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CXCR7 is up-regulated in human and murine hepatocellular carcinoma and is specifically expressed by endothelial cells

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ABSTRACT

Development of hepatocellular carcinoma (HCC) is a complex and progressive disease that involves cycles of liver cell death, inflammation, and tissue regeneration/remodelling. Chemokines and chemokine receptors play numerous and integral roles in the disease progression of HCC. Here we investigated the novel chemokine receptor CXCR7/RDC1 in HCC progression, its two known ligands CXCL12 and CXCL11, as well as the other CXCL12 receptor, CXCR4. Our results show that in a cohort of 408 human HCCs, CXCR7 and CXCL11 were significantly higher in tumours compared to normal liver controls (5- and 10-fold, respectively). Immunohistochemical (IHC) staining on human HCC sections confirmed that both CXCL11 and CXCR7 were much higher in cancer tissues. Furthermore, IHC staining revealed that CXCR7 protein was only expressed in endothelial cells whereas CXCL11 exhibited a much broader tissue expression. At the cellular level we observed that *in vitro*, human microvascular endothelial cells (HMEC-1) up-regulated CXCR7 under hypoxic and acidic pH conditions, which are well known characteristics of the HCC tumour micro-environment. As for its ligand, we observed that IFNγ robustly induced CXCL11 in hepatic stellate cells, hepatocytes, and HMEC-1s. In addition, in the mouse Diethylnitrosamine model of hepatocarcinogenesis we observed a very strong induction of CXCR7 and CXCL11 transcripts, confirming that CXCR7/CXCL11 up-regulation is conserved between human and mice liver cancer. Altogether, our results strongly support the hypothesis that the CXCL11/CXCR7 pathway is involved HCC progression.

1. Introduction

Liver cancer represents a worldwide disease; it is the fifth most common cancer and the third most prevalent cause of cancer mortality.[1] One of the hallmarks of HCC development is that a great majority (70–90%) of all HCC cases arise on a background of cirrhosis, which is the result of chronic liver damage caused by a variety of aetiologies, notably; Hepatitis

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B virus, Hepatitis C virus, alcoholic liver disease, non-alcoholic steatohepatitis and aflatoxin.[2]

Chronic liver injury is typically associated with a high remodelling activity, as well as massive leucocyte infiltration and a non-resolving inflammation.[3] Migration of leucocytes from the blood into the liver during homoeostasis and during liver injury is mainly controlled by chemokines, a family of small chemotactic cytokines. All though initially identified as mediators of leucocyte trafficking and homing, chemokines have been clearly demonstrated to have pleiotropic roles in pathological liver, including growth and survival of hepatocellular carcinoma cells.[4] Indeed, several chemokines and their receptors has been associated with evolution of hepatocarcinoma, notably the CCL20–CXCR6 axis, CX3CL1–CX3CR1, and the CXCL12–CXCR4 axis which have received the most attention.[5] Nevertheless, the CXCL12–CXCR4 pathway is still considered the major chemokine pathway involved in cancer, and is known to promote paracrine tumour growth, tumour cell invasiveness, tumour-angiogenesis and metastasis in numerous cancers.[6,7] However, the role of CXCL12–CXCR4 in the context of hepatocellular carcinoma is still controversial.[5,8]

Recently, a novel receptor for CXCL12, called CXCR7, has been identified and it has been hypothesised as a new molecular link in the chain of connections between inflammation and cancer.[9] In recent years, up-regulation of CXCR7 has been reported in numerous malignancies. It has been reported to promote lung and breast tumour growth,[10] to increase prostate cancer metastasis,[11] to modulate CXCR4 signalling in Rhabdomyosarcomas,[12] and to drive anti-apoptotic effects in human glioma cells.[13] There are still many unanswered questions concerning the functional role of CXCR7. There is evidence that in the context of tumour development, CXCR7 is implicated in cell growth/survival and adhesion, as well as promotion of tumour growth.[9,10] Other reports show that CXCR7 functions as a scavenging receptor for CXCL12 and CXCL11.[14,15] In addition it has been demonstrated that CXCR7 can heterodimerize with CXCR4 and modulate CXCL12 induced migration in trans-endothelial migration assays.[16,17]

Here, we have hypothesised that CXCR7 could be involved in human hepatocellular progression. In the present study, we explored the regulation and expression of CXCR7, and its natural ligands, CXCL12 and CXCL11, in human and mouse hepatocellular carcinoma.

2. Materials and methods

2.1. Patients

Liver tissue samples were obtained from 28 patients with HCC undergoing surgical resection of the tumour. Patients with HCC were 25 males and three females (59.9 ± 9.0 years). HCCs were developed on fibrosis ($n = 8$) and on cirrhosis ($n = 20$) from various aetiology including hepatitis C ($n = 22$), hepatitis B ($n = 7$) and alcohol abuse ($n = 14$). All tumour samples were taken within the tumour, and only tissue with anatomic pathology features that allow a matching diagnosis with the pathology report of each patient were used for RNA extraction. Controls were 10 histologically normal liver samples obtained from metastatic livers of colorectal cancer.[18] Additional human HCC samples ($n = 380$) and controls samples ($n = 16$) were provided by Prof. J. Zucman-Rossi as described by Boyault et al.[19] Access to this material was in agreement with French laws and satisfied the requirements of the local Ethics Committee. All tissue sections were routinely analysed after staining with haematoxylin–eosin–saffran.

2.2. Animal experiments

In the model of liver carcinogenesis, male C57BL/6J-129Sv mice received a single intraperitoneal injection of diethylnitrosamine (DEN) (Sigma–Aldrich Co., St. Louis, MO) at a dose of 10 μg/g body weight at the age of 15 days as initially reported by Vesselinovitch and colleagues.[20] Non-injected males were used as controls. Mice were maintained on normal diet and water *ad libitum* and they received human care in compliance with the national ethical guidelines for the care and use of laboratory animals. Liver samples were taken from several lobes and either snap frozen in liquid nitrogen, or in cooled isopentane, or fixed in buffered formalin. Liver sections 5 μm thick from formalin fixed, paraffin embedded liver tissue were prepared and the sections were routinely analysed after staining with haematoxylin–eosin–saffran.

