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ORIGINAL ARTICLE

Large (>3.8 cm) clear cell renal cell carcinomas are morphologically and immunohistochemically heterogeneous

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Abstract Heterogeneity is an inherent event to tumour development that is lately receiving much attention in oncologic research. The topic is being addressed primarily at the molecular level, and results are promising. However, translation to practical medicine is still pending. Our intention in this study is to approach the problem in a series of clear cell renal cell carcinomas with the tools that pathologists use in routine practice. Three randomly selected areas of 48 clear cell renal cell carcinomas prospectively collected in two different institutions were analysed for intratumour heterogeneity. The evaluated parameters were tumour size, cell type (clear vs. eosinophilic), Fuhrman's grade and immunohistochemical expression of carbonic anhydrase IX, BRCA1-associated protein-1 (BAP-1), cyclooxygenase-2 (COX-2) and Ki67. Intratumour heterogeneity was detected in 26 cases (54 %). Cell type, grade and Ki67 index were the parameters more frequently heterogeneous amounting, respectively, 44, 42 and 38 %. Tumour size was a significantly discriminative factor to predict tumour heterogeneity, with a cut-off of 3.8 cm ($p<0.001$). Aside from tumour size, the most relevant parameters related with intratumour heterogeneity were cell type (clear vs. eosinophilic), Fuhrman's grade and Ki67 and COX-2 expression patterns. Carbonic anhydrase 9 and BAP-1 did not show statistical relevance. We conclude that heterogeneity is a common event in clear cell renal cell carcinomas that may be overlooked in cases insufficiently sampled. Tumour size appears as a reliable tool in identifying this situation since clear cell renal cell carcinomas under 3.8 cm in diameter are always homogeneous. This point may help the pathologist to make decisions in tumour sampling.

Keywords Clear cell renal cell carcinoma · Intratumour heterogeneity · Tumour size · Fuhrman's grade · Immunohistochemistry

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Introduction

Renal cancer belongs to the top ten lists of the most common malignancies in males and females in Western countries [1, 2] and remains a problem of major concern for health authorities worldwide [1]. Only surgery has proven to be useful in improving survival rates, but its success is limited and related to early diagnosis. Unfortunately, radio- and chemotherapy are not effective treatments. For this reason, many resources are being implemented to discover biomarkers that may offer more efficient therapeutic alternatives to these patients [3–5].

Molecular analysis is the most efficient tool to investigate neoplasia. Multiple complex disturbances of the cellular metabolic network still not completely understood are the basis of carcinogenesis and tumour development [6, 7]. Renal cancer has been proposed as a model of the metabolic shift that promotes malignancy, an event known as the Warburg effect [8]. As a consequence of this intracellular turmoil, tumour heterogeneity takes place and is increasingly recognized at

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molecular level [9]. New findings in this area might eventually have potential therapeutic implications relevant in the near future [10]. However, the translation of this emerging body of knowledge to clinical practice is still pending.

Clear cell renal cell carcinoma (CCRCC) accounts for more than 70 % of renal neoplasms [11]. Pathologists have known for a long time that CCRCC is frequently microscopically heterogeneous, but studies trying to quantify this issue are rare [12–14]. Clinical and molecular data [12–15] suggest that conventional sampling of kidney tumours [16] probably allows only a partial and insufficient perception of tumour histology. In this context, minor foci of high grade as well as tumour areas with different genetic/epigenetic alterations [17] with impact on tumour behaviour could eventually be missed in some cases. While waiting for more clinically efficient approaches related to recent findings in molecular profiling [8], more extensive sampling of kidney tumours might clear up this issue. However, this might not be easily implemented in daily pathology practice.

Our aim was to learn more about intratumour heterogeneity of CCRCC from a clinical and morphological point of view, not taking molecular aspects into consideration. For this purpose, we studied by immunohistochemistry zonal variations in the expression of several markers related to tumour aggressiveness, in a series of organ-confined CCRCC.

