Carbonic Anhydrase IX Is Highly Expressed in Hereditary Nonpolyposis Colorectal Cancer

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Abstract

Carbonic anhydrase (CA) II, CA IX, and CA XII are expressed in various neoplasias and have been linked to tumorigenesis. We examined their expression in three different groups of colorectal cancer [i.e., microsatellite stable (MSS), microsatellite instable (MSI), and hereditary nonpolyposis colorectal cancer (HNPPC)]. First, we analyzed gene expression profiles of 113 specimens by a microarray method to study the expression of various CA isozymes in the subgroups of colorectal cancer. The results indicated that mRNAs for CA II and CA XII are down-regulated and CA IX mRNA is up-regulated in all three tumor categories when compared with the normal tissue. The up-regulation of CA IX was greatest in the HNPPC group. For more information, 77 specimens were immunohistochemically stained to study the levels of CA II, CA IX, and CA XII. Immunohistochemical analyses further confirmed that the subgroups express CA II, CA IX, and CA XII differentially, and the HNPPC group express higher levels of CA IX. Expression of these CAs did not correlate to Dukes stage or grade of differentiation. Our results show that CAs are differentially expressed in the subgroups of colorectal cancer, and CA IX expression seems to be very high in most cases of HNPPC. CA IX could be a potential diagnostic and therapeutic target in HNPPC. (Cancer Epidemiol Biomarkers Prev 2007;16(9):1760–6)

Introduction

Colorectal cancer is diagnosed in ~150,000 people in the United States and 500,000 worldwide each year (1). From all cases, about 3% to 4% are linked to the familial cancer syndromes. The most common form is hereditary nonpolyposis colorectal cancer (HNPPC; also called the Lynch syndrome), which is caused by a germ-line mutation in one of four DNA mismatch repair genes. However, many colorectal cancers show microsatellite instability without evidence of germ-line abnormalities (2). In these cases, the cause is biallelic methylation of the promoter sequences of MLH1—an epigenetic, not inherited, change that leads to a deficiency of DNA mismatch repair (2). The prognosis for HNPPC is better than for the sporadic form of cancer (3, 4), although the patients with HNPPC have an increased risk for cancer development in certain extracolonic sites such as the endometrium, ovary, stomach, small intestine, hepatobiliary tract, ureter, and renal pelvis.

Genetic instability, which is common in colorectal cancer and often targets the DNA mismatch repair genes, is one hallmark of carcinogenesis and can be promoted by cell stress factors within the tumor microenvironment such as cell hypoxia (5). What is less known is the mechanism through which hypoxia contributes to genetic instability (6). The transcription factor called hypoxia-inducible factor (HIF)-1α is crucial for the cellular response to hypoxia and is frequently overexpressed in hypoxic regions of human cancers, resulting in activation of genes essential for cell survival. One of these HIF-inducible genes is called CA9, which encodes the carbonic anhydrase (CA) IX isozyme that is over-expressed in certain carcinomas (7, 8). CA XII is another member of the CA isozyme family that has been reported to be inducible by hypoxia (8), although the evidence is not yet as convincing as for CA IX.

CAs have been classically considered zinc-containing metalloenzymes, which catalyze a very fundamental chemical reaction wherein carbon dioxide is hydrated to carbonic acid, which then dissociates to bicarbonate and proton (CO2 + H2O ⇌ H2 CO3 ⇌ H+ + HCO3). There are at least 13 active α-CA isozymes in mammals including five cytoplasmic (CA I, CA II, CA III, CA VII, and CA XIII), two mitochondrial (CA VA and CA VB), one secreted (CA VI), and five membrane-associated...
The study was approved by the Ethics Committee of the Jyväskylä Central Hospital. The samples included in the immunohistochemical staining were not the same tumors that were studied by the microarray analyses. There were 77 colorectal tumor samples, including 43 MSS, 18 MSI, and 16 HNPCC. The histologic grade was low in 29 lesions, moderate in 26 lesions, and high in 8 lesions. The lesions had been isolated from the ascending colon (N = 10), transverse colon (N = 4), descending colon (N = 2), sigmoid colon (N = 10), rectum (N = 19), and rectosigmoid (N = 1). Additional 14 samples were obtained from the colon, but the information about the exact location was not available. Of the carcinomas, 14 were at Dukes stage A, 28 at stage B, 16 at stage C, and 3 at stage D.

**Immunohistochemistry.** Tissue samples were washed briefly with PBS, fixed with 4% neutral-buffered formaldehyde, and embedded in paraffin. Sections were cut at 5 μm and placed on SuperFrost Plus microscope slides (Menzel). Immunoperoxidase staining was done using an automated Lab Vision Autostainer 480 (Immunovision Technologies Co.).

