

# Chapter 7

## An Overview of Carbonic Anhydrase-Related Neoplasms



Martina Takacova and Silvia Pastorekova

**Abstract** Aberrant control of acid–base homeostasis is an emerging cancer hallmark that results from adaptation to oncogenic metabolism and tumor microenvironment. It has dramatic consequences, contributing to acquisition of aggressive tumor phenotype and disease progression. Regulation of pH is executed by transporters, exchangers, and pumps mediating ion fluxes in rates dictated by actual physiological needs of tumor cells. However, full performance of the pH-regulation machinery requires carbonic anhydrases (CAs) catalyzing a reaction key to acid–base balance: reversible conversion of carbon dioxide to bicarbonate ion and proton. Here, we provide an overview of known and predicted links of the human CA isoforms to various types of neoplasms and summarize the mechanisms, through which they functionally contribute to cancer. We use the approach, in which data from published papers are complemented by publicly available metadata processed by the GEPIA2 instrument. This allows not only for aligning bioinformatics to experimental evidence (supporting a prominent position of CA IX), but also for prediction of novel correlations that remain to be experimentally proven (such as for CA XIV). With a continuous accumulation of the knowledge, it is now becoming clear that CAs are broadly expressed in tumors and actively participate in cancer development and/or progression.

**Keywords** Carbonic anhydrase · pH regulation · Cancer · Prognosis · GEPIA2

### Abbreviations

BRAF	B-Raf oncoprotein
CA	carbonic anhydrase
HIF	hypoxia-inducible factor

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IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
MCT	monocarboxylate transporter
MET	hepatocyte growth factor receptor
NF1	neurofibromatosis 1
NHE	sodium-proton exchanger
NSCLC	non-small cell lung carcinoma
PG	proteoglycan
RTK	receptor tyrosine kinase
RAS	c-Ras oncoprotein
TPM	transcripts per million
VHL	von Hippel Lindau

## 7.1 Introduction

Vital functions of cells, tissues, and organs in the human body crucially depend, among other factors, on proper pH regulation, especially in situations of high metabolic performance, gas exchange, and/or fluid production. Such processes occur under physiological conditions in virtually all organs and are particularly intense in stomach, intestine, liver, pancreas, kidney, lung, and brain. However, many pathological conditions including cancer are characterized by aberrant control of acid–base homeostasis as a result of perturbed ion transport and abnormal metabolism. Especially in cancer cells, this has very dramatic consequences, supporting acquisition of an aggressive tumor phenotype and contributing to disease progression (Corbet and Feron 2017).

Regulation of pH is executed by a number of ion transporters, exchangers, and pumps that carry out ion extrusion and/or ion import in a rate dictated by actual physiological needs of the cell and by status of its intracellular and extracellular microenvironment. Among these, lactate and proton exporters, and bicarbonate importers play a major role as pH-modulating mediators of ion fluxes across the plasma membrane, while proton pumps also control pH of certain intracellular organelles (endosomes, lysosomes). However, in addition to these “ion transport managers,” full performance of the pH regulation machinery requires enzymes of the carbonic anhydrase family catalyzing the reaction that is fundamental for acid-base balance, namely, the reversible conversion of carbon dioxide to carbonic acid that spontaneously dissociates to bicarbonate ion and proton (Supuran 2016). The human body contains 15 carbonic anhydrase (CA) isoforms of the  $\alpha$ -CA family, namely CA I-IV, CA VA and CA VB, CA VI-XIV. Twelve isoforms are active enzymes, with catalytic activity ranging from low to very high (CA II and CA IX belonging to the most active enzymes in the nature), while three isoforms are inactive (CA VIII, X, and XI). CAs also differ by subcellular localization: CA I-III, VII, VIII, X, XI, XIII are cytoplasmic, CA VA

and VB are mitochondrial, CA IV, IX, XII, XIV are membrane-associated, and CA VI is secreted (Pastorekova et al. 2004; Mboge et al. 2018). The diversity of the human CAs is further widened by their variable expression levels and heterogeneous expression pattern in organs and tissues ranging from almost ubiquitous (e.g., CA II and CA XIII), to limited to certain tissue (e.g., CA VA and CA VI). This makes their complex understanding very difficult and development of general statements very challenging.

It has been long believed that the nature “invented” these enzymes primarily to drive pH-related physiological processes in normal tissues, since the initial efforts to link CAs to cancer did not bring any clear-cut data. However, everything changed in the early 90 's of the twentieth century, with the discovery of two, at that time novel, CA isoforms. First it was CA IX, which showed strong association with a broad range of tumors contrasting to limited expression in normal tissues (Pastorek et al. 1994) followed by CA XII, which was detected in many normal tissues, but showed overexpression in certain tumors (Tureci et al. 1998). Later on, thorough examination of some of the “old” isoforms (CA I, II, IV) revealed that their expression patterns exhibit either positive or negative correlations with cancer within specific contexts. Moreover, inactive isoforms CA X and CA XI, have also been implicated in tumor phenotype. In fact, with continuous accumulation of data, it is now becoming clearly apparent that CAs can actively participate in cancer development and/or progression.

Here we present an overview of known and predicted links of CA isoforms to various types of neoplasms and summarize the mechanisms through which they functionally contribute to cancer. In this overview, data from published papers are complemented by publicly available RNA-seq metadata from TCGA and GTEx databases processed by the GEPIA2 instrument, which uses Transcripts Per Million (TPM) values of transcription levels normalized to transcript's length and divided by the scaling factor to allow for comparison of gene expression levels among different types (Tang et al. 2019). This combined approach allows not only for aligning bioinformatics to experimental evidence, but also for prediction of some correlations that still remain to be experimentally proven. In this way, it is also possible to demonstrate that the cancer-related expression pattern, in some cases, does not necessarily translate to clinically relevant information, suggesting that the experimental evidence including functional studies is an imperative prerequisite for understanding the roles of genes/proteins including carbonic anhydrases in cancer biology and of their potential usefulness for clinical applications. Note that CA genes are written in italic style with Arabic numbers, while CA proteins are written in normal style with Roman numbers, in line with the official nomenclature, and that abbreviations used to designate different cancer types were used according to GEPIA2 (Table 7.1).

## 7.2 Expression of Cytoplasmic CA Isoforms in Neoplasms

Metadata analysis revealed that all of the five genes encoding the cytoplasmic CAs are primarily expressed in normal tissues, with CA2 exhibiting the highest transcription levels followed by CA1, CA3, CA7, and CA13 (Fig. 7.1). Notably, CA II protein

**Table 7.1** An overview of TCGA/GTEX data available within GEPIA2 that were used for the expression and survival analysis of carbonic anhydrase genes

TCGA	Detail	Tumor	Normal	GTEX	Num
ACC	Adrenocortical carcinoma	77	-	Adrenal Gland	128
BLCA	Bladder Urothelial Carcinoma	404	19	Bladder	9
BRCA	Breast invasive carcinoma	1085	112	Breast	179
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma	306	3	Cervix Uteri	10
CHOL	Cholangio carcinoma	36	9	-	-
COAD	Colon adenocarcinoma	275	41	Colon	308
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	47	-	Blood	337
ESCA	Esophageal carcinoma	182	13	Esophagus	273
GBM	Glioblastoma multiforme	163	-	Brain	207
HNSC	Head and Neck squamous cell carcinoma	519	44	-	-
KICH	Kidney Chromophobe	66	25	Kidney	28
KIRC	Kidney renal clear cell carcinoma	523	72	Kidney	28
KIRP	Kidney renal papillary cell carcinoma	286	32	Kidney	28
LAML	Acute Myeloid Leukemia	173	-	Bone Marrow	70
LGG	Brain Lower Grade Glioma	518	-	Brain	207
LIHC	Liver hepatocellular carcinoma	369	50	Liver	110
LUAD	Lung adenocarcinoma	483	59	Lung	288
LUSC	Lung squamous cell carcinoma	486	50	Lung	288
MESO	Mesothelioma	87	-	-	-
OV	Ovarian serous cystadenocarcinoma	426	-	Ovary	88
PAAD	Pancreatic adenocarcinoma	179	4	Pancreas	167
PCPG	Pheochromocytoma and Paraganglioma	182	3	-	-
PRAD	Prostate adenocarcinoma	492	52	Prostate	100
READ	Rectum adenocarcinoma	92	10	Colon	308
SARC	Sarcoma	262	2	-	-
SKCM	Skin Cutaneous Melanoma	461	1	Skin	557
STAD	Stomach adenocarcinoma	408	36	Stomach	175
TGCT	Testicular Germ Cell Tumors	137	-	Testis	165
THCA	Thyroid carcinoma	512	59	Thyroid	278

