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Synchronous Metastatic Clear-Cell Renal Cell Carcinoma: A Distinct Morphologic, Immunohistochemical, and Molecular Phenotype

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1 **TITLE PAGE**

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3 **Synchronous metastatic clear cell renal cell carcinoma: a distinct morphological,**4 **immunohistochemical and molecular phenotype**

5

6 Short title: **Synchronous metastatic renal cell carcinoma**

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51 **ABSTRACT**

52

53 **Micro abstract**

54 **In order to compare synchronous and metachronous metastatic clear cell renal cell**
55 **carcinoma (ccRCC), we performed a pathological, immunohistochemical and molecular**
56 **study on primary tumors in a retrospective series of 48 consecutive patients with up to**
57 **ten years of follow-up. Synchronous metastatic ccRCC had a distinct phenotype that may**
58 **explain their worse prognosis.**

59

60 **Introduction:** Clear cell renal cell carcinomas (ccRCC) are highly metastatic tumors with
61 metastases detected at diagnosis (synchronous) or during follow-up (metachronous). To date,
62 there have been no reports comparing primary ccRCC of synchronous and metachronous
63 metastatic patients, yet different in terms of prognosis. Determining whether there is a
64 phenotypic difference between these two groups could have important clinical implications.

65 **Patients and Methods:** In a retrospective consecutive cohort of 98 patients with ccRCC, 48
66 patients had metastases including 28 synchronous and 20 metachronous presentations with a
67 follow-up of 10 years. For each primary tumor in these metastatic patients, pathological
68 criteria, expression of VEGF, PAR-3, CAIX and PD-L1 as detected by immunohistochemistry,
69 and complete *VHL* status were analyzed. Univariate analysis was performed and survival was
70 assessed using Kaplan-Meier curves compared by log-rank test.

71 **Results:** Compared to primary ccRCC in metachronous metastatic patients, primary ccRCC in
72 synchronous metastatic patients were significantly associated with a poorer ECOG
73 performance ($p=0.045$), higher pT status ($p=0.038$), non-inactivated *VHL* gene ($p=0.01$),
74 sarcomatoid component ($p=0.007$), expression of PAR-3 ($p=0.007$), and overexpressions of
75 VEGF ($>50\%$) ($p=0.017$) and PD-L1 ($p=0.019$). Patients with synchronous metastases had a

76 worse cancer-specific survival than patients with metachronous metastases even from
77 metastatic diagnosis (median survival 16 months versus 46 months, respectively, $p=0.01$).

78 **Conclusion:** This long-term study is the first to support the notion that synchronous m-
79 ccRCC has a distinct phenotype. This is probably linked to the occurrence of oncogenic
80 events that could explain their worse prognosis. These particular metastatic patients could
81 benefit from specific therapy.

82

83 **Keywords:** clear cell renal cell carcinoma; synchronous and metachronous metastases;
84 phenotype; clinical outcome

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101 **TEXT**

102

103 **INTRODUCTION**

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105 Clear cell renal cell carcinoma (ccRCC) is the most common histologic subtype of renal cell
106 carcinoma.¹ Tumor cells are characterized by inactivation of the *VHL* gene leading to HIF
107 stabilization, which induces the transcription of genes such as vascular endothelial growth
108 factor (*VEGF*).² As a consequence, the tumor microenvironment is highly vascularized.

