12, pT3 in seven and pT4 in 12 cases. Twelve patients (26%) had distant metastases (M+ at the time of surgery.

The median (range) follow-up of the patients was 48 (1–168) months. The survival time was calculated from the date of radical nephrectomy to the date of death or to the date of the last follow-up. Twenty-nine patients had died at the end of clinical follow-up; 23 deaths were related to the disease, with a median survival time of 13 (1–60) months, and six were attributable to causes other than tumour relapse. All patients with metastases at time of surgery died in the 2 years after surgery. Nineteen patients showed no evidence of disease during the clinical follow-up.

IMMUNOSTAINING

Ki-67 antigen labelling was localized to the nucleus with a fine, strong and homogenous brown granularity (Fig. 1), with a median (range) value of 13 (0–60)%. The median value of CD44H LI was 24 (0–95)%. Positive staining for CD44H was defined as a membranous and/or cytoplasmic staining pattern of epithelial tumour cells, even if the staining was diffuse or focal in tumour cells (Fig. 2). The median pattern of VEGF immunostaining, determined semiquantitatively, was 0.86 (0–2+) (Fig. 3).

MVI was considered present when tumour was seen in a vessel (Fig. 4); 23 tumours (48%) had MVI, while 25 (52%) had none on microscopic examination. Ten RCC (44%) from 23 RCC with MVI had distant metastases and 13 (57%) had not. While two tumours (8%) from 25 RCC with no MVI had metastases, 23 (92%) had not. Ten tumours from 12 metastatic patients had MVI, but two had not, and 13 (36%) from 36 nonmetastatic patients had MVI, but 23 (64%) had not. There was a statistical correlation between the presence of metastases and MVI (r = −0.40, P = 0.01). There was a significantly greater metastatic rate in patients who had RCC with MVI than in those

FIG. 1. Positive reactions in the nuclei with Ki-67 antibody in the tumour cells of Fuhrman III nuclear grade (original ×300; ABP).

FIG. 2. Immunostaining of CD44H showing a positive membranous and cytoplasmic reaction in the tumour cells with Fuhrman II nuclear grade (original ×300; ABP with CD44H).
who had RCC with no MVI (chi-square 8.04, P = 0.005).

UNIVARIATE ANALYSIS

To assess cancer-specific survival six patients who died from causes other than RCC were excluded from the analysis. The other patients’ characteristics are shown in Table 1.

Among the different clinicopathological and immunohistochemical variables analysed, tumour stage (P < 0.001), tumour size (P = 0.005), metastasis, MVI, Ki-67 LI, CD44H LI and VEGF expression (all P < 0.001) were associated with shorter survival. Although there was a statistical correlation between nuclear grade and survival (Spearman rank correlation, r = –0.43, P = 0.01), it was not a predictor of prognosis in Kaplan–Meier survival analysis (log-rank, P = 0.14). The median [50] cancer-specific survivals for each group are shown in Table 1 and Fig. 5. There was also a statistical correlation between CD44H LI and each of Ki-67 LI, expression level of VEGF and presence of MVI. CD44 LI was significantly greater in groups with a high-Ki-67 LI, high VEGF expression and MVI (Spearman rank correlation, respectively, 0.61, 0.72 and 0.71, all P = 0.01).

MULTIVARIATE ANALYSIS

Cox regression analysis, including variables significantly associated with survival in the univariate analysis, showed that MVI and VEGF expression were independent prognostic factors for cancer-specific survival (Table 1). In addition MVI (P = 0.01) in patients with no metastases, VEGF (P = 0.04) in patients with no MVI, and Ki-67 LI (P = 0.003) in patients with MVI, were independent predictors of cancer-specific survival in the multivariate analysis. No other independent predictor was identified in patients with metastases.

