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TITLE PAGE

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

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

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ABSTRACT

Micro abstract

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

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

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

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

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

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

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

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TEXT

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the most common histologic subtype of renal cell carcinoma.[1] Tumor cells are characterized by inactivation of the *VHL* gene leading to HIF stabilization, which induces the transcription of genes such as vascular endothelial growth factor (*VEGF*).[2] As a consequence, the tumor microenvironment is highly vascularized. Another critical component of the tumor microenvironment is the immune system as some tumors have a high density of tumor infiltrating lymphocytes (TIL).[3] Moreover, tumor cells were shown to express programmed death ligand 1 (PD-L1) to escape the immune system.[4, 5]

With approximately 40% of patients dying of metastases, ccRCC are highly aggressive tumors.[6] The most common sites of metastasis are the lungs, distant lymph nodes, liver, bones, brain and adrenal gland. Twenty to 30% of patients are diagnosed with metastatic disease (synchronous presentation) whereas 20% of patients with non-metastatic disease at diagnosis will later develop metastases during follow-up (metachronous presentation).[7] Metastases generally arise in the first six years after surgery.[8]

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

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

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

MATERIALS AND METHODS

Patients

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

Tissue sample management

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

Pathological analysis

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

Immunohistochemistry

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

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

VHL gene analysis

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

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

Statistical analysis

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

RESULTS

Patient and tumor characteristics

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

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

Synchronous and metachronous m-ccRCC: a distinct phenotype

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

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Worse clinical outcome with synchronous m-ccRCC

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

DISCUSSION

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

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

Sarcomatoid ccRCC may represent a completed epithelial-mesenchymal transition (EMT).[21] Invasion of the basement membrane and extracellular matrix is an essential event in tumor

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progression and considered a critical step during metastasis.[22] Partitioning-defective 3 (PAR-3), a crucial component of partitioning-defective complex proteins, was recently described as an independent prognostic factor in ccRCC.[17, 23] It is implicated in the development and maintenance of cell polarity. As it modulates cell–cell communication and promotes collective cell migration, PAR-3 may be involved in cancer cell EMT and metastasis formation in ccRCC.[23] Angiogenesis is also considered a crucial step in the progression of cancer. VEGF expression is a key molecular mechanism underlying the initiation and maintenance of the entire tumor process.[24, 25] Tumor progression is often paralleled by higher levels of VEGF expression as cancer cells gradually acquire their malignant potential.[25]

Interestingly, PD-L1 and the non-inactivated *VHL* gene were associated with the primary ccRCC in synchronous metastatic patients. The interaction between PD-1, an inducible inhibitory receptor expressed on lymphocytes and dendritic cells, and PD-L1 ligand, expressed by tumor cells, results in down-regulation of the T-cell response.[26] PD-L1 overexpression may reflect the ability of metastatic tumor cells to evade immune surveillance during migration. *VHL* gene inactivation is likely considered as an initial event in the ccRCC carcinogenic process.[2] Non-inactivation of the *VHL* gene more commonly seen in synchronous m-ccRCC may suggest early involvement of non-dependent VHL pathways such as MAP kinase and PI3K-AKT-mTOR pathways that are also implicated in PD-L1 expression.[27-29]

Compared to primary tumors of synchronous m-ccRCC, metachronous m-ccRCC probably acquire less oncogenic events. During the period between nephrectomy and symptomatic metastasis, initially dormant tumor cells acquire oncogenic events that are not predictable in primary tumors.[30] This explains the heterogeneity between primary tumors and metastasis

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

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

CONCLUSION

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

Clinical practice points:

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

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

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DISCLOSURE

The authors of this article have no relevant financial relationships with commercial interests to disclose and no funding to declare.

