Case Report

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Epithelial-to-mesenchymal transition and acquired resistance to sunitinib in a patient with hepatocellular carcinoma

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Background & Aims: Based on the success of sorafenib, several anti-angiogenic therapies are currently evaluated in advanced hepatocellular carcinomas. Few biological data are currently available from patients that may help understanding mechanisms of acquired resistance to these drugs. Herein, we report translational data from a post-treatment surgical specimen in a patient who experienced acquired resistance to sunitinib.

Methods: Clinical, radiological, and pathological data were collected before treatment, under treatment, and at the time of tumor progression. In addition, a biomolecular analysis was performed at the time of progression.

Results: In this patient with non-alcoholic steatohepatitis, initial response to sunitinib was followed by tumor progression within 6 months of treatment, requesting salvage surgical resection. Surprisingly, pathological examination on post-treatment specimens revealed the presence of two juxtaposed tissue components containing either sarcomatoid-like mesenchymal cells or well- to moderately-differentiated hepatocellular carcinoma cells. Cancer cells retain a high α-fetoprotein expression in both components. However, while cells from carcinoma expressed E-cadherin but no vimentin, cancer cells from the mesenchymal component highly expressed vimentin and lost E-cadherin protein expression as measured by immunostaining. *HMGA2* and *Ki67* mRNA were also expressed at higher levels in mesenchymal than in carcinoma cells.

Conclusion: This case report suggests the occurrence of an epithelial-to-mesenchymal transition in discrete areas of hepatocellular carcinomas developing resistance to sunitinib.

Keywords: Epithelial-to-mesenchymal transition; Acquired resistance; Anti-angiogenics; Sunitinib; Advanced HCC.

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Abbreviations: HCC, hepatocellular carcinoma; VEGF, Vascular Endothelial Growth Factor; PDGFR, platelet derived growth factor receptor; SHARP, Sorafenib HCC Assessment Randomized Protocol; α-FP, α-fetoprotein; qRTPCR, quantitative Real Time Polymerase Chain Reaction; HMGA2, High-Mobility Group AT-hook 2; EMT, epithelial-to-mesenchymal transition.

Introduction

Hepatocellular carcinoma (HCC) ranks worldwide as the 5th cause of cancer and the 3rd reason of cancer mortality. While chemotherapy demonstrated limited effects in HCC, VEGF (Vascular Endothelial Growth Factor), and its corresponding VEGF receptors (VEGFR) as well as platelet derived growth factor receptors (PDGFR) have been shown to be attractive molecules for several targeted therapies. Several drugs directed toward VEGF (bevacizumab) and VEGFR/PDGFR (sorafenib and sunitinib) demonstrated activity in a number of malignancies. In HCC, the SHARP (Sorafenib HCC Assessment Randomized Protocol) study was the first to show a 40% improvement of overall survival in patients with advanced HCC treated with sorafenib compared to placebo [1], leading to the approval of sorafenib in advanced HCC and paving the way for further development of VEGFR/PDGFR inhibitors in this disease. Sunitinib is another multitarget tyrosine kinase inhibitor showing promising activity in phase II studies with sustained progression-free survival [2] and leading to the launch of a large multicenter phase III trial comparing sorafenib to sunitinib. Despite the activity of multitarget tyrosine kinase inhibitors, most patients who initially benefit from treatment will finally experience secondary resistance to these targeted therapies. A better understanding of mechanisms associated with resistance may lead to the discovery of novel therapy strategies and/or optimal combinations. The following report illustrates the case of a patient who developed progression under sunitinib and for whom pathological examination provided some clues into understanding the mechanisms of resistance to sunitinib.

Case report

A 71-year-old man was admitted in the hospital for abnormalities of liver tests. Medical history revealed obesity (body mass index = 37), with no history of alcohol abuse, grade 1 arterial hypertension and myasthenia. Morphological aspect of liver tumor mass observed in computerized tomography scan and elevation of the α-fetoprotein (α-FP) were typical of HCC. This diagnosis was further confirmed by a liver biopsy, showing a

