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Carbonic Anhydrases II, IX, and XII in Barrett's Esophagus and Adenocarcinoma

Minna Nortunen[1], Heikki Huhta[1], Olli Helminen[1], Seppo Parkkila[3,4], Joonas H Kauppila[1,2], Tuomo J Karttunen,[1]* and Juha Saarnio[1]*

*indicated authors contributed equally as last author

Affiliations:

[1]Cancer and Translational Medicine Research Unit, Medical Research Center Oulu, University of Oulu and Oulu University Hospital, 90014 Oulu, Finland, [2]Upper Gastrointestinal Surgery, Department of Molecular Medicine and Surgery, Karolinska Institutet and Karolinska University Hospital, 17176 Stockholm, Sweden, [3]Faculty of Medicine and Life Sciences, University of Tampere, Finland, and [4]Fimlab Ltd, Tampere University Hospital, 33520 Tampere, Finland

Corresponding author: Minna Nortunen

minna.nortunen@gmail.com

+358 8 3152875

16-digit ORCID id: 0000-0003-0928-150X

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Abstract

The aim of our retrospective study was to investigate the expression and clinical significance of the cancer-associated carbonic anhydrases (CAs) II, IX, and XII in Barrett's esophagus and esophageal adenocarcinoma (EAC). We evaluated 101 archival specimens from patients with EAC as well as seven and 26 samples from patients with high-and low-grade dysplasia, respectively. In addition, normal esophageal squamous epithelium, gastric and intestinal metaplasia were analyzed when present. The expression patterns of isozymes were detected by immunohistochemistry. CAII and CIX expression levels were lower in the squamous epithelium than in the columnar cells while CAXII showed an opposite pattern and was present mainly in squamous epithelium. Expression patterns in benign, dysplastic, or malignant esophageal columnar lesions were not significantly different. The assessment of clinicopathological associations showed that CAII was significantly downregulated in metastatic disease ($p=0.026$). CAIX showed no association with prognosis, although there appeared to be an association ($p=0.056$) between high expression and nodal spread. In conclusion, CAII, CAIX, and CAXII do not serve as biomarkers for different phases in the development of EAC.

Introduction

The incidence of esophageal adenocarcinoma (EAC) is rapidly increasing in Western countries. In the U.S., the incidence rose nearly 5-fold from 1975 to 2009 [1]. The prognosis is still unsatisfactory despite initial curative surgery, with an overall 5-year survival of only 22% [2]. Barrett's esophagus, a complication of gastroesophageal reflux disease, is a well-recognized precursor to EAC via columnar epithelium dysplasia [3, 4]. Carbonic anhydrases (CAs) are zinc-containing metalloenzymes catalyzing the reversible hydration of CO_2 to bicarbonate and hydrogen ions. So far 12 catalytically active isoforms have been found in humans [5]. Cytosolic CAII and the membrane bound isoforms, CAIX and CAXII, are often considered tumor-associated CAs [6]. The basic function of these enzymes is to regulate the pH homeostasis of the body [7], but they also have other important physiological roles, such as regulation of glycolysis, gluconeogenesis, and CO_2 transport [8]. The majority of primary and metastatic cancers primarily show glycolytic metabolism even in the presence of oxygen [9]. Another key feature in cancer pathophysiology is tumor acidity [10-12]; CAII, CAIX, and CAXII may play important roles in acidifying the tumor microenvironment and maintaining upregulated tumor glycolysis. Recently, a dual role was proposed for CAII in intracellular pH regulation and exportation of excess lactate by supporting monocarboxylate transporters, namely MCT1 and MCT4 [13]. Extracellular acidosis on the other hand is largely due to extracellular facing CAIX and CAXII leading to CO_2 hydration and subsequent hypercapnic acidosis [10]. In addition, CAIX and CAXII play an important role in the regulation of intracellular pH [14] together with cytosolic CAII. Thus, crucial features of tumor progression, such as energy transport and pH regulation, are partly CA-dependent processes [14, 15].