2.3. Cell lines and primary culture cells

MCF-7 and Jurkat were purchased from American Type Culture Collection. Human micro-vascular endothelial cell line (HMEC-1) was provided from the Centre for Disease Control and Prevention (Atlanta, Georgia). All cell lines were cultured in appropriated medium in presence of FCS as recommended by the supplier. To mimic hypoxic conditions, the HMEC-1 cell line was treated with 250 μM of $CoCl_2$ (Sigma Aldrich) for different times and only during the exponential growth phase of the cells. To decrease and maintain an acidic pH of 6.5, the medium was changed 24 h before analysis with new medium containing 3 mM $NaHCO_3$. For the primary cultures; human hepatic cells were isolated from histologically normal specimens from partial hepatectomy in patients undergoing hepatic resection for liver metastases. Hepatocytes and activated HSC were isolated and cultured as previously described.[21]

2.4. RNA isolation and RT-PCR

Total RNA was extracted from isolated human liver cells or snap frozen liver tissue using the SV Total RNA isolation Kit® (Promega, Charbonnieres-lesbains, France) and 1 μg of total extracted RNA was subjected to a reverse transcription reaction using high capacity cDNA archive kit® (Applied Biosystem, Foster City, CA). A total of 12.5 ng total complementary DNA was used as a template for amplification with 250 ng of primers specific (all listed in Table 1). For real-time quantitative PCR, the mRNA levels were assayed using the 7000 sequence detection system ABI Prism® sequence detector (Applied Biosystems), using the double strand specific dye SYBR® Green system (Applied Biosystems). The PCR condition and cycle were as follows: initial DNA denaturation

Table 1 – Primers used in real time RT-PCR.

Gene	Forward	Reverse
Hum18S	5′GTAACCCGTTGAACCCCATT3′	5′CCATCCAATCGGTAGTAGCG3′
HumCXCL12	5′CTCGGGATGTGTAATGGCT3′	5′GCCTCCATGGCATACATAGA3′
HumCXCL11	5′CAGAGAGGCTGAGACCAACC3′	5′GCTGAAGGTGTGAGCTTTGG3′
Hum CXCR4	5′GATGAAGTCGGGAATAGTCAGC3′	5′AGCATGACGGACAAGTACAGG3′
Hum CXCR7	5′ACGAGACTGACCACCCAGAC3′	5′ACAGGCTATGACGCAACT 3′
Mouse18S	5′TTGGCAAATGCTTTCGCTC3′	5′CGCCGCTAGAGGTGAAATTC3′
MouseCXCL11	5′GTGAGTGCTTTCACCTTCG3′	5′GGAATTCCCCTCCTTGACTC3′
MouseCXCL12	5′CTCTCTGCTTGCCTCCAAAC3′	5′ATTGGTCCGTCAGGCTACAG3′
MouseCXCR4	5′TTCTCATCCTGGCCTTCATC3′	5′CTTTTCAGCCAGCAGTTTCC3′
MouseCXCR7	5′GAGGTCACTTGGTCGCTCTC3′	5′GTGTCCACCACAATGCAGTC3′
MouseVEGF	5′CACGACAGAAGGAGAGCAGAA3′	5′ACACAGGACGGCTTGAAGATG3′

10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, follow by an annealing step, and then extension at 60 °C during 1 min. Each point was performed in triplicate. To ensure that the primers produced a single and specific PCR amplification product, a dissociation curve was performed during the PCR cycle and only primers with a unique dissociation peak were selected, followed by migration on a 2% agarose gel to ensure that the PCR product was unique. The expression level of each gene was adjusted by the level of 18S mRNA and expressed as the ratio to 18S mRNA or as the ratio to the average gene expression level in normal liver.

2.5. *Flow cytometry*

CXCR7 and CXCR4 expressions on the surface of either MCF-7, Jurkat or HMEC-1 was evaluated by flow cytometry using monoclonal antibody against CXCR7 (clone 11G8 from RnD systems) or against CXCR4 (PE-CXCR4, clone 12G5 from BD Pharmingen), and IgG1 isotype was used as a negative control. Cell labelling for flow cytometry was performed in PBS/BSA 1% at 4 °C. Cells were incubated for 45 min at 4 °C with primary antibodies at 5.5 μg/ml for CXCR7 and 5 μl for CXCR4 (solution ready to use). Following washing, cells were incubated with phycoerythrin conjugated goat anti mouse IgG (2.5 μg/ml, Jackson Immunotech, Sulfolk, England) for 45 min at 4 °C in PBS/BSA 1%. After a final washing step, cells were analysed by flow cytometry using cytomics FC500 (Beckman, France).

2.6. *Immunohistochemical staining*

Paraffin embedded liver sections (5 μm) were rehydrated and heated in an antigen retrieval solution (citrate buffer pH 6.0) for 40 min. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide (10 min, RT) and the non-specific sites were blocked with PBS/BSA 2% (1 h, RT). CXCR7, and CXCL11 detection was performed by incubating sections with primary antibodies at RT for 1 h using 2 μg/ml monoclonal anti-CXCR7 (clone 11G8 from RnD Systems) or 0.1 μg/ml rabbit polyclonal anti-CXCL11 (Peprotech, Rocky Hill, NJ). Sections were processed with appropriate biotinylated secondary antibody and a streptavidin biotin peroxidase amplification kit (Vectastain, Vector Laboratories, Burlingame, CA). The peroxidase reaction was finally developed with diaminobenzidine (Dako) and sections were counter stained with Mayer's haematoxylin. Irrelevant serum was used instead of primary antibodies as the negative controls.

2.7. *Statistical analysis*

Statistical comparisons for significance were calculated using first non-parametrical Kruskall–Wallis ANOVA to exclude that difference between groups was due to random sampling. Exact group to group differences was calculated using Wilcoxon's test for paired samples, and with Mann–Whitney *U*-test for un-paired samples. The correlation between continuous variables was examined by means of Spearman's rank-order coefficients. A level of $p < 0.05$ was considered significant. Calculations were made with the commercially available software Statistica.

