HIILIHAPPOANHYDRAASIT II, VII, IX JA XII PAKSU- JA PERÄSUOLEN SYÖVISSÄ

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Paksu- ja peräsuolen syöpä (CRC) on ruuansulatuskanavan yleisin syöpä. Sen esiasteita, adenoomia, on löydettävissä 30-40 %:lla länsimaisesta väestöstä. Adenoomista suurin osa ei kuitenkaan koskaan etene pahanlaatuisiksi. CRC:n arviontiin on käytössä lukuisia luokittelumenetelmiä. Kuitenkin uusia merkkiaineita tarvitaan potilaille sopivimpien hoitojen löytämiseksi.
Alfa-hiilihappoanhydraasit (CA) ovat entsyymiperhe, jonka tärkein tehtävä on ylläpitää kehon happo-emästasapainoa. Syöpäsoluissa nämä entsyymit pitävät solun sisäisen pH:n neutraalina happamoittaen samalla soluvälitilaa, mikä lisää kasvaimen kasvua ja auttaa kasvainsolujen tunkeutumisessa normaaliin kudokseen.

Tutkimuksessamme selvitimme immunohistokemiallisesti neljän CA-isoentsyymmin esiintymistä CRC:stä otetuissa kudosnäytteissä ja esiintymisen mahdollista yhteyttä potilaiden ennusteeseen ja kasvainten kliinispatologisiin ominaisuuksiin.
Tilastollisessa analyysissä merkitsevää korrelaatio vaarasuhteessa havaittiin CA II ja CA XII entsyymien värjäytyvyyden voimakkuudessa ja CA II:n levinneisyydessä. Vastaavaa ei havaittu CA VII ja CA IX entsyymeillä, eikä CA XII:n levinneisyydessä.
Löydöstemme perusteella CA II:n ja CA XII:n esiintyvyydellä voi olla merkitystä paksu- ja peräsuolen syövän kehittymisessä. Niiden käyttöä diagnostiikassa olisi syytä tutkia jatkossa enemmän.

SISÄLLYS

Carbonic anhydrase enzymes II, VII, IX, and XII in colorectal carcinomas

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Abstract

Background, Colorectal adenocarcinoma is the most common cancer of the gastrointestinal tract. It usually develops from precursory adenomas, most of which are benign and do not proceed to cancer. Histopathological diagnostics of colorectal cancer is still based on tumor staging and grading, and unfortunately, there are very few biomarkers which provide useful information for assessment of the most suitable treatment in each case. Alpha-carbonic anhydrases (CAs) play an important role in maintaining acid-base homeostasis of the body. Importantly, several cancer types overexpress some CA isozymes, most remarkably the transmembrane isoforms CA IX and CA XII. These enzymes maintain intracellular pH within normal limits and simultaneously contribute to acidification of tumor microenvironment, which in turn, promotes tumor growth and malignant cell invasion; **Methods,** In this study, we defined expression of four CA isozymes, CA II, CA VII, CA IX, and CA XII, in colon carcinomas to find if there is any correlation between the enzyme expression levels and survival rate of patients. We also analyzed possible correlations to some important clinicopathological parameters, such as cell proliferation, tumor grade and stage; **Results,** Our results showed significant changes in hazard ratios, correlating to CA II and CA XII intensities. No correlations were found between CA VII or CA IX immunostaining results and the other parameters; **Conclusions,** The present findings indicate that CA II and CA XII could have some role as predictive factors in the diagnostics of colorectal cancer. Even though the significant findings were only associated to the staining intensity, this research line deserves further investigations because of the shortage of suitable biomarkers in clinical cancer diagnostics.
Keywords: biomarker, carbonic anhydrase, colorectal cancer, immunohistochemistry, prognosis, survival

Background

Colorectal cancer is the most common malignant disease of the gastrointestinal (GI) tract, and it is the second most common cancer in women and third in men [1]. Its incidence rate shows high regional variation, resulting in a 10-fold difference between countries [1]. The number of patients with colorectal cancer and precancerous lesions or polyps is increasing, which causes significant challenges to national healthcare systems and their possibilities to recognize early enough different tumor types to apply the most effective treatment. According to recent data the incidence of colorectal cancer is rising in many historically low-incidence countries like Japan, Korea, China and Eastern Europe, which is thought to be a result of cultural and dietary changes associated with Western lifestyle [1]. Even though the overall incidence is increasing, there has been some positive development in many previous high-risk countries at the same time. This is thought to be mainly because of more effective diagnostics and treatment.