109 Another critical component of the tumor microenvironment is the immune system as some
110 tumors have a high density of tumor infiltrating lymphocytes (TIL).³ Moreover, tumor cells
111 were shown to express programmed death ligand 1 (PD-L1) to escape the immune system.^{4,5}

112

113 With approximately 40% of patients dying of metastases, ccRCC are highly aggressive
114 tumors.⁶ The most common sites of metastasis are the lungs, distant lymph nodes, liver,
115 bones, brain and adrenal gland. Twenty to 30% of patients are diagnosed with metastatic
116 disease (synchronous presentation) whereas 20% of patients with non-metastatic disease at
117 diagnosis will later develop metastases during follow-up (metachronous presentation).⁷

118 Metastases generally arise in the first six years after surgery.⁸

119

120 Synchronous and metachronous ccRCC have a different prognosis. Currently, one of the
121 criteria of MSKCC and Heng risk criteria models for predicting survival in metastatic patients
122 is a time from initial diagnosis (including original localized disease) to treatment of less than
123 one year.⁹⁻¹¹ This risk factor includes synchronous metastatic patients. The difference in
124 prognosis between the two groups of patients may be linked to their primary tumors having
125 different phenotypes. To our knowledge, there are no reports comparing primary ccRCC in

126 synchronous and metachronous metastatic patients. Determining whether there is a phenotypic
127 difference between these two groups could have important clinical implications.

128

129 This is the first study to conduct an in-depth analysis of primary ccRCC in correlation with
130 pathological criteria, *VHL* status and long-term clinical outcome with a view to seeking
131 differences depending on synchronous or metachronous metastatic status.

132

133 **MATERIALS AND METHODS**

134

135 **Patients**

136 Between 2002 and 2005, 98 consecutive patients were operated for sporadic ccRCC in the
137 Department of Urology at Rennes University Hospital. Twenty-eight patients had
138 synchronous metastases at initial diagnosis and underwent nephrectomy in accordance with
139 international guidelines.¹² They received no prior therapy. Twenty patients had a nephrectomy
140 before developing metastases which were detected during follow-up (first CT scan 6 months
141 after surgery) and defined as metachronous. Consequently, a total of 48 patients presented
142 metastases between 2002 and 2012 and were included in our study. Patient charts were
143 retrospectively reviewed to assess pretreatment ECOG performance status, methods of
144 detection (incidental or symptomatic), involved metastatic sites with retrieved first staging CT
145 scan, MSKCC score, Heng criteria and therapies^{9, 11}. The outcome was specific death which
146 we assessed with a 10-year follow-up. The study protocol was approved by the local advisory
147 board and informed consent was obtained from each patient for the study.

148

149 **Tissue sample management**

150 Tumor samples were obtained from the processing of biological samples by the Rennes
151 Biological Resources Center-Health (CRB-Health) (BB-0033-00056). The research protocol
152 was conducted under French legal guidelines and fulfilled the requirements of the local
153 institutional ethics committee. All consecutive ccRCC and paired renal cortex samples were
154 analyzed. Immediately after macroscopic examination, small samples were collected from
155 surgical specimens, frozen in liquid nitrogen and stored at -80°C until DNA extraction.
156 Genomic DNA was extracted from 25 to 35 mg of frozen tissue sections using a QIAamp
157 DNA minikit (Qiagen, Courtaboeuf, France). DNA quantity and quality were estimated by
158 optical density (OD 260/280) measurement and 0.8% agarose gel electrophoresis using
159 standard protocols.

160

161 **Pathological analysis**

162 After fresh tissue sampling, surgical specimens were formalin-fixed. Paraffin sections were
163 stained with hematoxylin and eosin-safran for light microscopy. All slides were reviewed by a
164 dedicated uropathologist (NRL). The macroscopic and histological parameters analyzed were:
165 tumor size, multifocality, nucleolar grade according to the International Society of Urological
166 Pathology (ISUP) grading system, sarcomatoid component, tumor necrosis, granular
167 component, lymphocyte infiltrate and microvessel invasion.¹³ Sarcomatoid component was
168 defined as more than 10% involvement of the tumor. Tumor stage was defined by the latest
169 International Union Against Cancer classification (2009).¹⁴

170

171 **Immunohistochemistry**

172 For each ccRCC case, a representative slide of the tumor with the highest nucleolar grade and
173 the corresponding paraffin block were selected. VEGF (anti-VEGF antibody, sc-152, dilution
174 1/100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CAIX (anti-CAIX antibody,