The polyclonal rabbit antibodies against human CA II and XII have previously been characterized and produced (15, 23, 24). The monoclonal antibody M75 against human CA IX has also been described previously (15, 23, 25).

The automated immunostaining was done using Power Vision+ Poly-HRP IHC Kit (Immunovision Technologies Co.) reagents and included the following steps: (a) rinsing in wash buffer; (b) treatment in 3% H2O2 in double-distilled water for 5 min and rinsing with wash buffer; (c) blocking with cow colostrum diluted 1:2 in TBS containing 0.05% Tween 20 for 30 min and rinsing in wash buffer; (d) incubation with primary antibody (rabbit anti-human CA II, monoclonal M75 antibody against human CA IX or rabbit anti-human CA XII) for 30 min; (e) rinsing in wash buffer for 3 × 5 min; (f) incubation in poly-horseradish peroxidase–conjugated anti-rabbit/mouse immunoglobulin G for 30 min and rinsing in wash buffer for 3 × 5 min; (g) incubation in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (one drop of DAB solution A and one drop of DAB solution B in 1 mL) double-distilled water for 6 min; (h) CuSO4 treatment for 5 min to enhance the signal; and (i) rinsing with double-distilled water. All procedures were done at room temperature. The mounting of the sections was done with Entellan Neu (Merck). For the examination and photography, we used Zeiss Axioskop 40 microscope (Carl Zeiss).

The staining for CA isozymes was scored by three investigators (S.P., P.H., and A.M.N.) on a scale from 0 to 3. Intensity (INT) was scored as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; and 3, strong reaction. Tumor endothelial staining for CA II was scored using the same scoring values. The extent (EXT) of staining was scored as 0 when no evidence of specific detectable levels in majority (>50%) of the samples, which were removed from further analyses (22).

**Materials and Methods**

**Microarray Preparation and Statistical Analyses.** The preparation of the Human Genome U133A GeneChip arrays (Affymetrix, Inc.) and patient material used on the expression arrays has previously been described in detail (22). Informed consent was obtained from all patients and the study was approved by the ethics committees of the participating institutes. The hybridized expression arrays were quantified with Affymetrix Software MAS 5.0 and then normalized by truncating small values to 0.01 and centering both array and gene intensities to the corresponding median in GeneSpring software (Agilent Technologies). A Student’s t test for unequal variances was used for the probe sets of interest (CAs and HIF) to analyze the gene expression between the MSI, MSS, and HNPCC groups. The Affymetrix Detection Algorithm assigned flag calls (Present, Marginal, Absent) were used to identify probe sets with expression values below.
immunostaining was present; 1 when 1% to 10% of the cells were positive; 2 when 11% to 50% of the cells were positive; and 3 when 51% to 100% of the cells were positive. Staining indices were calculated for all studied isozymes using the formula \( \frac{INT \times EXT}{ref. \ 26} \). The statistical analyses were done with one-way ANOVA and Bonferroni’s \( t \) test.

The tissue sections immunostained for CA IX were also photographed with \( \times 100 \) magnification and subjected to digital image analysis. The staining extent was analyzed with analySIS software (Soft Imaging System Gmbh). From each section, two rectangular regions were scored using color threshold values of 242 (red and green colors) and 153 (blue color). Each analyzed region (1,300 \( \times 1,000 \) \( \mu m \)) covered representative tumor sample. The obtained relative area value indicated the mean percentage of stained area within the analyzed regions. Bonferroni’s \( t \) test was used to evaluate the significance of differences in protein expression between HNPCC, MSI, and MSS groups detected by immunohistochemical staining and digital image analysis.

**Results**

**Expression Microarray Analyses.** The microarray data were produced earlier (22) and reanalyzed in the present study for CA mRNA expression levels. In total, 113 colorectal samples were screened for CA I to CA XII and CA XIV mRNA expression. Twenty-three samples were classified as HNPCC; 17, sporadic MSI; 56, sporadic MSS; and 15, normal. Four samples were not classified as HNPCC; 17, sporadic MSI; 56, sporadic MSS; and 15, normal. Four samples were not classified.

The microarray data were analyzed with analySIS software (Soft Imaging System Gmbh). From each section, two rectangular regions were scored using color threshold values of 242 (red and green colors) and 153 (blue color). Each analyzed region covered a representative tumor sample. The obtained relative area value indicated the mean percentage of stained area within the analyzed regions. Bonferroni’s \( t \) test was used to evaluate the significance of differences in protein expression between HNPCC, MSI, and MSS groups detected by immunohistochemical staining and digital image analysis.

