doi:10.4149/neo_2009_04_298

Soluble form of carbonic anhydrase IX (CAIX) in transitional cell carcinoma of urinary tract

L. HYRSL[1*], J. ZAVADA[2], Z. ZAVADOVA[2], I. KAWACIUK[1], S. VESELY[1], P. SKAPA[3]

[1] Department of Urology, Charles University 2nd Faculty of Medicine and Hospital Motol, Prague, [2] Institute of Organic Chemistry and Biochemistry, Academy of Science, Prague, Czech Republic, e-mail: lhyrsl@volny.cz, [3] Department of Pathology and Molecular Medicine, Charles University 2nd Faculty of Medicine and Hospital Motol, Prague

Received November 26, 2008

We investigated the expression of cell-associated CAIX protein in histological sections of the transitional cell carcinoma (TCC) of the urinary tract and of the soluble form of CAIX (s-CAIX) shed by the tumor into the serum and urine of TCC patients. A total of 23 patients with histologically confirmed TCC or squamous cell carcinoma (SCC) were enrolled in the pilot study. Sixteen healthy individuals served as controls. Membrane-bound CAIX was present in the tumor cells near the endoluminal surface. Necrosis was observed in only 4 samples. Using Western blots, s-CAIX concentrated from urine was visualized as a double band at 50 and 54 kDa. In most cases, the presence of s-CAIX in the urine correlated with CAIX expression in the tumor. On the other hand, s-CAIX did not exceed the normal level in the serum of TCC patients. Urine from patients with TCC of the urinary bladder and renal pelvis contained s-CAIX, allowing the detection of tumors in approximately 70% of the patients. Moreover, two additional patients with suspected, but unconfirmed bladder tumor, with s-CAIX detected in urine, developed tumors identified as TCC within six months. We suggest that after a simple, rapid and sensitive test, monitoring s-CAIX levels in urine will be developed, it may be useful for early detection of relapse in patients following transurethral tumor resection.

Keywords: Carbonic anhydrase IX, diagnostic antigen, transitional cell carcinoma

Carbonic anhydrase IX (CAIX) appears to play an important role in pathogenesis of many types of human carcinomas. It is often expressed as a response of tumor cells to hypoxia (inactivation of VHL and upregulation of other proteins like HIF, VEGF, etc.), which all together escalate invasivity of the tumor. In consequence, CAIX was proposed as a marker of poor prognosis (1-4). Cell-associated CAIX can conveniently be detected by IHC staining of paraffin-embedded sections with MAb M75 as described in more than 200 publications.

Previously, we investigated the presence of soluble protein s-CAIX in the blood and urine of patients with renal clear cell carcinomas (RCC). This proved to be a more difficult task (5): while permanent tumor cell lines or short-term tumor explants shed an easily detectable amount of s-CAIX *in vitro,* the soluble antigen is rapidly cleared from the blood *in vivo* by absorption to unidentified deposits or by excretion in the urine. As a consequence, s-CAIX concentration in the serum or urine of RCC patients is only a few picograms per ml, which is not readily detectable by current methods such as ELISA. Therefore, we introduced a very highly sensitive and specific method. Immunoprecipitation of s-CAIX from larger volumes of blood (1 ml) or urine (10 ml) was followed by Western blotting in combination with enhanced chemiluminiscence (ECL). However, in many patients with RCC, s-CAIX was undetectable in the blood or urine, in spite of the fact that almost 100% of the tumors expressed high levels of CAIX in the cell membranes. In contrast, other tumor markers like PSA or CEA reach concentrations of several nanograms per ml in the sera of patients with carcinoma of

* Corresponding author

Abbreviations: Carbonic anhydrase IX (CAIX); Carcinoembryonic antigen (CEA); Enhanced chemiluminiscence (ECL); Enzyme-Linked ImmunoSorbent Assay (ELISA); Hypoxia inducible factor (HIF); Immunoglobulin G (IgG); Immunohistochemistry (IHC); Monoclonal antibody (Mab); Prostate specific antigen (PSA); polyvinylidene fluoride (PVDF); Renal cell carcinoma (RCC); Squamous cell carcinoma (SCC); transitional cell carcinoma (TCC); Transurethral resection of bladder tumor (TURBT); Vascular endothelial growth factor, (VEGF); von Hippel Lindau (VHL); Western blotting (WB).

prostate or colon and can be easily detected and quantitated by ELISA.

