EUROPEAN JOURNAL OF CANCER 46 (2010) 3141–3148

available at www.sciencedirect.com

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journal homepage: www.ejconline.com

Review

Carbonic anhydrase 9 in clear cell renal cell carcinoma: A marker for diagnosis, prognosis and treatment

Jacques Tostain [a,*], *Guorong Li* [a], *Anne Gentil-Perret* [b], *Marc Gigante* [a]

[a] *Department of Urology–Andrology, University Jean Monnet, Teaching Hospital, Saint Etienne, France*
[b] *Department of Pathology, University Jean Monnet, Teaching Hospital, Saint Etienne, France*

ARTICLE INFO

Article history:
Received 30 June 2010
Accepted 14 July 2010
Available online 13 August 2010

Keywords:
Renal cancer
Biomarkers
Carbonic anhydrase 9
Diagnosis
Prognosis
Immunohistochemistry
ELISA
RT-PCR
Treatment

ABSTRACT

Carbonic anhydrase 9 (CA9) is a transmembrane member of the carbonic anhydrase family. It catalyses the reversible hydration of carbon dioxide into bicarbonate and a proton, thus enabling tumour cells to maintain a neutral pH despite an acidic microenvironment. CA9 is not expressed in healthy renal tissue but is expressed in most clear cell renal cell carcinomas (CCRCC) through HIF-1α accumulation driven by hypoxia and inactivation of the VHL gene. CA9 expression can be detected in the tumour by immunohistochemistry (IHC), in blood and tissue by ELISA assay and RT-PCR. It has a 100% diagnostic specificity in solid renal tumours, while ELISA assays on aspiration fluids may help in atypical cysts. Blood-based assays, ELISA for CA9 antigen and RT-PCR for CA9 mRNA are promising for the prognosis and follow-up of localised CCRCC. In metastatic disease, high CA9 expression by IHC was reported to be a powerful prognostic marker with better survival and sensitivity to IL-2, but this is still debated. Almost no data are currently available on the association of CA9 expression and outcome to targeted drugs. The prognostic value of CA9 in CCRCC could be explained by the frequent VHL gene inactivation driving an early activation of the HIF pathway. The poorer prognosis associated with low CA9 expressing tumours could be due to the simultaneous overexpression of EGFR contributing to the activation of AkT and mTOR pathways. Targeting CA9 by inhibitors, radioimmunotherapy, monoclonal antibodies or vaccination is promising and offers new avenues for clinical research.

1. Introduction

Renal cell carcinoma incidence is increasing and represents 2–3% of adult malignancies, with more than 200,000 new cases and more than 100,000 deaths every year throughout the world.[1] About 80% are clear cell renal cell carcinomas (CCRCC) that are more aggressive than papillary or chromophobe carcinomas. One third of patients have metastases at diagnosis and among those with a clinically localised disease, 30–40% will develop metastases after surgery.[2] TNM staging and nuclear grade are major prognostic factors, but tumours with similar TNM stage and grade may still have very different outcomes. Radical or partial nephrectomy remains the gold standard for the management of clinically localised disease. Using a risk stratification nomogram, such as the Mayo nomogram[3] allows more tailored follow-up. Intermediate and high-risk patients are followed-up by repeated, expensive imaging. For metastatic patients, targeted therapies have

* *Corresponding author:* Tel.: +33 (0)477828331; fax: +33 (0)477828464.
E-mail address: jacques.tostain@chu-st-etienne.fr (J. Tostain).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.07.020

improved response rates compared to immunotherapy but are indicated only when imaging shows metastases.

Research into biomarkers is a very active domain in renal oncology,[4] seeking to improve diagnosis, identify tumours with a high risk of recurrence, allow easier follow-up, suggest earlier adjuvant treatment and select the best treatment option for metastatic patients. Carbonic anhydrase 9 (CA9), one of the most studied surface antigens in CCRCC, is to date the most promising biomarker to meet these requirements.

2. Ca9 in CCRCC

2.1. *Function of carbonic anhydrases – identification of CA9*

Carbonic anhydrases (CAs) are metalloenzymes expressed in almost all tissues. Their main function is to catalyse the reversible reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3$. Bicarbonate is actively incorporated and neutralised by a proton with the catalytic action of intracellular CAs; this generates carbon dioxide which then passively diffuses into the extracellular space where it is captured by extracellular CAs to be converted into bicarbonate and a proton. The result, typical of solid tumours, is a neutralisation of the intracellular milieu with a concomitant acidification of the extracellular milieu.

The monoclonal antibody (mAb) G250 (Grawitz 250) that recognises an antigen expressed in the membranes of CCRCC cells but which is not expressed in normal renal tissue was described in 1986.[5] The MN antigen was later detected in cervical carcinoma cells[6] and was shown to be homologous to a carbonic anhydrase[7] named MN/CA9. The antigen targeted by mAb G250 was shown to be identical to MN/CA9.[8] The denomination CA9 (or CA IX) is now commonly used. CA9 is a 58/54 kDa protein with an extracellular portion composed of an N-terminal proteoglycan-like region (PG) and a large central carbonic anhydrase domain (CA) linked via a single transmembrane anchor to a short C-terminal intracytoplasmic tail.[9] CA9 has a cytoplasmic expression in some normal tissues, mostly in larger bile ducts and the gastric mucosa,[5,7] but is not expressed in healthy renal tissue.[10]