The expression levels of Ki-67, CD44H and VEGF were then compared between metastatic and nonmetastatic tumours, and with or without MVI. Because all patients with metastases and MVI died from RCC, although...
FIG. 5.
Kaplan–Meier cancer-specific survival curves in patients with RCC according to: a, MVI (present in 23, green) and not present (19, red; log-rank test, \( P < 0.001 \)); b, metastases present (12, green) and not present (26, red; log-rank test, \( P < 0.001 \)); c, Ki-67 LI (\( \geq 15 \) in 18, green; 11 metastatic, seven nonmetastatic; and <15 in 24, red; one metastatic and 23 nonmetastatic; log-rank, \( P < 0.001 \)); d, CD44 LI (\( \geq 20 \) in 20, green, 12 metastatic, eight nonmetastatic and <20 in 22, red, none metastatic and 22 nonmetastatic; log-rank, \( P < 0.001 \)); e, VEGF expression level (score 0 in 20, green, all nonmetastatic, 1+ in 11, red, three metastatic and eight nonmetastatic; 2+ in 10, black, nine metastatic and one nonmetastatic). The survival time decreased when VEGF expression was 0, 1+, and 2+, although not significantly (log-rank, \( P = 0.15 \)); f, MVI and Ki-67 LI (\( \geq 15 \) in 18, green, 17 with MVI, and one no MVI; and <15 in 24, red, six with MVI and 18 no MVI; log-rank, \( P < 0.001 \)); g, MVI and CD44 LI (\( \geq 20 \) in 20, green, 18 with MVI, two no MVI; <20 in 22, red, five with MVI and five no MVI; log-rank, \( P = 0.001 \)); h, MVI and VEGF expression level (0 in 21, green, eight with MVI, 13 no MVI; 1+ in 11, red, six with MVI, five no MVI; 2+ in 10, black, nine with MVI, one no MVI; log-rank, \( P < 0.001 \)). In all plots the crosses show censored data.
Ki-67 LI was ≥15, CD44H ≥20 and VEGF expression level >1+, these three markers are not necessary to predict the prognosis in metastatic RCC with MVI. However, there were differences in patients with no metastases or with and with no MVI for all the markers (Table 2 and Fig. 5).

**DISCUSSION**

RCC is well recognized as a malignancy with an unpredictable course [1]. Some patients with comparable or the same histological tumours can show a wide variation in biological behaviour and clinical outcome [1–3]. Therefore, prognostic factors are particularly important in RCC. Tumour stage and nuclear grade are usually considered the main pathological prognostic factors [2,3,17,18], but improved prediction is needed and attempts to find better prognostic criteria remain under investigation [6,10,15,19]. Although some results from these studies are discordant, individual studies of Ki-67 LI, CD44 LI, VEGF expression and MVI provide significant survival information [5–8,10,11,14,15,20]. One study evaluated the relationship between Ki-67, CD44 and vascular density in RCC [5], but none of the studies has evaluated the relationship between Ki-67, CD44, VEGF expression and MVI in such tumours.

In the present study the prognostic value of classical histological and some immunohistochemical variables in tissues specimens of RCC was evaluated retrospectively in 48 patients with a long-term follow-up. Tumour stage, size, metastases, MVI, Ki-67 LI, CD44 LI and VEGF expression were significant markers of a poor prognosis in the univariate analysis. Although there was a statistical correlation between nuclear grade and survival, it was not a predictor of cancer-specific survival. MVI and VEGF expression were the only independent prognostic factors in the multivariate analysis in all patients with RCC. In addition, MVI patients with no metastases, VEGF with no MVI, and a high Ki-67 LI with MVI were independent predictors of cancer-specific survival in the multivariate analysis. In metastatic patients and those with no MVI, Ki-67 LI, CD44H LI and VEGF expression were higher and unnecessary for the prognosis, but those with no metastases or MVI had a significantly shorter survival time with higher Ki-67 LI, CD44 LI and VEGF expression. Moreover, CD44H LI correlated with each of Ki-67 LI, VEGF expression and the presence of MVI.

Cell proliferation is the simplest and most commonly used variable in evaluating tumour progression and prognosis. Ki-67 is a nuclear antigen expressed in the G1, G2, G3, and M phases of the cell cycle, but not in G0 cells [20]. Ki-67 immunohistochemistry has been widely used to evaluate cell proliferation activity in clinical archival tumour material, and the expression of Ki-67 has been accepted as an excellent indicator of tumour proliferation. In previous studies, high proliferation rates detected by Ki-67 expression were associated with advanced clinical stage, poor histological differentiation (high nuclear grade), and a poor prognosis in RCC [5,19,20]. One study showed that CD44 expression was associated with a high rate of cell proliferation and poor prognosis, and an inverse correlation with microvessel density [5].

Cell-adhesion molecules are thought to participate in tumour cell invasion and metastases by mediating interactions between tumour cells and their environment. Altered expression of particular cell-adhesion molecules on tumour cells may not only suggest pathogenetic mechanisms for tumour metastases but also provide prognostic information for particular tumours [10,13,21]. CD44 is one of a family of transmembrane glycoproteins that recognize hyaluronan, a widely distributed component of extracellular matrix. CD44H and its several variant isoforms have been associated with diverse physiological functions such as cell-cell and cell-matrix interactions as well as lymphocyte homing [22]. Previous studies suggested a role for CD44 in the invasive and metastatic behaviour of tumour cells [12,13,23]. The increased expression of CD44H and its splicing variants may be associated with unfavourable clinical behaviour, e.g. in non-small cell lung carcinoma and gastric carcinomas [12,13]. On the contrary, loss of CD44 expression has been associated with aggressive behaviour of prostate and laryngeal cancers [24,25]. The association between CD44 and clinical behaviour of tumours is probably tissue- and tumour type-dependent [12]. Recently, the expression of CD44H and its variants was reported in RCC [5,10,11,26]. Terpe et al. [26] showed a strong correlation between the expression of CD44 isoforms and tumour differentiation (grade) in RCCs. Paradis et al. [10] also showed that CD44H expression correlated with the nuclear grade, size and stage of the tumour. Furthermore, in that series of conventional RCCs, multivariate analysis showed that CD44H expression was associated with both patient survival and the disease-free period, indicating that this marker is a prognostic factor independent of grade and stage. Glisce et al. [11] found that the association between CD44H expression and progression or recurrence of RCC is important, as it suggests that CD44H may be important pathogenetically in tumour progression.