Our previous report on s-CAIX (5) was based on RCC patients with primary tumors. However, recent results on patients with metastatic or recurrent RCC indicate higher levels of serum s-CAIX, which can be detected by ELISA. Patients who had high serum s-CAIX had a significantly higher rate of recurrence compared to those who had a low s-CAIX value (6).

In the present experiments, we concentrated on urine samples from patients with bladder carcinomas, which indeed turned out to be more suitable for assaying s-CAIX. Their main advantage is that these tumors are bathing directly in urine, with most of the cells expressing CAIX located at the luminal surface. Therefore, the s-CAIX they are shedding has little opportunity to get lost and we were even able to detect s-CAIX in the urine of patients with very small tumors (7).

At first presentation, most TCC are superficial, they can be multifocal, intraepithelial, and sometimes very small; therefore, some of the tumors can escape endoscopic detection and surgical removal. This increases the risk of recurrence and so warrants monitoring the presence of a marker protein in urine.

Patients and methods

1. Patients and controls. The pilot study included 23 patients (20 men and 3 women, mean age 67.9 years, range 24-88) with endoscopic suspicion of TCC mainly of the urinary bladder, in one case of the renal pelvis. All patients routinely underwent intravenous pyelography, cystoscopy, or uretheroscopy before surgery to obtain information for the standard staging protocol. Urine and serum samples were taken before surgery and stored at -80 °C until further processing. Surgically removed tissue samples from all the patients were fixed in 10% buffered formalin, embedded in paraffin, and processed according to standard histopathological procedures with hematoxylin-eosin staining.

In total, 16 individuals served as controls; they were healthy volunteers or patients admitted to the hospital for other urological complaints (infections, urinary stones, etc). The study was carried out with the approval of the Departmental Ethics Committee.

2. Immunohistochemistry (IHC). Representative tissue sections of 4 μm thickness were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol to water. After standard blocking of endogenous peroxidase activity for 20 min at room temperature, the tissue sections were incubated overnight at 4 °C with M75 anti-CAIX antibody, hybridoma TC fluid diluted 1:50 (8). The antigen-antibody complexes were visualized with universal immunoperoxidase polymer detection kit N-Histofine, Simple Stain MAX PO multi (Nichirei Biosciences, Tokyo, Japan) with 3,3'-diaminobenzidine (Fluka Chemie, Buchs, Switzerland) as chromogen.

Only membranous staining was considered as a positive result. The CAIX immunostaining was scored under low magnification using a semiquantitative method, and a 4-level evaluation system of the percentage of CAIX positive tumor cells was applied (score 0 – 0%, score 1 – <10%, score 2 – 10-50%, score 3 – >50%). The distribution and extent of CAIX positivity was noted with an emphasis on the assessment of perinecrotic areas of tumors.

3. Western blot analysis. We used an extremely sensitive method described previously (5). No modification was introduced; thus, the present results with TCC patient samples are comparable to previous findings with RCC patient samples. Briefly, 10 ml samples of urine (or 1 ml of serum) were clarified by centrifugation, and s-CAIX was concentrated by precipitation with MAb V10 followed by Sepharose beads with linked anti-mouse IgG. The whole amount of antigen from individual specimens was eluted from the beads with sample buffer at 100 °C and loaded onto single lanes of an SDS polyacrylamide gel. The proteins were separated by SDS PAGE and transferred to PVDF membrane. The blots were developed with M75 IgG directly conjugated to peroxidase and visualized by ECL exposed on a sensitive film. For calibration purposes, each blot included 4 standard amounts of purified complete CAIX or s-CAIX. M75 (9) and V10 (10) were the CAIX specific Mabs used.

4. ELISA. The procedure was described previously (5); it was a sandwich method employing V10-IgG as capture antibody and peroxidase-conjugated M75 IgG as detector antibody.

Results

Out of a group of 23 patients, seven had undergone radical cystectomy, one had undergone nephroureterectomy for TCC of the renal pelvis and the rest had undergone transurethral resection (TURBT). Histopathological analysis confirmed the diagnosis of TCC in 22 specimens and of SCC in one sample. Eighteen tumors displayed a papillary growth pattern; flat lesion was found in one case and in three cases the type of growth could not be exactly evaluated. pT stage and expression of CAIX antigen by IHC and detection of s-CAIX in urine by WB are listed in Table 1.