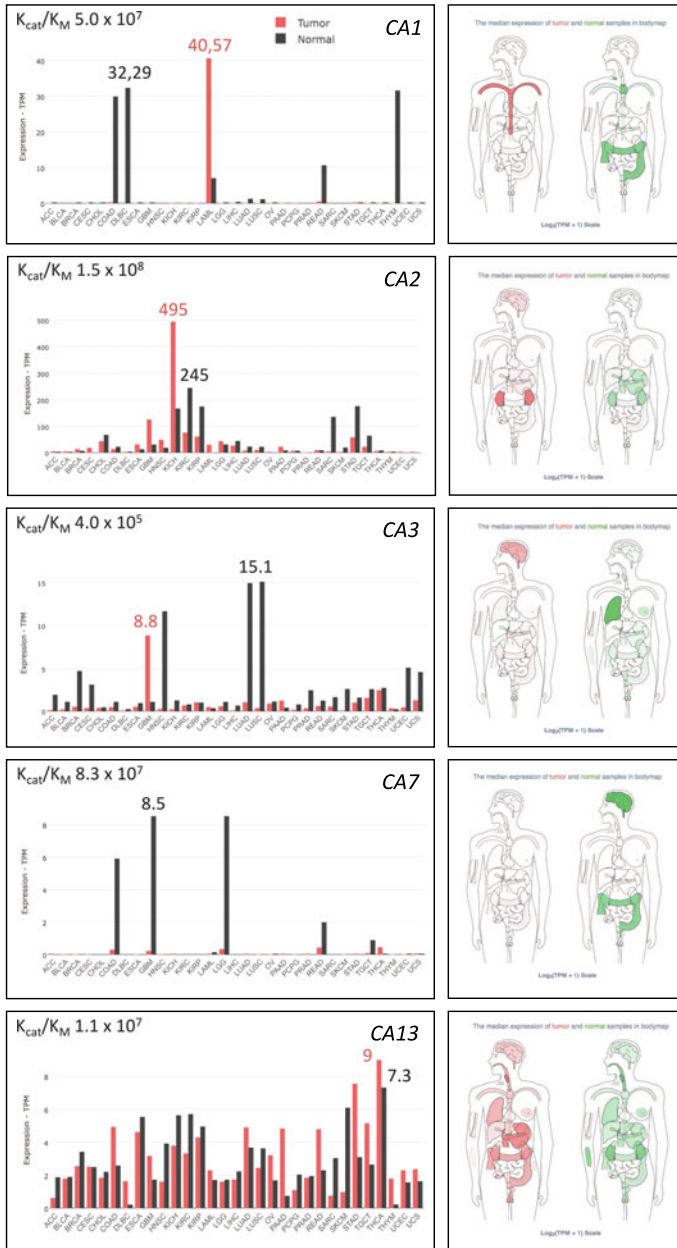
(continued)

**Table 7.1** (continued)

TCGA	Detail	Tumor	Normal	GTEx	Num
THYM	Thymoma	118	2	Blood	337
UCEC	Uterine Corpus Endometrial Carcinoma	174	13	Uterus	78
UCS	Uterine Carcinosarcoma	57	-	Uterus	78
UVM	Uveal Melanoma	79	-	-	-

displays also the highest catalytic activity, while the activity of CA III is the lowest among all active CA isoforms (Mboge et al. 2018). In line with these facts, most of the research related to cytoplasmic CAs in cancer has been focused on CA II as described in more detail further below.

As shown in Figs. 7.1 and 7.5, *CAI* gene transcription is relatively low, reaching TPM values of around 30 in normal colon, B-cells, and T-cells, and is not expressed in their neoplastic counterparts. However, *CAI* expression is increased in acute myeloid leukemia (LAML, TPM 40.57) compared to normal myeloid cells (TPM 7). In spite of this differential expression, GEPIA2-performed Mantel-Cox Log-rank test of meta-data from LAML failed to show a significantly better overall survival in the high *CAI* expression cohort, irrespective of whether cutoff was set to median (50%) or whether cutoff-high was set to 75% and cutoff-low to 25% (Fig. 7.6). Nevertheless, the literature contains dozens of papers dealing with the relationship of *CAI* to cancer. CA I protein expression was demonstrated by immunohistochemistry (IHC) in both normal and malignant endocrine tissue of pancreas, with intense positive staining observed particularly in the cells expressing glucagon (Parkkila et al. 1995b). IHC comparison of CA I distribution in normal large intestine versus colorectal tumors showed high CA I expression in normal colon, decreased intensity in benign lesions, and very weak staining in malignant tumors (Kivela et al. 2001). This finding was confirmed by an independent proteomic study suggesting that downregulation of CA I is an early event in colorectal carcinogenesis (Wang et al. 2012). In contrary, no *CAI* expression-based difference in overall survival was predicted by log-rank test of COAD cohorts from TCGA database. CA I protein was also detected by proteomic methods in sera of patients with non-small cell lung carcinoma (Wang et al. 2016). Noteworthy, sera of patients suffering from both acute myeloid leukemia and chronic lymphocytic leukemia were found to contain CA I (and CA II) autoantibodies (Mentese et al. 2017, 2018). Lakota and colleagues observed elevated CA I autoantibodies in sera of patients with spontaneous regression of tumors developing an aplastic anemia-type syndrome after a high-dose therapy with autologous stem cell transplantation (Skultety et al. 2010; Jankovicova et al. 2013). Silencing of CA I in prostatic (PC3) tumor cells was shown to change the composition of exosomes secreted by these cells indicating their enhanced malignant potential (Banova Vulic et al. 2019). However, due to many open questions and generally inconsistent data, it is currently impossible to make any conclusion on the relationship of CA I to cancer.



**Fig. 7.1** Expression of genes coding for the cytoplasmic carbonic anhydrases in tumors and normal samples. Data extracted from TCGA/GTEX databases were analyzed and converted to graphics using GEPIA2 instrument (Tang et al. 2019). Data in the graphs relate to TPM units and intensities of colors in the bodymaps correspond to Log/TPM+1 Scores in particular tissues. Numbers above the columns highlight the highest TPMs for each CA isoform.  $K_{cat}/K_M$  values in the upper left corners inform about the catalytic efficiency of CA isoforms {adopted from (Mboge et al. 2018)}

The *CA3* gene is mostly transcribed in several normal tissues (mainly breast, lung, and uterus), with a TPM value up-to 15 (Fig. 7.1). It is generally not expressed in tumors, except relatively low expression in glioblastoma (GBM, TPM 8.8), which is about 8-times higher than in normal brain (Fig. 7.5). However, this does not translate into the correlation to overall survival of GBM patients, but such correlation exists in LAML (Fig. 7.6). Interestingly, there are only two published studies dealing with CA III expression or function in cancer. Despite metadata showing only very low expression of CA III in liver and liver-derived neoplasms (1 TPM vs 0.1 TPM), an immunohistochemical analysis suggests that CA III is expressed in normal hepatocytes and that it is reduced in hepatocellular carcinoma (Kuo et al. 2003). The second study explored the effects of manipulated CA III expression on invasiveness of HCC (in TCGA abbreviation LIHC) cell line SK-Hep1 (Dai et al. 2008). The authors found that CA III suppression decreases, while CA III overexpression increases cell invasiveness possibly via activation of FAK signaling and extracellular acidification. Taking into account that there is no extension/confirmation of this observation and that CA III activity is very low, biological relevance of that observation remains elusive.

*CA7* transcription is detectable only in normal tissues (namely intestine, brain, and testes, TPM up to 8.5) but not in neoplasms (Figs. 7.1 and 7.5). Albeit absent or low, *CA7* levels in tumors derived from these tissues (COAD, READ, GBM, LGG) do not correlate with overall survival in any group of patients. In the published study of Yang and colleagues (Yang et al. 2015), reduced *CA7* mRNA and protein expression in colorectal carcinoma was significantly correlated with poor differentiation, positive lymph node metastasis, advanced TNM stage, and unfavorable clinical outcome. However, no such correlation was confirmed by immunostaining of colorectal carcinoma specimens from an independent cohort of patients, when both extent and intensity of staining were taken into account (Viikila et al. 2016). Thus, further studies are needed to resolve these conflicting results.

*CA13* gene transcription is detected both in normal tissues and in tumors, although it appears to be increased in some normal tissues and decreased in other ones when compared to tumors (Figs. 7.1 and 7.5). The significance of this differential expression remains unknown, but taking into account the fact that the levels of *CA13* transcription are rather low (up to 9 TPM), differences are too minor to have a biological relevance. There is only a single paper using immunohistochemistry to detect CA XIII protein expression in colorectal carcinoma (Kummola et al. 2005). The study results suggest that the expression of CA XIII is downregulated in tumor cells compared to the normal tissue, which is actually opposite to RNA-seq data acquired from TCGA database where significant correlation to overall survival was found in KIRC, LAML, LGG, and SKCM.