3. Results

3.1. *In hepatocellular carcinoma, transcripts of CXCR7 and CXCL11 are significantly over-expressed whereas CXCR4 or CXCL12 are not*

Messenger RNA levels of CXCR7 and CXCL11 were measured in 28 HCC samples and compared to 10 normal livers by using real time PCR; CXCR7 and CXCL11 were observed to be significantly induced 5- and 10-fold, respectively in HCC tissues compared to normal liver (Fig. 1A). CXCR4 and CXCL12 mRNA did not show any significant difference between HCC and normal liver. In collaboration with the team of Prof. Zucman-Rossi at the University of Paris VII, we looked at CXCR7 expression on an affymetrix data set that included 58 additional HCC samples. In addition, RNA induction of CXCR7 was measured by qPCR, (using a different primer set then Fig. 1A), in a very large cohort of 322 HCC additional samples, bringing the total number of HCC samples measured to 408. Interestingly, all data sets showed a strong up-regulation of CXCR7 in HCC (Fig. 1B and C).

3.2. *CXCR7 protein is expressed specifically by endothelial cells within HCC tumours as well as in the adjacent fibrotic liver*

To confirm that the mRNA induction of CXCR7 in HCC is also observed at the level protein level, we stained normal liver and HCC paraffin embedded sections using an anti-CXCR7

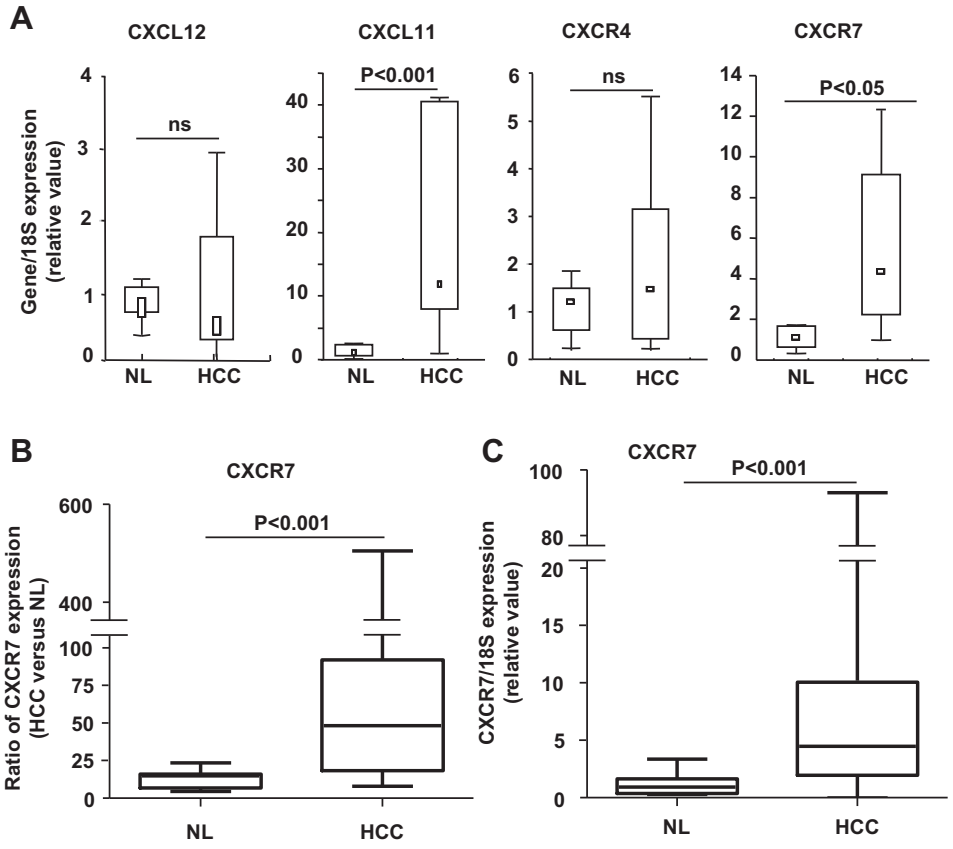

Fig. 1 – Expression of CXCL11, CXCL12, CXCR4 and CXCR7 in human normal liver and in human hepatocarcinoma (HCC). (A) Box plot representing the mRNA expression of CXCL12, CXCL11, CXCR4 and CXCR7 measured by real time PCR in NL (Normal Liver, *n* = 10) and HCC (*n* = 28). The y-axis values represent the induction of each gene relative to the control after normalisation by the 18S RNA expression. Each point was performed in triplicate. (B) Data mining of CXCR7 expression in an affymetrix data set of 58 HCC compared to seven normal livers (NL). (C) In collaboration with Dr. Zucman-Rossi CXCR7 mRNA expression was measured in an additional 322 HCC and compared to 9 NL. Statistical comparisons for significance were made with Mann–Whitney *U*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

specific antibody (11G8 clone-R&D systems) (Fig. 2C–E). It is important to underline that we tested several other CXCR7 antibodies; two rabbit polyclonal antibodies against human CXCR7 (Genetex and Abcam) and found that they did not stain specifically. A recent paper published by Berahovich and colleagues confirmed that most CXCR7 antibodies commercially available were not specific, except for CXCR7 11G8 clone, confirming our IHC observations.[22] Irrelevant IgG showed no staining in normal livers (Fig. 2A) and in HCC samples (Fig. 2B). In normal livers, CXCR7 was sparsely expressed by a few large vessels (Fig. 2C). In HCC the number of endothelial cells positive for CXCR7 dramatically increases (Fig. 2D) compared to normal liver. Interestingly, endothelial cells positive for CXCR7 are distributed throughout both the adjacent non-tumoural fibrotic liver and in the tumour itself (Fig. 2E and F). Furthermore, in Supplementary data S1, we looked at CXCR7 expression by flow cytometry on human primary hepatocytes (HPH), HCC cell lines (HepG2, HuH-7, Hep3b and B16A2), gall bladder adenocarcinoma cell line (huGB), and a cholangiocellular carcinoma cell line (huCCT1). No staining was observed in all cell lines examined. This confirms our observation IHC staining where only endothelial cells were positive for CXCR7.