1. IHC staining. The M75 antibody bound to CAIX on the tumor cell membranes, and a clear signal was observed upon addition of the chromogenic substrate. Variation in staining intensity between particular cases was minimal and differences were seen primarily in the extent and distribution of immunopositivity (Fig. 1). The luminal epithelial cells of the neoplastic papillary structures bound M75 antibody more effectively than the basal areas of the epithelium. IHC analysis of tumors with a score 1 staining pattern revealed focal, superficial localization of CAIX. In contrast, analysis of lesions classified as score 3 revealed a diffuse luminal localization of CAIX as well as immunopositivity for CAIX in deeper layers of the epithelium.

Necrosis was found in only three TCCs and one SCC. The necrotic area in no case exceeded 10% of the evaluated tissue

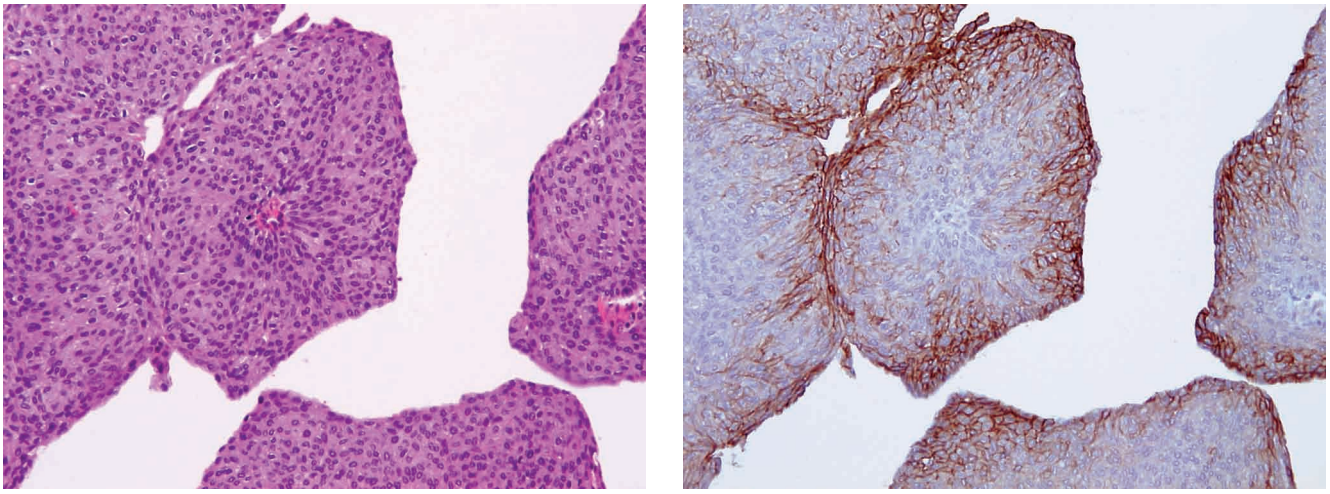

Figure 1. Transitional cell carcinoma (TCC) of the urinary bladder, grade 2 (original magnification 200x). A: hematoxylin-eosin staining; B: cell membrane immunoreactivity for CAIX predominantly in the superficial areas of the neoplastic papillary structures (score 2 staining pattern).

section of the tumor. One TCC with focal necrosis was completely negative for CAIX; the other three tumors containing necrotic areas displayed a score 3 or 2 staining pattern. No significant change in CAIX immunostaining was observed in the perinecrotic tumor zones.

Table 1. Human TCC and expression of CAIX antigen (semiquantitative) by IHC and detection of s-CAIX in urine by WB

Patient	Tumor stage/grade	IHC	WB
1.	T1G1	+++	-
2.	TXG1	+	-
3.	T4G4	+	-
4.	T1G3	+	++
5.	T2bG2	+++	+++
6.	T1G2	++	+++
7.	T1G2	++	+
8.	T3bG3	++	++
9.	T4G3	-	-
10.	TaG2	++	+
11.	T1G2	-	-
12.	TaG2	-	
13.	TXG2	+	+
14.	TXG2	-	++
15.	T3aG2	-	++
16.	T1G3	+++	+++
17.	Ta,TisG2	nt	++
18.	T1G2	+	+++
19.	T1G2	++	+++
20.	TXG2	++	-
21.	T3aG2	+	++
22.	TXG2	++	++
23.	TXG3	+	++

In normal areas of urothelium CAIX staining by IHC was negative.