Colorectal adenocarcinoma usually develops from benign precursor lesions i.e. colonic adenomas [2]. Prevalence of adenomas clearly increases by age. Postmortem studies have indicated that 30-40 % of individuals from Western countries have adenomas and most of them are asymptomatic [3]. Majority of these are benign and do not transform to malignant adenocarcinomas [2]. There are also familial syndromes where younger patients develop these adenomas, which proceed to cancer with a much higher percentage [2-4]. Precancerous lesions are often removed during colonoscopy, which prevents them to proceed to cancer, and thus, an early diagnostics combined with a complete tumor removal are the key factors for the most successful outcome.

The diagnostic armamentarium of colorectal cancer includes several clinicopathological parameters, which can be utilized to determine the malignancy and prognosis of each case. They include TNM (tumor, nodes, metastasis), Dukes or Astler-Coller staging, tumor grade, microsatellite instability, and some molecular markers which have been recently reviewed by Marzouk and Schofield [5]. Earlier some other molecular markers, such as tumor suppressor gene p53 and its mutations as well as antigen Ki-67, have also been associated with tumor proliferation and cancer prognosis [6, 7]. However, new biomarkers are urgently needed to improve the stratification of colorectal cancer patients for different treatment options.

Carbonic anhydrases (CAs) constitute a group of zinc-binding enzymes which catalyze the reversible hydration of CO2 to bicarbonate. This reaction is crucial for maintenance of pH homeostasis of the body. Through this chemical reaction they are involved in several downstream physiological processes, such as bone resorption, vision, and production of saliva, bile, pancreatic juice and gastric juice [8-10]. The mammalian alpha-CA family includes 16 known isoforms of which 15 can be found in humans. These isoforms show marked differences in their kinetics and cellular and subcellular distributions [8, 9]. Although the functions of CA enzymes in cancer cells have been extensively studied, their exact roles are still incompletely understood.

CA proteins have been studied extensively during the last 20 years as potential markers for various cancers. Cytosolic CA II is the most widely expressed isozyme in normal tissues, such as gastric, pancreatic, biliary, and intestinal epithelia [10, 14]. It is often absent or only weakly expressed in malignant tumors. Recently, CA II was shown to be highly overexpressed in gastrointestinal stromal tumors and was suggested as a potential biomarker for this mesenchymal tumor type [15]. CA VII is another cytosolic isozyme which shows a more restricted tissue distribution than CA II. It is predominantly expressed in the brain where it contributes to bicarbonate-driven GABAergic excitation [16]. A recent study showed that CA VII is overexpressed in glioblastomas, suggesting that it may represent another tumor-associated CA isoform [17].

CA IX has attracted lots of attention, because its expression is limited to few normal tissues, such as gastric, intestinal and gall bladder epithelia, and it is highly overexpressed in hypoxic tumors

[10-12]. CA XII is another isoform which is overexpressed in several cancers, even though it is also present in various normal tissues. It has been demonstrated in both the normal intestinal epithelium and malignant colorectal tumors [10, 12, 13]. CA IX and XII, are known to be regulated via von Hippel Lindau (vHL) / hypoxia inducible factor (HIF) pathway [18].

Under hypoxic conditions cells produce acidic metabolic products via anaerobic glycolysis. This pathway is inhibited in the presence of enough oxygen. Notably, tumor cells have a tendency to upregulate glucose intake and increase the rate of anaerobic glycolysis even if the amount of oxygen is sufficient [19]. Tumor cells need CA enzymes among many other proteins, such as ion transporters, to maintain the neutral intracellular pH [20]. During this process extracellular pH decreases, which in turn, disturbs physiological processes of the surrounding normal tissue and promotes cancer growth [19, 21]. Indeed, increased glucose intake and hypoxia are often signs of more aggressive and invasive tumors, which also correlate with a poor prognosis [19]. It has been suggested that partial hypoxia may contribute to cell selection, favoring a shift from a pre-malignant phenotype to more malignant forms where the oxygen free metabolism plays a major role and the cells can survive in challenging hypoxic environments [19].