175 ab15086, dilution 1/1500, Abcam, Cambridge, UK), PAR-3 (anti-PAR-3, HPA0300443,
176 dilution 1/50, Sigma-Aldrich, Saint Louis, USA) and PD-L1 (anti-PD-L1 antibody, clone
177 130021, dilution 1/200, R&D Systems, Minneapolis, MN, USA) expression was assessed by
178 immunohistochemistry as previously described.¹⁵⁻¹⁷ The cut-off for positive cases was 85% of
179 tumor cells for CAIX as described in a previous study.^{16, 18} The percentage of tumor cells for
180 VEGF was reported. Only cytoplasmic PAR-3 expression in tumor cells was considered
181 positive.¹⁷ PD-L1 was overexpressed when intensity of membranous or cytoplasmic staining
182 in tumor cells was moderate to strong as previously described.¹⁹ Regarding tumor infiltrating
183 lymphocytes, CD3 (anti-CD3 antibody, clone SP7, dilution 1/100; Thermo Scientific,
184 Waltham, MA, USA) and CD20 (anti-CD20 antibody, clone L26, dilution 1/25; Dako,
185 Glostrup, Denmark) expression was assessed. The inflammatory extent was coded as one (few
186 sparse lymphocytes in the tumor) or two (marked dense lymphocytes or lymphoid nodules).
187 IHC scoring was independently assessed by two pathologists (SFKJ and NRL) blinded to the
188 clinical grouping of the specimens. Discordant cases were reevaluated collegially to reach a
189 consensus score.

190

191 ***VHL* gene analysis**

192 We determined the complete *VHL* status for each tumor by analyzing *VHL* gene mutation,
193 deletion and promoter methylation. *VHL* mutations were detected by sequencing using
194 denaturing high-performance liquid chromatography (DHPLC). All mutations were confirmed
195 in a second round of PCR and sequencing reactions. *VHL* gene deletions and promoter
196 methylation were detected by Multiplex Ligation-dependent Probe Amplification (MLPA)
197 analysis using the SALSA MLPA P016B *VHL* probe kit and the SALSA MS-MLPA kit,
198 respectively.²⁰ As *VHL* is a tumor-suppressor gene, *VHL* gene impairments necessarily
199 involve biallelic alterations in tumor cells as two hits are required to be inactivated. Tumors

200 with two alterations of the *VHL* gene were defined as inactivated for that gene (in*VHL*). Those
201 with no or only one alteration were defined as non-inactivated *VHL* tumors (ni*VHL*).

202

203 **Statistical analysis**

204 χ^2 , Fisher's exact and Mann-Whitney tests were performed to compare qualitative and
205 quantitative parameters, respectively between the synchronous and metachronous metastatic
206 patient groups. Cancer-specific survival (CSS) was calculated from metastasis diagnosis to
207 death from cancer. The Kaplan-Meier method was used to represent CSS, and the resulting
208 curves were compared using log-rank tests. All p-values were 2-sided, and p-values less than
209 0.05 were considered statistically significant. All statistical analyses were performed using
210 Stata 11.1 (College Station, TX, USA) software.

211

212 **RESULTS**

213

214 **Patient and tumor characteristics**

215 The population characteristics and pathological parameters are summarized in Table 1. The
216 median age at diagnosis was 61 years (42-80). Twenty-three patients (47.9%) had an ECOG
217 performance status of 0. The mean tumor size was 9cm with tumors ranging from 2 to 18 cm.
218 In 6 cases (12.5%), nodal invasion was present. Twenty-eight patients (58.3%) had
219 synchronous metastases at diagnosis whereas 20 patients (41.7%) developed metastases after
220 initial diagnosis with a 32-month mean (6-78 months). The most common metastatic sites
221 were the lungs in 32 cases (66.7%), bones in 27 cases (56.3%), distant lymph nodes in 16
222 cases (33.3%) and liver in 7 cases (14.6%). Less common sites were the brain in three cases
223 (6.3%), soft tissue in four cases (8.3%), adrenal gland in five cases (10.4%), pancreas in two
224 cases (4.1%), peritoneum in one case (2.1%), contralateral kidney in two cases (4.1%) and