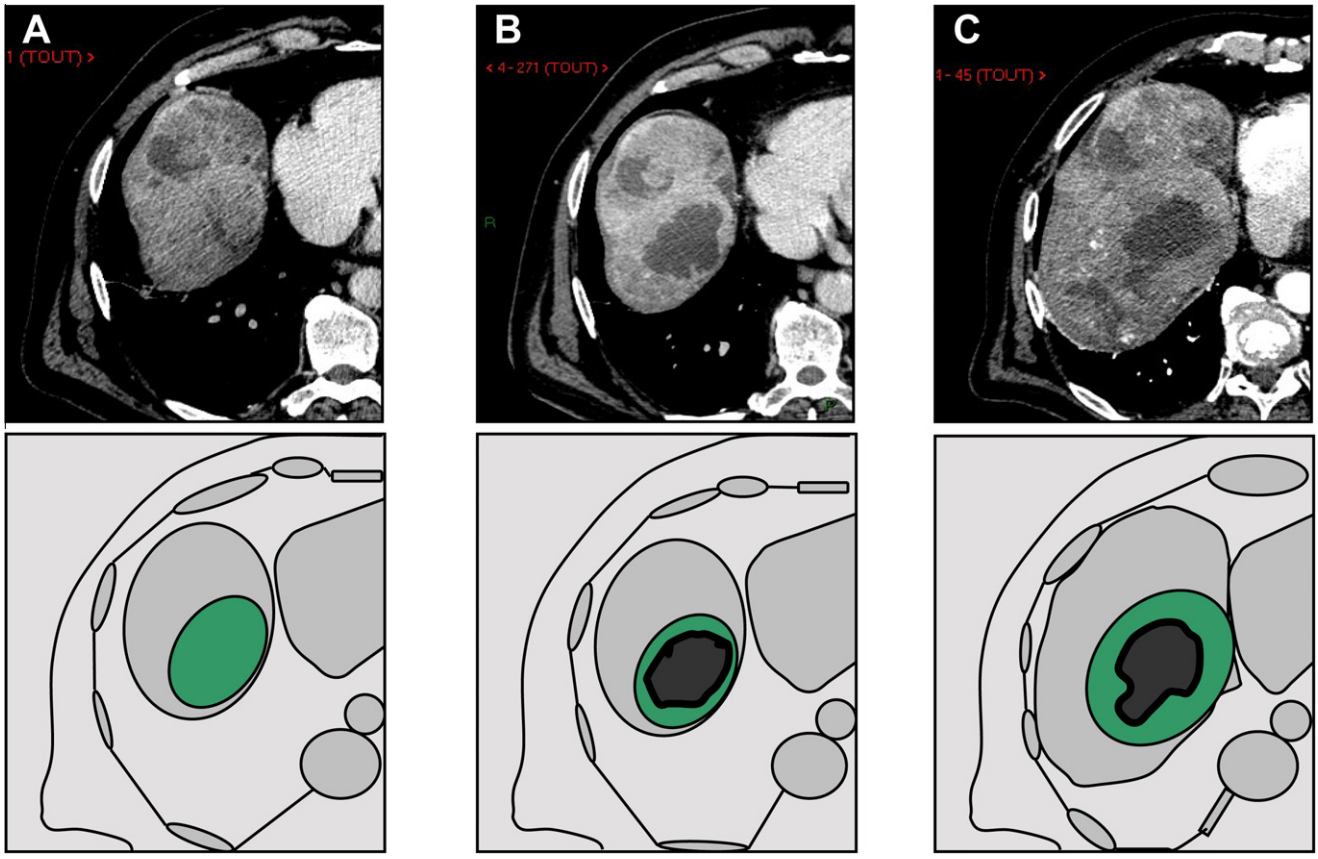

Fig. 1. HCC tomodensitometric aspect at baseline (A), under anti-angiogenic treatment (B), and at progression (C). Lower images are schemes representing the tumor in green and necrosis in gray. Exposure to sunitinib was associated with occurrence of central tumor necrosis and regrowth from peripheral part of the tumor at the time of progression.

well-differentiated HCC associated with liver fibrosis (F3). Viral HBV and HBC serologies were negative and the diagnosis of HCC developed from non-alcoholic steatohepatitis was established. After a multidisciplinary evaluation with surgeons, radiologists, and oncologists, neither surgical resection nor chemoembolization (portal vein obstruction) were easily possible and, since sorafenib was not approved at that time, with the patient's consent, he entered a phase II study evaluating the antitumor activity of sunitinib.