Carbonic anhydrase II is the most widely distributed isoform in the human body and is present throughout the gastrointestinal tract [7]. Significant downregulation of CAII expression is

associated with poor survival in various cancers, such as pancreatic and gastric adenocarcinomas [16, 17]. The CAIX and CAXII isoforms are hypoxia-inducible and strongly regulated by HIF-1alpha [14]. The expression of CAIX is very limited in normal, non-malignant human tissues [7]. In most solid tumors investigated [14, 18-20], high CAIX expression is associated with a poor prognosis, and CAIX inhibitors are being avidly researched as a cancer treatment. Although a similar association has been reported in EAC by Birner et al. [21], other studies have had restricted sample sizes [22, 23] or the results have been controversial [24]. CAXII is expressed in many tissues, such as breast, renal, large intestine, and to some extent gastric mucosa [25]. It is also found in several cancers [25-27] with a variable association with the overall survival. In squamocellular esophageal carcinoma, CAXII has been described as a marker of poor prognosis [26].

No previous studies have examined the role of CAII or CAXII in EAC or its precursor lesions. The role of CAIX is controversial in the light of previous reports. The aim of this study was to describe the expression patterns of CAII, CAIX, and CAXII in Barret's esophagus with or without dysplasia and in EAC. We also analyzed whether the expression of any of these isozymes is associated with the prognosis of EAC patients.

Materials and methods

Patients

The paraffin-embedded archival specimens from patients with EAC and associated Barrett's esophagus between the years 1987-2013 were obtained from the Department of Pathology, Oulu University Hospital, Oulu, Finland. A major part of the same series has been previously reported in

studies describing Toll-like receptors (TLRs) [28,29]. For the current report, we updated the follow-up data. The series consisted of 101 patients with EAC, seven patients with high-grade dysplasia, and 26 patients with low-grade dysplasia as the most advanced lesion. The median age of the patients was 63 years (range 43-91). There were 71 (70%) patients who died during follow-up. The median follow-up time of the surviving patients was 20 months (range 0-288 months). The patients' survival data were acquired from the Statistics Finland and the other relevant data from the patients' records at Oulu University Hospital. The patients' data are summarized in Table 1. The use of the patients' samples and the data inquiry were approved by the Oulu University Hospital Ethics Committee and by the National Authority for Medicolegal Affairs (VALVIRA).

Immunohistochemistry

Immunohistochemistry was performed on sections of tissue blocks that were first selected by an expert gastrointestinal pathologist to be representative using hematoxylin-eosin stained sections. The CA immunostaining was conducted at the Department of Anatomy, University of Tampere using polyclonal rabbit anti-human CAII and CAXII sera and monoclonal anti-human CAIX antibody (M75), as described previously for polymer-based detection [5]. The antibodies have been previously characterized and are specific for each isozyme [30-32].

Assessment of CA expression

The histological sample slides were digitized using an Aperio AT2 Console (Leica Biosystems Imaging Inc., Nussloch, Germany). Different lesions in the specimens were identified and marked by an expert gastrointestinal pathologist (T.J.K.). CA immunoreactivity was analyzed by three

independent researchers who were blinded from the clinical data [28]. In assessment of staining intensity, internal positive controls were used; parietal cells for CAII and CAIX, red blood cells for CAII and suprabasal cells of the normal squamous epithelium for CAXII. The staining intensity (0-3) and the proportion (percentage) of stained cells (0-100%) were separately assessed in the epithelial cells of all evaluated lesions. Mean values of the three independent estimates were used if the estimates did not differ by more than 1 in the intensity assessment or more than 30% in the percentage assessment. If the estimates differed over these limits, a consensus was reached after re-evaluation. The mean intensity and mean percentage were then multiplied together and the CA histoscore (0-300) was obtained. Histoscore values were further dichotomized into two equally sized groups by the median value for each CA isozyme.

Statistical analysis

For statistical analyses, we used IBM SPSS Statistics 23.0 (IBM Corp., Armonk, NY, USA). To compare CA expression levels between different lesions, we used one way ANOVA and we used the Tukey honest significant difference test in post hoc analysis. The chi-squared test was used to calculate the statistically significant differences between prognostic and clinicopathological variables. Life tables were calculated according to the Kaplan–Meier method and the survival curves were compared using the log rank test.