Lastly, the apparently most important cytoplasmic isoform is encoded by the *CA2* gene. The catalytic activity of CA II is among the highest not only within the CA family but also generally among all known enzymes. Its expression assessed by immunodetection was found to correlate with intracellular CA activity measured in a range of intact human cancer- and fibroblast-derived cells and in their membrane-free lysates. Moreover, genetic knockdown of CA II in HCT116 colon carcinoma

cells demonstrated that majority of intracellular CA activity was attributable to CA II. These data support the view that CA II is a crucial intracellular CA isoenzyme (Hulikova et al. 2014). Studies based on manipulated CA II expression and on use of small molecule inhibitors suggested that CA II activity participates in intracellular pH regulation (irrespective of normal or tumor cell phenotype) through cooperation with ion transporters, namely, by facilitating proton or bicarbonate transport across membranes, or mediating proton diffusion in cytoplasm. Initially, it was proposed that CA II forms a transport metabolon with chloride/bicarbonate exchangers that exploit the CA catalytic activity (Sterling et al. 2001). Later on, this concept was extended to the sodium/proton exchanger NHE1 (Li et al. 2002) and to the sodium/bicarbonate co-transporter (Villafuerte et al. 2014). CA II was also shown to cooperate in a non-catalytic manner with the monocarboxylate transporter MCT1, acting as an antenna collecting and transferring protons to MCT1 in order to facilitate its transport activity (Becker et al. 2005; Noor et al. 2018).

However, these scenarios are partly questioned by the finding of Swietach and colleagues who demonstrated that intracellular CA activity in cancer cell lines does not correlate with resting intracellular pH (pHi), NHE1 flux, or bicarbonate transporter flux measured in these cells, and that proton and bicarbonate fluxes produced by cells over the physiological pHi range are not of sufficient magnitude to require intracellular CA catalysis, with the exception of acid loading by bicarbonate export at high pHi (over 7.4) that is not typical for cancer cells (Hulikova et al. 2014). This study also suggests that high intracellular CA activity (mostly attributable to CA II) is associated with faster and larger pHi oscillations, larger pH-dependent intracellular calcium ion oscillations, and stronger inhibition of mTORC1 pathway in response to extracellular  $p\text{CO}_2$  fluctuations (Hulikova et al. 2014). In contrast, pHi of cells exhibiting low intracellular CA activity is less responsive to  $p\text{CO}_2$  fluctuations, which might be of key importance for cells' survival especially in tumor tissues with dynamic temporal and/or regional changes of blood flow, delivery of oxygen and nutrients as well as removal of metabolic waste due to aberrant tumor vasculature (Gillies et al. 2018).

Actually, the above-described view can explain why expression of the CA2 gene (as well as the levels of other genes encoding the cytoplasmic CAs with lesser contribution to overall intracellular CA activity) is primarily detected in non-cancerous tissues (with TPM values up to 245) and downregulated in related tumors. In line with this, TCGA/GTEX databases processed by GEPIA2 show that CA II levels are decreased in KIRC, KIRP, SARC, STAD, and TGCT when compared to their normal counterparts. In contrary, CA2 expression is increased in GBM and KICH (with TPM of 126 and 495, see Figs. 7.1 and 7.5). While this increased tumor expression does not predict overall survival of cancer patients in case of GBM and KICH, reduced CA2 transcription is significantly associated with reduced overall survival in patients with KIRC and SARC (Figs. 7.6 and 7.7), but not KIRP, STAD, and TGCT. Interestingly, significantly worse overall survival predicted by GEPIA2 is also linked with decreased CA II levels in COAD (with cutoff-high 75% and cutoff-low 25%) that is principally in line with IHC and proteomic studies of independent patients' cohorts showing that loss of expression of CA II (and CA I) accompanies progression to



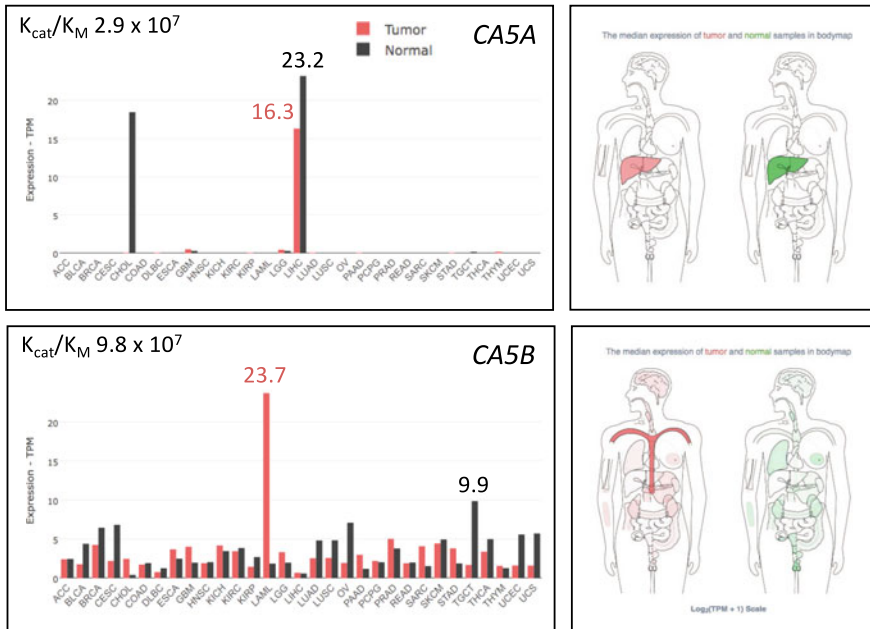
malignant phenotype (Kivela et al. 2001; Wang et al. 2012). Moreover, CA II is the most widely distributed isoform in the central nervous system, and its expression was also detected by IHC in different brain tumors (Parkkila et al. 1995a), medulloblastomas and neuroectodermal tumors (Nordfors et al. 2010), hematological malignancies (Leppilampi et al. 2002), esophageal carcinoma (Nortunen et al. 2018), pancreatic tumors (Parkkila et al. 1995b), gastrointestinal stromal tumors (Parkkila et al. 2010; Liu et al. 2013), hepatocellular carcinomas (Kuo et al. 2003), uterine tumors (Hynninen et al. 2012), and urinary bladder cancers (Tachibana et al. 2017). In meningiomas, CA II was found in endothelial cells in association with increasing malignancy grade and tumor proliferation rates (Korhonen et al. 2009). CA II expression also was observed in tumor vessel endothelia of melanoma and esophageal, renal, and lung cancers (Yoshiura et al. 2005).

There are also several papers describing the functional aspects of CA II. It was shown that forced CA II expression in colorectal cancer cells remarkably suppresses tumor cell growth both in vitro and in vivo (Zhou et al. 2013). Similarly, CA II overexpression inhibits cell migration and invasion by reversing EMT in hepatocellular carcinoma cells, while its downregulation promoted invasiveness and metastasis (Zhang et al. 2018). Thus, the link of CA II to neoplastic phenotype is tumor tissue- and cell type-dependent and may also reflect the physiology of tumor microenvironment.

### 7.3 Expression of Mitochondrial CA Isoforms in Cancer

Mitochondrial CA isoforms are encoded by two phylogenetically-related genes *CA5A* and *CA5B*. Since mitochondria are impermeable to bicarbonate ions, activity of CA VA and CA VB enzymes is required for production of bicarbonate that is utilized by mitochondrial enzymes for metabolic processes, namely, by pyruvate carboxylase for gluconeogenesis and carbamoyl phosphate synthase I for ureagenesis. Targeted disruption of the murine *Car5A* and *Car5B* genes and their double knock-out implicated both enzymes in ammonia detoxication and glucose metabolism (Shah et al. 2013). Despite glucose metabolism being of high importance for tumor biology, so far there are no thorough studies linking *CA5A* and/or *CA5B* to cancers, except a single paper identifying the *CA5A* gene within a signature of genes predicting decreased overall survival in patients with head and neck carcinoma (Bornstein et al. 2016).

Thus, publicly available RNA-seq metadata can provide at least basic insight into possible relationships of *CA5A* and *CA5B* expression to human tumors. GEPIA2 testing showed that *CA5A* gene is transcribed in normal liver (in accord with expectation) at the TPM level of 23.2 and was slightly decreased in liver neoplasms (TPM 16.3). In addition, *CA5A* expression was detected in bile ducts, but not in cholangiocarcinoma (Figs. 7.2 and 7.5). In contrary, *CA5B* is transcribed in many normal tissues (namely in the breast, ovaria, uterus, cervix, testes, TPM up to 9.9) and at lower levels also in the corresponding cancers. The highest *CA5B* expression is



**Fig. 7.2** Expression of genes coding for the mitochondrial carbonic anhydrases in tumors and normal samples. Data extracted from TCGA/GTEX databases were analyzed and converted to graphics using GEPIA2 instrument (Tang et al. 2019). Data in the graphs relate to TPM units and intensities of colors in the bodymaps correspond to Log/TPM+1 Scores in particular tissues. Numbers above the columns highlight the highest TPMs for each CA isoform.  $K_{cat}/K_M$  values in the upper left corners inform about the catalytic efficiency of CA isoforms {adopted from (Mboge et al. 2018)}

present in acute myeloid leukemia (Fig. 7.2), but it has no impact on overall survival (Fig. 7.6). However, these data do not take into consideration the potential role of CA VA/CA VB catalytic activities, which might be involved in adaptation to metabolic demands of tumor cells. Clearly, additional studies are warranted to resolve whether mitochondrial CAs are involved in tumor biology.