3.3. *CXCL11 protein is strongly expressed by both parenchymal and non-parenchymal cells in HCC tumours as well as in the adjacent fibrotic liver*

CXCL11 staining in normal liver was faint, cells positive for CXCL11 were essentially liver lymphocytes and a very small percentage of hepatocytes (Fig. 3C). Specificity of such staining was demonstrated by controls performed with the appropriate pre-immune rabbit serum and showed no specific staining (Fig. 3A and B, respectively). In HCC, CXCL11 staining was very intense and located to several types of cells: in the HCC nodules, cancerous hepatocytes showed intense staining but only at the periphery of the nodules (black arrow), lymphocytes presented both in the tumour and in the adjacent fibrotic tissue were positive (dotted arrow) (Fig. 3D, E and F). Moreover, endothelial cells located in the fibrous tissue of the liver also stained positively for CXCL11.

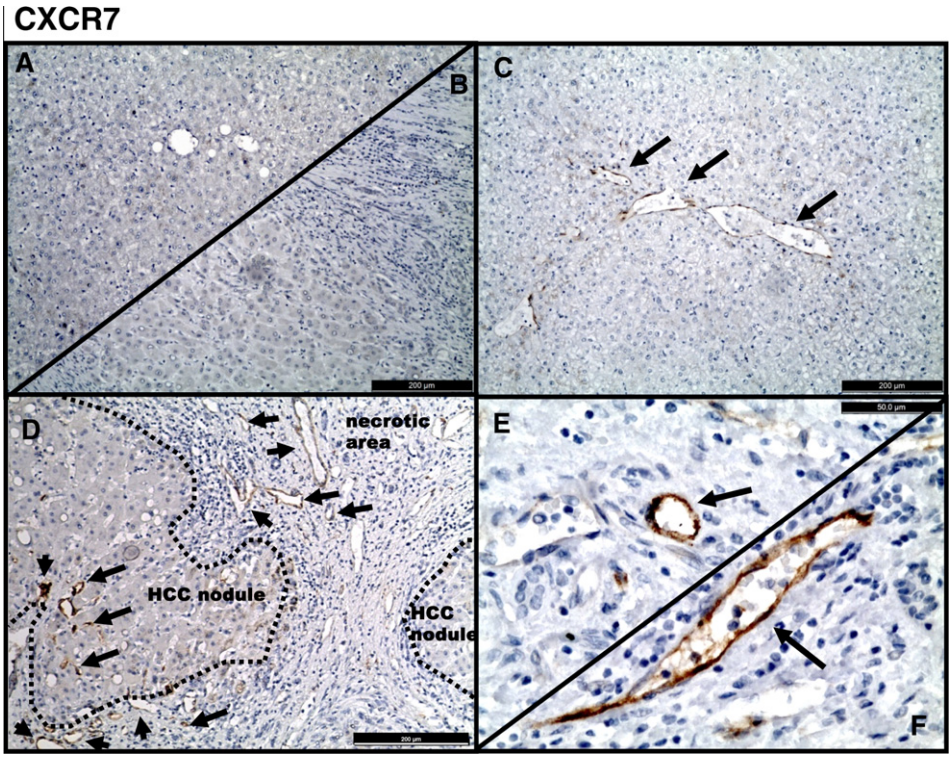

Fig. 2 – CXCR7 localisation in human normal liver and in human hepatocellular carcinoma (HCC). Immunohistochemistry was performed with isotype controls (A and B) or with anti-CXCR7 antibody (C–F) in normal liver (A and C) and liver tissues from patients with hepatocellular carcinomas (B, D, E and F). Cells staining positive for CXCR7 are indicated with full arrows (endothelial cells) and HCC nodule area are surrounded with dotted line. Sections were counterstained with haematoxylin. Scale bars represent 200 μm (A–D) or 50 μm (E and F).

3.4. *IFN-γ positively regulates CXCL11 mRNA and protein in human hepatic cells*

A panel of several cytokines (IFN-γ, TGF-β, IL-10 or IL-4) known to be highly expressed in human hepatocellular carcinoma was tested for their ability to induce CXCL11, CXCL12, CXCR4 and CXCR7 in several types of human hepatic cells; human primary hepatic stellate cells (HSC) and human primary hepatocytes.[23] Our results show that IFN-γ induced CXCL11 mRNA in both HSC and hepatocytes (Fig. 4A). CXCL12, CXCR4, and CXCR7 mRNA showed no significant induction (data not shown). CXCL11 protein secretion was measured by ELISA in the conditioned media of either HSC or hepatocytes, after stimulation with the indicated cytokines. The results of this assay showed that only IFN-γ induced CXCL11 protein secretion. Normalisation of the CXCL11 concentration to mg of protein showed that human hepatocytes were the largest contributors to secreted CXCL11 after IFN-γ stimulation (Fig. 4B). Dose response experiments with increasing concentration of IFN-γ were performed in HSC, hepatocytes and in a third cell type; human endothelial micro-vascular cells (HMEC-1). In all cell types studied CXCL11 responded in a dose dependent manner to increasing concentrations of IFN-γ (Fig. 4C). CXCL12, CXCR4, and CXCR7 mRNA showed no significant induction (data not shown).

3.5. *Hypoxia and low pH up-regulates CXCR7 in human microvascular-endothelial cells*

During HCC tumour development, it is well described that numerous environmental changes occur in the liver tissue micro-environment which include changes in oxygen distribution and pH.[24,25] Here we tested several hypoxia mimicking agents as well as low pH which for their ability to induce CXCR7 in human endothelial micro-vascular cells (HMEC-1). HMEC-1s were exposed to a chemical hypoxia inducer, $CoCl_2$ (250 μM), for several timepoints. Our results show that CXCR4, CXCR7 and VEGF (positive control for hypoxia) mRNA, measured by real time PCR, were all up-regulated in a time related manner in response to $CoCl_2$ treatment (Fig. 5). Confirmation of CXCR7 induction was performed by flow cytometry using anti-CXCR7 (11G8 clone). Protein induction of CXCR7 was observed to be maximal at 24 h after treatment with 250 μM $CoCl_2$ (Fig. 6A). Furthermore, HMEC-1 cells were also observed to up-regulate significantly CXCR7 at the protein level when shifted from a neutral pH (7.4) to a low pH media (6.5) (Fig. 6B).