Results

Expression patterns of CAII, CAIX, and CAXII in esophageal squamous epithelium, Barrett's esophagus, dysplasia, and adenocarcinoma

Expression levels of CAII, CAIX, and CAXII in different esophageal lesions are summarized in Table 2, and examples of the characteristic staining patterns are shown in Figure 1. Epithelial expression of CAII was present in all the esophageal lesions assessed (Table 2; Figure 1). The expression was mostly cytoplasmic, as expected, but was also found in nuclei occasionally. In the normal squamous epithelium, CAII typically showed diffuse expression throughout (Figure 1; D, E). In gastric metaplasia of the esophagus, the staining intensity was significantly stronger compared with normal epithelium and adenocarcinoma. The strongest staining was present in the parietal cells and superficial parts of the gland (Figure 1; A-B). When comparing gastric and intestinal metaplasia, CAII expression appeared weaker in the latter and the staining intensity grew stronger towards the surface of the mucosa (Figure 1; B, C, E). In low- and high-grade dysplastic regions, the staining was generally spread very diffusely, but no significant differences emerged compared with non-dysplastic columnar epithelium or carcinoma. In adenocarcinomas, the intensity of the staining was weaker than in gastric metaplasia. It typically represented diffuse cytoplasmic staining with a weak-to-moderate intensity (Figure 1; A, D).

Expression of CAIX was membrane bound, as reported previously [31]. There was practically no staining of CAIX in the normal squamous epithelium (Figure 1; I, J). Expression in gastric metaplasia was strong and present in all cell types across the epithelium (Figure 1; F, G). There was a decrease in the expression from gastric metaplasia to intestinal metaplasia to dysplastic lesions to adenocarcinoma (Figure 1; F-J).). In intestinal metaplasia, the expression was still strong and showed the highest basal staining (Figure 1; H, J). A similar pattern was seen in the dysplastic lesions, which showed the staining intensifying towards the basal region of the gland. However, the overall expression was weaker than in the non-dysplastic columnar epithelium. The differences in histoscores between gastric metaplasia and high- and low-grade dysplastic lesions were significant

(Figure 1; H ;Table 2). The CAIX expression in the adenocarcinoma samples was variable. It was mostly weak or absent (Figure 1; F, I), but a few cancers stained very intensely. Yet, the mean histoscore for adenocarcinomas was significantly lower than for metaplasias and low-grade dysplasia (Table 2).

The CAXII expression was mostly limited to the normal squamous epithelium, where the staining was present in the cell membranes and concentrated in the basal parts of the epithelium (Figure 1; N-O). Metaplastic lesions, low-grade dysplasia, and adenocarcinomas showed weak or absent expression for CAXII (Figure 1; K-M, O). In high-grade dysplastic lesions, expression of CAXII tended to more abundant, but no significant differences emerged (Figure 1; N).

Correlation of CA expression with clinicopathological variables and survival in EAC

A low CAII histoscore was associated with the presence of distant metastases (Table 3; p=0.026). A similar association was also observed with the staining intensity and percentage of positive cells. The expression of CAIX tended to be higher with lymph node positive disease (Table 3; p=0.056). Expression of CAII, CAIX, or CAXII showed no other significant associations with clinicopathological features, including age, gender, tumor node metastasis (TNM) classification, or the stage or grade of differentiation of the tumor (Table 3). To emphasize the early stages of carcinogenesis, we also analyzed CA expression levels in pT1a (n=7) and pT1b (n=8) tumors and found again no significant differences. The CAII, CAIX, or CAXII expression patterns did not associate with survival in Kaplan-Meier analyses and therefore multivariable tests were not performed.

Discussion

This study describes the expression patterns of the cancer-associated CAII, CAIX, and CAXII, in normal esophageal squamous epithelium, precancerous lesions, and EAC. There was expression of CAII and CAIX in all the epithelial types, except for the normal squamous epithelium where expression of CAIX was weak or absent. The CAXII staining was strong in the normal squamous epithelium, but absent or only very weakly present in the columnar lesions. In carcinomas, low CAII levels were associated with distant metastases ($p=0.026$). The CAIX expression was consistently decreased in more malignant lesions. In carcinomas there seemed to be an association between high CAIX expression and nodal spread ($p=0.056$). CAII, CAIX and CAXII did not associate with prognosis.