VEGF is a general endothelial growth factor that acts on all processes involved in vascularization, extravasation, proliferation, migration, tube formation and the differentiation of endothelial cells. VEGF is expressed by many different cell types during various normal and pathological angiogenesis-related processes, e.g. embryogenesis, wound healing, ischaemic heart disease, tumour growth and metastasis [27]. In contrast to other angiogenic growth factors, e.g. basic fibroblast growth factor

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### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SEM) survival, months</th>
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<tbody>
<tr>
<td>Ki-67 LI, %</td>
<td>No metastases</td>
</tr>
<tr>
<td>&lt;15</td>
<td>77 (9)*</td>
</tr>
<tr>
<td>≥15</td>
<td>27 (8)</td>
</tr>
<tr>
<td>CD44 LI, %</td>
<td>No metastases</td>
</tr>
<tr>
<td>&lt;20</td>
<td>32 (8)*</td>
</tr>
<tr>
<td>≥20</td>
<td>78 (10)</td>
</tr>
<tr>
<td>VEGF grade</td>
<td>No metastases</td>
</tr>
<tr>
<td>0</td>
<td>75 (10)</td>
</tr>
<tr>
<td>1+</td>
<td>47 (15)</td>
</tr>
<tr>
<td>2+</td>
<td>18 (10)</td>
</tr>
</tbody>
</table>

**Observed cancer-specific survival in patients with no metastases (Kaplan-Meier survival model)**
(bFGF) and TGF-β, the activity of VEGF is tightly restricted to vascular endothelial cells [28]. Conventional RCCs are highly vascularized tumours, and increased levels of VEGF mRNA have been found in most such RCCs [15,28]. Furthermore, enhanced secretion of VEGF has been correlated with inactivation of the von Hippel-Lindau tumour suppressor gene, which is common in RCCs [16]. Paradis et al. [15] reported that VEGF expression was positively correlated with the size of the tumour in conventional RCCs. This result supported the hypothesis that VEGF is associated with tumour growth and progression. Furthermore, they showed that cytoplasmic VEGF expression was an independent prognostic factor, suggesting that although most conventional RCCs express VEGF, only a high level of expression has prognostic significance. These cases were of a significantly higher grade and stage, and a worse prognosis, than those with no cytoplasmic VEGF immunostaining.

A recent report showed that CD44 is involved in tumour angiogenesis; more specifically, up-regulation of CD44 by bFGF and VEGF has been detected in vitro [14]. In the present study the positive correlation between CD44 and VEGF expression also verified this autocrine regulation mechanism in vivo. The precise mechanism whereby CD44 may contribute to tumour progression or recurrence in RCC is unclear. CD44 expression by RCCs may allow tumour cells to adhere more firmly to hyaluronan, thereby aiding tumour cell implantation [11,29].

The haematogenous spread of RCC is the most important and frequent mechanism of distant metastases [8]; such metastasis from a primary site is supposed to proceed through a series of steps. The first step is MVI by the primary tumour; patients with metastatic disease at diagnosis had tumours with MVI. Taking all these findings, they suggest that MVI is a marker of different biological behaviour of the tumour. In another report, disease-free survival was calculated in relation to pT category, tumour grade, tumour diameter and MVI; the most significant factor determining disease-free survival was MVI [8].

In conclusion, the prognosis of RCC depends on at least four closely linked mechanisms; Ki-67, a tumour cell proliferation marker, the expression of CD44, an adhesion molecule, and VEGF, an angiogenic marker, and the presence of MVI. In addition to classical histological markers (tumour stage, tumour size, nuclear grade), Ki-67 LI, CD44 LI and VEGF expression appear to be useful markers to identify patients with a poor outcome. These variables might also be useful to change the follow-up schedule, to decrease the intervals in selected patients, and might help to clarify which patients need adjuvant therapy.

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CONFLICT OF INTEREST
None declared.

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Abbreviations: VEGF, vascular endothelial growth factor; LI, labelling index; MVI, microvessel invasion; ABP, avidin-biotin-peroxidase; bFGF, basic fibroblast growth factor.