**Table 1. The relative mRNA expression levels of CA I to CA XII and CA XIV in HNPCC, sporadic carcinomas, and normal tissue**

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<th>Probe set ID</th>
<th>Gene symbol</th>
<th>Mean normal</th>
<th>Mean HNPCC</th>
<th>Mean sporadic</th>
<th>( P ), HNPCC vs sporadic</th>
<th>( P ), HNPCC vs normal</th>
<th>( P ), sporadic vs normal</th>
<th>( P ), MSI sporadic vs normal</th>
<th>( P ), MSS sporadic vs normal</th>
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<td>0.846</td>
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<td>0.166</td>
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<td>1.21</td>
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<td>1.33</td>
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<td>1.99</td>
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<td>1.54</td>
<td>1.17</td>
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<td>1.29</td>
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<td>0.183</td>
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<td>0.800</td>
<td>0.952</td>
<td>0.780</td>
<td>0.663</td>
<td>0.690</td>
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</table>

**NOTE:** Comparisons between expression levels in HNPCC, sporadic carcinomas, and normal tissue were done using Student’s \( t \) test.

\( *P < 0.01. \)

\( 0.01 < P < 0.05. \)

\( 0.001 < P < 0.01. \)
There was a slight tendency toward increased endothelial staining when the dysplasia grade became higher. However, this observation did not reach statistical significance \((P = 0.092)\). Furthermore, endothelial staining for CA II did not show any correlation with any specific subgroups of colorectal cancer (i.e., MSS, MSI, and HNPCC) nor did it correlate with the Dukes stages. In the tumor cells themselves, CA II was generally very weakly expressed. The most advanced and malignant tumors according to the Dukes classification and tumor grade showed almost negligible staining. However, the CA II staining index, which represents the evident intensity and extent (positive area), was significantly different between MSS, MSI, and HNPCC \((P = 0.002)\). Figure 2 shows the mean staining indices for CA II. It shows that staining index was highest in MSI followed by HNPCC and MSS. No significant differences for CA II staining were found between the groups classified according to the Dukes classification or grade of dysplasia (Figs. 3 and 4).

A total of 77 samples were stained with CA IX antibody; 43 of them were MSS; 18, MSI; and 16, HNPCC. CA IX immunostaining was moderate or high in most tumor samples. Only few showed no evidence of staining. The staining index for CA IX in HNPCC was significantly higher than in MSS \((P = 0.044)\); Fig. 2). Digital image analysis of CA IX immunostaining also confirmed the highest expression levels of CA IX in HNPCC. It showed significant statistical differences between HNPCC and the other groups. In HNPCC tumors, the mean positively stained area covered \(\sim 28\%\) of the tumor (Fig. 5). In MSS and MSI tumors, the corresponding values were \(\sim 12\%\) \((P = 0.003)\) and \(14\%\) \((P = 0.04)\), respectively. Grade of dysplasia did not show any correlation to CA IX index. However, the staining index showed a tendency to be higher when Dukes classification was B or C than when it was A or D.

Seventy-six samples consisting of 44 MSS, 15 MSI, and 17 HNPCC were stained with anti–CA XII antibody. CA XII staining seemed to be slightly stronger than CA II staining in the more malignant and advanced cases (i.e., in MSS subgroup, Dukes stages C and D and grade 3), whereas CA XII staining was weaker than CA IX staining in all classifications except for Dukes stage D. The staining index for CA XII was highest in MSI and lowest in MSS. There was a significant statistical difference between MSI and MSS \((P = 0.036)\) but no significance was observed when either of these two was compared with HNPCC. No significant difference was found in CA XII staining between various grades or Dukes stages.
Ten of 16 HNPCC cases were found to carry the common Finnish founder mutation in exon 16. Similar to the microarray results, immunohistochemical data revealed that the pattern of CA expression was the same for the cases with or without the common founder mutation.

Discussion

The hereditary cancer syndromes are characterized by germ-line mutations in the mismatch repair genes, tumor suppressor genes, or oncogenes. Due to inherited basis of these mutations, the syndromes are often associated with multiple cancers occurring in the same patient. To the best of our knowledge, our report is the first to show CA expression in HNPCC, which is one of the hereditary cancer syndromes. von Hippel-Lindau disease was the first hereditary cancer syndrome in which overexpression of CA IX and CA XII was reported (18, 28). The overexpression was found to be due to a mutant von Hippel-Lindau protein, which fails to polyubiquitinate HIF-1α transcription factor under normoxic conditions whereby HIF-1α escapes the normal proteosomal degradation (29). HIF-1α is a key factor that can bind to the promoter region of CA9 gene, inducing its expression (8). Transcriptional regulation of CA12 gene has not been carefully analyzed to date, and even basic data about the promoter region are still lacking (7). What is known is that, similarly to CA9, CA12 transcription in renal cancer cell lines is under the negative control of von Hippel-Lindau protein (18).