2.2. *Regulation of CA9 in CCRCC*

The tumour environment is characterised by hypoxia and extracellular acidosis. CA9 expression is dependent on activation of the transcription factor Hypoxia-Inducible Factor-1α (HIF-1α).[11] Because of the strong cell surface activity of CA9, tumour cells maintain a near neutral intracellular pH, thereby favouring cell-survival. The extracellular acidosis activates metalloproteinases and release of growth factors, thus facilitating tumour growth and metastases.[12]

Besides hypoxia, the expression of CA9 in CCRCC is triggered by the inactivation of the tumour suppressor gene von Hippel–Lindau (VHL) by mutation, hypermethylation or deletion[13,14] with subsequent loss of VHL protein function or expression. CA9 is thus expressed in almost all CCRCCs. A positive relationship exists between the presence of a mutated VHL gene and high tumour expression of CA9.[15] CA9 expression is down-regulated by the reintroduction of the wild-type VHL gene.[14] In other subtypes of renal carcinomas, CA9 expression is only dependent on hypoxia.

The VHL protein (pVHL) is a component of a protein complex (VBC-CUL-2) formed with pVHL, Elongin B, Elongin C, Cullin and *Ring box* 1. In normoxia this VHL complex binds to HIF-1α, allowing its ubiquitination and rapid degradation by the proteasome.[16] During hypoxia or with an abnormal VHL gene, HIF-α cannot bind to the VHL complex which accumulates in the cell and binds to the HIF-1β factor. The dimeric complex HIF-1α–HIF-1β translocates into the nucleus and binds to HRE (HIF Responsive Element), driving a transcription sequence of hypoxia-induced genes,[11] notably those implicated in energy metabolism (glucose transporters, glycolytic enzymes), angiogenesis (VEGF and VEGFR-1) and surface transmembrane CAs.

CA9 expression in CCRCC is strictly dependent on HIF-1α,[17] but with weaker expression in high grade (Fuhrman 3–4) than in low grade tumours (Fuhrman 1–2).[18] The fact that some CCRCCs do not show VHL mutation and/or HIF-1α stabilisation despite expressing CA9 may suggest that an additional factor is involved in CA9 regulation.[17]

2.3. *Detection of CA9 expression*

2.3.1. *CA9 antigen*

Most studies have used the mouse mAb G250/MN75 which detects the CA9 antigen either by flow cytometry,[19] or more commonly, using IHC in CCRCC tissue and fine needle aspiration cytology.[20] The commercially available rabbit polyclonal antibody seems to give similar results,[20] but a cross-reactivity at high dilutions with beta-tubulin has also been described.[21]

CA9 is expressed in 94–97% of CCRCCs[15,22,23]; it is seen much less frequently in papillary carcinomas, and is not expressed in normal healthy renal tissue, in chromophobe and collecting ducts carcinoma or in oncocytoma.[20,23–25] It is detectable in sarcomatoid CCRCCs.[26]

The extracellular domain of CA9, including PG and CA domains, is released in the culture medium of CCRCC cells, but also *in vivo* in the body fluids (blood, urine) of patients harbouring the tumour: this soluble form of 50/54 kDa is named s-CA9.[26] It is cleared from the blood some days after nephrectomy and its concentration is extremely low in healthy subjects.[26,27] The CA9/s-CA9 ratio is about 10% and should not be influenced by hypoxia which increases the absolute value of CA9 and thus s-CA9 shedding.[9,26] s-CA9 release is a metalloprotease-dependent process regulated by TACE (ADAM 17).[9] s-CA9 can be assayed by ELISA[27] in serum, plasma and tissue. Values in serum and plasma are similar[28] and could provide prognostic information.[27,28] Zhou reported that serum values were correlated neither to IHC nor to tissue ELISA, but with tumour size.[29] Conversely, CA9 levels in tissue detected by ELISA correlated with CA9 expression measured by IHC.[29]

2.3.2. *CA9 mRNA*

CA9 mRNA expression by RT-PCR (reverse transcription polymerase chain reaction) has been shown in 97% of CCRCCs and in 56% of papillary carcinomas but in neither chromophobe carcinomas nor oncocytomas.[30] CA9 mRNA is a promising molecular marker of CCRCC,[30] highly correlated with IHC.[25]

2.3.3. CA9 DNA

The CA9 gene is located in the p12–p13 region of chromosome 9 and comprises 11 exons encoding the CA9 protein.[31] It is one of the genes positively regulated by HIF-1α which has a fixation site on the promoter gene HRE. Some polymorphisms have been described mainly on exon 1.[32]

3. CA9 as a diagnostic marker

The increasing usefulness of imaging techniques has resulted in a considerable increase in the diagnosis of small asymptomatic renal masses, 20% of which are benign.[33] Percutaneous biopsy of small solid renal masses is sensitive and specific but has been shown to provide insufficient material for histopathological examination in as much as 37% of tumours $\leqslant$3 cm.[34] The Bosniak classification of atypical cysts gives poor specificity for category II or III tumours, malignant in 24% and 41% of cases, respectively,[34,35] while the sensitivity of fine needle aspiration cytology is low.[36] CA9 might prove to be a useful diagnostic tool.

3.1. *In solid tumours*

Several groups have surveyed normal tissues for the presence of CA9.[5,10,19,24] CA9 expression has never been reported in non-cancerous renal tissue. Thus, CA9 expression in a renal sample is pathognomonic for malignancy.