3.6. *Up-regulation of CXCL11, CXCR7 and CXCR4 in the DEN mice model of hepatocellular carcinoma*

DEN (diethylnitrosamine) is a common liver carcinogen used to model the development of hepatocellular carcinoma in

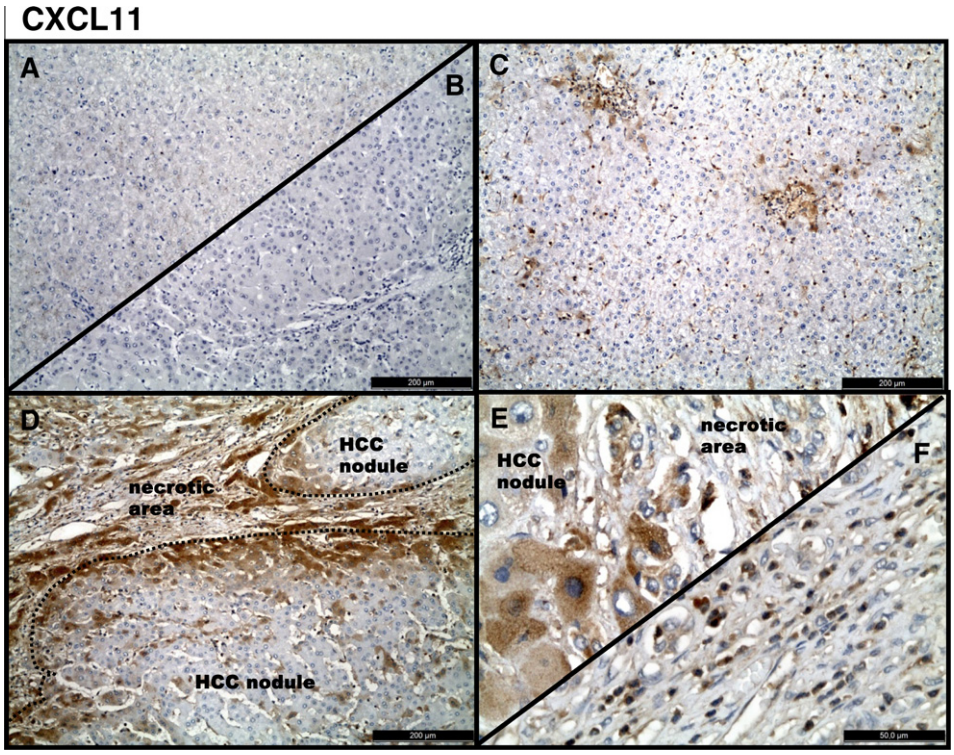

Fig. 3 – CXCL11 localisation in normal human liver and hepatocellular carcinoma (HCC). Immunohistochemistry was performed with antibodies pre-immune rabbit serum (A and B) or with anti-CXCL11 rabbit polyclonal antibody, (C–F) in normal liver (A and C) and liver tissues from patients with hepatocellular carcinomas (B, D, E and F). Sections were counterstained with haematoxylin. Cells staining positive for CXCL11 are indicated with either full arrows (cancerous hepatocyte) or dotted arrow (lymphocytes). HCC nodule area is surrounded with a dotted line and partly zoomed in E, fibrotic and necrotic area beside HCC nodule is zoomed in F. Scale bars represent 200 μm (A–D) or 50 μm (E and F).

mice. DEN was injected one time at 15 days of age, and 12 months later the mice developed HCC. In this model, no lesion was detected by histological analysis before 6 months. Basophilic foci of altered hepatocytes were identified at 6 months, hepatocellular adenomas at 8 months, and multifocal HCC at 12 months.[26] Here we collected liver samples from mice injected with DEN at 3, 5, 7, 9 and 12 months and we measured the RNA induction of CXCL11, CXCL12, CXCR4, CXCR7 and VEGF. Our data shows that no significant up-regulation was observed until the 12 month time-point, at which we observed a robust up-regulation of both CXCR4 and CXCR7. In addition, CXCR4 and CXCR7 mRNA expression showed a very strong correlation as show by a Spearman rank analysis ($r = 0.6435$, $p = 0.0007$) (Fig. 7C). With concern to CXCR7 ligands, we observed that CXCL11 was up-regulated in DEN induced HCC, as well as VEGF but not CXCL12 (Fig. 8). The overexpression of CXCR4 and CXCR7 was also observed in another murine model of HCC (ASV-induced HCC) (*Supplementary* data S2).

4. Discussion

In this report, we evaluated the expression of the novel chemokine receptor CXCR7, as well as CXCR4, CXCL12 and CXCL11 in human and murine hepatocarcinogenesis. Our results show a strong mRNA induction of CXCR7 and of CXCL11 in HCC compared to normal liver, which was confirmed at the protein level by immunohistochemistry. Interestingly, neither CXCL12 nor CXCR4 showed a significant mRNA induction between control and HCC samples. This result is consistent with numerous reports that have also observed either no difference or a down-regulation of CXCR4 and CXCL12 in HCC, suggesting that in liver CXCL11 is potentially CXCR7s major ligand.[27,28]

At the protein level, CXCR7 expression was restricted to only endothelial cells in all samples studied. Tumour feeding endothelial cells positive for CXCR7 have also been observed in numerous other human cancer tissues, such as breast carcinoma, lung adenocarcinoma, ovary mucinous adenocarcinoma, bladder transitional cell carcinoma and kidney renal cell carcinoma.[10] We also screened a large number of human liver HCC cell lines, and found no staining confirming our IHC results. Zhen and colleagues recently reported that HCC cells were positive for CXCR7; this difference in results is most likely due to their use of a different antibody.[29] Regarding the protein distribution of CXCL11, we observed that capillaries, hepatic stellate cells, infiltrating lymphocytes, hepatocytes were positive in the fibrotic tissue adjacent to the tumour nodules. Within tumour nodules, only malignant hepatocytes at the periphery of the tumour nodule were markedly positive for CXCL11.