225 digestive organs in three cases (6.3%). Several sites were frequently involved in 32 cases
226 (66.6%) whereas only one site was involved in 16 cases (33.3%). Before systemic treatment,
227 most patients had intermediate or high risk according to MSKCC or Heng models. When
228 eligible for systemic treatment, the patients received various therapies such as cytokines in 14
229 cases, sunitinib (first-line or second-line therapy) in 13 cases, sorafenib in two cases, hormone
230 therapy in three cases and standard chemotherapy in two cases. Eleven patients were referred
231 to supportive care specialists and eight patients were not treated in our center. With a follow-
232 up of 10 years, 43 patients (89.6%) from our study died of their cancer.

233

234 **Synchronous and metachronous m-ccRCC: a distinct phenotype**

235 The synchronous and metachronous metastatic patient and tumor characteristics are
236 summarized and compared in Tables 2 and 3. Synchronous and metachronous m-ccRCC
237 shared the following features: aggressive tumors with symptomatic detection (75%), a median
238 tumor size of 9cm (2-18 cm), a high nucleolar grade (grade 3-4 in 87.5%), tumor necrosis
239 (70.8%), granular component (72.9%), microvascular invasion (60.4%) and overexpression of
240 VEGF (79.2%). However, compared to metachronous m-ccRCC, synchronous m-ccRCC had
241 a poorer ECOG score ($p=0.045$), a worse risk group in both MSKCC and Heng models
242 ($p=0.007$ and $p=0.010$ respectively) and corresponded to even more aggressive tumors with
243 higher pT status ($p=0.038$) and sarcomatoid component ($p=0.007$). In the
244 immunohistochemistry analysis, they were associated with overexpression of VEGF with a
245 50% cut-off ($p=0.017$) as defined by a receiver operating characteristic (ROC) curve,
246 expression of PAR-3 ($p=0.007$) and overexpression of PD-L1 ($p=0.019$). At the molecular
247 level, they were associated with a non-inactivated *VHL* gene ($p=0.01$). The pathological and
248 immunohistochemical phenotype of synchronous m-ccRCC is shown in Figure 1.

249

250 Worse clinical outcome with synchronous m-ccRCC

251 Patients with synchronous metastases had a worse CSS from the date of metastasis diagnosis
252 compared to patients with metachronous metastases without statistical differences between the
253 two groups in care management. They had a median survival of 16 months versus 46 months
254 for the patients with metachronous metastases ($p=0.01$), Figure 3. Five-year survival was 3.6%
255 for patients with synchronous metastases and 20% for patients with metachronous metastases.

256

257 DISCUSSION

258

259 To our knowledge, this is the first study to compare synchronous and metachronous primary
260 m-ccRCC. While our results may be seen to be limited in terms of sample size, we present a
261 highly detailed study at the pathological, immunohistochemical and molecular levels in a
262 retrospective series of consecutive ccRCC patients with a long-term clinical follow-up of up
263 to ten years. Due to the period of our study, treatments were quite heterogeneous without
264 statistical difference between the two groups. Besides, patients received no prior systemic
265 therapy before nephrectomy.

266

267 Primary ccRCC in both synchronous and metachronous metastatic patients is characterized by
268 aggressive and disseminating tumors. However, we report a distinct pathological and
269 molecular phenotype of the primary tumors. In the m-ccRCC group, synchronous m-ccRCC
270 was particularly associated with a higher pT status, sarcomatoid component, cytoplasmic
271 expression of PAR-3, overexpression of VEGF and PD-L1 and a *niVHL* gene.