Our results indicated that CA IX is the only CA isoform that is clearly overexpressed in HNPCC. The induction of CA IX expression was shown to be greater in HNPCC than in sporadic cancers both at the mRNA and protein levels. This is an important observation because overexpression of CA IX has frequently been associated with poor prognosis in types of cancer other than renal cell carcinoma (30–33). There is, however, good evidence that HNPCC has a better prognosis than sporadic colorectal cancer (3, 4). Ten-year survival rates of HNPCC patients and those with sporadic tumors are 87.5% and 44.8%, respectively (34). The finding of high CA IX levels in HNPCC led us to analyze the HIF-1α expression levels from the microarray data. We found that HIF-1α mRNA levels were also higher in HNPCC compared with sporadic tumors. The mean expression values for HIF-1α mRNA were 0.84, 1.13, and 1.36 in normal mucosa, sporadic tumors, and HNPCC, respectively. Although HIF-1α mRNA levels are principally constitutive and not induced by hypoxia, it has been...
shown that different breast carcinoma cell lines differ by intrinsic levels of HIF-1α mRNA, and that in some of them increased level of mRNA corresponds with increased HIF-1α protein level (35). This is also supported by Jiang et al. (36) who showed that up-regulation of the basal mRNA could lead to higher HIF-1α protein expression. Therefore, higher HIF-1α mRNA levels could potentially contribute to higher expression of CA IX in HNPCC.

Interestingly, CA IX has shown higher expression levels in Dukes stages B and C when compared with stages A and D. At the moment, it is difficult to make any conclusion about weaker CA IX staining in stage D tumors due to a low number of samples in this category. However, stage A tumors are generally of a smaller size and presumably contain less hypoxic areas than tumors in stages B and C. On this basis, it could be anticipated that the expression of CA IX in the stage A tumors is lower because of weaker activation/stabilization of HIF-1α transcription factor. This view is supported by the studies showing positive correlation of HIF-1α protein expression to increasing tumor stage as well as to increasing invasion and metastasis (37, 38). Although the biological role of CA IX has not been fully clarified, it is quite conceivable that the hypoxic cells of higher stage colorectal tumors are more dependent on pH regulation due to acidosis caused by anaerobic metabolism and thus need higher expression of CA IX for adaptation to hypoxic stress.

The microarray data on various CA isozyme mRNA expression levels in the normal colon were found to be consistent with the previously published results on the mRNA and protein levels of CAs in the colon (15, 17, 39–43). The expression microarray results from this study suggested that CA I, CA II, and CA IV have the highest transcript levels in the normal human intestine. CA XIII mRNA levels were not analyzed in this study due to absence of CA13 probes on the microarray. Based on the previously published data, CA XIII is considered one of the highly expressed isozymes in the colon (27, 44). CA I, CA II, and CA IV isozymes showed clearly decreased mRNA expression levels in both sporadic and HNPCC tumors. The decrease in sporadic carcinomas has previously been documented for CA I and CA II (16, 27), but the decreased CA IV expression is a novel finding.

CA I, CA II, and CA IV are developmentally regulated and not expressed until late embryonic or fetal life and, in some organs, turned on only postnatally (45–47). Loss of expression with dedifferentiation in gastrointestinal tumors may be the reverse of the induction of “carcinoembryonic antigens,” which are expressed normally only in embryonic life but induced postnatally when differentiated cells expressing them undergo malignant transformation and dedifferentiation. In that sense, CA IX behaves in the opposite fashion in parallel with the carcinoembryonic antigens induced by dedifferentiation and, in this case, hypoxia.

It has been proposed that different CA isozymes present in the tumor cells may contribute to acidification of the extracellular milieu, thereby creating a microenvironment that is conducive to tumor growth and spread (18). The acidification might be a contributing factor promoting genetic instability (20), which is one of the characteristic features of HNPCC tumors. If so, the high expression of CA IX in most of these tumors may increase the risk for further genetic alterations in HNPCC cancer cells. From that perspective, the presence of CA IX in HNPCC may be advantageous in terms of diagnostics and therapy of this disease. CA IX protein contains a large extracellular part that includes the active site of the enzyme. Being outside of the cell, this active site is readily accessible to antibodies or drugs. A number of recent studies show promise in the development of specific chemical inhibitors or biomarkers targeting CA IX (7, 48). It is hoped that this line of research will provide useful tools for diagnostics and therapy of HNPCC.

References

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