Interestingly, we observed that only one cytokine, IFNγ was sufficient in inducing CXCL11 at massive levels in numerous primary liver cells and endothelial cells (human hepatic

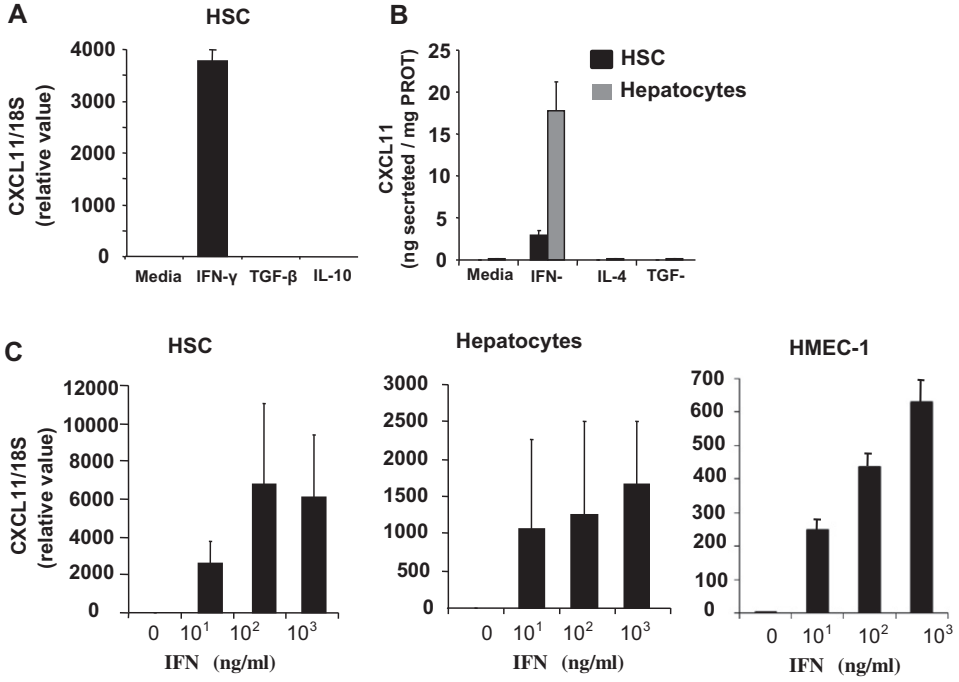

Fig. 4 – Regulation of CXCL11 by cytokines in isolated human liver cells and endothelial cells. (A) Transcript production of CXCL11 was measured by real time PCR on primary hepatic stellate cells (HSC) stimulated by 100 ng/ml IFNγ, 5 ng/ml TGFβ and 10 ng/ml IL-10 during 8 h. (B) CXCL11 proteins were measured by ELISA on conditioned media of primary hepatic stellate cells (black column) and human primary hepatocytes (grey column) stimulated by 100 ng/ml IFNγ, 50 ng/ml IL-4 and 5 ng/ml TGFβ and during 24 h. (C) Transcript production of CXCL11 was measured by real time PCR on primary hepatic stellate cells (HSC), human primary hepatocytes and human micro-vascular endothelial cells (HMEC-1) stimulated by 10, 100 and 1000 ng/ml IFNγ during 8 h. The y-axis values represent the induction of each gene relative to the untreated control culture, after normalisation by the 18S RNA expression. Standard deviation was representative of the variation of expression between at least three independent experiments.

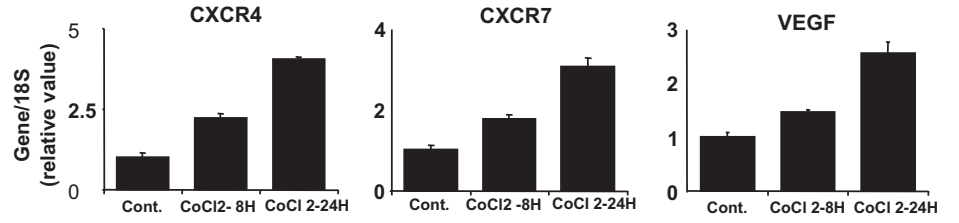

Fig. 5 – Regulation of CXCR4, CXCR7 and VEGF in HMEC-1 treated by $CoCl_2$. Transcript production of CXCR4, CXCR7 and VEGF was measured by real time PCR in human micro-vascular endothelial cells (HMEC-1) stimulated by 250 μM $CoCl_2$ for 8 and 24 h. The y-axis values represent the induction of each gene relative to the control untreated, after normalisation by the 18S RNA expression. Standard deviation was representative of the variation of expression between at least three independent experiments.

stellate cells, primary human hepatocytes and human micro-vascular endothelial cells (HMEC-1)). Furthermore, we observed that human hepatocytes in primary culture produced 5-fold more CXCL11 than hepatic stellate cells and thus represent possibly the major source of CXCL11 in inflamed liver. This result is not-surprising since, CXCL11 is part of the class of IFN-γ inducible chemokine which also includes CXCL9 and CXCL10 which are clearly up-regulated during liver inflammation.[30] Similarly, Helbig and colleagues demonstrated that stimulating the hepatocarcinoma cell line HuH-7 with IFNγ also resulted in a robust production of CXCL11 *in vitro*, suggesting that IFNγ could be a major regulator of CXCL11 both in malignant and in inflamed liver cells.[31]

In regards to CXCR7 regulation, we observed that $CoCl_2$, a hypoxia mimicking agent, as well as acidic pH robustly induced CXCR7 in only human microvascular endothelial cells (HMEC-1). Interestingly, a recent report observed *in vivo* a significant induction of CXCR7 in hypoxic lung, suggesting that hypoxia could be a major positive regulator for CXCR7.[25] In addition, Liu and colleagues recently reported that both $CoCl_2$ also triggered the up-regulation of CXCR7 on mesenchymal stem cell, suggesting that hypoxia might a general regulator

