PIRITTA HYNNINEN

Expression of Carbonic Anhydrases II, IX and XII in the Normal Female Reproductive Tract, Gynecological Tumors and Lynch Syndrome

ACADEMIC DISSERTATION
To be presented, with the permission of the Board of the School of Medicine of the University of Tampere, for public discussion in the Small Auditorium of Building M, Pirkanmaa Hospital District, Teiskontie 35, Tampere, on August 29th, 2014, at 12 o’clock.

UNIVERSITY OF TAMPERE
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:


ABBREVIATIONS

ARID1A AT-rich interactive domain 1A gene
BOT borderline ovarian tumor
BRAF B-Raf proto-oncogene
BRCA breast cancer 1, early onset
BSA bovine serum albumin
CA carbonic anhydrase
CA9 carbonic anhydrase 9 gene
CA12 carbonic anhydrase 12 gene
CA125 carbohydrate antigen 125
ccRCC clear-cell renal cell carcinoma
CAH complex hyperplasia with atypia
CH complex hyperplasia
CIN cervical intraepithelial neoplasia
CRC colorectal cancer
CTNNB1 catenin (cadherin-associated protein), beta1
DNA deoxyribonucleic acid
EC endometrial cancer
EnOC endometrioid ovarian carcinoma
ER estrogen receptor
ERBB2 v-erb-b2 avian erythroblastic leukemia viral oncogene homolog2
ESS extent of staining
FGFR2 fibroblast growth factor receptor 2
FIGO International Federation of Gynecology and Obstetrics
GIST gastrointestinal stromal tumor
H2AX H2A histone family, member X
HE4 human epididymis protein 4
HER2 human epidermal growth factor receptor 2
HGSC high grade serous carcinoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>HNF</td>
<td>hepatocyte nuclear factor</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia-responsive element</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IC</td>
<td>intracellular</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>INT</td>
<td>intensity of staining</td>
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<tr>
<td>Ki-67</td>
<td>nuclear antigen Ki-67</td>
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<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
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<tr>
<td>LGSC</td>
<td>low-grade serous carcinoma</td>
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<tr>
<td>LM</td>
<td>leiomyoma</td>
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<tr>
<td>LMS</td>
<td>leiomyosarcoma</td>
</tr>
<tr>
<td>LS</td>
<td>Lynch syndrome</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBOT</td>
<td>mucinous borderline ovarian tumor</td>
</tr>
<tr>
<td>MED12</td>
<td>mediator complex subunit 12</td>
</tr>
<tr>
<td>mf/hpf</td>
<td>mitotic figures/high power field</td>
</tr>
<tr>
<td>MLH</td>
<td>mutL homolog</td>
</tr>
<tr>
<td>MMMT</td>
<td>mixed Müllерian Mesodermal Tumor</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MN</td>
<td>carbonic anhydrase IX</td>
</tr>
<tr>
<td>MOC</td>
<td>mucinous ovarian cancer</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MSH</td>
<td>mutS homolog</td>
</tr>
<tr>
<td>MSI</td>
<td>microsatellite instable</td>
</tr>
<tr>
<td>MSS</td>
<td>microsatellite stable</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rabbit serum</td>
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<tr>
<td>OC</td>
<td>ovarian cancer</td>
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<tr>
<td>OCCC</td>
<td>ovarian clear-cell carcinoma</td>
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<tr>
<td>OSCC</td>
<td>ovarian serous cystadenocarcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PG</td>
<td>proteoglycan-related region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PHD</td>
<td>prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>phosphatidylinositol 3-kinase, regulatory subunit 1</td>
</tr>
<tr>
<td>PMS</td>
<td>postmeiotic segregation increased</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
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<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>SBOT</td>
<td>serous borderline ovarian tumor</td>
</tr>
<tr>
<td>SH</td>
<td>simple hyperplasia</td>
</tr>
<tr>
<td>SI</td>
<td>staining index</td>
</tr>
<tr>
<td>SP</td>
<td>signal peptide</td>
</tr>
<tr>
<td>STUMP</td>
<td>smooth-muscle tumors of uncertain malignant potential</td>
</tr>
<tr>
<td>US</td>
<td>uterine sarcoma</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and tween</td>
</tr>
<tr>
<td>TIC</td>
<td>tubal intraepithelial carcinoma</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TP53</td>
<td>tumor protein p53</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
</tr>
<tr>
<td>VIN</td>
<td>vulvar intraepithelial neoplasia</td>
</tr>
<tr>
<td>VSCC</td>
<td>vulvar squamous cell carcinoma</td>
</tr>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Carbonic anhydrases (CAs) are a family of metalloenzymes whose main function is to catalyze the conversion of carbon dioxide to bicarbonate ion and proton. The twelve active CAs in humans are widely localized and thus participate in a variety of physiological processes, including CO₂ transport, pH regulation, bone resorption, ureagenesis, gluconeogenesis, production of biological fluids, metabolic processes and fertilization. In addition to a variety of normal biological processes, CAs participate in several pathological conditions and they are also functionally involved in growth and invasion of cancer cells. Although there have been many studies published related to CAs in different tumors, very little is known about their expression in gynecological tumors. CAIX has been found to correlate with a poor prognosis in several tumor categories. In cervical cancer, CAIX seems to be a potent diagnostic biomarker for endogenous hypoxia. Previous studies have shown expression of CAXII in normal endometrial cells, suggesting a role in reproductive function. The many findings of CAs in cancer cells have led to some clinical investigations where different cancer-associated CAs were evaluated as potential treatment targets.