In the esophageal squamous epithelium, CAII was constantly present, as described previously [7, 33, 34]. It showed a diffuse, cytoplasmic staining pattern with the strongest signal in the suprabasal layer. This expression pattern is consistent with the role of CAII in epithelial defense against acid reflux [34]. No previous data have been reported on CAII in esophageal, gastric, or intestinal metaplasia or dysplasia and adenocarcinoma. We found extensive expression of CAII in esophageal gastric metaplasia and especially in metaplastic parietal cells, mostly in a pattern similar to that reported in the gastric mucosa [33]. Expression of CAII was slightly weaker in esophageal intestinal metaplasia and the staining tended to accentuate in the superficial parts of the metaplastic area. This could point to CAII playing a role in cell maturation in intestinal metaplasia, since CAII is similarly concentrated in the apical parts of the intestinal villi [33]. In the dysplastic columnar metaplasia, the expression intensity of CAII was comparable to non-dysplastic columnar metaplasia. Interestingly, the staining was more widely distributed across both the bases and superficial parts of dysplastic columnar metaplasia (Figure 1C), correlating with either the extension of the cell proliferation zone

or lesser surface maturation in dysplasia [35, 36]. However, due to the overlap in its staining distribution, CAII does not appear to be a biomarker of Barrett's dysplasia.

In adenocarcinomas, the level of CAII expression was significantly lower than in columnar metaplasia, but did not differ from dysplasia. Downregulation of CAII was significantly associated with metastatic disease, although there was no association with survival. In gastric cancer, low CAII expression correlates with tumor size, distant metastasis, TNM-stage, and poorer overall survival [16, 37]. In addition, expression of CAII has been reported significantly downregulated in metastatic gastric cancer cells [38]. *In vitro* studies have suggested that the enzyme has inhibitory effects on the growth of colorectal cancer cells [39] and similary as in gastric cancer, CAII has been identified as a metastasis-associated factor in colorectal carcinoma by analysis of gene expression profiles [40]. The mechanisms linking low or absent CAII expression with metastasis are unknown. To point out, a lack of CAII protects tumor cells from the extracellular pH fluctuations common in carcinomas, possibly stabilizing carcinoma cell functions [41]. In *Xenopus* oocytes, CAII has a non-catalytic function as a facilitator of transmembrane ion shuttles [13], such as the monocarboxylate transporters MCT1 and MCT4. These transporters of high energy metabolites, such as lactate, are upregulated in EAC [42] and various other carcinomas. Altogether, these data suggest the mechanisms linking downregulation of CAII and metastatic behaviour should be investigated further.

Expression of CAIX in the normal esophageal squamous epithelium was nearly absent in our samples, similar to what Turner et al. reported previously [23]. In esophageal gastric metaplasia, the CAIX staining was strong and covered the full depth of the epithelium, corresponding with CAIX expression in the gastric mucosa [31]. The expression showed a non-significant decrease in esophageal intestinal metaplasia, where there was also a decreasing gradient towards the surface. A

similar pattern of CAIX expression has been reported in gastric intestinal metaplasia [43] and large intestinal mucosa [44], corresponding with the location of proliferating cells. The functional significance of the observed decrease in expression on the surface is unknown. However, a recent animal study found that CAIX plays an important role in the gastric barrier function against luminal acid [45], suggesting that downregulation in esophageal intestinal metaplasia could be involved in acid reflux-related damage. The decreasing trend in CAIX expression continued in low- and high-grade dysplasia, again in the same manner as has been described for low- and high-grade dysplastic adenomas in the stomach [43]. In dysplasia, as in intestinal metaplasia, the staining was confined to the basal and suprabasal parts. The expression of CAIX in preneoplastic lesions and Barrett's esophagus has been reported in two previous studies concerning EAC [21, 24]. Both these reports state, congruent to our results, that precancerous lesions and especially non-dysplastic metaplasias had higher expression levels of CAIX than adenocarcinomas.

The CAIX expression in carcinomas showed no significant correlation with clinicopathological variables or survival; although, high CAIX expression appeared to associate (p=0.056) with nodal spread. The expression of CAIX in EAC has been studied before with controversial results [21-24]. Turner et al. originally reported CAIX (MN antigen) expression in EAC in 1997 with only 10 patients and suggested loss of CAIX expression was associated with cancer progression [23]. In 2008, Driessen et al. [22] published a population of 39 EAC patients and found a survival benefit for CAIX-negative cancers. However, gastric and cardiac cancer patients were included in their survival analysis. Birner et al. [21] showed in a larger study (n=182) that overexpression of CAIX in EAC is strongly associated with shorter overall and disease-free survival. In 2015, Huber et al. [24] reported on a series of 123 EAC patients and found no association between the expression of CAIX and prognosis. For gastric cancer, the published results are also ambiguous, reporting a survival benefit for CAIX-negative [22] cancers or no association with survival [46, 47] at all.