The patient received sunitinib for four consecutive weeks every 6 weeks, initially at the dose of 50 mg/day for 4 weeks then subsequently at 37.5 mg daily dosing due to skin toxicity and asthenia. The first tumor evaluation performed after 4 weeks of therapy showed tumor stabilization according to RECIST criteria. As illustrated in Fig. 1A and B, exposure to sunitinib was associated with an occurrence of central tumor hypodensity on CT-scan as compared to baseline. Hypodensity has been frequently described under targeted therapy and was thought to reflect the occurrence of tumor necrosis. Criteria including both changes in tumor size and measuring tumor hypodensity in gastrointestinal stromal tumors were recently proposed by Choi and coworkers [3] and were found to be applicable in HCC [2]. In our patient both clinical and radiological assessments encouraged us to maintain therapy for up to 24 weeks.

At week 25, this patient was referred to hospital for abdominal pain, lombalgias, and anorexia. At registration, the α-FP level had increased significantly and the CT-scan showed an increase in tumor size corresponding to a disease progression according to RECIST criteria (Fig. 1C). Furthermore, this increased tumor size was associated with changes in tumor density at the periphery of the tumor with an increased thickness of a well-vascularized tumor rim. Those radiological features were consistent with the occurrence of an acquired resistance to sunitinib. In the absence of other possible therapy and the tumor remaining restricted to the liver with no distant metastasis, it was decided to propose a salvage surgical resection and the patient underwent a right hepatectomy.

Macroscopic pathological examination of the liver resection showed a 28 cm-long heterogeneous tumor with fleshy beige areas associated with cholestatic, necrotic, and hemorrhagic areas (Fig. 2A), associated with liver fibrosis (F3). Microscopic analysis led to the identification of two different tumor components. The first component was mainly restricted to the inner part of the tumor and was made by well- to moderately-differentiated HCC (Fig. 2B). In this patient, pathological and immunohistochemical aspects of this well-differentiated component obtained after surgical resection were similar to those obtained on the liver biopsy at diagnosis. The patient also displayed a second tumor component made by non-cohesive, fusiform cells, with marked cellular mesenchymal dedifferentiation, consistent with a sarcomatous aspect (Fig. 2C). This second component was located on surrounding areas of the tumor, invading adjacent