Material and methods

We prospectively collected 48 CCRCCs in two pathology laboratories during 2013. Only organ-confined tumours were considered for the study because our aim was to evaluate tumour heterogeneity in early phases of tumour development. Age, sex, tumour diameter, AJCC staging [18] and Fuhrman's grade [19] were recorded in every case. Immunohistochemistry was performed on formalin-fixed paraffin-embedded material following standardized methods in the Cruces University Hospital Pathology Lab, University of the Basque Country, Barakaldo, Spain. As antibodies, we used anticarbonic anhydrase IX (CAIX; NCL-L-CAIX, dilution 1:100, Novocastra, Newcastle, UK), BRCA1-associated protein-1 (BAP-1; sc-28383, dilution 1:30, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Ki67 (MIB-1, ready-to-use, Dako, Glostrup, Denmark) and cyclooxygenase-2 (COX-2; CX-294, dilution 1:100, Dako, Glostrup, Denmark). Immunohistochemical staining was performed in an automated immunostainer (EnVision FLEX, Dako Autostainer Plus; Dako, Glostrup, Denmark). Tris-EDTA was used for antigen retrieval. Negative controls were slides not exposed to the primary antibody, and these were incubated in PBS and then processed under the same conditions as the test slides. A histological and immunohistochemical analysis was performed using a Nikon Eclipse 80i microscope (Tokyo, Japan).

Seven parameters were considered in the evaluation of intratumour heterogeneity in this study: tumour diameter, cells (clear vs. eosinophilic), grade (Fuhrman) and CAIX, BAP-1, COX-2 and Ki67 immunostaining patterns. Three macroscopically similar areas separated from each other were randomly selected in every tumour for histological and immunohistochemical analysis. Gross haemorrhage and necrosis were not included in the sampling process for this purpose. The slides were evaluated at the microscope randomly. Positive and/or negative immunostaining was recorded for every stained slide of every case. Results were correlated between cases and with age, sex and tumour diameter.

In a preliminary analysis, an independent 2-group *t* test was performed to check if age, sex and tumour size were different in the two conditions, heterogeneous vs. homogeneous (*t* test function in R, r-project.org).

A data-mining analysis was performed using Waikato Environment for Knowledge Analysis (WEKA) [20]. Multiple parameters were selected in order to obtain the classification rules for the heterogeneity response. Next, a classification and regression tree (CART) [21] was performed using 66 % of the data for training and the remainder 44 % for testing.

The use of WEKA also allowed attribute selection, turning out both the most relevant and irrelevant parameters. To this aim, different search methods were used such as the best-first, rank-search or random-search algorithms [21].

Results

A total of 26 (54 %) tumours were heterogeneous in at least one of the seven parameters evaluated (Table 1), and most of them (22 cases, 85 %) showed intratumour heterogeneity in more than one parameter. On the other hand, 22 (46 %) CCRCCs were histologically and immunohistochemically homogeneous in this series. Cell type, grade and Ki67 index were the parameters more frequently heterogeneous with 44, 42 and 38 % of the cases, respectively.

Cell heterogeneity included conventional clear and eosinophilic cells in all cases (Fig. 1a) and was detected in 21 cases (44 %). Focal sarcomatoid cell change was found in one case. Rhabdoid and syncytial cells [14] were not detected.

Table 1 Evaluation of heterogeneity in 48 CCRCCs

Heterogeneity	*n*	Percent
Cell type	21	43.75
Grade	20	41.66
CAIX	11	22.91
BAP-1	5	10.41
COX-2	7	14.58
Ki67	18	37.50

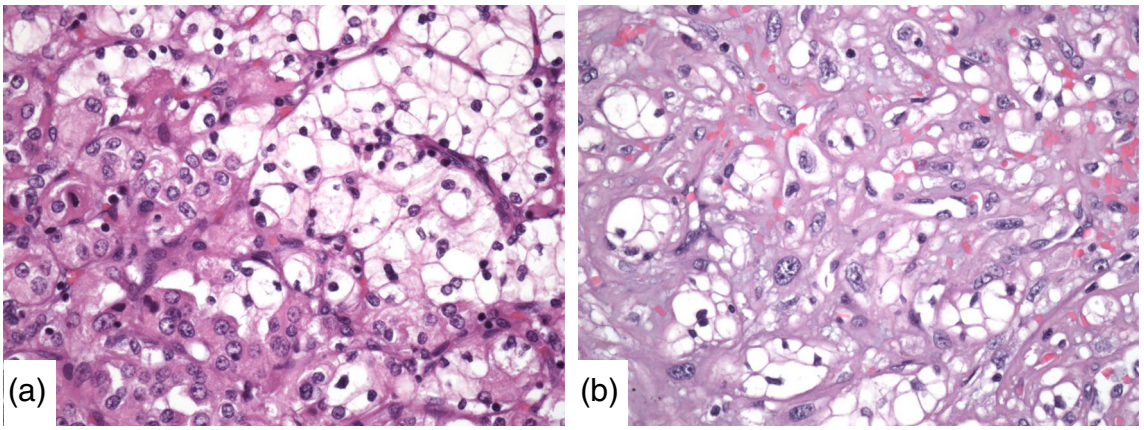

Fig. 1 Intratumor heterogeneity [clear and eosinophilic cells (**a**) and low and high grade cells (**b**)] in clear cell renal cell carcinoma in routine histological sections