IHC membranous staining is usually uniform and similar whether from a diagnostic biopsy or a surgical specimen, even if a tendency for reduced expression from metastases as compared to the primary tumour has been described.[37] Staining intensity has been used in composite scores,[29] but may be dependent on too many factors to be considered accurate.[10]

Fine needle aspiration cytology can be improved by mRNA CA9 detection since there is no overlap between the absolute signal values of tumour and normal parenchyma.[28] In 35 patients with a small indeterminate renal tumour, sensitivity and specificity were 53% and 71%; sensitivity and specificity of mRNA detection in these smears were 68% and 100%; overall sensitivity increased to 93% when mRNA detection was associated with conventional cytology.[36]

Renal masses have been explored by PET-scan imaging with ^{124}I-labelled chimeric mAb cG250.[38] Sensitivity was 94% and specificity was 100% for diagnosis of CCRCC.

Blood-based assays may be the future for non-invasive diagnosis of renal tumours. Two studies showed similar values for serum s-CA9 in CCRCC (114.0–126.1 pg/mL), higher than in other types of renal cancer.[27,29] A different ELISA kit reported significantly higher values (180 pg/mL), but unfortunately there was a considerable overlap between healthy individuals and CCRCC patients.[28] It is therefore unlikely that s-CA9 could be considered as a reliable diagnostic tool in the near future. Serum quantitative RT-PCR for mRNA CA9 may perform better since a pilot study comparing 84 patients with CCRCC with 44 healthy controls showed 93.7% sensitivity and 100% specificity.[39] More research and large prospective studies are needed before a blood-based diagnosis becomes available.

3.2. *In atypical cysts*

Due to the paucity of cells in cyst fluid, the detection of CA9 mRNA is disappointing. In a pilot study on 64 renal cystic masses (Bosniak I: 23; II: 3; IIF: 6; III: 27; IV: 8), the percutaneous aspiration of cystic fluid in 36 patients showed that s-CA9 was high (>1000 pg/ml) in the fluid obtained from the 20 malignant cysts (median: 2140 pg/ml; range: 1112–2140 pg/ml), whereas all but one of the benign cysts had no or very low s-CA9 levels (median: 0 pg/ml; range: 0–2140 pg/ml).[40] Using IHC, no benign cyst was found to express CA9 whereas 95.2% of the malignant cysts showed strong expression of CA9. In this report, no epithelial cell could be seen in the necrotic calcified wall of the cyst classified as 'benign' despite a high s-CA9 level in the fluid, so that it was impossible to differentiate a totally necrotic cystic RCC from a true benign cyst modified by ischaemia. This suggests that CA9 could provide a non-invasive way to differentiate malignant cystic renal tumours from atypical but benign cysts.

4. CA9 as a prognostic marker

One of the most important challenges in oncology is the early prediction of recurrence and metastasis and sensitivity to systemic therapies. It has become even more important with the availability of new drugs for the treatment of mCCRCC. Using IHC, the correlation between CA9 expression and pathologic features gave controversial results: no correlation with T stage, grade and tumour size,[22,23,41] inverse correlation between a low expression and tumour size, grade and node involvement.[15] In the series by Leibovich et al., low expression was significantly associated with the presence of symptoms, a high nuclear grade (68.1% grade 3–4 versus 41.8% with a high expression), necrosis and sarcomatoid differentiation (9.8% for low expression versus 3.9% for high expression).[10] The debate is now onto the prognostic value of CA9 expression which may vary in localised or mCCRCC.

4.1. *Clinically localised CCRCC*

4.1.1. *Immunohistochemistry*

Bui et al. studied the prognostic value of CA9 expression in 172 M0 CCRCC.[22] With a threshold value of 85% of tumour cells expressing CA9, they found no survival difference between patients with low or high expression. Nevertheless, median survival for high-risk patients ($\geqslant$pT3 and Fuhrman grade $\geqslant$2) was 30 months for those with high CA9 expression compared to 10 months for those with low expression, a figure similar to that for patients with metastases.[22] Other studies have reported a worse prognosis for M0 patients with low CA9 expression.[23,41]

Leibovich et al. used the same threshold value with 730 CCRCC patients of which only 81 had metastases.[10] Compared to patients with high CA9 expression, univariate analysis (RR: 1.65; $p < 0.001$) gave patients with low expression a 65% increased risk of dying from cancer. However, multivariate analysis did not show CA9 expression to be an independent prognostic factor over the whole series. No definitive conclu-

sion can therefore be drawn from these retrospective studies on the prognostic value of CA9 expression using IHC in localised CCRCCs.

Additional molecular prognostic factors have been reported: VEGF – when combined with CA9 – was an independent prognostic factor of specific survival[41]; a mutated VHL gene associated with high CA9 expression was an indicator of lower aggressiveness in almost all T1 tumours, whereas the association of a non-mutated VHL gene with low CA9 expression was predictive of worse specific survival.[15]

4.1.2. *Blood-based assays*

In 91 CCRCC patients, serum s-CA9 levels were correlated with T stage ($T_{1–2}$ versus T_3), grade ($Fuhrman_{1–2\ versus\ 3-4}$) and metastatic status.[27] With a median follow-up of 38 months, a high preoperative s-CA9 level in non-metastatic patients was a prognostic factor for recurrence. In another study with localised disease, serum s-CA9 levels were correlated with tumour size but not with Fuhrman grade.[29] In general, post-operative s-CA9 levels decrease, except in metastatic or progressive disease.[28]

Detection of CA9 gene expression in peripheral blood cells is predictive of a higher risk of recurrence.[42] Serum CA9 mRNA might prove to be more convenient and sensitive for follow-up. In 25 CCRCCs (23 N0M0 and 2 NxM1), CA9 mRNA was high preoperatively yet close to zero 7 days after curative surgery in the N0M0 patients whereas it remained high in the NxM1 patients.[39] With a median follow-up of 29 months in 31 N0M0 CCRCC patients, two showed CA9 mRNA recurrence and developed metastases.