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TABLES

Table 1. Summary of the clinical and histopathological characteristics of the 48 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%)

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Table 2. Clinical features and their association with synchronous or metachronous 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‡

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

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Table 3. Histological, immunohistochemical and molecular features and their 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**†

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

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

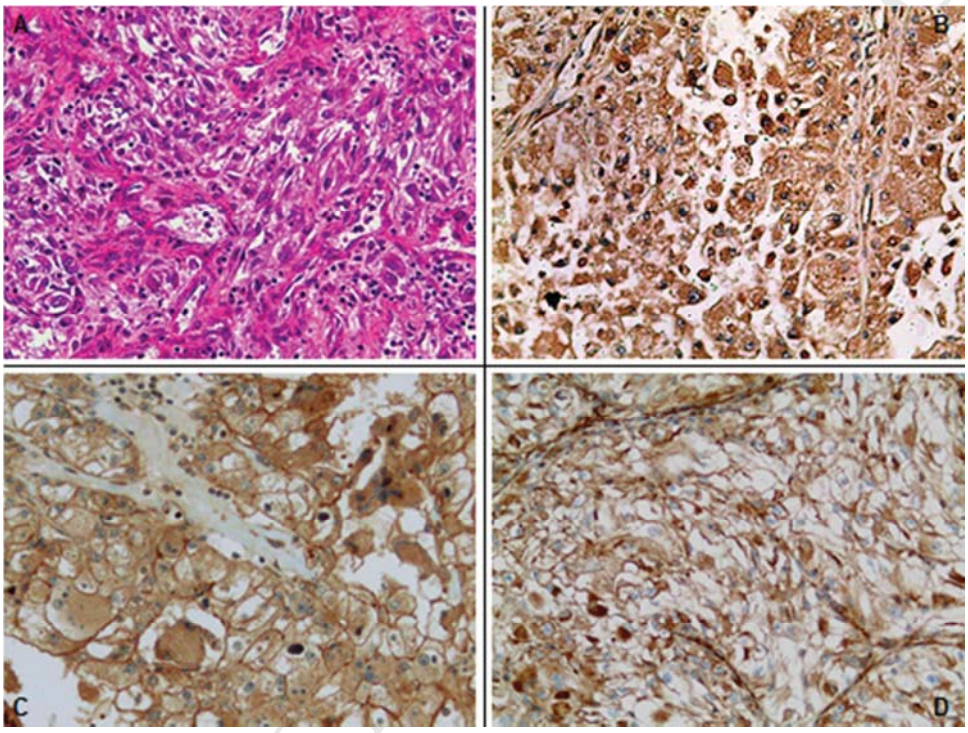

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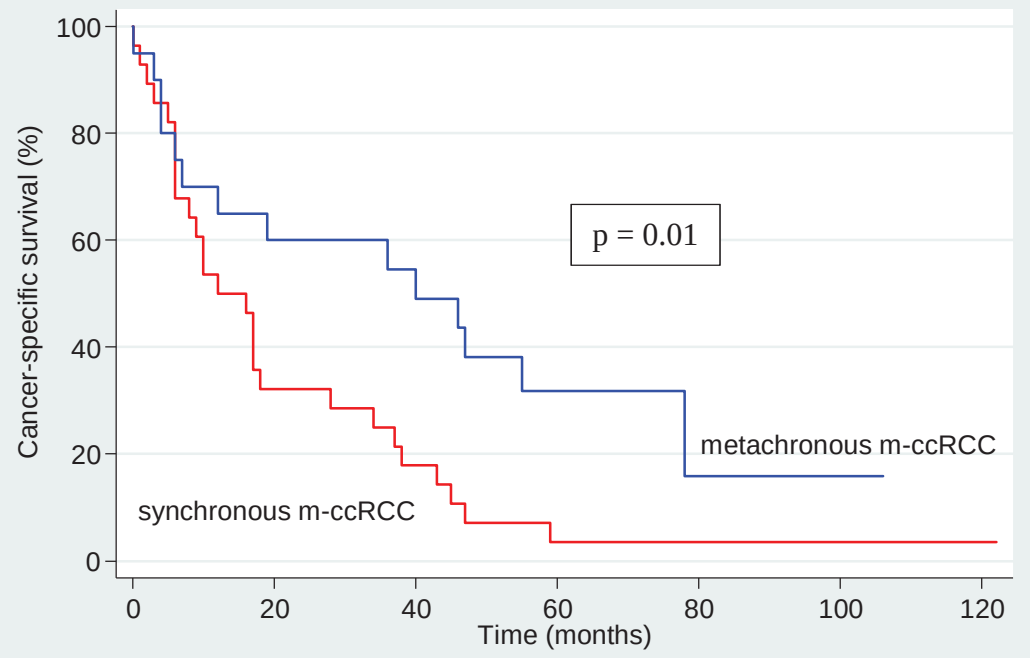
Figure 2. Cancer specific survival in patients from metastasis diagnosis according to metastatic status.



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