### 7.4 Expression of Membrane-Bound and Secreted CA Isoforms in Cancer

Four membrane-bound isoforms (CA IV, IX, XII, and XIV) and one secreted isoform (CA VI) are active enzymes with the catalytic sites facing the extracellular space. Their catalytic performance is relatively high, with CA IX exhibiting the highest activity (similar to CA II), then followed by CA VI, CA IV, CA XIV, and CA XII, activity of which is approximately 4-times lower than that of CA IX. All these

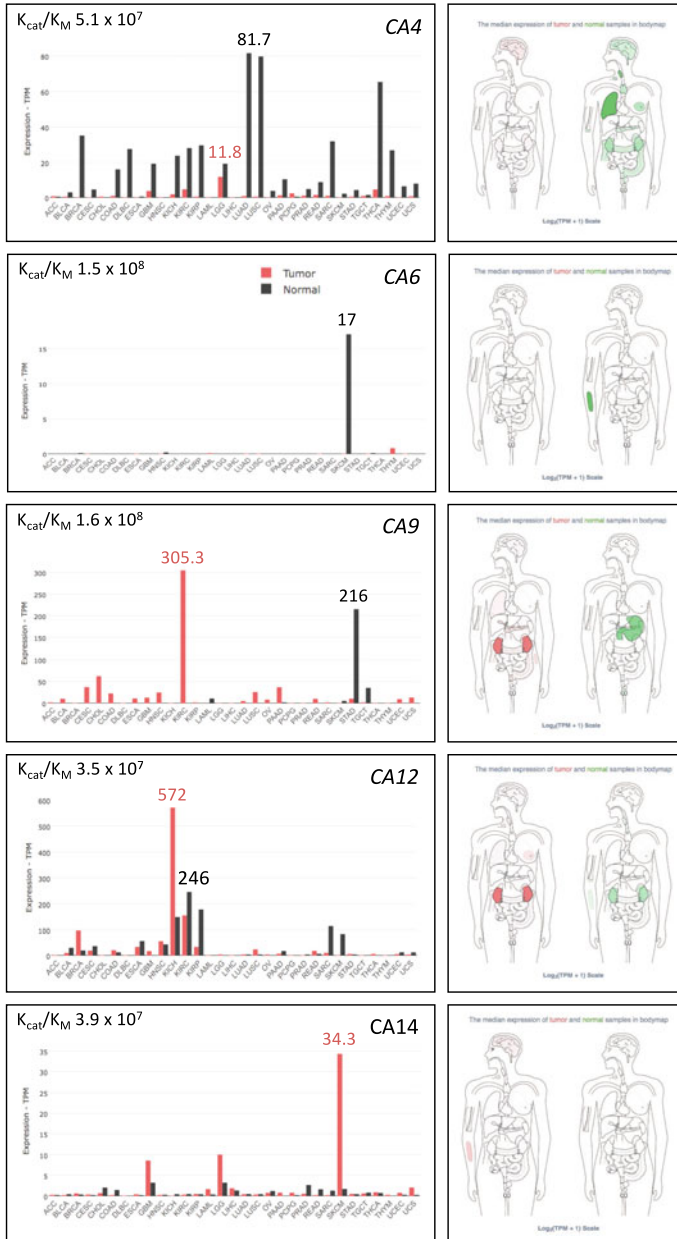
enzymes appear to support extracellular CO<sub>2</sub> diffusion, and the membrane-bound isoforms also participate in bicarbonate import and proton extrusion.

While CA IV is bound to the plasma membrane by the GPI anchor, three other exofacial CAs are type I membrane proteins, with the N-terminal side at the cell surface and C-terminal side in the cytoplasm (Pastorekova et al. 2004). In addition, CA IX has an extra N-terminal proteoglycan-like domain (PG) linked by the hinge region to the CA domain (Opavsky et al. 1996). This PG domain contains a repetitive stretch of proline-perturbed basic amino acids, and has an intrinsically disordered structure with conformational flexibility that is implicated in non-catalytic functions of CA IX relevant for tumor biology (Zavada et al. 2000; Langella et al. 2018). CA IX isoform was shown to cooperate with extracellular structures of bicarbonate transporters and sodium/proton exchangers facilitating ion transport via its catalytic activity (Morgan et al. 2007; Orłowski et al. 2012; Svastova et al. 2012), and with monocarboxylate transporters MCT1 and MCT4 via a non-catalytic mechanism, in which the PG domain serves as a proton-conducting antenna interacting with MCT4 chaperone CD147 (Jamali et al. 2015; Ames et al. 2018, 2019). Similar model was demonstrated for CA IV, which facilitates lactate export via interaction with chaperons of MCTs (Klier et al. 2014; Forero-Quintero et al. 2019).

TCGA/GTEX metadata-derived expression patterns of the membrane-bound and secreted CAs provided by GEPIA2 instrument are very distinct and remarkable (Figs. 7.3 and 7.5), and generally have a lot of support in the published papers, albeit some previously unknown and potentially interesting connections have also been disclosed as described below.

Noteworthy, the *CA4* gene is mostly expressed in normal tissues, and only occasionally and at a low level in some tumors (Figs. 7.3 and 7.5). The highest TPM values of *CA4* transcription (up to 81.7) are exhibited by normal counterparts of LUAD, LUSC, THCA, BRCA, SARC, DLBC, KIRP, KIRC, KICH, and THYM. However, only KIRC and LUAD show a highly significant correlation between high expression of *CA4* and better overall survival, with hazard risk reduced up to 3-times compared to tumors with low *CA4* (Figs. 7.6 and 7.7). Interestingly, these significant correlations exist independently of whether the analysis includes all *CA4* transcript variants together, or only individual protein-coding transcript variants identified by GEPIA2, namely, *CA4-001*, *CA4-005* (both in KIRC and LUAD), and *CA4-006* (only in KIRC). In addition, both median and quartile cutoffs produced similar probability values.

Experimental data are in agreement with predictions based on bioinformatics. In patients with renal clear cell carcinoma, decreased expression of *CA4* mRNA in tumors was associated with poor survival (Takenawa et al. 1998). Loss of *CA4* was also proposed as a biomarker distinguishing follicular thyroid carcinomas from follicular adenomas (Davidov et al. 2014). *CA4* expression determined by Q PCR was also found to be significantly downregulated in non-small cell lung carcinomas (NSCLC) as well as in six NSCLC-derived cell lines, and lower *CA4* levels were correlated with lymph node metastasis and shorter overall survival (Chen et al. 2017). Importantly, *CA4* was proposed to be a novel tumor suppressor in colorectal cancer (CRC). This proposal was based on the observation that *CA4* gene is silenced in



**Fig. 7.3** Expression of genes coding for the membrane-bound and secreted carbonic anhydrases in tumors and normal samples. Data extracted from TCGA/GTEX databases were analyzed and converted to graphics using GEPIA2 instrument (Tang et al. 2019). Data in the graphs relate to TPM units and intensities of colors in the bodymaps correspond to Log/TPM+1 Scores in particular tissues. Numbers above the columns highlight the highest TPMs for each CA isoform.  $K_{cat}/K_M$  values in the upper left corners inform about the catalytic efficiency of CA isoforms {adopted from (Mboge et al. 2018)}

more than 90% of CRC tumors primarily by the promoter hypermethylation (Zhang et al. 2016). In support of the tumor suppressor concept, re-expression of *CA4* in CRC cells inhibits cell proliferation and induces apoptosis via downregulation of the WNT pathway and degradation of  $\beta$ -catenin (Zhang et al. 2016).

Based on GEPIA2 analysis, *CA6* expression is confined to normal skin (TPM 17, Figs. 7.3 and 7.5), and its reduced level in SKCM predicts worse overall survival (Figs. 7.6 and 7.7). In the published literature, CA VI isoform is primarily known as a component of saliva expressed in serous acinar cells of human salivary glands. Immunostaining revealed its potential diagnostic utility for discrimination of acinic cell carcinoma from mammary analogue secretory carcinoma of the salivary gland (Hsieh et al. 2016). No further information on the relationship of CA VI to cancer is available to date.