4.1.3. *Perspectives for follow-up*

These preliminary results suggest that molecular factors may help identify a subgroup of patients with clinically localised CCRCC but with a high risk of recurrence, and so requiring close follow-up or even adjuvant treatment after surgery before metastasis becomes detectable. Although it is too early to imagine the routine use of s-CA9 and/or CA9 mRNA serum, one can hope in the future for a simple, non-invasive molecular follow-up of clinically localised CCRCC after surgery or ablative treatment. The integration of biomarkers, notably CA9, in clinico-pathologic algorithms for predicting post-surgical evolution is still limited[4] and needs to be independently validated over a large patient cohort.

4.2. *Metastatic CCRCC*

CA9 expression seems to be a promising marker for selecting which patients with mCCRCC are suitable for systemic or vaccination therapy.

4.2.1. *CA9 and IL-2 therapy*

Using IHC, Bui et al. showed that a threshold value of 85% of cells expressing CA9 in the primary renal tumour could be used to separate patients with metastases into two groups with different prognoses.[22] Below 85% (low expression), median specific survival was 5.5 months versus 24.8 months above 85% (high expression) ($p < 0.001$). Specific survival was also lower in the low expression group when stage, grade and ECOG status were analysed separately. In their patients with metastases who were treated with IL-2 (84% having high CA9 expression), the overall response rate was worse in the low CA9 expression group (14%) compared to the high expression group (27%), and all complete responses (8%) were in the latter group. The conclusion was that low CA9 expression was an independent prognostic factor for reduced survival in patients with metastases (HR: 3.10; $p < 0.001$) and that there was a lower probability that they would respond to IL-2 therapy. Subsequent studies by the same group confirmed high CA9 tumour expression and/or the presence of an allelic variant rs12553173 as an independent favourable prognostic factor in mCCRCC, with better sensitivity to IL-2.[4,32] Conversely Leibovich et al., using the same threshold level and multivariate analysis could not confirm that CA9 staining was an independent prognostic factor either in all their patients or in only those with metastases.[10] These two studies were different in the proportion of metastatic patients (46%[22] versus 11%[10]) and in those receiving IL-2 (60%[22] versus 6%[10]).

A case-control study of 66 mCCRCC treated by high doses of IL-2 confirmed the prognostic value of CA9 immunostaining, with a higher response rate for tumours with high expression (OR: 3.3):78% of responders had high expression compared to only 51% of non-responders ($p = 0.04$), and longer survival in the high expression group ($p = 0.03$), including all patients surviving for more than 5 years.[43] Combining histologic features with CA9 immunostaining, Atkins et al. developed a two-compartment model with distinct rates of response to IL-2.[43] The good risk group included 96% of responders to IL-2. Here again, survival was longer in the high expressors than in the low expressors and survival of more than 5 years was only observed among the high expressors. The predictive value of CA9 expression was not affected by IL-2 dosages.

The prognostic relevance of CA9 expression was also confirmed by the rapid progression of metastatic CCRCC patients with poor fixation of mAb ^{131}I-cG250 on their metastatic sites.[44]

From these studies, CA9 tissue expression might appear predictive of mCCRCC prognosis and response to IL-2. Some treatment algorithms for metastatic disease have already included CA9 expression.[4] Nevertheless, these data come from retrospective series and need to be confirmed prospectively before the possibility can arise of selecting patients for treatment giving their molecular individualisation. A prospective clinical trial of high dose IL-2 treatment « SELECT» (http://www.ClinicalTrials.gov identifier: NCT00554515) is currently being run to investigate the validity of the model developed by Atkins.[43]

4.2.2. *CA9 and targeted therapies*

Despite the importance of targeted drugs (VEGFR-TKIs, anti-VEGF mAbs, mTOR blockers) in today's standard treatment of mCCRCC, virtually nothing is known of the predictive nature of CA9 expression with patient response to these drugs. With a threshold value of 85% CA9+ tumour cells, Cho did not observe any correlation between CA9 expression and response to temsirolimus even though there was a tendency for patients with very low expression not to respond at all.[45] In a pilot study in 60 patients whose disease had progressed after cytokine therapy, the benefits of sorafenib for tumour

shrinkage and progression free survival compared to a placebo appeared stronger in tumours with high CA9 expression.[46] Obviously, research is needed on the correlation between CA9 expression and response to targeted therapies in mCCRCC patients.

4.2.3. *Is CA9 expression modified by the treatment of metastatic disease?*

Hulick reported that palliative nephrectomy does not decrease s-CA9 levels.[28] In the same study, four patients with mCCRCC who had had a palliative nephrectomy received an antiangiogenic therapy. Over a 12-month follow-up period one patient responded to treatment; his s-CA9 levels were stable. The other three patients showed progression of the disease and rising s-CA9 levels.[28]

Jensen compared the expression of CA9 in patients receiving IL-2, with or without IFN-α, with successive core-needle biopsies taken from the same tumour site, either the primary tumour or a metastatic lesion.[37] Some of the individual tumours had varying expressions of CA9 during treatment, but as a group, there was no difference. Neither was there any correlation between the time from treatment start to the biopsy procedure and the change in CA9 expression.

To date, data are too sparse to argue for a role for CA9 in the follow-up of responses to immuno or targeted therapies.