The aim of this research is to investigate the expression of isozymes CA II, CA VII, CA IX, and CA XII in colon carcinomas. The expression levels of these enzymes assessed by immunohistochemistry are correlated to the data obtained from clinical and pathological analyses. Our results show that both CA II and CA XII staining intensities correlate with the survival rate of colorectal cancer patients, suggesting a potential role for these enzymes as prognostic biomarkers.

Methods

Overall 840 patients with colorectal carcinoma were operated in Helsinki University hospital during years 1983-2001 and the specimens and clinical data from 645 patients were available for our study. The ethical committee of the University Hospital (Dnro 226/E6/06) and National Supervisory Authority for Welfare and Health (Dnro 3990/04/046/07) had granted permission for the use of these samples. Survival data were available for all patients and obtained from patient records, the Finnish Cancer Registry and Statistics Finland. The clinicopathological characteristics of the patients are described in detail in table 1.

Tumor samples were arranged as multitissue arrays. Of 645 patient samples we obtained, 106 ended up with no scoring results. This was mainly because these samples were either washed out or displaced during the staining process. 539 samples were considered representative enough to be graded and analyzed. The microscope slides containing the tissue specimens were immunostained with rabbit anti-human CA II, CA VII and CA XII sera or with monoclonal anti-human CA IX antibody (M75). These antibodies have been previously utilized in numerous studies and have been shown to be specific for each isozyme [22-25]. Five µm sections were processed for immunoperoxidase staining, which was performed using an automated Lab Vision Autostainer 480 (LabVision Corporation, Fremont, CA, USA). Automated immunostaining was performed using the Power Vision+ Poly-HRP Immunohistochemistry kit (ImmunoVision Technologies Co) reagents and included the following steps: (1) rinsing in wash buffer; (2) treatment in 3% H_2O_2 in ddH_2O for five minutes and rinsing with wash buffer; (3) blocking with cow colostrum diluted 1:2 in Tris-buffered saline (TBS) containing 0.05% Tween-20 for 30 minutes and rinsing in wash buffer; (4) incubation with primary antibody (polyclonal antibodies diluted 1:2000 and monoclonal M75 diluted 1:100) for 30 minutes; (5) rinsing in wash buffer three times for five minutes; (6) incubation in poly-HRP-conjugated anti-rabbit/mouse IgG for 30 minutes and rinsing in wash buffer three times for five minutes; (7) incubation in DAB (3,3`-diaminobenzidine tetrahydrochloride) solution (one drop of DAB solution A and one drop of DAB solution B in 1 ml of ddH_2O) for six minutes and rinsing in ddH_2O; (8) $CuSO_4$ treatment for five minutes to enhance the signal and rinsing in ddH_2O; (9) treatment with hematoxylin for one minute and (10) rinsing with ddH_2O. All procedures were performed at room temperature. The mounting of the sections was performed using Entellan Neu (Merck; Darmstadt, Germany) and was finally examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany). The intensity of the staining was scored on a scale of 0 to 3 as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; and 3, strong reaction. The extent of the staining was also scored as 1 when 1-10% of the cells stained, 2 when 11-50% of the cells stained and 3 when 51-100% of the cells stained. A negative score (0) was given to tissue sections that had no evidence of specific immunostaining.

To get at least one representative scored value for each sample, two to three series of tissue array slides were stained for each isozyme. All unidentifiable samples and those which contained no tumor tissue were excluded from scoring. In addition to the analysis of tumor cells, CA II immunostaining was also evaluated in endothelial cells and CA VII in tumor stroma.

Statistical analysis

Results are announced as number of patients and percentage of patients, the Kaplan-Meier mean survival time with 95 % confidence intervals and the Cox regression hazard ratios and their 95 % confidence intervals (CI). p-values < 0.05 were considered statistically significant. Two-tailed tests were used.

The Kaplan-Meier analysis and the Cox regression hazard ratio model were used to analyze the survival data. The assumption of constant hazard ratios over time was tested by including time dependent covariate for each testable variable. Because both Dukes classification and differentiation status did not follow the Cox model assumption, stratified analyses for Dukes class and differentiation were used. For statistical analyses, Dukes classifications A and B, as well as C and D were combined to reduce the number of groups to two. Same was done to tumor differentiation status, in which low and medium low differentiation were combined as well as medium high and high differentiation status. In the Cox proportional hazards model we included variables for age, sex and, side of the tumor. Separate analyses were made for each CA staining.