272

273 Sarcomatoid ccRCC may represent a completed epithelial-mesenchymal transition (EMT).²¹

274 Invasion of the basement membrane and extracellular matrix is an essential event in tumor

275 progression and considered a critical step during metastasis.²² Partitioning-defective 3 (PAR-
276 3), a crucial component of partitioning-defective complex proteins, was recently described as
277 an independent prognostic factor in ccRCC.^{17, 23} It is implicated in the development and
278 maintenance of cell polarity. As it modulates cell–cell communication and promotes
279 collective cell migration, PAR-3 may be involved in cancer cell EMT and metastasis
280 formation in ccRCC.²³ Angiogenesis is also considered a crucial step in the progression of
281 cancer. VEGF expression is a key molecular mechanism underlying the initiation and
282 maintenance of the entire tumor process.^{24, 25} Tumor progression is often paralleled by higher
283 levels of VEGF expression as cancer cells gradually acquire their malignant potential.²⁵
284
285 Interestingly, PD-L1 and the non-inactivated *VHL* gene were associated with the primary
286 ccRCC in synchronous metastatic patients. The interaction between PD-1, an inducible
287 inhibitory receptor expressed on lymphocytes and dendritic cells, and PD-L1 ligand,
288 expressed by tumor cells, results in down-regulation of the T-cell response.²⁶ PD-L1
289 overexpression may reflect the ability of metastatic tumor cells to evade immune surveillance
290 during migration. *VHL* gene inactivation is likely considered as an initial event in the ccRCC
291 carcinogenic process.² Non-inactivation of the *VHL* gene more commonly seen in
292 synchronous m-ccRCC may suggest early involvement of non-dependent VHL pathways such
293 as MAP kinase and PI3K-AKT-mTOR pathways that are also implicated in PD-L1
294 expression.²⁷⁻²⁹
295
296 Compared to primary tumors of synchronous m-ccRCC, metachronous m-ccRCC probably
297 acquire less oncogenic events. During the period between nephrectomy and symptomatic
298 metastasis, initially dormant tumor cells acquire oncogenic events that are not predictable in
299 primary tumors.³⁰ This explains the heterogeneity between primary tumors and metastasis

300 already described.⁴ Our study emphasizes the need to assess metastatic sites rather than the
301 primary tumor to identify predictive biomarkers for targeted therapies.

302
303 In our study, we confirmed the poor prognosis of synchronous metastatic patients.⁹ As
304 expected, most of them had time from initial diagnosis to treatment of less than one year that
305 is a risk factor in both MSKCC and Heng models. In a recent publication by Beuselinck *et al.*,
306 synchronous m-ccRCC were particularly linked to poor sunitinib response.³¹ This poor
307 prognosis may rely on advantageous oncogenic events acquired in their primary ccRCC such
308 as PD-L1 expression. If confirmed by further studies, these patients may be good candidates
309 for PD1/PDL1 immunotherapy.

310

311 CONCLUSION

312

313 In this long-term study of metastatic patients, we revealed that synchronous m-ccRCC had a
314 distinct phenotype which is likely explained by the occurrence of oncogenic events. Patients
315 with synchronous m-ccRCC have a worse prognosis and could benefit from specific therapy.

316

317 Clinical practice points:

- 318 • Patients with synchronous metastatic clear cell renal cell carcinoma (ccRCC) are
319 already known to have a worse prognosis than patients with metachronous metastatic
320 ccRCC.
- 321 • For the first time, we compared synchronous and metachronous primary tumors at
322 pathological, immunohistochemical and molecular levels and found a different
323 phenotype of synchronous primary ccRCC.

- 324 • Patients with synchronous metastasis overexpressed PD-L1 and may be good
325 candidates for PD1/PDL1 immunotherapy.