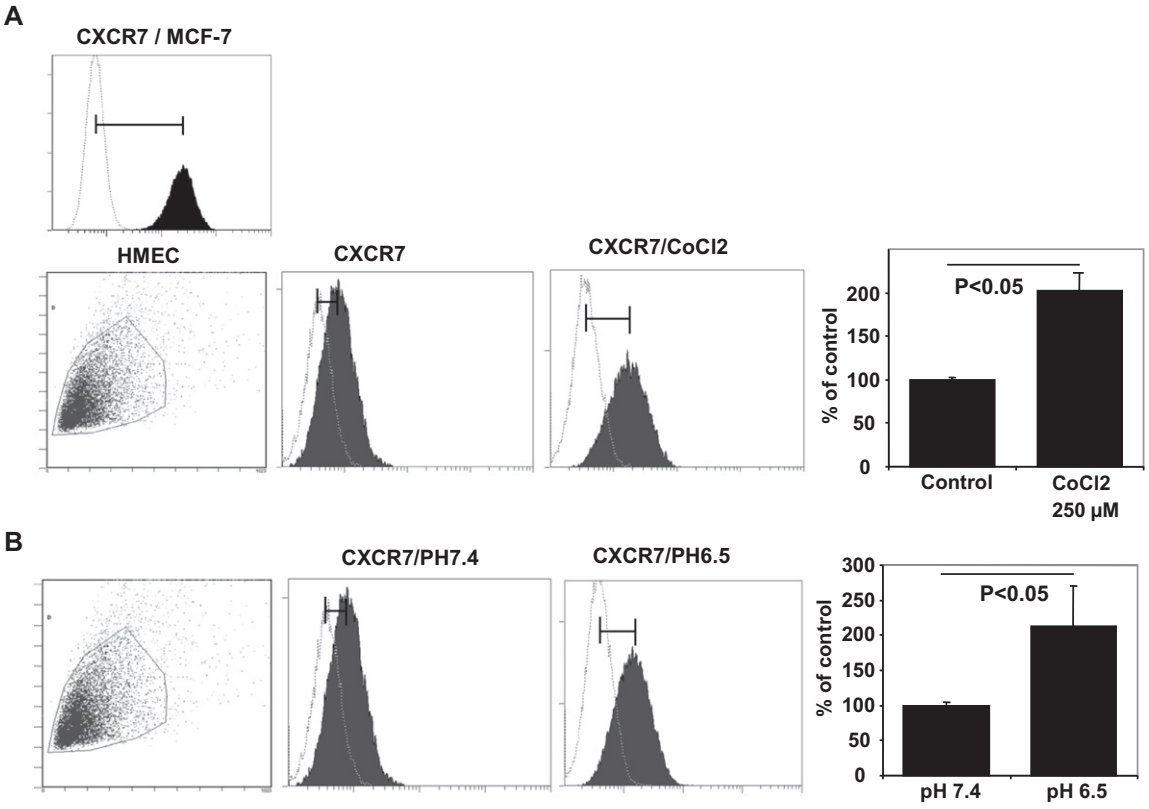

Fig. 6 – Regulation of CXCR7 in HMEC-1 treated by $CoCl_2$ or pH 6.5. Expression of CXCR7 on the surface of HMEC-1 was characterised by flow cytometry using anti-CXCR7 11G8 monoclonal antibody. Cells were cultured in the presence of 250 µM $CoCl_2$ (A) or at pH 6.5 (B) during 24H before flow cytometry analysis. Positive control experiment for CXCR7 staining was performed on MCF-7 cells. Representative SSC/FSC graphs and fluorescence intensity histograms were presented. In parallel, fluorescence intensity ratio of CXCR7 labelled-HMEC-1 versus isotype control were calculated and expressed as a percentage of control (i.e. treated versus untreated cells) (mean value ± SEM, $n = 4$ independent experiments). * Indicates statistical significance using the t-test with $p < 0.05$.

of CXCR7.[32] In addition, there is also a well-known link between hypoxia and acidification of the micro-environment during cancer progression.[33,34] It has been well documented that the HCC micro-environment is strongly hypoxic and have high activation levels of hypoxia inducible factors.[35] Hence, our results showing that low pH and hypoxia are able to induce CXCR7 on endothelial cells *in vitro* are compatible with the actual microenvironment in the liver during HCC progression, and provide a novel mechanism by which CXCR7 could be regulated *in vivo* during hepatocellular carcinoma progression.

Based on our results we hypothesise that the functional role of CXCR7 on inflamed endothelial cells in the liver, is to regulate the chemokine gradient of CXCL11, thereby controlling the migration of CXCR3+ T-cells into the inflamed liver tissues. Numerous articles have reported a crucial function of CXCR7 in controlling the migration of CXCR4+ cells towards CXCL12 gradients by sequestering excess CXCL12. Excess chemokine will result in desensitization of the chemokine receptor and block cell migration, and, therefore, CXCR7 sequestration of CXCL12 can be of vital importation for the migration of CXCR4+ cells.[36–38] Similarly, a recent paper by Cruz-Orengo and colleagues showed that by scavenging and controlling the CXCL12 gradient, CXCR7 expression on inflamed endothelial cells of the CNS was essential in controlling CXCR4+ leucocytes entry into the tissue.[39] Furthermore, several reports have demonstrated that CXCR7 expression on endothelial positively influenced the trans-endothelial migration of CXCR4+ cells.[17–40] Therefore, in the context of hepatocellular carcinoma, CXCL11 which is being massively produced by numerous cell types and released in the blood could be scavenged by CXCR7+ endothelial cells. It has already been well documented that CXCR3+ T-cells are highly present in HCV-infected livers and are also have been detected within HCC nodules.[41,42] Hence, by controlling the CXCL11 gradient, CXCR7 could participate in facilitating the migration of CXCR3+ T-cells (the signalling receptor for CXCL11), into HCC tissues. Moreover, we demonstrate that CXCL11 and CXCR7 are also highly expressed in several murine model of hepatocellular carcinoma (DEN and ASV). The CXCR7-CXCL11 axis seems to be conserved between murine and human hepatocellular carcinoma and, therefore, the murine hepatocellular carcinoma models could represent good models for studying the relative contribution of CXCR7 and CXCL11 to liver cancer.

In conclusion, our data demonstrate that CXCR7 and CXCL11 are highly expressed in human and mouse HCC, and that CXCR7 is specifically expressed on endothelial cells.