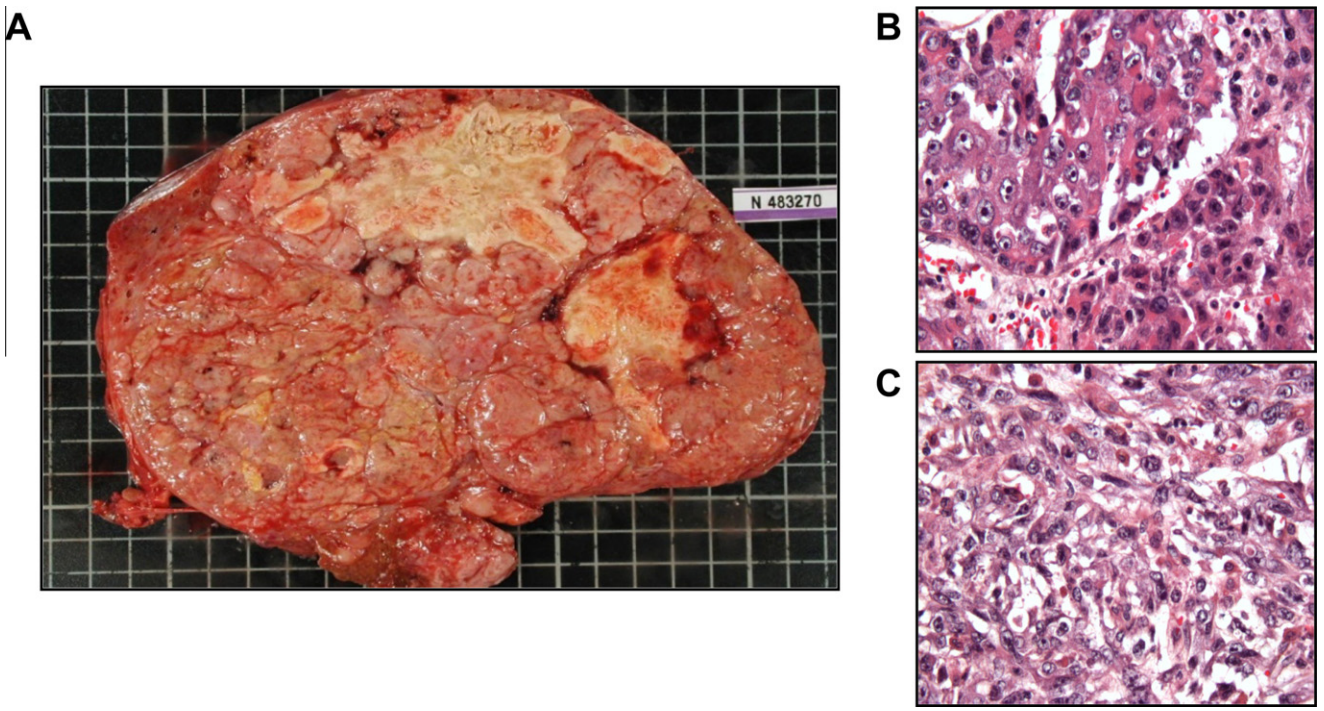

Fig. 2. Pathological analysis at the time of progression under sunitinib. Macroscopic examination (A) showed heterogeneous tumor with fleshy beige areas associated with cholestatic, necrotic, and hemorrhagic areas. Microscopic examination identified one component made by well- to moderately-differentiated HCC (B) and one component made by sarcomatoïd-like cells (C).

non-tumor tissues. This analysis was completed by immunohistochemistry. Cells in both components expressed strong α-FP immunostaining; but while the well-differentiated component highly expressed E-cadherin and did not express vimentin, the mesenchymal section displayed a strong vimentin immunostaining and lost E-cadherin expression (Fig. 3). By immunohistochemistry, N-cadherin expression was found poorly expressed in both component of the tumor without conspicuous differences.

In order to provide additional data consistent with mesenchymal dedifferentiation, we further analyzed this tumor from available remaining frozen tissues by qRT-PCR comparing mRNA expression of a selected panel of genes involved in cell proliferation and differentiation with that of the adjacent liver (Fig. 3). Comparing tumor tissues and adjacent non-tumor hepatic tissues, we found no difference for E-cadherin and N-cadherin mRNA expressions. We previously showed that in cancer cell acquiring a mesenchymal phenotype the gene expression was down-regulated for *S100A4*, also known as the fibroblast-specific protein 1, *Claudin 4* a protein involved in tight junctions of epithelial cells, and mucin *MUC1*, a gene encoding for a membrane bound glycosylated phosphoprotein that anchored to the apical surface of epithelia, [4]. In this study, mRNA expression levels of *Claudin 4*, *S100A4*, and *MUC1* were $\geqslant$4-fold decreased whereas *HMGA2* (High-Mobility Group AT-hook 2) and *Ki67* mRNA levels were, respectively, 5- and 2.5-fold increased in the tumor as compared to non-tumor liver. Comparing the mesenchymal and the epithelial components, we observed no difference in mRNA expressions for *E-cadherin*, *N-cadherin*, *S100A4*, *Claudin 4*, and *MUC1* but slight differences in *KI67* and *HMGA2* mRNA expression levels. In this report, qRT-PCR data strongly suggested that under treatment with sunitinib, cancer cells repressed the expression of genes involved in epithelial differentiation, which along with the strong vimentin and the loss of E-cadherin immunostaining, suggested that cells might have initiated EMT in some area of the tumor.

Discussion

Sarcomatoid differentiation is a growth pattern characterized by spindle-shaped histology i.e. fibroblast-like appearance that can be observed across all subtypes of renal cell as well hepatocellular carcinoma, typically yielding a poor prognosis. Interestingly, fibroblast-like cancer cells with sarcomatoid features may express various levels of epithelial and mesenchymal markers including cytokeratin, vimentin, and serum response factor in HCC – a phenotype consistent with the definition of EMT [5,6]. EMT is a physiological process involved during normal embryonic development. Typically, epithelial cells are (apico-basally) polarized closely joining each other through tight junctions, whereas mesenchymal cells are less structured, having no intercellular junctions and greater motility. EMT was recently described in tumoral tissues and this phenomenon is thought to be a major factor in tumor growth, angiogenesis, and metastasis [7]. During EMT, carcinoma cells undergo a loss of epithelial markers and acquire mesenchymal properties. Vimentin is the mesenchymal marker most commonly associated with EMT. Vimentin expression is associated with a gain in cell motility and invasiveness [8]. High expression of vimentin is usually associated with down-regulation of E-cadherin, a protein involved in tight junctions, and up-regulation of N-cadherin, a protein being expressed essentially during cell migration. In the last years, several *in vitro* studies have shown that tumor cells that acquire an EMT-phenotype may become resistant to several anticancer agents such as