In analysis male patients were compared to females and 65 years-old or older patients were compared to younger ones. Tumor location was also considered in the analysis: right side of the colon versus left side of the colon and rectum.

When analyzing the expression of each CA enzyme, level 0 was considered the baseline and all other grading levels were compared to it. Announced hazard ratio rises exponentially every time when grading value rises one unit. Expression levels of CAs were also compared separately to the well-known tumor markers, p53 and Ki67. For statistical analysis p53 and Ki67 reactions were each divided into two groups (0-5 and >5).

Statistical analyses were performed by SPSS v 10© (IBM corp, New York).

Results

Four CA isozymes, CA II, VII, IX, and XII were selected for the immunohistochemical analysis of colorectal cancer. Summary of the immunohistochemical staining results is presented in table 2. Figure 1 demonstrates representative images of positive immunostaining of each isozyme in colon carcinoma specimens. Normal colon and pancreas specimens are shown as positive controls. Our main results, shown in figure 2, include Kaplan-Meier plots for the staining intensity and extent of each isozyme. In Kaplan-Meier analysis, there was a significant decreasing trend in survival as the intensity of CA II or CA XII, or the extent of CA II increased. The mean survival time decreased from 17.7 years (95% CI, 14.8-20.6) to 12.3 years (95% CI, 10.5-14.2) as the CA II intensity increased from 0 to 3. The mean survival time decreased from 18.3 years (95% CI, 15.5-21.1) to 13.5 years (95% CI, 12.0-15.0) as the extent of CA II increased from 0 to 3. The mean survival time decreased from 16.5 years (95% CI, 13.7-19.2) to 9.9 years (95% CI, 7.3-12.4) as the intensity of CA XII increased from 0 to 3. In the Cox regression analysis, the most significant results were found for CA II staining intensity as shown in table 3. Increase in CA II intensity from 0 to 1 increased the age, sex, Dukes, differentiation and tumor side corrected hazard ratio to 1.19 fold (CI: 1.04-1.37 p = 0.009). No significant interaction was, however, found between the CA II intensity and age, sex or location of tumor. When comparing the extent of the CA II staining to the survival rate, the result did not reach statistical significance. However, a significant interaction was found between the CA II staining extent and patients´ age, the hazard ratio being 1.59 fold (CI: 1.22-2.09, p = 0.001), suggesting that the CA II staining extent correlated better with survival in older patients. Notably, CA II-positive staining was often induced in the endothelium of tumor capillaries in addition to the actual tumor cells. In Kaplan-Meier analysis the vascular endothelial staining of CA II did not show any significant correlation to survival (p = 0.676).

CA VII immunostaining results showed no significant correlation to patients´ survival which can be seen in figure 2. In the Kaplan-Meier survival analysis p-values were 0.566 for the staining intensity and 0.495 for the extent. Negative results were also observed for stromal staining (p=0.816 for intensity and p=0.591 for extent). There was a significant correlation (p=0.013) between the epithelial staining extent and Dukes classification. Over 50 % extent was found in Dukes classes 3 C and 4 D more frequently than expected, while it was more rarely found in classes 1 A and 2 B. These results are also shown in the table 4. The extent was 0 or under 10 % more frequently in class 4 D than expected. No significant correlation was found between the epithelial or stromal CA VII immunostaining and other parameters.

In Kaplan-Meier analysis no significant correlation was found between the survival data and intensity (p = 0.879) or extent (p = 0.315) of CA IX immunostaining. Additionally, no correlation was found when the CA IX immunostaining results were compared with the other parameters.

CA XII was another enzyme in this study, which showed a significant correlation when its staining intensity was compared to the survival data of colorectal cancer patients. In the Cox regression analysis increased intensity of CA XII immunostaining showed a slightly increased hazard ratio of 1.18 (95% CI, 1.01-1.38, p=0.036, table 3). Gender or location of the tumor did not show any significant correlations. The extent of CA XII immunostaining showed no significant correlation to the patients´ survival in Kaplan-Meier analysis (p=0.242). Comparison of CA XII immunostaining results with the other clinicopathological parameters showed no significant correlations.