326

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328

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335

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337 The authors of this article have no relevant financial relationships with commercial interests
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339

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- 429

1 TABLES

2 **Table 1. Summary of the clinical and histopathological characteristics of the 48**
 3 **metastatic patients.**

Variables	Number of patients (%)
Sex	
M	27 (56.3%)
F	21 (44.7%)
ECOG	
0	23 (47.9%)
1	25 (52.1%)
Age (years)	61 (42-80)
Mode of detection	
Incidental	12 (25%)
Symptomatic	36 (75%)
Tumour size (cm)	9 (2-18)
ISUP grade	
2	6 (12.5%)
3	18 (37.5%)
4	24 (50%)
Tumour stage (pT)	
1	5 (10.4%)
2	11 (22.9%)
3	28 (58.3%)
4	4 (8.3%)
Lymph node status (pN)	
0	42 (87.5%)
1-2	6 (12.5%)
Metastasis status (pM)	
0	20 (41.7%)
1	28 (58.3%)
MSKCC score	
Favorable prognosis	8 (16.7%)
Intermediate prognosis	27 (56.3%)
Poor prognosis	13 (27.0%)
Heng criteria	
Favorable prognosis	9 (18.7%)
Intermediate prognosis	19 (39.6%)
Poor prognosis	20 (41.7%)

5 **Table 2. Clinical features and their association with synchronous or metachronous**
 6 **metastatic patients.**

Variables	Patients with synchronous m-ccRCC (n=28)	Patients with metachronous m-ccRCC (n=20)	p-value
Sex			p=0.559 [†]
Male	17 (60.7%)	10 (50%)	
Female	11 (39.3%)	10 (50%)	
Age	62	59	p= 0.216 [‡]
ECOG			p=0.045[†]
0	10 (35.7%)	13 (65%)	
1	18 (64.3%)	7 (35%)	
Mode of detection			p=0.198 [†]
Incidental	5 (17.9%)	7 (35%)	
Symptomatic	23 (82.1%)	13 (65%)	
Metastatic localization			
Lung	11 (39.3%)	5 (25%)	p=0.301 [†]
Bone	10 (35.7%)	11 (55%)	p=0.184 [†]
Distant lymph node	18 (64.3%)	15 (75%)	p=0.430 [†]
Liver	25 (89.3%)	16 (80%)	p=0.429 [‡]
Unique site	17 (60.7%)	14 (70%)	p=0.507 [†]
MSKCC score			
Favorable prognosis	1 (3.6%)	7 (35%)	p=0.006 [‡]
Intermediate prognosis	15 (53.6%)	12 (60%)	p=0.658 [†]
Poor prognosis	12 (42.8%)	1 (5%)	p=0.007 [‡]
Heng criteria			
Favorable prognosis	1 (3.6%)	8 (40%)	p=0.002 [‡]
Poor prognosis	11 (39.3%)	8 (40%)	p=0.960 [†]
Favorable prognosis	16 (57.1%)	4 (20%)	p=0.010 [†]
Treatment			
Cytokines	8 (19.6%)	6 (30%)	p=0.914 [†]
Sunitinib	5 (17.9%)	8 (40%)	p=0.086 [†]
Others	5 (17.9%)	3 (15%)	p=0.793 [†]
Supportive care	8 (19.6%)	3 (15%)	p=0.473 [‡]
Unknown	4 (14.3%)	4 (20%)	p=0.703 [‡]

7 [†] Pearson chi2 test, [‡] Fisher's exact test, [§] Mann-Whitney test

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10 **Table 3. Histological, immunohistochemical and molecular features and their**
 11 **association with synchronous or metachronous m-ccRCC.**