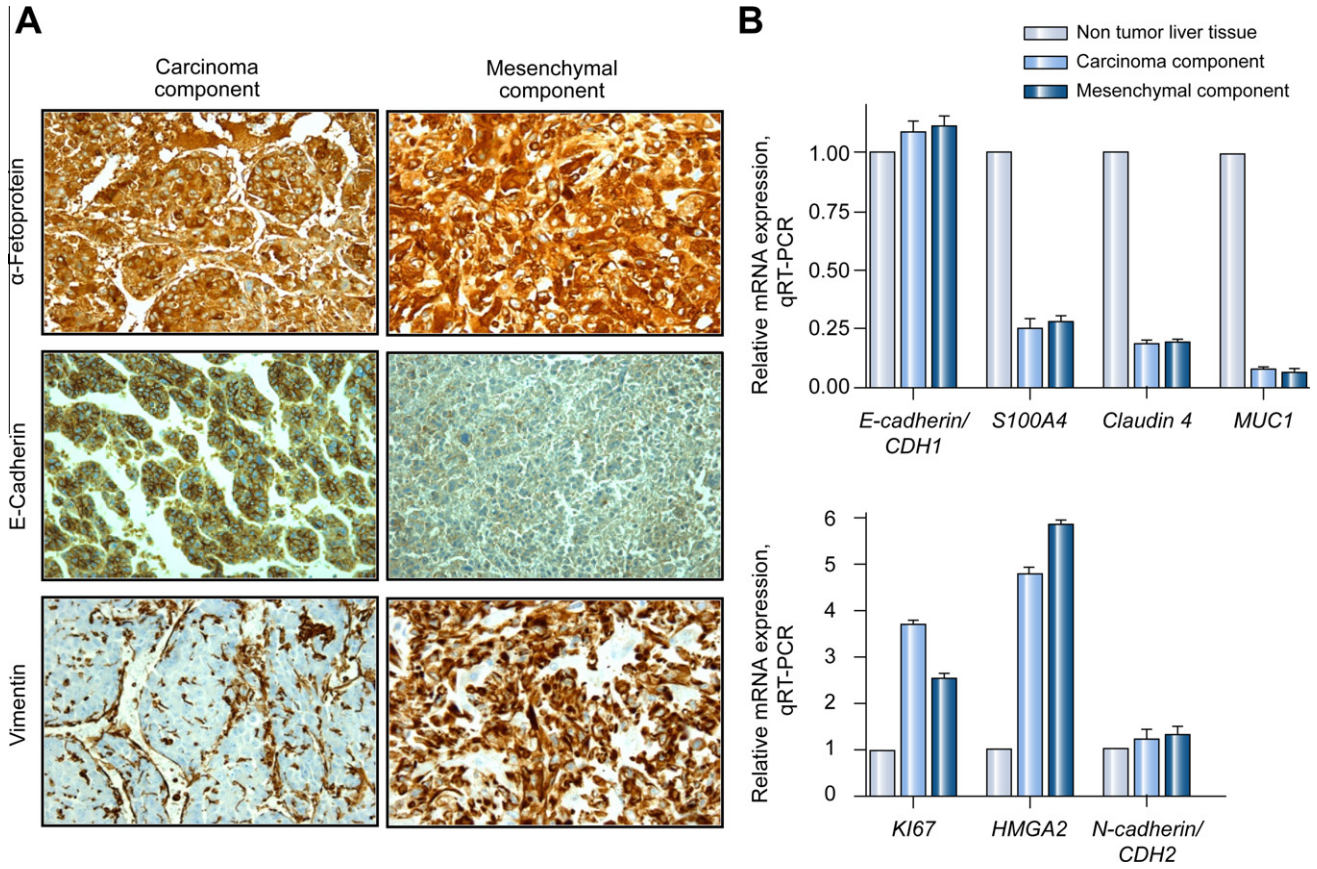

Fig. 3. Proteins (immunohistochemistry) and mRNA (qRT-PCR) expressions of biomarkers in the carcinoma and the mesenchymal components of a hepatocellular carcinoma at the time of sunitinib progression. (A) Cells in both components expressed strong α-FP immunostainings. The well-differentiated carcinoma component expressed E-cadherin and did not express vimentin, while the mesenchymal section displayed no E-cadherin expression and a strong vimentin immunostaining. (B) mRNA of *S100A4*, *Claudin 4*, and *MUC1* were down regulated in both tumor components compared to adjacent liver, whereas *Ki67* and *HMGA2* were up-regulated. mRNA of N-cadherin remained unchanged.