2. Western blots. Soluble CAIX concentrated from the urine and sera of 23 patients enrolled into this study (22 identified as TCC, 1 as SCC) and from 16 control healthy individuals was analyzed by Western blotting (WB) with M75 Ab (presented in Fig. 2).

The antigen was detected in 16 out of 23 urine samples from the patients (69.6%). Two samples (No. 57 and F) from TCC patients and one from a SCC patient (No. 50) displayed very high levels of s-CAIX. The amount of s-CAIX in these samples far exceeds the calibration standards. The urine samples contained the following concentrations of s-CAIX as measured by ELISA: No. 50 = 325 ng/ml, No. 57 = 6.2 ng/ml, and sample F = 595 ng/ml. The concentration of s-CAIX in the other urine samples could be visually assessed by comparison with the calibration standards included in the blot. In most of the urine specimens from TCC patients it was 50 – 400 pg/10ml (= 5 – 40 pg/ml). These were scored as "positive". In seven TCC patients s-CAIX was not detectable; three of them were IHC positive and one was not tested by IHC because the tissue sample was too small. Distribution of s-CAIX level in urine of TCC patients and of controls is indicated in Figure 3.

Remarkably, serum from TCC patients was s-CAIX negative in all instances, even the serum from patients with CAIX-positive urine. Figure 2 presents randomly selected samples of s-CAIX analyses we performed from bladder carcinoma patients and from controls. Control urine samples were all CAIX negative, with the exception of one positive sample obtained from an apparently healthy young male.

3. Brief analysis of the overall results. The materials obtained from all individuals, subjected to surgery (mostly to TURBT) on the ground of endoscopic findings and of other clinical tests were examined by three methods: by histology (H.E. staining),

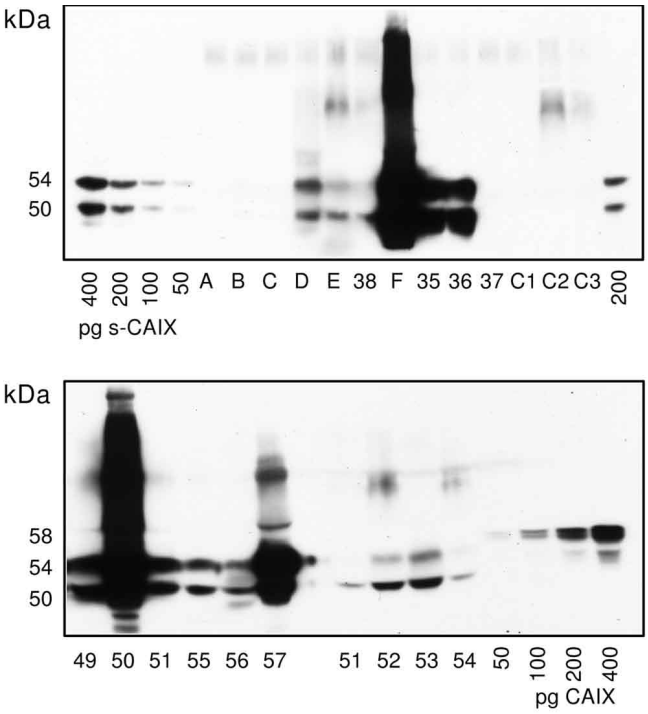

Figure 2. Detection of s-CAIX in urine and sera by Western blot analysis. Lanes A, B, C, D, E, F, 35, 36, 37, 38, 49, 50, 51, 55, 56, 57 = urine of TCC patients; C1, C2, C3 = urine of healthy controls; 51, 52, 53, 54 = serum of RCC patients. (Patient 51 had a combination of both RCC and TCC).