4.2.4. *How can the prognostic value of CA9 be explained?*

Contrary to CCRCC, a strong CA9 expression by IHC is usually correlated to poor prognosis for various tumours including cervical, ovarian, colorectal, head and neck, bladder and non-small lung carcinomas.[24] In these cancers, CA9 expression is only linked to hypoxia. In CCRCC, the very frequent VHL gene inactivation drives an early activation of the HIF pathway and CA9 expression. This may explain the evolving role of CA9 during tumour progression.

Intact CA9 antigen and s-CA9 have chaperone-like functions.[47] HSP110 and HSP70 are classical chaperones and are also powerful immunoadjuvants. Since CA9 and s-CA9 can be internalised and processed by dendritic cells, they may stimulate an early adaptative immune response against tumour antigens.[47] Moreover, CA9 expression and s-CA9 shedding are increased in response to IL-2, providing a mechanism that may contribute to the IL-2 response.[47] CA9 plays a complex role in cell adhesion. Very low concentrations of CA9 antigen enable *in vitro* adhesion of CGL1 and HeLa cells, abrogated by mAb M75, whereas s-CA9 does not support cell adhesion.[48] On the other hand, CA9 decreases E-cadherin-mediated cell adhesion via interaction with β-catenin, which could be of potential significance in hypoxia-induced tumour progression.[49]

CA9 expression has been found to be lower in high grade tumours than in low grade tumours[18] and lower in metastatic sites than in the primary tumour of the same individual[22,37] suggesting a loss of CA9 with disease progression. It might be a consequence of a gradual switch to a HIF-2α response during tumour progression,[13] explaining lower CA9 expression in the higher stages and the poor prognosis cases. Since high serum s-CA9 levels have been correlated with an increased risk of recurrence,[27] one could hypothesise that this loss might be partly due to a higher shedding rate. TACE is a metalloprotease which regulates CA9 shedding but is also involved in the ectodomain shedding of membrane-bound TGF-α producing the soluble transforming growth factor-α (sTGF-α), a mitogen of renal epithelial cells and a ligand of the epidermal growth factor receptor (EGFR).[50] Overexpression of EGFR is hypoxia-dependent and correlated to a worse prognosis of CCRCC.[51] With the activation of EGFR, tumour cells engage in a classic TGF-α/EGFR autocrine-signalling pathway. This may be reinforced by the transduction capacity of the cytoplasmic tail portion of CA9 which can be phosphorylated in an EGFR dependent manner, contributing through the PI-3K signalling to activation of the AkT and mTOR pathways.[52] Apart from this vicious cycle, as CCRCC progresses it may become less dependent on HIF-related factors and be driven more by mutations controlling other pathways, making it less responsive to immunotherapy and possibly more aggressive.[43]

5. Targeting CA9 for therapy of metastatic CCRCC

According to these properties, interfering with CA9 could have therapeutic implications.

5.1. *CA9 inhibitors*

Nanomolar CA9 inhibitors bind specifically to cells expressing CA9 and by maintaining a neutral intracellular pH and decreasing acidification of the microenvironment might decrease cell survival and invasion. Indisulam (E7070) is a sulphonamide derivative exhibiting a significant antitumour effect and is currently in Phase I and II clinical trials for the treatment of solid tumours.[53] Another class of compounds are membrane-impermeable and do not inhibit intracellular CAs. They may therefore exhibit less side-effect as compared to acetazolamide, which indiscriminately inhibits all CAs.[54]

5.2. *Vaccination*

The CA9 antigen encodes a human leucocyte antigen (G250:254-262) recognised by cytotoxic T lymphocytes in HLA-A2.1 individuals[55] and is internalised and processed by dendritic cells.[47] This makes CA9 a putative candidate for vaccination.

A phase I trial of vaccination of CA9-derived peptides in HLA-A24-positive patients with cytokine-refractory metastatic renal cell carcinoma showed partial responses in three patients with multiple lung metastases and a stabilisation of the disease with a median duration of 12.2 months in six patients.[56] Overall, the median survival time was 21.0 months for these patients with progressive disease who were enrolled in the trial following the failure of immunotherapy.

In another phase I trial, renal tumour RNA-transfected dendritic cells were administered to 10 patients with mCCRCC.[57] The vaccine-induced T-cell reactivity was directed against a number of renal tumour-associated antigens, including CA9, and only three patients died from the disease after a mean follow-up of 19.8 months.

5.3. *Immunotherapy*

Accumulating evidence indicates that the restricted surface expression makes CA9 a very promising target for tumour immune therapy. Only one humanised anti-CA9 mAb has been evaluated in a clinical setting (WX-G250, [Rencarex®; WILEX, Munich, Germany]). Non-randomised phase II clinical trials have used weekly intravenous infusion given to patients with mCCRCC. In one of these, infusions were scheduled for 36 patients over 12 weeks.[58] Patients with stable disease (SD) or response were eligible to receive additional treatment for 8 weeks. One complete response and a significant regression were observed during the follow-up. Five patients with progressive disease at study entry were stable for more than 6 months after study entry. The median survival after the treatment start was 15 months. In a second trial, 35 patients with progressive disease were given 11 weekly infusions of WX-G250 combined with a daily low dose IL-2 regimen.[59] With a median survival of 22 months this combination was superior to WX-G250 monotherapy and was comparable to current non-specific cytokine regimens in patients with advanced mCCRCC. A phase III-randomised double-blind study is in progress to evaluate WX-G250 as an adjuvant therapy after nephrectomy for patients with localised high-risk disease. (ARISER: Adjuvant RENCAREX® Immunotherapy trial to Study Efficacy in non-metastatic RCC, (http://www.ClinicalTrials.gov identifier: NCT00087022)). Results will be available in 2013.