It might be worth considering whether there is any biological plausibility in the controversial observations of the prognostic value of CAIX in EAC and gastric cancer. Chen et al. [48] found that CAIX expression is frequently lost in gastric cancers; however, the expression can be restored and the tumors re-expressing CAIX have a poorer prognosis. This sequence supports the findings from studies where CAIX overexpression indicated poor prognosis [21, 22]. Thus, could be suggested, that the expression of CAIX is dynamic, fluctuating during carcinogenesis and tumor progression, and may explain its varying prognostic value [21-24]. There is a question raised whether immunohistochemistry at any given time is the best way to assess CAIX expression in tumors. For instance, the methylation status of CAIX [48] in gastric cancer was associated with lymph node metastasis; whereas, expression analyzed by immunohistochemistry was not associated with clinicopathological parameters. The same study also demonstrated that although HIF1-alfa is induced, the expression of CAIX in gastric cancer is probably also regulated by site-specific methylation and not merely by hypoxia. This could explain, for instance, Birner's [21] finding that HIF1-alpha and CAIX levels had no clear correlation and explain why CAIX is not expressed or associated with survival categorically in all hypoxic tumors.

We consistently found strong CAXII expression only in the parabasal cells of normal esophageal squamous epithelium with a membrane-bound pattern, as has been described before[26]. In other esophageal lesions studied, including adenocarcinomas, the expression was practically absent with no association with survival or clinicopathological parameters. There are no previous studies about CAXII in esophageal columnar metaplasia or adenocarcinoma. Our findings suggest that CAXII does not play any important role in the pathogenesis of dysplastic metaplasia or EAC. In other cancer types, the correlation of CAXII with prognosis has varied, with some reports linking CAXII

overexpression with poor survival [26, 27] and some with a good prognosis [49] or better radiotherapy outcomes [50].

In conclusion, the cancer-associated CAII, CAIX, and CAXII all have characteristic expression patterns in EAC, its precursor lesions, and normal squamous epithelium. In EAC, CAII downregulation is associated with metastatic disease. CAIX expression is lost towards the more malignant lesions, but in lymph node positive disease, the expression seems to be again higher. Finally, CAXII is only expressed in the normal esophageal squamous epithelium and there is no evidence for its role in EAC or precursor lesions.

Figure Captions:

Fig.1 Examples of typical expression patterns of CAII (left panel; A-E), CAIX (middle panel, F-J), and CAXII (right panel, K-O) in different esophageal lesions. Each row shows a corresponding field of a case. A, F, and K represent a sample showing gastric metaplasia with cardiac-type mucosa (right) and adenocarcinoma (left). B, G and L show esophageal gastric body type metaplasia. C, H and M demonstrate a high-grade dysplasia lesion (right) bordering with non-dysplastic intestinal metaplasia (left). D, I, and N show the border of esophageal squamous epithelium and adenocarcinoma. E, J, and O show squamous epithelium (left) and intestinal metaplasia (right).

Compliance with Ethical Standards:

Regional Ethical Committee approval No: EETTMK: 81/2008

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Conflict of Interest:

The authors have no conflicts of interest to declare.

Contribution statement:

Tuomo Karttunen and Juha Saarnio designed the study, the data was acquired by Minna Nortunen, Heikki Huhta, Olli Helminen, Joonas Kauppila and analyzed by Tuomo Karttunen, Juha Saarnio, Minna Nortunen, Heikki Huhta, Olli Helminen and Joonas Kauppila. Seppo Parkkila contributed to the interpretation of the data. Minna Nortunen wrote the manuscript which was reviewed and edited by all the other authors. All authors have approved the final version to be published and have agreed to be accountable for every aspect of the work.