In contrast to the other CA genes, *CA9* is predominantly expressed in tumor tissues (CESC, CHOL, COAD, ESCA, GBM, HNSC, KIRC, LUSC, OV, PAAD, READ, UCEC, UCS, TPM up to 305.3) and virtually not in their normal counterparts (Figs. 7.3 and 7.5). According to RNA-seq data from TCGA/GTEX databases, the only normal tissues that exhibit *CA9* transcription levels higher than tumors are stomach (TPM 216) and testes (TPM 35.7), albeit published IHC studies detected CA IX protein also in the epithelia of pancreas, gallbladder, intestinal crypts, and basal skin cells (Pastorekova et al. 1997; Kivela et al. 2001).

In tumors, expression of *CA9* is directed primarily by a HIF-1 transcription factor composed of an oxygen-regulated  $\alpha$  subunit and a constitutive  $\beta$  subunit. The  $\alpha$  subunit is degraded in proteasome following hydroxylation of its critical prolyl residues and recognition by pVHL tumor suppressor protein acting as E3 ubiquitin ligase, in conditions of normal oxygen delivery (Kaelin and Ratcliffe 2008). When metabolic and proliferative demands of tumor cells exceed the availability of oxygen, which is reduced due to aberrant tumor vasculature, resulting hypoxia inhibits prolyl hydroxylases (including the asparaginyl hydroxylase that hampers HIF- $\alpha$  transcriptional activity) and leads to escape of an  $\alpha$  subunit from pVHL recognition and degradation. As a consequence, HIF- $\alpha$  is stabilized and activated, accumulates in the cytoplasm, enters the nucleus, dimerizes with HIF- $\beta$ , and forms a competent transcription factor that induces expression of hundreds of genes coding for proteins involved in adaptation of tumor cells to hypoxia (Ratcliffe 2013; Semenza 2012). *CA9* is one of the key targets of HIF-1 that drives its transcription in hypoxic tumor cells to a very high magnitude through binding closely to the transcription initiation site of the *CA9* gene (Wykoff et al. 2000). Thus, expression of *CA9* in the majority of tumor types is heterogeneous and regionally distributed in hypoxic or post-hypoxic areas. However, in some cancer entities, *CA9* expression is induced due to activation of pathways driven by oncogenes (such as SRC or RET, Takacova et al. 2010, 2014).

The situation is rather different in KIRC, which express the highest levels of *CA9* transcript as well as CA IX protein (this can be observed both in metadata and IHC studies). These renal clear cell carcinomas are characterized by inactivating mutations of VHL tumor suppressor gene, resulting in constitutive stabilization of HIF- $\alpha$  subunit and sustained activation of the HIF-governed hypoxia-related pathways even in the absence of physiological hypoxia (Wiesener et al. 2001). Since *CA9* is one of the

most highly HIF-1-induced genes, it is expressed in a very high percentage of VHL-defective renal cancer clear cells (Ivanov et al. 1998; Bui et al. 2003; Stillebroer et al. 2010).

When including all four *CA9* alternative transcripts (two protein-coding and two non-translated) into GEPIA2 survival analysis, *CA9* expression shows significant associations with overall survival of patients with several types of cancers, namely, GBM, LUAD, PAAD, and SKCM (Fig. 7.6). However, when solely the full-length transcript *CA9-001* is taken into account, the spectrum of tumor types is extended to KIRC and SARC (Fig. 7.7). *CA9-001* is the only full-length transcript, which is induced by hypoxia and translated to a functional and active CA IX protein (Barathova et al. 2008).

In all mentioned tumor types except KIRC, higher *CA9* expression is related to significantly poorer prognosis (Figs. 7.6 and 7.7). In the case of KIRC, higher *CA9-001* levels (but not total *CA9* levels) show correlation with significantly better overall survival (Figs. 7.6 and 7.7). This appears to be a consequence of a shift from HIF-1 that principally governs the *CA9* transcription in early stages of this disease, towards HIF-2, which drives a kidney cancer progression and determines malignant tumor phenotype in renal clear cell carcinoma, but induces *CA9* to much lower degree (Raval et al. 2005).

Numerous immunohistochemical studies of various tumor tissue specimens suggest significant relationships to clinical variables in a broad spectrum of tumors other than those revealed by GEPIA2 analysis, including BRCA, ESCA, COAD, MESO, OV, READ, STAD, UCEC, etc. {reviewed in (Pastorek and Pastorekova 2015; Pastorekova and Gillies 2019)}. Thorough meta-analysis of the results of 147 publicly available clinical studies (excluding KIRC) reveals a strong significant association between CA IX expression and all survival endpoints: overall, disease-free, loco-regional, disease-specific, metastasis-free, and progression-free survival (van Kuijk et al. 2016). Absence of correlations of TGCT/GTex *CA9* data to survival in these additional cancer types can be explained by a regionally and temporally heterogeneous, hypoxia-induced *CA9* gene expression pattern, as well as by relatively short half-time of the *CA9* transcripts contrasting with very long half-time of the CA IX protein (Rafajova et al. 2004). Moreover, the CA IX ectodomain can be cleaved by metalloproteinases and released to extracellular space and body fluid, which can affect the CA IX protein expression in tumors (Zatovicova et al. 2005).

In addition to the value of *CA9* gene and/or CA IX protein expression as a surrogate marker of tumor hypoxia {which is the topic of extensive translational research, e.g., for bioimaging (Tafreshi et al. 2012)}, a lot of attention has been paid to the role of CA IX protein in tumor biology. Numerous studies employing genetic manipulation or pharmacologic inhibition of CA IX show that it is a key component of the pH-regulating machinery that enables tumor cells to survive hostile hypoxic and acidotic conditions in the tumor microenvironment and to gain invasiveness and metastatic propensity (Svastova et al. 2004, 2012; Swietach et al. 2009; Chiche et al. 2009; Radvak et al. 2013; Csaderova et al. 2013; Chafe et al. 2015; Swayampakula et al. 2017; Lee et al. 2018; Debreova et al. 2019). In addition, CA IX was found to contribute to stemness and chemoresistance (Ledaki et al. 2015; Vidlickova

et al. 2016; Gibadulinova et al. 2020). Interestingly, CA IX has been associated with invasive phenotype pioneering in a novel and sometimes hostile environment (Lloyd et al. 2016). Moreover, CA IX interacts or communicates with a number of regulatory proteins (besides ion transporters mentioned above), which are involved in important cellular processes, such as  $\beta$ -catenin,  $\alpha 2\beta 1$  integrin, CD98hc, MMP14, and other actin-regulatory proteins, hERG1 potassium channel, NCX1 sodium/calcium exchanger, PKA, PI3 kinase, etc. (Svastova et al. 2003; Swayampakula et al. 2017; Debreova et al. 2019; Lastraioli et al. 2019; Liskova et al. 2019; Ditte et al. 2011; Dorai et al. 2005). These interactions can have profound effects on enzymatic and signaling functions of CA IX and also on its accessibility to antibodies and small molecules.

The expression pattern in a broad range of tumors as well as functional involvement of CA IX in tumor biology have raised enormous interest in the development of CA IX-targeting drugs, including inhibitors and specific monoclonal antibodies for immunotherapeutic applications {reviewed in (Neri and Supuran 2011; Oosterwijk-Wakka et al. 2013; Pastorek and Pastorekova 2015; Singh et al. 2018; Pastorekova and Gillies 2019)}. These efforts are continuously ongoing and novel combination strategies are being investigated (McIntyre et al. 2012; Dubois et al. 2013; Boyd et al. 2017; Chafe et al. 2019).

*CA12* expression evaluated by GEPIA2 is present in several tumor types including KICH, KIRC, BRCA, and HNSC, but also in the normal counterparts of KIRC, KIRP, KICH, SARC, and SKCM (Figs. 7.3 and 7.5). The highest level is detected in KICH (TPM 572), but the relationship of a high *CA12* level to better overall survival is significant only in SKCM (both for sum of all three protein-coding transcripts and solely for *CA12-001* transcript) (Fig. 7.6). In KIRC, only the *CA12-002* transcript relates to better overall survival while the other transcript variants do not show any significant correlations. Based on the available experimental data, *CA12* expression is regulated by hypoxia, albeit to a lower magnitude than *CA9*, and a direct role of the HIF transcription factor in its regulation has not been clearly defined (Wykoff et al. 2000). However, *CA12* expression appears to be driven by differentiation factors at least in some tumor types (Barnett et al. 2008; Waheed and Sly 2017; Franke et al. 2019). In invasive BRCA, CA XII protein expression assessed by IHC is associated with a lower grade, a lower relapse rate, and a better overall survival (Wykoff et al. 2001; Watson et al. 2003; Li et al. 2019). The link between CA XII expression and better prognosis was demonstrated also in cervical cancer {in association with superior disease-free survival, (Yoo et al. 2010) and resectable NSCLC (Ilie et al. 2011)}. In contrast, IHC staining for CA XII increases with increasing grade of colorectal tumors (Kivela et al. 2000), high CA XII expression is linked to lower survival rate of patients with esophageal squamous cell carcinoma (Ochi et al. 2015). Interestingly, a shorter, alternatively spliced *CA12* transcript has been associated with poor prognosis of patients with diffusely infiltrating astrocytic gliomas (Haapasalo et al. 2008). In cell culture models with genetically manipulated expression, CA XII was shown to participate in pH regulation, tumor growth, and chemoresistance (Chiche et al. 2009; Kopecka et al. 2015). Based on these relationships, also CA XII

is investigated as therapy target for CA inhibitors and monoclonal antibodies (Gondi et al. 2013; Kopecka et al. 2015; Boyd et al. 2017).