conventional cytotoxics (oxaliplatin [9] and paclitaxel [10]) as well as targeted therapies (protein kinase C modulators [4] and epithelial growth factor receptor 1 inhibitors [11]). To our knowledge, the acquisition of an EMT phenotype during a process leading to acquire resistance to targeted therapy has never been observed in tumor biopsy from a hepatocellular patient in the context of treatment with a multikinase inhibitor such as sunitinib. The presence of mesenchymal cells coexisting with well-differentiated carcinoma cells at diagnosis has been previously described in HCC but remains an uncommon feature. In our case report, the high α-FP immunostaining in both tumor components suggests that cancer cells in the mesenchymal component still express some characteristics typically observed in hepatocytes and, therefore, may derive from carcinoma cells that were present at diagnosis prior to sunitinib treatment.

As expected during EMT, mesenchymal cancer cells in this tumor were poorly differentiated and presented high proliferative capacity, as demonstrated by the high number of cells expressing Ki67. In this tumor, the strong expression of vimentin and the loss of markers of cellular adhesion such as E-cadherin and Claudin 4 in mesenchymal cancer cells are other common features typically observed in cells undergoing EMT. Therefore, it is likely that sunitinib treatment either (1) selected an already preexisting subpopulation of mesenchymal cells that existed in the tumor prior treatment and progressively became predominant or (2) induced cell signaling changes that progressively stimulated the transcriptional EMT machinery toward a mesenchymal differentiation in an increasing number of cancer cells over time.

In this case-report, we detected major transcriptional changes between tumor and non-tumor tissues, mainly oriented with a reduced expression of genes involved in epithelial differentiation. However, we were surprised to observe that most transcriptional changes observed in mesenchymal cells were also observed in well-differentiated carcinoma cells. This suggested that cancer cells becoming resistant to sunitinib, although still bearing a differentiated carcinoma phenotype, already started activating transcription factors of EMT. Upon investigating factors known to activate transcription, we found that tumor exposed to sunitinib over-expressed HMGA2. The high-mobility group A proteins (HMGA1 and HMGA2, formerly HMGI/Y and HMGI/C, respectively) are non-histone chromatin architectural proteins acting on the promoter/enhancer regions of several genes and promoting the recruitment of transcription factors participating in cellular growth, differentiation, and EMT. The over-expression of HMGA proteins correlates with the occurrence of metastasis and poor prognosis in several human cancers. HMGA2 is a nuclear factor that binds AT-rich DNA sequences, contributing

to transcriptional regulation in tumors that has recently emerged as a transcriptional organizer of key signaling molecules such as SNAIL involved in EMT [12], making HMGA2 a major factor in tumor growth and invasion [13]. Data presented in this report supports the important role of HMGA2 in EMT occurring in HCC during treatment with sunitinib.

Interestingly, despite a lower E-cadherin protein expression in the mesenchymal components, we found no difference in the *E-cadherin* mRNA levels between the epithelial and mesenchymal components. This feature may be either due to contaminations of mesenchymal crunch tissues by well-differentiated carcinoma cells and non-tumor hepatocytes during mRNA extraction or may be related to higher E-cadherin protein degradation in mesenchymal cells. Another explanation would be that cancer cells might not have completely silenced the expression of all EMT genes. This later feature has been observed in patients with renal cell carcinomas resistant to sunitinib where cytokeratin and vimentin expression may sometimes coexist together within cancer cells [14]. Furthermore, as observed in renal carcinoma, EMT may be fully reversible, suggesting some plasticity in the transcriptional activation of epithelial and mesenchymal genotypes that could allow the presence of both phenotypes in some tumors [14].