Lamers et al. have used CA9 as a target for adoptive cell therapy.[60] Three mCCRCC patients were treated with scFv(G250)-autologous-transduced T-cells. The surface of these cells expresses the scFv(G250) receptor which recognises CA9 and enables them to exert antigen-specific effector functions, such as the killing of CA9+ cells. Grade 2 and 4 liver enzymes disturbance was also observed. Liver biopsy suggested that the scFv(G250)+ T-cells had specifically attacked the bile duct epithelial cells, known to strongly express CA9.[24] To prevent liver toxicity in the future, a single low dose injection of cG250 antibody will limit the uptake by liver tissue before injecting the transfected T-cells.

5.4. *Radioimmunotherapy*

Due to the high expression of CA9 in CCRCC, cG250 has been used as a carrier molecule. A phase I/II radioimmunotherapy trial with ^{131}I-labelled mAb G250 was conducted in 33 mCCRCC.[61] Seventeen had stable disease and partial responses were observed in two patients. Unfortunately, the human antimouse immunoglobulin antibody was detected in all patients 4 weeks after infusion and restricted therapy to a single infusion.

A phase I radioimmunotherapy escalation study with ^{131}I-labelled chimeric mAb cG250, presumed to be immunosilent, showed a partial response.[62] These results were not confirmed in a subsequent phase II study consisting of two sequential high-dose treatments with ^{131}I-cG250 in 29 patients.[63] In 19 patients who received two infusions the disease stabilised in five and continued to progress in 14; eight patients only were able to receive one radio-immunotherapy infusion because of grade 4 haematologic toxicity, formation of human antichimeric antibodies, or disease progression.

Because animal studies have shown that tumour growth was more efficiently delayed by cG250 labelled with other radionuclides, a phase I/II ^{177}Lu-cG250 dose-escalation radioimmunotherapy study is currently ongoing (http://www.ClinicalTrials.gov identifier: NCT001142415).

6. Conclusion

CA9 is to date the most studied and promising molecular marker for diagnosis, prognosis and therapy of CCRCC. Despite encouraging results, it must be emphasised that most of the literature on CA9 has reported the results on retrospective studies.

For diagnosis, it seems now accepted that CA9 expression by IHC in a renal biopsy is pathognomonic of malignancy. For cystic renal masses, the importance of s-CA9 levels in the cystic fluid must be confirmed in larger series.

Beyond these invasive techniques, research is needed on the relationship between CA9 expression by IHC and the serum values of s-CA9 and CA9 mRNA which may become useful tools for diagnosis, prognosis and follow-up.

Prospective data on the medical management of CCRCC will be available in the future from two ongoing phase III clinical trials: the ARISER study using adjuvant anti-CA9 mAb after nephrectomy in localised high-risk disease, and the SELECT study investigating the predictive value of CA9 expression on sensitivity to IL-2 in mCCRCC. Since targeted drugs are today's first line treatment of mCCRCC, the relevance of CA9 in this clinical setting should be promptly addressed. Other studies targeting CA9 by inhibitors, vaccination, immunotherapy or radioimmunotherapy are only in phases I–II.

Obviously, CA9 will not be the only molecular marker of CCRCC in the future but we have evidence that it will remain one of the best.

Conflict of interest statement

J. Tostain and G. Li are patent inventors on the use of CA9 mRNA in biological fluids for the diagnosis or prognosis of cancer.

Acknowledgements

This research was supported by the French Ministry of Health (PHRC), the INCa Canceropole CLARA and the Comité Départemental de la Loire de la Ligue Nationale Contre le Cancer. The study sponsors where in no way involved in the collection and interpretation of data nor in the writing of the manuscript or in the decision to submit it for publication.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;**55**:74–108.