Expression of *CA14* has not been experimentally linked to cancer so far, but GEPIA2 analysis disclosed its increased levels in GBM, LGG, and SKCM (TPM up to 34.3), Figs. 7.3 and 7.5. Even more interestingly, higher *CA14* expression (for all transcript variants together and also separately for *CA14-001* and *CA14-005*) shows a significant correlation to worse overall survival of patients with LGG and SKCM, but to better prognosis in case of GBM (Figs. 7.6 and 7.7). This is an unexpected finding that does not have any supporting clinical data in the published literature. Additional links of CA XIV to cancer coming from other publicly available databases are described by Mboge and colleagues (Mboge et al. 2018), but those data also require experimental/clinical validation.

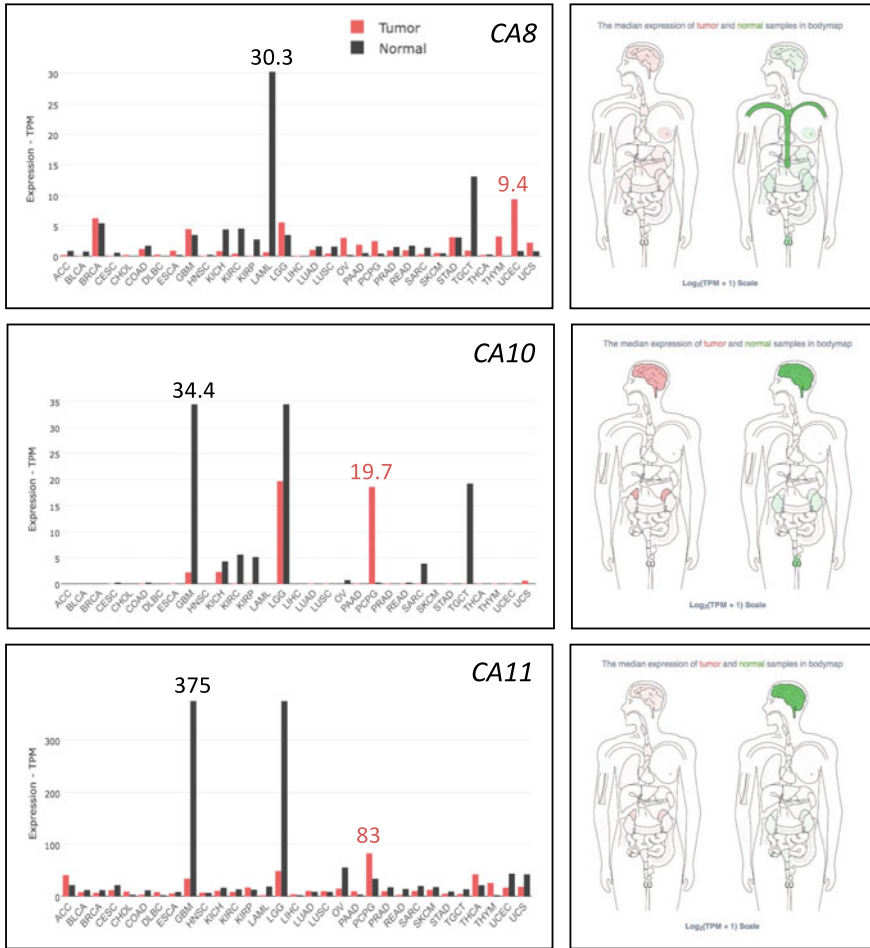
## 7.5 Expression of Inactive CA Isoforms in Cancer

Three genes coding for inactive CA isoforms, namely, *CA8*, *CA10*, and *CA11* contain mutations affecting the critical residues involved in the coordination of a zinc ion, which is crucial for the CA catalytic activity. This precludes participation of these isoforms in the canonic pH regulation-related metabolons involving bicarbonate transporters and sodium/proton exchangers. However, the non-catalytic cooperation with MCTs can still be feasible and was recently demonstrated for all three proteins CA VIII, CA X, and CA XI (Aspatwar et al. 2019). The authors suggest that the inactive CAs may function as a proton antenna for MCT1, to drive proton-coupled lactate transport across cell membranes.

GEPIA2 shows that the *CA8* gene is differentially transcribed particularly in LAML and TGCT (TPM up to 30.3) where it is strongly decreased in comparison to normal counterparts, but does not show any relationship to overall survival (Figs. 7.4, 7.5, 7.6). On the other hand, *CA8* transcription is elevated in tumor versus normal tissues in UCEC, THYM, OV, PAAD, and PCPG (TPM up to 9.4). Out of these tumor types, only in SKCM higher *CA8* relates to poor prognosis, whereas in UCEC and PAAD (with quartile cutoffs), higher *CA8* expression is linked to better overall survival (Fig. 7.6). This is corresponding to the observation that CA VIII immunopositivity in 13% of astrocytomas and 9% of oligodendrogliomas is associated with more benign behavior (Karjalainen et al. 2018). However, the opposite relationship was found in colorectal and lung carcinomas (Miyaji et al. 2003; Akisawa et al. 2003), and experiments using cell models suggest that CA VIII promotes progression and invasiveness of lung cancer cells (Lu et al. 2004; Ishihara et al. 2006) and growth of colon cancer cells (Nishikata et al. 2007).

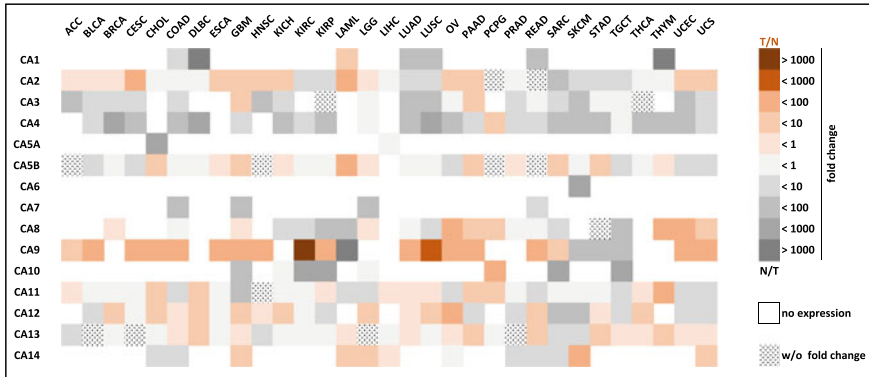
*CA10* gene expression is increased in PCPG (TPM 19.7) and decreased in GBM, LGG, TGCT, KIRC, KIRP, SARC, and KICH, with no association to overall survival, except a significant correlation of high *CA10* transcription with a better overall survival in LGG patients (Figs. 7.4–7.7). The *CA11* gene expression pattern (tumor vs normal) is similar to *CA10* and includes increased levels in PCPG (TPM 83) and





**Fig. 7.4** Expression of genes coding for the inactive carbonic anhydrases in tumors and normal samples. Data extracted from TCGA/GTEX databases were analyzed and converted to graphics using GEPIA2 instrument (Tang et al. 2019). Data in the graphs relate to TPM units and intensities of colors in the bodymaps correspond to Log/TPM + 1 Scores in particular tissues. Numbers above the columns highlight the highest TPMs for each CA isoform

decreased levels in GBM, LGG, OV, UCEC, and UCS (Figs. 7.4,7.5). A significant relationship between high *CA11* level and better overall survival was observed only in LGG (Fig. 7.6). In line with this, levels of CA X and CA XI isoforms (that are secreted synaptic proteins inhibiting the growth of glioma cell lines) were reduced in clinical glioma samples and negatively associated with high histological grade (Tao et al. 2019). In preclinical experiments of the same study, *CA11* knockdown promoted cell growth, clone formation and migration, and increased tumor size in xenografted mice. In contrast, CA XI immunostaining was observed in the small