Discussion

In our study, we found two isozymes, CA II and CA XII, which significantly correlated with the patients´ survival rate. Our results suggest that these proteins might have some value in the prognostic assessment of colorectal cancer patients when deciding which patients should undergo a more aggressive and demanding cancer treatment in order to curate or postpone cancer progression. On the other hand, enzyme expression can also define which patients have better prognosis and can be treated with less aggressive treatments. This can diminish side effects and increase life quality during treatments. Even though CA IX represents a promising biomarker for several cancers, our results suggested that it has no major role in histopathological diagnostics of colorectal carcinoma nor does it present any value as a prognostic factor in this tumor category.

The role of different CA isozymes has been under intensive research during the last decade. These studies have been focused on the association between CA expression and tumor aggressiveness and patient survival, role in tumor metastasis as well as their possibility to act as potential targets to anticancer drugs [21, 26-28]. CA isozymes have shown abnormal expression in various malignant tumors compared to normal cells and tissues [17, 19, 21, 28-30]. In one of the early studies, CA IX was shown to be diffusely expressed in colorectal cancer, whereas the normal or adenomatous mucosa showed a more limited distribution [11]. Immunostaining of serial sections for Ki-67 confirmed that CA IX was expressed in areas with high proliferative capacity. Similarly, CA XII showed a more diffuse immunostaining reaction in colorectal carcinomas compared to the normal colon or adenomas [13]. Cytosolic CA II isozyme has shown a very different distribution. It is highly expressed in several normal gastrointestinal tract tissues, such as gastric and intestinal mucosa [14], but is clearly downregulated in most colorectal tumors [31]. The same phenomenon may also occur in the case of the other cytosolic CAs [32]. CA VII expression was found to be 4-fold downregulated in sigmoid or rectosigmoid carcinomas compared to the normal tissue according to Birkenkamp-Demtroder´s study [33]. Recently, Niemelä et al. [34] reported cDNA microarray results on the expression of all CAs, except for CA XIII, in both normal and malignant colorectal specimens. The fold-changes for our target CA isozymes were: CA II (-7.4 (normal mucosa vs. sporadic carcinoma)), CA VII (-4.3), CA IX (+2.4) and CA XII (-3.2). In our figure 1 the increase of expression is clearly demonstrated in the case of CA IX, whereas the interpretation is more difficult with the other isozymes because of high staining intensities in both the normal and tumor tissues.

Even though the pathogenesis of colorectal cancer has been intensively studied during the last decades, there are still molecular mechanisms that clearly warrant more research. For example, it was recently shown that polyps with any advanced neoplastic features are smaller in the right side than in the left side of the colon, and the gene expression is also different from side to side [33, 35]. In our study, we analyzed the possible correlation between the enzyme expression and the side of the colon. According to this analysis, the reactivity of CA enzymes showed no correlation to the tumor location. The main clinicopathological factors affecting colorectal cancer patients' survival are the tumor grade, resection margins in the operation, and the presence or absence of lymph node metastases [3]. Other factors with prognostic significance include tumor budding, micrometastases, peritoneal carcinomatosis, lymphatic, perineural or venous invasion, and histological properties including the level of invasion [5]. In Marzouk and Schofield´s review article [5], a number of potential molecular markers, such as microsatellite instability, BRAF mutation, KRAS mutation, PIK3CA mutations, and PTEN deletion, were evaluated for their potential prognostic value. Although they have been considered promising, the clinical value of these markers is still uncertain. More knowledge is already available about the role of 18q deletions and thymidylate synthase expression which have been associated with unfavorable prognosis and tumor recurrence. Even though many of these molecular markers have been used when choosing

the patients with metastatic disease to chemotherapy [5], new markers are still needed to distinguish different cancer types or to stratify the patients for personalized chemotherapy according to the cancer properties.

Conclusions

Results of our study suggest that two isozymes, CA II and CA XII, significantly correlate with the patients´ survival rate. These proteins might have some value in the prognostic assessment of colorectal cancer patients when deciding which patients should undergo a more aggressive and demanding cancer treatment in order to curate cancer or postpone cancer progression.

List of abbreviations

CA, carbonic anhydrase; CI, confidence interval; DAB, 3,3`-diaminobenzidine tetrahydrochloride; HIF, hypoxia inducible factor; TNM, tumor, nodes, metastasis; vHL, von Hippel Lindau

Competing interests

None declared

Authors' contributions

PV, SPar, AJK and CH participated in the design of the study. Samples of this study were collected by CH. Immunohistochemial staining and light microscopy was performed by PV. AW, WSS, JP, SPas and SPar produced and characterized the primary antibodies. Statistical analysis was done by HM and CH. PV drafted the first version of the manuscript. All authors were involved in the writing process, read and approved the final manuscript.