2. Lam JS, Leppert JT, Figlin RA, Belldegrun AS. Role of molecular markers in the diagnosis and therapy of renal cell carcinoma. *Urology* 2005;**66**:1–9.
3. Leibovich BC, Blute ML, Cheville JC, et al. Prediction of progression after radical nephrectomy for patients with clear cell renal cell carcinoma: a stratification tool for prospective clinical trials. *Cancer* 2003;**97**:1663–71.
4. Kim HL, Seligson D, Liu X, et al. Using tumor markers to predict the survival of patients with metastatic renal cell carcinoma. *J Urol* 2005;**173**:1496–501.
5. Oosterwijk E, Ruiter DJ, Hoedemaeker PJ, et al. Monoclonal antibody G 250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney. *Int J Cancer* 1986;**38**:489–94.
6. Zavada J, Zavadova Z, Pastorekova S, et al. Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. *Int J Cancer* 1993;**54**:268–74.
7. Pastorek J, Pastorekova S, Callebaut I, et al. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. *Oncogene* 1994;**9**:2877–88.
8. Grabmaier K, Vissers JL, De Weijert MC, et al. Molecular cloning and immunogenicity of renal cell carcinoma-associated antigen G250. *Int J Cancer* 2000;**85**:865–70.
9. Zatovicova M, Sedlakova O, Svastova E, et al. Ectodomain shedding of the hypoxia-induced carbonic anhydrase IX is a metalloprotease-dependent process regulated by TACE/ADAM17. *Br J Cancer* 2005;**93**:1267–76.
10. Leibovich BC, Sheinin Y, Lohse CM, et al. Carbonic anhydrase IX is not an independent predictor of outcome for patients with clear cell renal cell carcinoma. *J Clin Oncol* 2007;**25**:4757–64.
11. Wykoff CC, Beasley NJ, Watson PH, et al. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res* 2000;**60**:7075–83.
12. McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* 2000;**6**:149–56.
13. Mandriota SJ, Turner KJ, Davies DR, et al. HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. *Cancer Cell* 2002;**1**:459–68.
14. Ivanov SV, Kuzmin I, Wei MH, et al. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel–Lindau transgenes. *Proc Natl Acad Sci USA* 1998;**95**:12596–601.
15. Patard JJ, Fergelot P, Karakiewicz PI, et al. Low CAIX expression and absence of VHL gene mutation are associated with tumor aggressiveness and poor survival of clear cell renal cell carcinoma. *Int J Cancer* 2008;**123**:395–400.
16. Ivan M, Kondo K, Yang H, et al. HIF alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* 2001;**292**:464–8.
17. Grabmaier K, AdW MC, Verhaegh GW, Schalken JA, Oosterwijk E. Strict regulation of CAIX(G250/MN) by HIF-1alpha in clear cell renal cell carcinoma. *Oncogene* 2004;**23**:5624–31.
18. Baldewijns MM, Thijssen VL, Van den Eynden GG, et al. High-grade clear cell renal cell carcinoma has a higher angiogenic activity than low-grade renal cell carcinoma based on histomorphological quantification and qRT-PCR mRNA expression profile. *Br J Cancer* 2007;**96**:1888–95.
19. Li G, Passebosc-Faure K, Lambert C, et al. The expression of G250/mn/CA9 antigen by flow cytometry: its possible implication for detection of micrometastatic renal cancer cells. *Clin Cancer Res* 2001;**7**:89–92.
20. Liao SY, Aurelio ON, Jan K, Zavada J, Stanbridge EJ. Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. *Cancer Res* 1997;**57**:2827–31.
21. Li Y, Wang H, Oosterwijk E, et al. Antibody-specific detection of CAIX in breast and prostate cancers. *Biochem Biophys Res Commun* 2009;**386**:488–92.
22. Bui MH, Seligson D, Han KR, et al. Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. *Clin Cancer Res* 2003;**9**:802–11.
23. Sandlund J, Oosterwijk E, Grankvist K, et al. Prognostic impact of carbonic anhydrase IX expression in human renal cell carcinoma. *BJU Int* 2007;**100**:556–60.
24. Ivanov S, Liao SY, Ivanova A, et al. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. *Am J Pathol* 2001;**158**:905–19.
25. Murakami Y, Kanda K, Tsuji M, Kanayama H, Kagawa S. MN/CA9 gene expression as a potential biomarker in renal cell carcinoma. *BJU Int* 1999;**83**:743–7.
26. Zavada J, Zavadova Z, Zat'ovicova M, Hyrsl L, Kawaciuk I. Soluble form of carbonic anhydrase IX (CA IX) in the serum and urine of renal carcinoma patients. *Br J Cancer* 2003;**89**:1067–71.
27. Li G, Feng G, Gentil-Perret A, Genin C, Tostain J. Serum carbonic anhydrase 9 level is associated with postoperative recurrence of conventional renal cell cancer. J Urol 2008;180:510–3 [discussion 13–4].
28. Hulick P, Zimmer M, Margulis V, et al. Blood levels of carbonic anhydrase 9 correlate with clear cell carcinoma activity. *Clin Proteom* 2009;**5**:34–45.
29. Zhou GX, Ireland J, Rayman P, Finke J, Zhou M. Quantification of carbonic anhydrase IX expression in serum and tissue of renal cell carcinoma patients using enzyme-linked immunosorbent assay: prognostic and diagnostic potentials. *Urology* 2010;**75**:257–61.
30. Li G, Cuilleron M, Gentil-Perret A, et al. Rapid and sensitive detection of messenger RNA expression for molecular differential diagnosis of renal cell carcinoma. *Clin Cancer Res* 2003;**9**:6441–6.
31. Nakagawa Y, Uemura H, Hirao Y, et al. Radiation hybrid mapping of the human MN/CA9 locus to chromosome band 9p12–p13. *Genomics* 1998;**53**:118–9.
32. de Martino M, Klatte T, Seligson DB, et al. CA9 gene: single nucleotide polymorphism predicts metastatic renal cell carcinoma prognosis. *J Urol* 2009;**182**:728–34.
33. Neuzillet Y, Lechevallier E, Andre M, Daniel L, Coulange C. Accuracy and clinical role of fine needle percutaneous biopsy with computerized tomography guidance of small (less than 4.0 cm) renal masses. *J Urol* 2004;**171**:1802–5.
34. Lechevallier E, Andre M, Barriol D, et al. Fine-needle percutaneous biopsy of renal masses with helical CT guidance. *Radiology* 2000;**216**:506–10.
35. Wolf Jr JS. Evaluation and management of solid and cystic renal masses. *J Urol* 1998;**159**:1120–33.
36. Li G, Cuilleron M, Cottier M, et al. The use of MN/CA9 gene expression in identifying malignant solid renal tumors. *Eur Urol* 2006;**49**:401–5.
37. Jensen HK, Nordsmark M, Donskov F, Marcussen N, Von der Maase H. Immunohistochemical expression of carbonic anhydrase IX assessed over time and during treatment in renal cell carcinoma. *BJU Int* 2008;**101**(Suppl. 4):41–4.
38. Divgi CR, Pandit-Taskar N, Jungbluth AA, et al. Preoperative characterisation of clear-cell renal carcinoma using iodine-124-labelled antibody chimeric G250 (124I-cG250) and PET in patients with renal masses: a phase I trial. *Lancet Oncol* 2007;**8**:304–10.
39. Tostain J, Li G. CA9 as a predictive marker of response to therapies of clear cell renal cell carcinoma. Oral presentation.