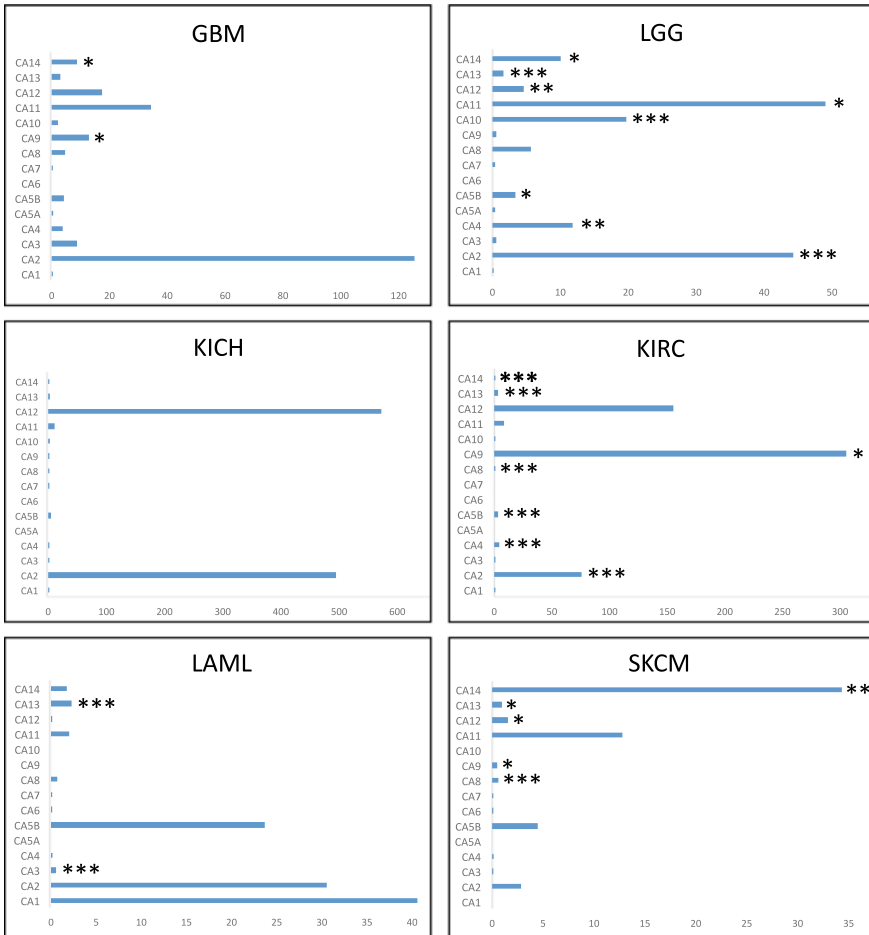
**Fig. 7.5** Heatmap visualizing differential expression of the genes coding for CA isoforms in all GEPIA2-analyzed tumors compared to normal samples. Tumor/normal ratio is expressed in color scale shown on the right side, with tumor-related expression illustrated in red shades and normal sample-related expression in grey shades. Lack of expression in both tumor and normal samples is represented by white color and similar expression level in tumor versus normal samples is represented by dotted pattern. The differential expression does not automatically translate to prognostic value as specified in the body text for each CA isoform

fraction of the astrocytic and oligodendroglial tumor specimens, but not in the most benign pilocytic astrocytomas (Karjalainen et al. 2018). These scarce data indicate that our knowledge related to expression and functions of inactive CA isoforms is still limited.

## 7.6 Carbonic Anhydrase-Related Neoplasms

Taking together all the information extracted from the TCGA/GTEX datasets by the GEPIA2 tool, we can identify few tumor entities that are characterized by expression of carbonic anhydrases, which also show relationship to overall survival (Figs. 7.6 and 7.7).

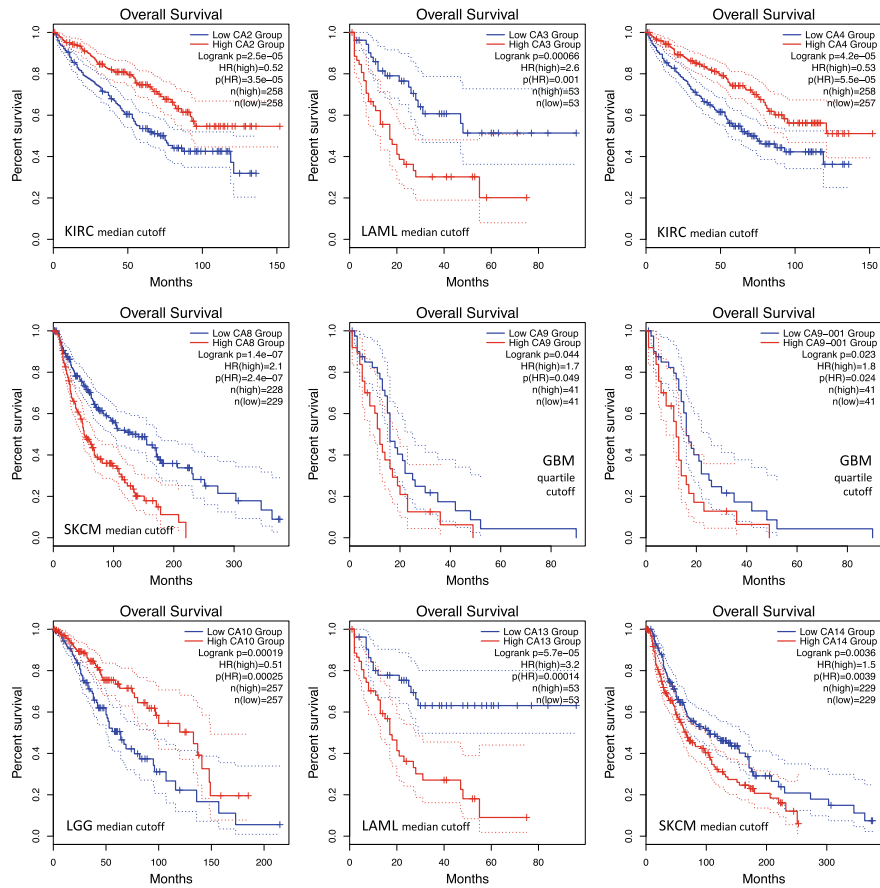
**GBM** (Glioblastoma multiforme) is a malignant brain tumor that accounts for about 15% of all brain tumors in adults. GBM patients have a poor prognosis and survive less than 15 months following diagnosis (information extracted from The Cancer Genome Atlas (TCGA), National Cancer Institute at the National Institutes of Health, USA). Recent integrated analysis of genetic alterations in main signaling pathways in tumors suggests that the most frequent alterations in GBM affect the pathways of RTK/Ras, cell cycle, PI3K, and p53 which control cell proliferation and survival and affect diverse aspect of tumor phenotype including metabolism and adaptation to stresses (Sanchez-Vega et al. 2018; Vander Heiden et al. 2009). Moreover, GBM tumors are characterized by hypoxia and acidosis, and thus, it is not surprising that these tumors express several carbonic anhydrases (Figs. 7.5 and 7.6),



**Fig. 7.6** Selection of carbonic anhydrase-related neoplasms with depicted expression levels of CA isoforms (expressed as TPM in the particular cancer type. Graphs do not show expression of the genes in the corresponding normal samples. Data extracted from TCGA/GTEX databases were analyzed using GEPIA2 instrument (Tang et al. 2019). Stars indicate significant relationship to overall survival (\* p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001) without discriminating positive and negative correlations

of which increased expression of *CA14* is linked to better prognosis, and increased expression of *CA9* is linked to poor prognosis, while the other isoforms' genes (including the highly expressed *CA2*) do not show any significant relationship to overall survival (Fig. 7.6).

**LGG** (Low grade glioma). Glioma develops in the brain's glial cells, which support the brain's nerve cells and keep them healthy. Tumors are classified into grades I, II, III, or IV based on standards set by the World Health Organization. Lower grade glioma consists of grades II and III. Regardless of grade, growing glioma tumor



**Fig. 7.7** Selected examples of Kaplan-Meier survival plots generated by the analysis of overall survival performed by GEPIA2 instrument (Tang et al. 2019) based on expression of CA genes. GEPIA2 uses Long-rank test (Mantel-Cox test) for hypothesis test. The Cox proportional hazard ratio (HR) and the 95% confidence interval are included in the survival plot

compresses the normal brain tissue, frequently leading to disabling or fatal effects (The Cancer Genome Atlas, NIH, USA). There are three molecular subtypes of LGG with distinct clinical outcomes depending on presence of mutations in *IDH1/IDH2* genes and a co-deletion of a short arm of chromosome 1 and longer arm of chromosome 19. The subtype with the poorest outcomes contains wild-type *IDH* and appears to be a precursor of the more aggressive GBM. *IDH* (isocitrate dehydrogenase) is an  $\text{NADP}^+$ -dependent enzyme that catalyzes a conversion of isocitrate to  $\alpha$ -ketoglutarate and  $\text{CO}_2$ . It is a tumor suppressor protein that functions at a crossroad of cellular metabolism in lipid synthesis, cellular defense against oxidative stress, oxidative respiration, and oxygen-sensing signal transduction (Reitman and Yan 2010). *IDH* mutations cause stabilization of HIF transcription factor and hypoxic reprogramming.