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Tables

Table 1, Patient characteristics

		Number of patients	Percentage (%)
Total		539	100
Gender	Male	294	54.5
	Female	245	45.5
Age	< 65 year	231	42.9
	≥ 65 year	308	57.1
Tumor location	Right side of colon	157	29.1
	Left side of colon	138	25.6
	Rectum	244	45.3
Stage	Dukes A	82	15.2
	Dukes B	191	35.4
	Dukes C	136	25.2
	Dukes D	130	24.1
Differentiation	High	19	3.5
	Medium high	352	65.3
	Medium low	141	26.2
	Low	27	5.0

Table 2, Summary of CA staining statistics

	Mean	SD	Median	Mode	25 %	75 %
CA II epithelium intensity	1.8	1.1	2	3	1	3
CA II epithelium extent	2.2	1.1	3	3	2	3
CA II endothelium	0.9	0.3	1	1	1	1
CA VII epithelium intensity	1.4	1.0	1	1	1	2
CA VII epithelium extent	2.0	1.2	3	3	1	3
CA VII stromal intensity	2.8	0.5	3	3	3	3
CA VII stromal extent	2.8	0.5	3	3	3	3
CA IX epithelium intensity	1.7	1.2	2	3		3
CA IX epithelium extent	1.6	1.2	2	3		3
CA XII epithelium intensity	1.5	0.9	1	2	1	2
CA XII epithelium extent	2.0	1.1	2	3	1	3

Table 3, COX regression analysis results of selected parameters.

	Hazard ratio	CI 95 % lower	CI 95 % upper	p -value
CA II epithelium intensity	1.19	1.04	1.37	0.0092
Sex	1.20	0.92	1.58	0.1777
Age over 65 years	1.78	1.35	2.35	0.0000
Side of colon	1.17	0.86	1.58	0.3170
CA II epithelium extent	0.88	0.73	1.05	0.1576
Sex	1.25	0.95	1.64	0.1133
Age over 65 years	0.64	0.32	1.25	0.1869
Side of colon	1.14	0.84	1.55	0.3876
Interaction between CA II epithelial intensity and age over 65 years	1.59	1.22	2.09	0.0007
CA XII epithelium intensity	1.18	1.01	1.38	0.0360
Sex	1.23	0.94	1.60	0.1301
Age over 65 years	1.67	1.27	2.19	0.0002
Side of colon	1.09	0.81	1.47	0.5571

Regression analyses are stratified by Dukes and differentiation status.

Separate analyses were done for each CA staining.

Table 4, CA VII and Dukes class

CA VII epithelial extent	Dukes 1A		2B		3C		4D	
	Count	%	Count	%	Count	%	Count	%
0	15	16.1 %	36	38.7 %	25	26.9 %	17	18.3 %
1	14	19.7 %	31	43.7 %	14	19.7 %	12	16.9 %
2	10	15.2 %	23	34.8 %	14	21.2 %	19	28.8 %
3	28	11.4 %	85	34.6 %	67	27.2 %	66	26.8 %

p= 0.013, linear by linear association test

Figure legends

Figure 1. **Distribution of carbonic anhydrases II, VII, IX, and XII in tissue sections**. CA II (A,B,C), CA VII (D,E,F), CA IX (G,H,I), and CA XII (J,K,L) were immunohistochemically localized in specimens of normal pancreas (A,D,G,J), colon (B,E,H,K) and colorectal carcinoma (C,F,I,L). The normal pancreas and colon sections were used for control purposes. As expected, CA II is located to the pancreatic ducts and centroacinar cells, and in the normal colon the positive signal is strongest in the enterocytes of deep crypts. In the present example image of colorectal cancer, CA II antibody strongly labels the cancer cells. CA VII seems to stain strongly the stromal tissue. This phenomenon is seen in both the normal pancreas and colon as well as in colorectal cancer, whereas the cancer cells show only minimal if any staining. CA IX antibody weakly stains the ductal cells and some acinar cells of pancreatic tissue and also the basolateral cell membranes in basal parts of the colonic crypts. In colorectal cancer, CA IX shows strong staining in malignant cells, whereas the necrotic part remains negative. CA XII shows positive staining in the basolateral membranes of the pancreatic acinar cells and colonic enterocytes, and the malignant cells of colon carcinoma are also strongly positive for this isozyme. Original magnifications x 400.