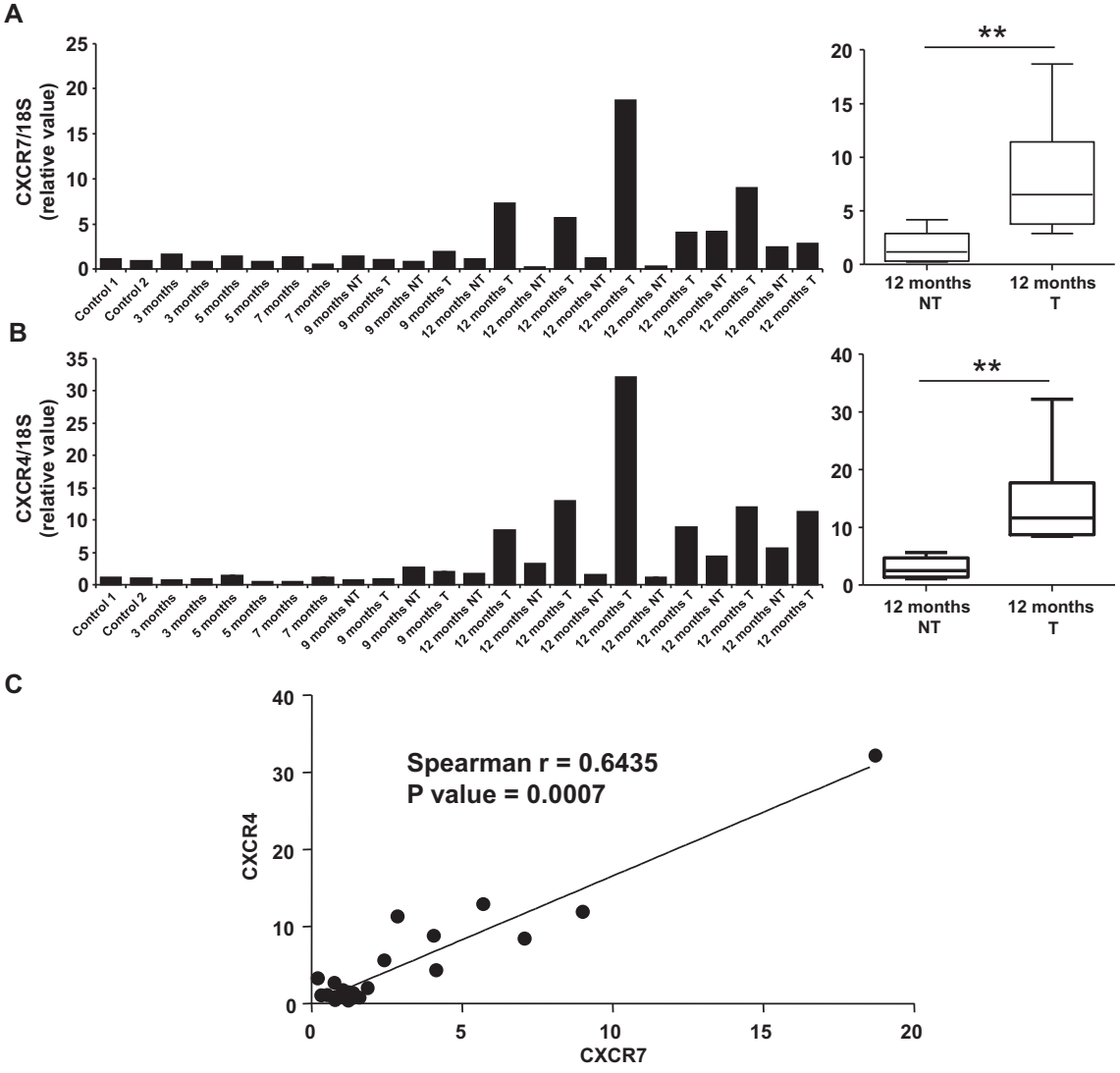

Fig. 7 – Expression of CXCR4 and CXCR7 in mice diethylnitrosamine DEN-induced liver tumours. mRNA expressions of CXCR4 (A) and CXCR7 (B) were evaluated by real time PCR in livers collected from mice given a single dose of DEN or PBS (control mice) and sacrificed after 3, 5, 7, 9 and 12 months of treatment. For time of 12 months, total RNA were extracted from HCC (T) and non-tumoural (NT) tissues. The y-axis values represent the induction of each gene relative to the control receiving PBS, after normalisation by the 18S expression. Each point was performed in triplicate. On the right panel, the boxplots represent levels of mRNA CXCR7 and CXCR4, in non-tumoural (NT) and HCC (T) pieces of liver from mice ($n = 6$) injected with DEN at 12 months, the bars correspond to the atypical variations, the boxes to the values between 25% and 75%, the band at the middle to the median. Statistical comparisons for significance were made with Mann–Whitney U-test. $^{**}p < 0.01$. (C) Correlations between CXCR4 and CXCR7 expression levels in all mice liver samples were calculated, Spearman's rank order coefficient and the p-value are indicated.

In addition, we identified that CXCR7 is up-regulated in human micro-vascular endothelial by hypoxia and low pH, environmental conditions which are present in HCC nodules. Based on the known biology of CXCR7 on endothelial cells we hypothesise that the CXCR7 could be participating in sequestering circulating CXCL11 and thereby controlling the CXCL11 gradient during liver HCC development.

Conflict of interest statement

None declared.

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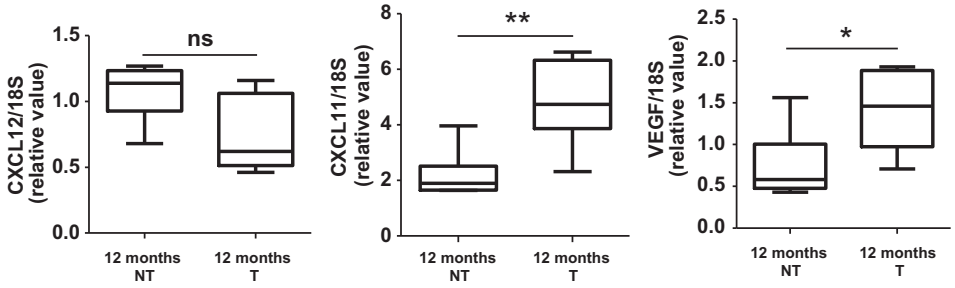

Fig. 8 – Expression of CXCL12, CXCL11 and VEGF in mice DEN-induced liver tumours. Box plot representing the mRNA expression of CXCL12, CXCL11 and VEGF measured by real time PCR in non-tumoural (NT) and tumoural (T) pieces of liver from mice ($n = 6$) injected with DEN at 12 months. The y-axis values represent the induction of each gene relative to the control after normalisation by the 18S expression. Each point was performed in triplicate. The bars correspond to the atypical variations, the boxes to the values between 25% and 75%, the band at the middle to the median. Statistical comparisons for significance were made with Mann–Whitney *U*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, ns: not significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.06.044.

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