In: Rein AplRslTd, editor. 3rd A.R.Tu.R Symposium 2009, Chantilly (France), 2009.
40. Li G, Billal I, Gentil-Perret A, et al. CA9 as a molecular marker for differential diagnosis of cystic renal tumors. *Urol Oncol* 2010, in press.
41. Phuoc NB, Ehara H, Gotoh T, et al. Prognostic value of the co-expression of carbonic anhydrase IX and vascular endothelial growth factor in patients with clear cell renal cell carcinoma. *Oncol Rep* 2008;**20**:525–30.
42. Gilbert SM, Whitson JM, Mansukhani M, et al. Detection of carbonic anhydrase-9 gene expression in peripheral blood cells predicts risk of disease recurrence in patients with renal cortical tumors. *Urology* 2006;**67**:942–5.
43. Atkins M, Regan M, McDermott D, et al. Carbonic anhydrase IX expression predicts outcome of interleukin 2 therapy for renal cancer. *Clin Cancer Res* 2005;**11**:3714–21.
44. Brouwers AH, Boerman OC, Oyen WJ. In vivo molecular prediction of carbonic anhydrase IX-G250MN expression on immunotherapy outcome in renal cancer. Clin Cancer Res 2005;11:8886 [author reply 86].
45. Cho D, Signoretti S, Dabora S, et al. Potential histologic and molecular predictors of response to temsirolimus in patients with advanced renal cell carcinoma. *Clin Genitourin Cancer* 2007;**5**:379–85.
46. Signoretti S, Regan M, Atkins M. Carbonic anhydrase IX as a predictive biomarker of response to kidney cancer therapy. *BJU Int* 2008;**101**(Suppl. 4):31–5.
47. Wang Y, Wang XY, Subjeck JR, Kim HL. Carbonic anhydrase IX has chaperone-like functions and is an immunoadjuvant. *Mol Cancer Ther* 2008;**7**:3867–77.
48. Zavada J, Zavadova Z, Pastorek J, et al. Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion. *Br J Cancer* 2000;**82**:1808–13.
49. Svastova E, Zilka N, Zat'ovicova M, et al. Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with beta-catenin. *Exp Cell Res* 2003;**290**:332–45.
50. Franovic A, Robert I, Smith K, et al. Multiple acquired renal carcinoma tumor capabilities abolished upon silencing of ADAM17. *Cancer Res* 2006;**66**:8083–90.
51. Franovic A, Gunaratnam L, Smith K, et al. Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. *Proc Natl Acad Sci USA* 2007;**104**:13092–7.
52. Dorai T, Sawczuk IS, Pastorek J, Wiernik PH, Dutcher JP. The role of carbonic anhydrase IX overexpression in kidney cancer. *Eur J Cancer* 2005;**41**:2935–47.
53. Supuran CT. Development of small molecule carbonic anhydrase IX inhibitors. *BJU Int* 2008;**101**(Suppl. 4): 39–40.
54. Svastova E, Hulikova A, Rafajova M, et al. Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. *FEBS Lett* 2004;**577**:439–45.
55. Vissers JL, De Vries IJ, Schreurs MW, et al. The renal cell carcinoma-associated antigen G250 encodes a human leukocyte antigen (HLA)-A2.1-restricted epitope recognized by cytotoxic T lymphocytes. *Cancer Res* 1999;**59**:5554–9.
56. Uemura H, Fujimoto K, Tanaka M, et al. A phase I trial of vaccination of CA9-derived peptides for HLA-A24-positive patients with cytokine-refractory metastatic renal cell carcinoma. *Clin Cancer Res* 2006;**12**:1768–75.
57. Su Z, Dannull J, Heiser A, et al. Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. *Cancer Res* 2003;**63**:2127–33.
58. Bleumer I, Knuth A, Oosterwijk E, et al. A phase II trial of chimeric monoclonal antibody G250 for advanced renal cell carcinoma patients. *Br J Cancer* 2004;**90**:985–90.
59. Bleumer I, Oosterwijk E, Oosterwijk-Wakka JC, et al. A clinical trial with chimeric monoclonal antibody WX-G250 and low dose interleukin-2 pulsing scheme for advanced renal cell carcinoma. *J Urol* 2006;**175**:57–62.
60. Lamers CH, Sleijfer S, Vulto AG, et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 2006;**24**:e20–2.
61. Divgi CR, Bander NH, Scott AM, et al. Phase I/II radioimmunotherapy trial with iodine-131-labeled monoclonal antibody G250 in metastatic renal cell carcinoma. *Clin Cancer Res* 1998;**4**:2729–39.
62. Steffens MG, Boerman OC, de Mulder PH, et al. Phase I radioimmunotherapy of metastatic renal cell carcinoma with ^{131}I-labeled chimeric monoclonal antibody G250. *Clin Cancer Res* 1999;**5**:3268s–74s.
63. Brouwers AH, Mulders PF, de Mulder PH, et al. Lack of efficacy of two consecutive treatments of radioimmunotherapy with ^{131}I-cG250 in patients with metastasized clear cell renal cell carcinoma. *J Clin Oncol* 2005;**23**:6540–8.