Glioma development in IDH-mutant and IDH wild-type tumors is driven by different oncogenic pathways that include mainly RTK/RAS pathway (IDHwt), p53 and Hippo pathways (IDHmut), and Wnt, TGF $\beta$ , and Hippo (IDHmut-codel), as illustrated in integrative analysis performed by Sanchez-Vega and colleagues (Sanchez-Vega et al. 2018). Interestingly, compared to IDHwt gliomas, IDHmut gliomas have distinct metabolic and microenvironmental characteristics correlated with tumor acidity and hypoxia (Yao et al. 2019). This might be one of the reasons, why LGG display variable expression of CA genes, including *CA2*, *CA4*, *CA5B*, *CA8*, *CA10*, *CA11*, *CA12*, *CA13*, and *CA14* (Fig. 7.5) and all of them, except *CA8*, are significantly associated with prognosis (Fig. 7.6). Tumor-related increases of *CA4*, *CA10*, and *CA11* correlate with better overall survival, increases of *CA2*, *CA5B*, *CA12*, *CA13*, and *CA14* correlate with worse prognosis. Because LGGs are strongly related with carbonic anhydrases, these neoplasms might be a suitable target for anticancer strategies exploiting CA inhibitors.

**KICH** (Chromophobe renal cell carcinoma) is a rare type cancer that originates in the distal regions of kidney and forms in the cells lining the small tubules, which help filter waste from the blood, making urine. It accounts for 5% of all kidney cancer cases and can have either hereditary or sporadic basis (The Cancer Genome Atlas, NIH, USA). Pathways altered in KICH relate to cell cycle, PI3K, and p53 and often translate to oncogenic metabolism and adaptation to hypoxia and acidosis (Sanchez-Vega et al. 2018; Linehan and Ricketts 2013). Only two CA isoforms show high expression in KICH and decreased expression in the corresponding normal tissues: *CA2* and *CA12*, but none of them is significantly associated with overall survival.

**KIRC** (Renal cell carcinoma) is the most common type of kidney cancer that forms in the cells lining the small proximal tubules in the kidney, which filter waste from the blood and make urine. When detected early, most cases of KIRC can be treated effectively, but survival rates are low when the cancer has spread from the kidney to other parts of the body. About 92% of KIRC are clear cell carcinoma (The Cancer Genome Atlas, NIH, USA). According to analysis of Sanchez-Vega and colleagues (Sanchez-Vega et al. 2018), altered pathways include RTK/RAS, cell cycle, and PI3K, but the primary genetic event is an inactivating mutation in *VHL* gene, which affects cellular oxygen sensing and leads to constitutive activation of the HIF pathway even in the absence of physiologic hypoxia (Kaelin and Ratcliffe 2008; Wiesener et al. 2001). This also includes metabolic changes associated with poor outcomes. Since *CA9* and *CA12* are upregulated by hypoxia, it is not surprising that these are the main isoforms present in this type of tumor, accompanied by *CA2*. As explained above, due to a HIF-1 to HIF-2 shift in the course of cancer progression, a higher level of HIF-1-regulated *CA9* is associated with a better prognosis, albeit its expression in later stages still remains relatively high and suitable for immunotherapeutic targeting (Bui et al. 2003). *CA12* exhibits a similar relationship, but only when *CA12-002* splicing isoform is tested, otherwise there is no clear relationship to overall survival. Similarly, increased *CA2* expression is associated with a better prognosis, while *CA4* shows a highly significant association with longer overall survival when absent or low (Figs. 7.6 and 7.7).

**LAML** (Acute myeloid leukemia) is a cancer of the blood and bone marrow, which can quickly worsen and result in death within months, when untreated. Survival decreases with older age because standard treatments are less tolerated (The Cancer Genome Atlas, NIH, USA). LAML cancers carry relatively few mutations compared to other frequently occurring solid tumors. About a half of AMLs include activation of the RTK/RAS pathway, and about one-sixth is affected in the cell cycle and Notch pathways (Sanchez-Vega et al. 2018). *CA1*, *CA2*, and *CA5B* are the most highly expressed CA genes in LAML, but they do not display any link to prognosis in contrast to less expressed *CA3* as well as *CA13*, both being positively associated with poor prognosis (Figs. 7.6 and 7.7).

**SKCM** (Skin cutaneous melanoma) is a cancer of melanocytes, skin cells that produce melanin. Melanoma is most often discovered because it has metastasized, or spread, to another organ, such as the lymph nodes. There are four major subtypes of cutaneous melanoma: BRAF mutant (most common), RAS mutant, NF1 mutant, and Triple Wild-Type. Mutations in each of the identified driver genes, *BRAF*, *RAS*, and *NF1* lead to uncontrolled cell growth (The Cancer Genome Atlas, NIH, USA). According to integrated analysis of genetic alterations in cancer, RTK/RAS and cell cycle oncogenic pathways predominate in SKCM (Sanchez-Vega et al. 2018). Based on experimental data, hypoxia and acidosis appear to be important components of melanoma progression (Moellering et al. 2008; Marino et al. 2012). Out of CA isoforms, only *CA14* exhibits a tumor-related expression linked to worse prognosis in SKCM (Figs. 7.6 and 7.7). The other CAs (*CA2*, *CA3*, *CA4*, *CA6*, *CA9*, *CA11*, *CA12*, and *CA13*) are expressed at higher levels in normal tissues compared to tumors. In a survival analysis, higher levels of *CA8* and *CA9* predict worse overall survival, while *CA12* and *CA13* show opposite relationships (Fig. 7.6).

Additional CA-related tumor types can be recognized on the basis of immunohistochemical staining of human tumor tissues that were published in hundreds of studies. These include, for example, breast carcinomas that express CA IX as a poor prognostic factor and CA XII as a good prognostic factor; colorectal carcinomas that express CA I, CA II, CA VII, CA XIII (all decreasing as cancer progresses), CA VIII, CA IX, CA XII (increasing with cancer progression); lung carcinomas (mainly NSCLC) expressing CA I and CA IV as good prognostic factors, CA VIII and CA IX as poor prognostic factors, and CA XII as a good prognostic factor (see the references related to the particular CA isoforms).

## 7.7 Conclusions

Both genomic metadata and immunohistochemical studies discussed here and described elsewhere in the literature indicate that there is virtually no cancer devoid of carbonic anhydrase, apparently because CA-facilitated pH regulation, ion transport, and/or CO<sub>2</sub> diffusion are really fundamental for cell survival, proliferation, and metabolic processes in tumor tissues {see also (Mboge et al. 2018)}. Naturally, histological and mutational diversity combined with all abnormalities characteristic

for malignant cancer, including oncogenic metabolism, aberrant proliferation, deregulated signaling, and adaptations to stresses in tumor microenvironment cause that the expression patterns of diverse CA isoforms in tumor tissues differ from those in normal tissues and are generally very complicated and context-dependent. This complexity of CA isoforms is further elaborated by the differences in the expression dynamics and stability of their transcripts compared to proteins, by the existence of alternative splicing variants of CA transcripts (with only some variants coding for functional proteins), by the posttranslational modifications of CA proteins affecting their functions, subcellular localizations, and regional tissue distribution, etc. Another level of complexity is added by a diversity of CA activities and interactomes. These numerous factors are behind the lack of full agreement between the data from RNA-seq, IHC/proteomic analyses, and clinical parameters. Despite this intricate picture, there are few clearly evident and undisputable relationships between certain CA isoforms and particular tumor types. Out of them, the most prominent position belongs to CA IX, which indicates an aggressive phenotype, poor prognosis, and poor response to therapy in a broad range of tumors (except renal clear cell carcinomas, as explained above), followed by CA XII (in a majority of cases indicating good prognosis), and CA IV that was proposed to be a tumor suppressor. Since these relationships are not new, the bioinformatic data included here can serve to support the existing studies.

In contrast, data mining and their integrated analysis using GEPIA2 instrument brought up very interesting and novel relationships, such as for CA XIV, which has not been previously linked to cancer through other than bioinformatic approaches (Mboge et al. 2018). In this case, but also regarding other *in silico* identified relationships of CAs to cancer, it is clearly evident that the RNA-seq data have to be supplemented and supported by the experimental evidence in order to obtain reliable and clinically useful information. To fulfill such requirement, the field of carbonic anhydrases requires further exploration followed by translation of knowledge to meaningful anticancer strategies.

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