Figure 2. **Kaplan-Meier figures of survival analysis of CA II, CA VII, CA IX and CA XII**. Both CA II staining intensity ($p=0.004$) and extent ($p=0.022$) significantly correlated with the patient survival. A weak correlation was also found between the CA XII staining intensity and survival ($p=0.020$).

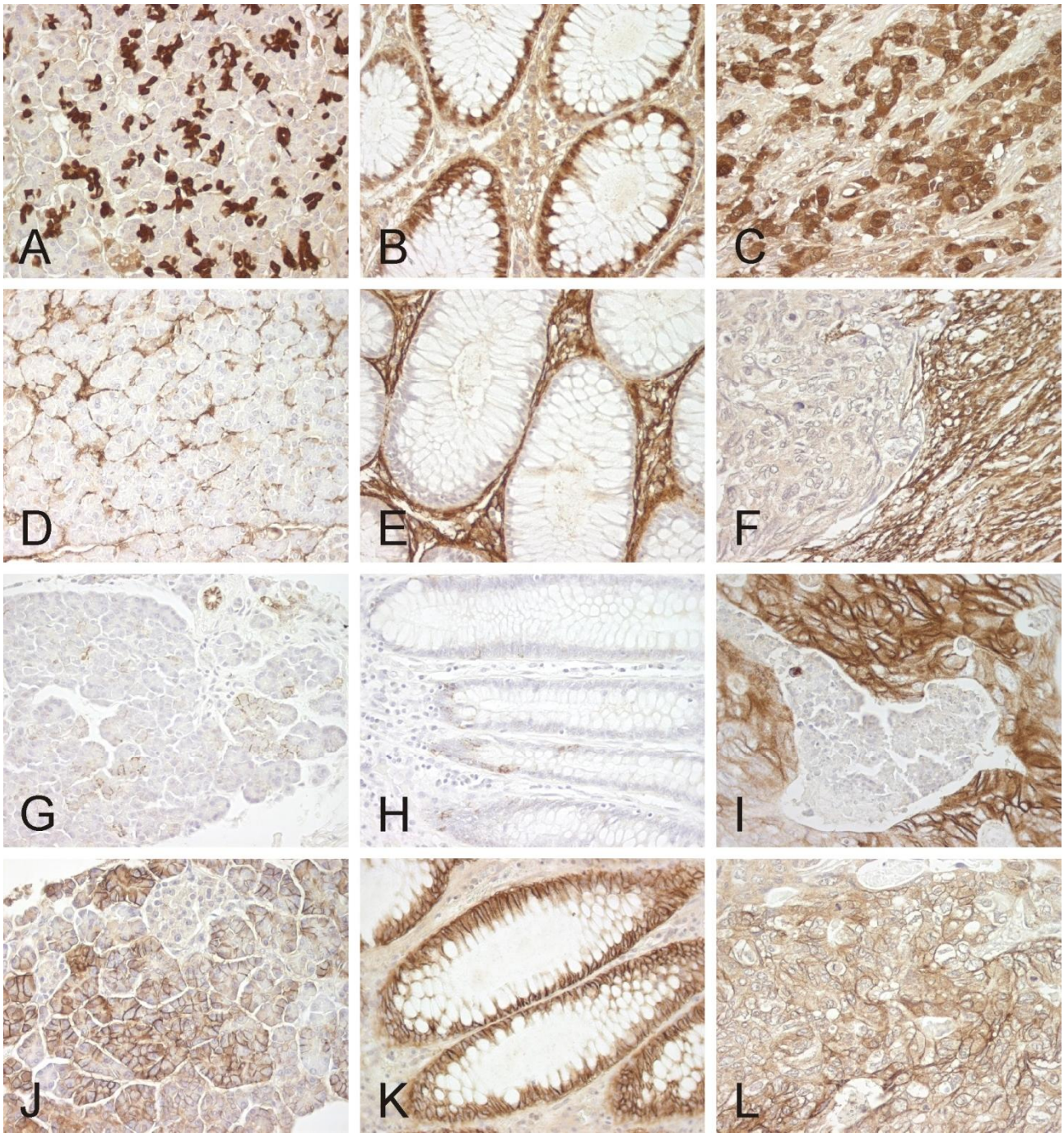

Figure 1. Distribution of carbonic anhydrases II, VII, IX, and XII in tissue sections