Grade heterogeneity was detected in 20 cases of the series (42 %) and always included high grade areas (grades 3 or 4) (Fig. 1b). Grade variability in these cases was high, with five tumours (25 %) displaying the whole spectrum of Fuhrman's system (grades 1 to 4) along the three randomly selected areas. Grade was homogeneous in 28 CCRCCs (58 %) all of them being low-grade tumours (grades 1 and 2).

Most CCRCCs in the series displayed homogeneous immunostaining for CAIX (36 cases, 75 %). Only one case was homogeneously negative (2 %). Heterogeneous immunostaining with CAIX was found in 11 cases (Fig. 2a). BAP-1 was consistently positive in 40 cases (83 %) and consistently negative in three (6 %). Five cases (10 %) showed heterogeneous immunostaining for BAP-1 (Fig. 2b). Finally, most tumours in the series were consistently negative for COX-2 (41 cases, 85 %), with only seven cases (15 %) displaying focal immunoreactivity (Fig. 2c). Ki67 index varied between <5 and 20 %

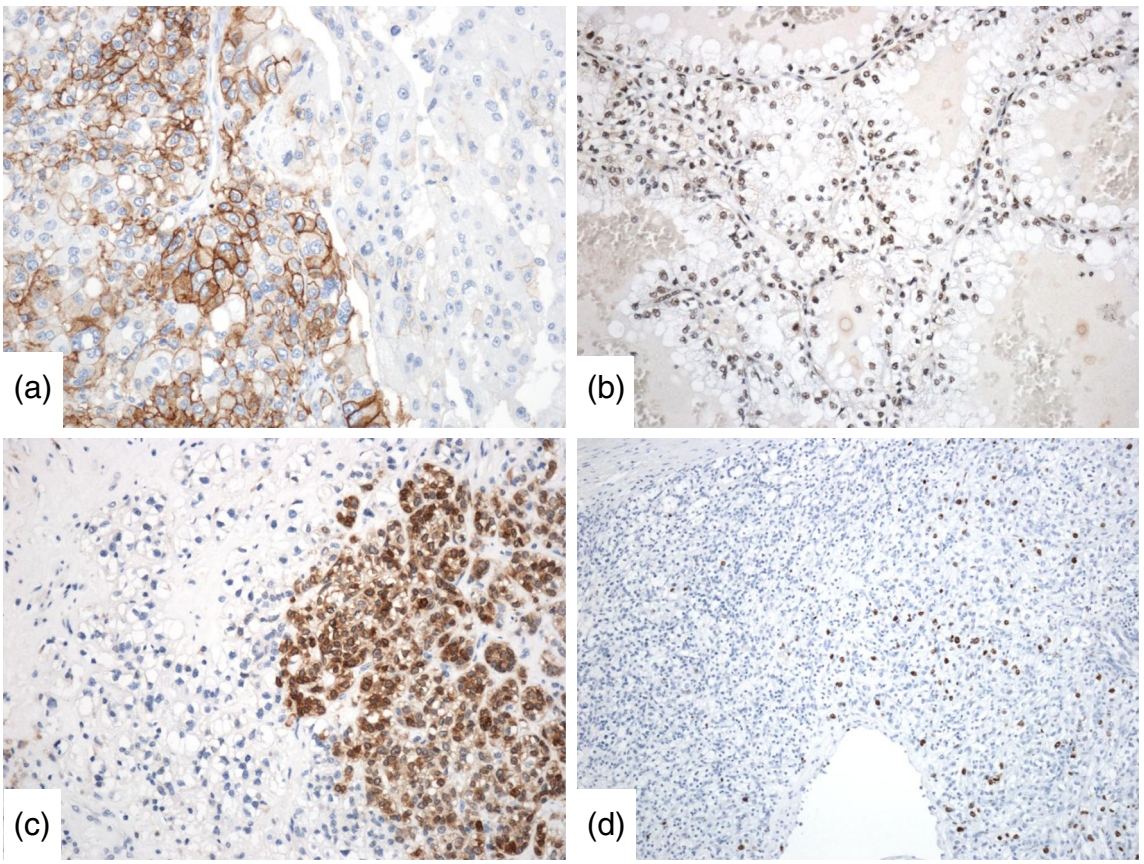

Fig. 2 Heterogeneous immunostaining with carbonic anhydrase IX (**a**), BAP-1 (**b**), COX-2 (**c**) and Ki67 (**d**) in clear cell renal cell carcinoma

(Fig. 2d). A homogeneous index under 5 % was detected in 31 cases (65 %). Two cases (4 %) had a consistent Ki67 index over 10 % in all selected areas. Variations ranging from <5 to 15 % were observed in 14 cases (29 %).