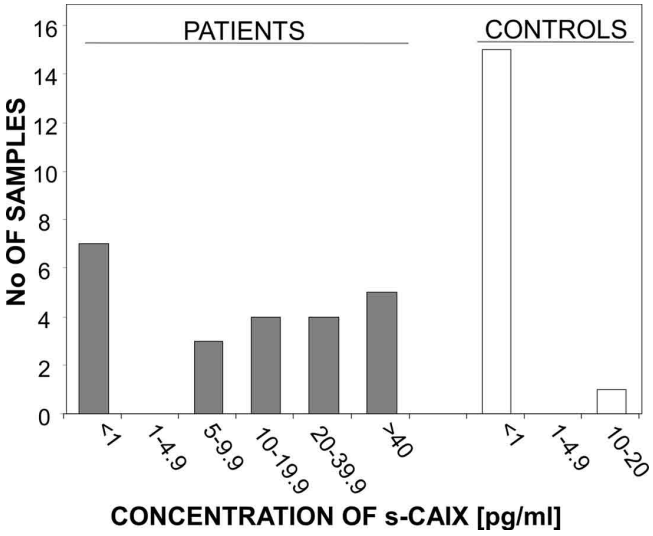

Figure 3. Distribution of s-CAIX level in urine of TCC patients and of controls.

by IHC of surgically removed tissues and by Western blotting of urine obtained before resection. Urine only was examined from 16 control subjects. According to the results of the three tests, the patients were divided into five groups (see Table 2). Group 1 – all three tests positive – was represented by thirteen (56.5%) patients. Into groups 2 and 3 fall six (26.1%) patients with histologically confirmed carcinoma, but with CAIX antigen detected either by IHC or by WB only. In all of these instances, the antigen level was low.

Group 4 – histologically confirmed carcinoma, but antigen was not detected by either method (three patients). These are simply patients with antigen-negative tumors. Group 5 – in one tumor positive patient IHC was not done, because of a very small tissue sample sufficient only for hematoxylin-eosin staining.

Out of 16 urine samples obtained from healthy donors (groups 6 and 7), only in one was detected a significant level of s-CAIX. We were unable to elucidate the cause of antigen presence.

Discussion

How do the present observations of s-CAIX in body fluids of TCC patients compare with our previous findings for RCC patients? There are several important differences between these two types of tumors, which are described in the following paragraphs.

TCCs of the urinary bladder are usually multifocal and relatively discrete neoplasms with papillary architecture. Another common feature is their superficial growth, affecting the mucosa and submucosa (Tis, Ta, T1). Expression of CAIX in neoplastic cells is considered to be a consequence of focal hypoxia, which induces ectopic CAIX synthesis (1-4). In our IHC experiments CAIX was not focal, but it was frequently observed in the superficial parts of neoplastic papillary structures. This staining pattern could be interpreted as a result of the low tension of oxygen in the areas relatively distant from the central vascular tree of the papillae and probably also in the urine. Since the three tumors containing necrosis and tested for CAIX were clearly positive, an eventual increase of CAIX expression in the perinecrotic tumor zones could be hardly

Table 2. An overview of the results obtained by histology (hematoxylin-eosin staining), CAIX antigen detection by immunohistochemistry (IHC) and Western blotting (WB)

		Method		
Group	**Histology**	**IHC**	**WB**	**No. of cases**
1.	+	+	+	13*
2.	+	+	-	4
3.	+	-	+	2
4.	+	-	-	3
5.	+	ND	+	1
Total				23
Controls				
6.	-	ND	-	15
7.	-	ND	+	1
Total				16

* 12 – TCC and 1 – SCC
ND – not done

detectable by the semiquantitave method used for evaluation. The complete IHC negativity of another TCC case with focal necrosis might reflect a diverse biological nature of this tumor with constitutional inability of CAIX expression.

A different situation is encountered with RCC, which grows deep in the renal parenchyma. Tumor size can be massive, even over 1000 g. CAIX stains as a diffuse membranous antigen, not induced by hypoxia, but by the loss or inactivation of the VHL (Von Hippel Lindau) gene, which has been identified as a tumor suppressor gene (11).

The differences between TCC and RCC provide a plausible explanation for our observations of s-CAIX in body fluids: in RCC patients the concentration of s-CAIX in blood and in the urine is extremely low, in some patients even below the detection limit of a highly sensitive test. The concentration of s-CAIX in parallel samples of blood and urine from individual patients was very similar. Extremely high s-CAIX was found in only a single RCC patient who was already *ante finem* (5).

On the other hand, s-CAIX was detected in the urine of 69.6% of histologically confirmed TCC patients. The concentration was relatively high, and in three instances it was extremely high. However, we did not detect s-CAIX in the serum of any of TCC patients.