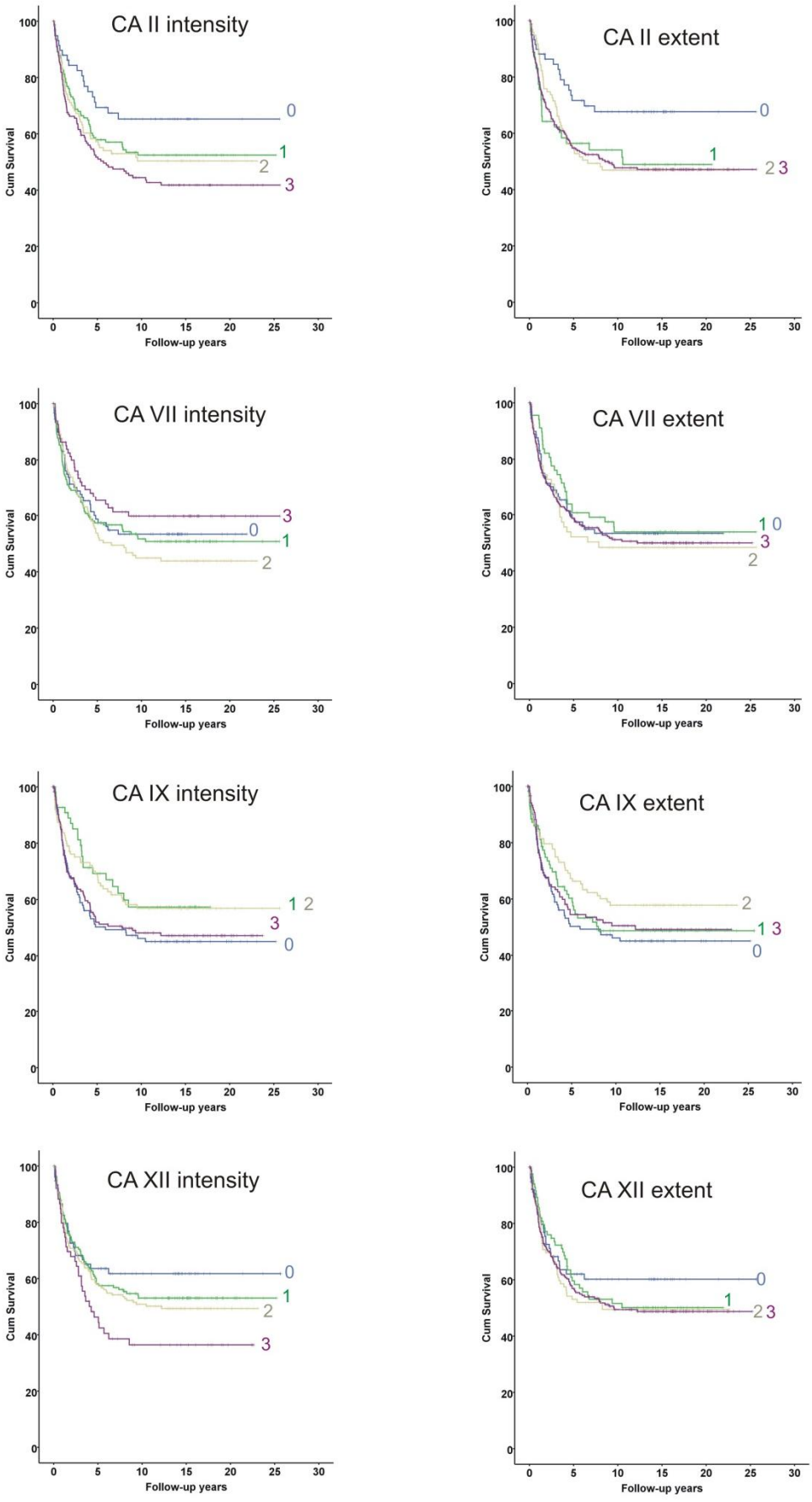

Figure 2. Kaplan-Meier figures of survival analysis of CA II, CA VII, CA IX and CA XII.