Tumour size was a statistically significant parameter ($p<0.001$) (Fig. 3) when comparing homogeneous vs. heterogeneous cases while age and sex were not. Selecting the heterogeneity to be the response, a first CART classification was performed for age, sex and size. Two results were found, firstly that both age and sex were irrelevant parameters and secondly that the only relevant parameter for the classifier was tumour size. Only tumours larger than 3.8 cm were heterogeneous, with 88 % correctly classified cases.

A second CART classification was performed considering all parameters. The decision tree for this classifier showed that if Fuhrman's grade was not the same in the three randomly selected areas chosen for the study, then the tumour was classified as heterogeneous. Additionally, if Fuhrman's grade was the same in the three areas, then the determinant variable was COX-2. If the COX-2 variable was not the same in all the three areas, then the tumour was heterogeneous. If the COX-2 was the same, the tumour was homogeneous. Using this classification scheme, 94 % of the cases were correctly classified.

Regarding attribute selection, the method showed that the most relevant parameters for intratumour heterogeneity were size (cut-off, 3.8 cm), cell component (clear vs. eosinophilic), Fuhrman's grade and Ki67 and COX-2 expression patterns. Strikingly, CAIX and BAP-1 expression patterns were not relevant for predicting intratumour heterogeneity in this series.

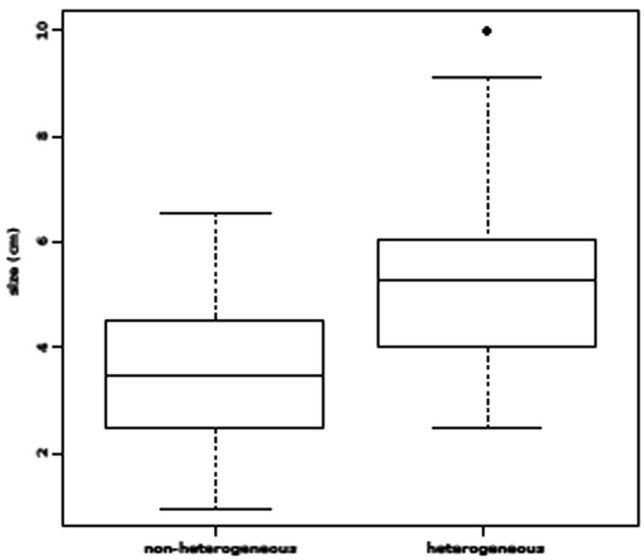

Fig. 3 Significant differences in tumour size between homogeneous and heterogeneous clear cell renal cell carcinomas

Discussion

Heterogeneity is a process inherent to the development of neoplasia and is presently a permanent matter of debate. Initially, heterogeneity was limited to morphology, and this is still used by pathologists worldwide in terms of intratumour variation of growth pattern, cell morphology and histological grade. Heterogeneity has been linked to aggressive tumour behaviour: with few exceptions, the more heterogeneous a neoplasm presents, the more likely the clinical outcome seems to be worse. The probability of finding more or less heterogeneity in a tumour is directly related to the amount of tissue examined. Tumour size is also a major determinant for developing heterogeneity in CCRCC: we show in this study that only tumours larger than 3.8 cm in maximum diameter are heterogeneous. This finding matches quite well with the current AJCC staging system [18] and implies that pT1a CCRCCs are always homogeneous. This is useful in terms of tumour sampling, because theoretically, one single sample of tumour tissue might be sufficient to obtain a full histological impression of any CCRCC smaller than 3.8 cm in diameter. In contrast, more than one block per centimetre of tumour would be necessary for pT1b or larger tumours.

Newell proposed in 1976 an equivalent of Darwinian selection as responsible for intratumour heterogeneity [22], the clonal evolution model. More recently, some authors have demonstrated genetically heterogeneous areas in a significant number of CCRCC [23]. Treatment implications of heterogeneity in terms of gene mutations and amplifications in combination with a variable microenvironment have been extensively reviewed in breast carcinoma, glioblastoma and other neoplasms [24]. However, although promising, the molecular approach to this issue can still not be easily translated to clinical practice because the involved molecular profiles must be first clarified and optimized.