Different mechanisms of sunitinib resistance have been reported such as the expression of additional proangiogenic growth factors, the recruitment of bone marrow-derived cells, increased pericyte coverage, as well as angiogenesis-independent growth patterns. Sunitinib appears as a paradigm drug capable of inducing tumor hypoxia and necrosis, as illustrated in our case report by the occurrence of a large area of tumor hypodensity on CT-scan suggesting either a reduced vascularisation and/or necrosis. Tumor reactions induced by sunitinib in hypoxic and necrotic condition may drive mechanisms of acquired resistance. Tumor cells may adapt to a hypoxic environment by activating specific pathways associated with hypermetabolism, glycolysis and resistance to acidosis-induced toxicity as well as secondary developed neoangiogenesis. Suggested mechanisms included a selection of cells that can better tolerate hypoxia and, in the setting of intrinsic resistance to VEGF inhibition, the recruitment of CD11b and Gr1-positive bone marrow-derived proangiogenic cells [15]. Hypoxia genes that are expressed within solid tumors may result in discrete changes within different areas of tumors, probably contributing to tumor heterogeneity. The link between hypoxia and EMT has been strengthened by the observed activation of EMT transcription factor expression by HIF-1. Another hypoxia-related gene, lysyl oxidase, was found to interact directly with SNAIL, another transcription factor of EMT [16]. Another specific feature of the tumor microenvironment is the stromal reaction through which epithelial–mesenchymal interactions activate or regulate several pathways involving integrins, cytokines, chemokines, and growth factors that are critical for tumor growth and metastasis. Sunitinib being first registered in kidney cancer, resistance to sunitinib has been first observed and described in renal cell carcinoma. Hammers et al. have described the de novo onset of an EMT-like phenotype in a patient with conventional clear renal cell carcinoma on sunitinib treatment. In this report, authors have observed an induction of HIF-1α staining in the skin metastasis as compared with the nephrectomy sample that resolved in the xenografts [14]. The reverted histological phenotype observed in the renal cancer xenografts suggested that the escape mechanisms against anti-VEGFR therapies might be transient. Proposed resistance mechanisms include an increase in alternative proangiogenic factors such as interleukin-8 (IL-8) and basic fibroblast growth factor as seen in the setting of anti-VEGFR2 antibody therapy. The empty basement membrane sheaths and pericyte changes [17,18] may provide the scaffold for tumor angiogenesis in the resistant setting. Bhatt et al. showed that CXCL9 treatment delays resistance to sunitinib in 786-O- and A498-derived tumors suggesting that angiostatic pathways are suppressed as a result of VEGFR inhibitors and set the stage for the subsequent development of resistance to therapy [19]. CXCR3, the receptor for CXCL9, is expressed on both tumor cells and endothelium. CXCR3 signaling in other tumor types has pro-invasive properties. The fact that these angiostatic molecules are expressed by tumor cells in untreated mice and even over-expressed in some tumors that have developed resistance to sunitinib indicates that their presence is not an absolute deterrent to tumor growth and vascularization. This observation also indicated that the early phases of tissue remodeling induced by VEGFR blockade are affected by the presence of CXCL9 and that the disappearance of this chemokine from the tissue facilitates the re-establishment of the tumor micro-circulation and the development of resistance to VEGFR antagonists. Huang et al. showed that the development of sunitinib resistance was accompanied by an increased expression of tumor-derived IL-8, another proangiogenic chemokine that may functionally compensate for the inhibition of VEGF/VEGFR-mediated angiogenesis [20]. Shojaei et al. also investigated modes of resistance to sunitinib in renal cell carcinoma showing that the hepatocyte growth factor and its related receptor c-MET pathway were involved in the development of resistance to anti-angiogenic VEGFR therapy [21]. Elucidation of the mechanisms underlying the acquired resistance to VEGFR blockade may contribute to the development of novel therapeutic approaches that could enhance the efficacy of VEGFR inhibitors in clinical trials.

Altogether, our data suggest that under protracted exposure to sunitinib, HCC may develop resistance that is associated with transcriptional, immunohistochemical, and morphological changes that are consistent with the activation of EMT.

Conflict of interest

Authors have no financial funding from industry to declare, except for S.F. and E.R. who are consultants for PFIZER and BAYER. This study was supported in part by A.A.R.E.C. (Association d'Aide à la Recherche et l'Enseignement en Cancérologie.)

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