We propose that, in patients with RCC, s-CAIX is first shed into the blood and circulates there for some time. Since this is a cell adhesion protein (12, 13), it is absorbed by tissues expressing putative CAIX-specific receptor(s) (or CAIX-binding protein(s)). The absorbed protein is eventually internalized by the cells and destroyed. A fraction of s-CAIX is filtered through the kidneys and eliminated in urine. Conceivably, the RCC patient in whom we previously observed a very high concentration of s-CAIX in both blood and urine already had all of the cell surface receptors in body saturated with s-CAIX and therefore, the newly synthesized s-CAIX remained in the blood and some was eliminated in the urine.

An entirely different situation was observed in TCC patients, in whom, most of the s-CAIX is shed directly into the urine. Shedding of the antigen into the blood is prevented by the basal membrane, which separates the tumor from the circulating blood. Therefore we were unable to detect the antigen in the serum, even when it was observed in urine.

The present experiments were not intended to provide statistics on s-CAIX shedding, since our procedure is quite time-consuming, laborious, and only semi-quantitative, although it is very specific and sensitive. Nevertheless, the present data support the view that measuring s-CAIX levels in urine may be a useful tool for monitoring patients after resection or another therapy for tumor recurrence. This will require the development of a simple, rapid, and versatile method.

Acknowledgements. This research was supported by Bayer Diagnostics, USA. The authors thank Hillary Hoffman for language correction.

References

[1] Pastorekova S, Zavada J. Carbonic anhydrase (CA IX) as a potential target for cancer therapy. Review article. Cancer Ther 2004; 2: 245–62.

[2] Pastorekova S, Parkkila S, Zavada J. Tumor associated carbonic anhydrases and their clinical significance. Adv Clin Chem 2006; 42: 167–216. doi:10.1016/S0065-2423(06)42005-9 Can't connect to PubMed

[3] Loncaster JA, Harris AL, Davidson SE et al. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: Correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 2001; 61: 6394–6399.

[4] Harris AL. Hypoxia – a key regulatory factor in tumour growth. Nature Reviews Cancer 2002; 2: 38–47. doi:10.1038/nrc704 Can't connect to PubMed

[5] Zavada J, Zavadova Z, Zatovicova M et al. Soluble form of carbonic anhydrase IX (CA IX) in the serum and urine of renal carcinoma patients. Brit J Cancer 2003; 89: 1067–71. doi:10.1038/sj.bjc.6601264 Can't connect to PubMed

[6] Li G, Feng G, Gentil-Perret A et al. Serum carbonic anhydrase 9 level is associated with postoperative recurrence of conventional renal cell cancer. J Urol 2008; 180: 510–514. doi:10.1016/j.juro.2008.04.024 Can't connect to PubMed

[7] Turner KJ, Crew JP, Wykoff CC et al. The hypoxia-inducible genes VEGF and CA9 are differentially regulated in superficial vs invasive bladder cancer. Brit J Cancer 2002; 86: 1276–82. doi:10.1038/sj.bjc.6600215 Can't connect to PubMed

[8] Liao SY, Brewer C, Zavada J et al. Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Amer J Pathol 1994; 145: 598–609.

[9] Pastorekova S, Zavadova Z, Kostal M et al. A novel quasi-viral agent, MaTu, is a two-component system. Virology 1992; 187: 620–26. doi:10.1016/0042-6822(92)90464-Z Can't connect to PubMed

[10] Zatovicova M, Tarabkova K, Svastova E et al. Monoclonal antibodies generated in CA IX-deficient mice recognize different domains of tumor-associated hypoxia--induced carbonic anhydrase IX. J Immunol Methods 2003; 282: 117–34. doi:10.1016/j.jim.2003.08.011 Can't connect to PubMed

[11] Ivanov SV, Kuzmin I, Wei Ming-Hui et al. Down-regulation of transmembrane carbonic anhydrases by wild-type von Hippel-Lindau transgenes. Proc Natl Acad Sci USA 1998; 95: 12596–12601. doi:10.1073/pnas.95.21.12596 Can't connect to PubMed

[12] 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. Brit J Cancer 2000; 82: 1808–1813. doi:10.1054/bjoc.2000.1111 Can't connect to PubMed

[13] Zavadova Z, Zavada J. Carbonic anhydrase IX (CA IX) mediates tumor cell interactions with microenvironment. Oncol Reports 2005; 13: 977–82.