Variables	Patients with synchronous m-ccRCC (n=28)	Patients with metachronous m-ccRCC (n=20)	p-value
Histological criteria			
Tumour size (cm)	8.9	9.2	p=0.722‡
ISUP nucleolar grade 4	18 (64.3%)	6 (30%)	p=0.019†
Tumour stage (T3-4)	22 (78.6%)	10 (50%)	p=0.038†
Lymph node status (N1-2)	5 (17.9%)	1 (5%)	p=0.214§
Tumour necrosis	19 (67.9%)	15 (75%)	p=0.591†
Sarcomatoid component	15 (53.6%)	18 (90%)	p=0.007†
Granular component	18 (64.3%)	17 (85%)	p=0.111†
Microvascular invasion	17 (60.7%)	12 (60%)	p=0.960†
Dense lymphocyte infiltrate	4 (14.3%)	1 (5%)	p=0.385‡
Immunohistochemical study			
VEGF ≥50%	22 (78.6%)	9 (45%)	p=0.017†
CAIX ≥ 85%	20 (71.4%)	14 (70%)	p=0.915†
Cytoplasmic PAR-3	23 (82.1%)	9 (45%)	p=0.007†
PD-L1 Intensity 2+ 3+	21 (75%)	7 (35%)	p=0.019†
VHL status			
Deletion	15 (53.6%)	16 (80%)	p=0.059†
Mutation	16 (57.2%)	16 (80%)	p=0.097†
Promoter methylation	2 (7.1%)	3 (15%)	p=0.636‡
Inactivation	12 (42.9%)	16 (80%)	p=0.010†

12 † Pearson chi2 test, ‡ Fisher's exact test, § Mann-Whitney test

13

FIGURES

Figure 1. Pathological parameters associated with synchronous m-ccRCC

A) Sarcomatoid component, HES x200

B) Diffuse cytoplasmic expression of VEGFA, IHC x200

C) Cytoplasmic and membranous expression of PAR-3, IHC x200

D) PD-L1 overexpression with intense membranous staining, IHC x200

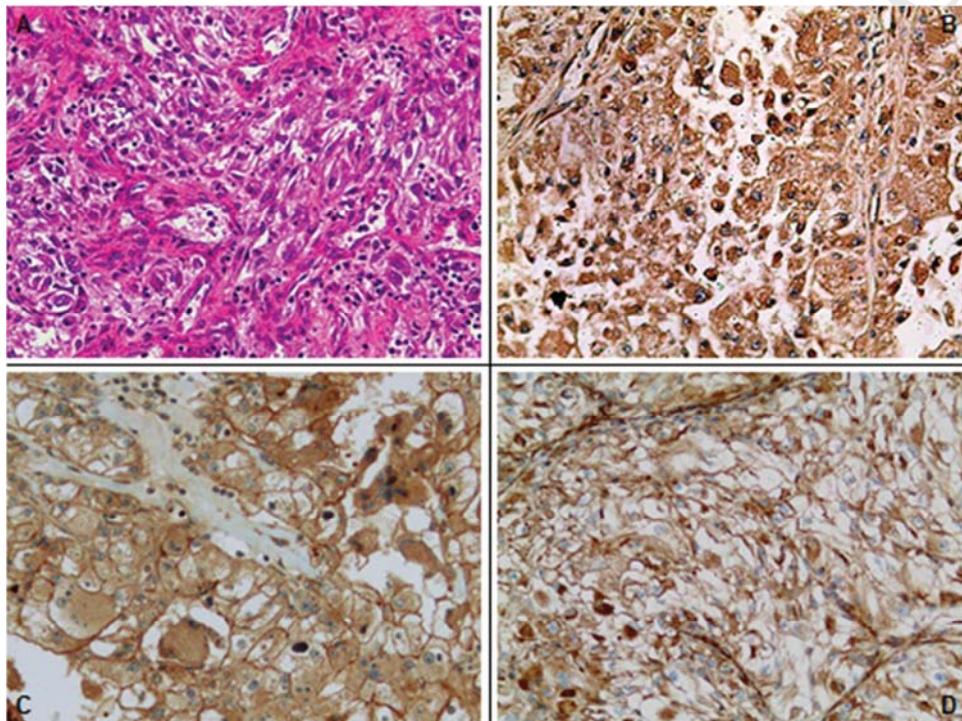


Figure 2. Cancer specific survival in patients from metastasis diagnosis according to metastatic status.