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Table 1. Baseline characteristics of the study population with esophageal adenocarcinoma (EAC), high-grade dysplasia (HGD), and low-grade dysplasia (LGD)

	EAC n=101	HGD n=7	LGD n=26
Age at diagnosis			
<60 years	37/101	2/7	7/26
60-65 years	19/101	2/7	3/26
>65 years	45/101	2/7	16/26
Sex			
Male	82/101	7/7	17/26
Female	19/101	0/7	9/26
Tumor			
T_1	17/100		
T_2	16/100		
T_3	51/100		
T_4	16/100		
Lymph nodes			
negative	41/100		
positive	59/100		
Organ metastases			
negative	69/100		
positive	31/100		
Grade			
1	35/98		
2	26/98		
3	37/98		
Stage			
I	14/100		
II	39/100		
III	15/100		
IV	32/100		
Tumor size			
small (<40mm)	40/93		
large (≥40mm)	53/93		

TNM-staging was available for 100, grade of differentiation for 98, and tumor size for 93 patients

Table 2. Baseline characteristics of CAII, CAIX, and CAXII expression in normal esophageal squamous epithelium and in different esophageal lesions

CAII	intensity mean	intensity 95% CI	statistical significance	histoscore mean	histoscore 95% CI	statistical significance
Normal epithelium	1.3	1.1-1.4		74	56-94	
Gastric metaplasia	1.9	1.7-2.0	a	116	95-138	
Intestinal metaplasia	1.4	1.2-1.7		72	48-98	
Low-grade dysplasia	1.7	1.5-1.9		95	72-121	
High-grade dysplasia	1.9	1.5-2.2		139	97-179	
Adenocarcinoma	1.4	1.2-1.6	b	81	62-101	
CAIX						
Normal epithelium	0.6	0.3-0.8		13	5-25	
Gastric metaplasia	2.4	2.2-2.5	a	211	191-232	a
Intestinal metaplasia	2.2	2.0-2.5	a	163	134-192	a
Low-grade dysplasia	1.9	1.6-2.1	a	144	116-172	ab
High-grade dysplasia	1.7	1.3-2.0	a	100	62-143	ab
Adenocarcinoma	1.8	1.5-2.0	ab	78	63-92	abcd
CAXII						
Normal epithelium	2.2	2.0-2.4		142	122-159	
Gastric metaplasia	0.5	0.3-0.7	a	13	7-21	a
Intestinal metaplasia	0.4	0.3-0.6	a	11	5-18	a
Low-grade dysplasia	0.4	0.2-0.5	a	12	5-21	a
High-grade dysplasia	0.8	0.5-1.0	a	37	21-56	a
Adenocarcinoma	0.4	0.3-0.5	a	15	8-23	a

The histoscore was calculated by multiplying the staining intensity (0-3) with the percentage of positive cells (0-100%), resulting in a value between 0 and 300. Values are presented as the mean and 95% confidential interval (95%CI). For statistical testing, we used one way ANOVA with Tukey test in post hoc analysis. a Compared with normal epithelium, $p<0.05$; b compared with gastric metaplasia, $p<0.05$; c compared with intestinal metaplasia, $p<0.05$; d compared with low-grade dysplasia, $p<0.05$

Table 3. The relationship between CAII, CAIX, and CAXII histoscores and the clinicopathological variables in esophageal adenocarcinoma

Variable	n/N(%)	CA II histoscore n(%)			CA IX histoscore n(%)			CA XII histoscore n(%)		
		weak	strong	p	weak	strong	p	weak	strong	p
pT										
T 1-2	33/100(33%)	9	24	0.465	16	17	0.537	21	12	0.39
T 3-4	67/100(67%)	27	40		27	40		50	17	
Lymph nodes										
negative	41/100(41%)	15	26	0.542	22	19	0.056	31	10	0.268
positive	59/100(59%)	21	38		21	38		40	19	
Organ metastases										
negative	69/100(69%)	20	49	0.026	27	42	0.172	49	20	0.587
positive	31/100(31%)	16	15		16	15		22	9	
Grade										
1	35/98(36%)	8	27	0.268	15	20	0.99	25	10	0.365
2	27/98(28%)	11*	15*		12	15		16	11	
3	37/98(38%)	16	21		16	21		29	8	
Stage										
I	14/100(14%)	4	10	0.184	6	8	0.387	10	4	0.775
II	39/100(39%)	13	26		16	23		29	10	
III	15/100(15%)	3	12		4	11		9	6	
IV	32/100(32%)	16	16		17	15		23	9	
Tumor size										
small (<40mm)	40/93(43%)	12	28	0.178	19	21	0.357	27	13	0.269
large (>40mm)	53/93(57%)	22	31		22	31		40	13	

*CAII staining only available for 26 grade 2 tumors due to paraffin blocks running out