For renal tumours, European practice guidelines consider one tissue sample per centimetre of tumour as appropriate [16], but this is not universally applied. Recent studies, however, have demonstrated that more extensive tumour sampling of CCRCC yields data significantly different in terms of tumour grade and tumour cellularity in comparison to conventional sampling [13, 14]. For instance, in a recent study, 28 CCRCCs were fully sampled and 17 tumour heterogeneities were found in terms of the presence of high grade areas while conventional sampling of the same tumours did show heterogeneity in 17 cases but high grade areas only in seven [13]. A recent study of 51 consecutively fully sampled CCRCCs reported foci of syncytial cells in eight cases and rhabdoid cells in five. The presence of these cells in CCRCC statistically correlated with higher grade and stage [14]. Syncytial cells have been associated with tumour aggressiveness in CCRCC in a very recent study [25], and rhabdoid elements are equivalent to sarcomatoid dedifferentiation in another

[26]. Considering the prognostic importance of these histological findings, current sampling protocols in renal carcinomas might not be appropriate. However, the issue is open for discussion because of reasons of cost; more extensive tumour sampling might not be universally applicable.

Immunohistochemistry may help in discovering tumour heterogeneity in CCRCC that morphologically appears monotonous, as has been shown in breast cancer [27]. We designed the study to look beyond histological monotony in selecting areas of CCRCC for immunohistochemical analysis that were histologically similar but from distant tumour areas. We chose markers of which earlier studies had reported correlation with tumour aggressiveness and weak response to treatment. For CCRCC, CAIX is one of the most promising markers [3]. CAIX is involved in the metabolic shift of cancer which is associated with acidification of the tumour microenvironment. CAIX expression is regulated by the von Hippel-Lindau protein (pVHL); it is overexpressed in many tumours and is involved in the regulation of metastatic spread of the tumour through activation of cancer-associated fibroblasts in adjacent stroma [28]. However, results are controversial as some authors have shown that high immunohistochemical expression of CAIX in CCRCC cells correlates with favourable prognosis [29], but others do not find statistically significant differences on multivariate analysis [30]. In addition, CAIX expression seems to predict good response to systemic therapy in metastatic CCRCC [31]. CAIX immunohistochemical staining pattern was not a feature predicting intratumour heterogeneity in our study.

BAP-1 is a tumour suppressor gene localized on chromosome 3. Its product is a deubiquitinating nuclear protein involved in double-stranded DNA repair, chromatin remodelling, cell cycle checkpoints, transcription and apoptosis [32]. The precise effect of BAP-1 on the BRCA-1 pathway is still under discussion. Some authors [33] argued that BAP-1 binds BRCA-1, cleaves ubiquitin and enhances the growth suppressor effects of BRCA-1. *BAP-1* mutations increase the susceptibility for the development of several tumours such as Spitz nevus, uveal melanoma, mesothelioma and clear cell renal cell carcinoma [32]. BAP-1 protein is inactivated in roughly 15 % of CCRCC, and its loss in this tumour seems to be associated with high tumour grade and rhabdoid phenotype [34, 35]. In our study, the BAP-1 immunohistochemical staining pattern did not predict intratumour heterogeneity.

COX-2 (cyclooxygenase-2) is an enzyme involved in the production of prostaglandins and arachidonic acid [36] and plays a role in inflammation, tumour growth, invasiveness and metastasis. COX-2 expression has been correlated with poor prognosis in CCRCC, and its inhibition via antiangiogenic drugs has antitumour properties [37]. We found the expression pattern of COX-2 to be associated with intratumour CCRCC heterogeneity.

Ki67 is a nuclear protein involved in cell proliferation widely used in pathology as a marker to determine the growth fraction of tumours. High rate of Ki67 immunostaining has been correlated with poor prognosis and with intratumour heterogeneity in CCRCC [38].

Conclusions

Intratumour heterogeneity in CCRCC remains poorly understood. We found that tumour size, cell type, Fuhrman's grade and Ki67 and COX-2 immunohistochemical staining patterns to be relevant parameters associated with tumour heterogeneity. We found CCRCC smaller than 3.8 cm in diameter to be invariably homogeneous. This parameter might be taken into account in determining how CCRCC should be sampled.

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Conflict of interest The authors declare that they have no conflict of interest.

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