The primary aim of this study is to investigate by immunohistochemistry (IHC) the expression of CAII, CAIX and CAXII in different gynecological tumors. Prior to starting the analyses of the tumor specimens, we first examined the expression of these isoenzymes in the normal mouse endometrium, ovary and placenta. Thereafter, we continued to study their expression in human ovarian cancer (OC), normal endometrium, endometrial cancer (EC), leiomyoma (LM) and different gynecological sarcomas. Because CAIX and CAXII are highly expressed in some hereditary cancers, we also studied the expression of these isoenzymes in colorectal or endometrial tumors from patients with Lynch syndrome (LS).

The first study indicated that CAXII is a highly expressed isoenzyme in mouse endometrium; our later study confirmed previous findings that human endometrium also contains high levels of CAXII. In the mouse ovary CAIX was completely absent and only a faint signal was observed for CAXII. The second study involved a series of epithelial ovarian tumors that we immunostained for CAIX and CAXII. Most cases of borderline mucinous cystadenomas and mucinous cystadenocarcinomas showed moderate or strong CAIX immunostaining. In addition to ovarian neoplasms, our
investigations included various endometrial tumors. CAIX expression in the normal endometrium and benign tumors was usually weak and sporadic, whereas overexpression was evident in most endometrial cancers (P<0.005). All LMs were negative for CAII and CAXII and a comparison between LMs and sarcomas showed statistical significant differences for all studied isoenzymes (P<0.001). In LS, CAIX was found to be upregulated in colorectal cancer. In LS-associated EC CAIX immunostaining was observed to a lesser extent. CAII expression was significantly stronger in LS-related endometrial cancer than in sporadic cancer and the staining increased from simple hyperplasia to more malignant forms (P<0.001).

The high expression of CAXII in endometrial cells supports the theory of CAXII being important for normal reproductive functions. CAIX has been already considered a potential therapeutic target in renal cell cancer. We demonstrate here that CAIX is upregulated in endometrial cancer, ovarian mucinous cystadenocarcinomas and in several uterine sarcomas, where it could also serve as a potential therapeutic and diagnostic target protein. CAII could be linked to malignant transformation of the endometrium in LS patients and can be considered a possible biomarker to predict the right time for prophylactic hysterectomy.
Hiilihappoanhydraasit (CA:t) ovat metalloentsyymejä, joiden pääasiallinen tehtävä on katalysoida reversibelisti hiilidioksidin hydraatiota bikarbonaatiiksi ja protoniksi. Ihmisellä on kuvattu 12 aktiivista hiilihappoanhydraasia, jotka osallistuvat useisiin fysiologisiin prosesseihin, kuten CO\textsubscript{2}:n kuljetukseen, pH:n säätelyyn, luun resorptioon, elimistössä olevien nesteiden tuotantoon, moniin aineenvaihduntareaktioihin ja hedelmöittymiseen. Normaalien fysiologisten prosessien lisäksi, hiilihappoanhydraasit osallistuvat useisiin patologisiin tapahtumiin, kuten syöpäsolujen kasvuun ja invaasioon. Vaikka hiilihappoanhydraasien esiintymisestä on useita tutkimuksia erilaisissa syövissä, on hyvin vähän tietoa niiden ilmentymisestä gynekologisissa kasvaimissa. CAIX entsyymin esiintymisen on todettu olevan yhteydessä huonoon ennusteeseen useissa eri kasvainryhmissä. CAIX on myös lupaava biomerkkiaine, joka kuvaa kasvainsolujen hapenpuutetta esimerkiksi kohdunkaulakanavan syövissä. Aikaisemmissa tutkimuksissa on todettu CAXII:n ilmentyvän voimakkaasti normaalin kohdun limakalvollat. Siten tällä entsyymillä voisi olla merkitystä lisääntymistoiminnassa. Hiilihappoanhydraasien ilmeneminen useissa syöpätyypeissä on johtanut moniin tutkimuksiin, joissa kasvaimiin liittyviä hiilihappoanhydraaseja tutkitaan mahdollisina syövän hoitotavoiteina.


Ensimmäinen tutkimus osoitti, että CAXII entsyymiä esiintyy runsaasti hiiren kohdun limakalvolla. Myöhempän tutkimus osoitti, että myös ihmisen kohdun limakalvolla tätä isoentsyymiä esiintyy runsaasti. Hiiren munasarjoissa CAIX:n esiintymistä ei ollut juuri lainkaan todettavissa. Toisessa tutkimuksessa tutkimme immunohistokemiallisesti CAIX ja CAXII entsyymin esiintymistä erityyppisissä munasarjakasvaimissa. Useimmat munasarjan rajalaatuiset (borderline) musinoottiset cystadenoomat
Piritta Hynninen

ja musinoottiset cystadenokarsinoomat osoittivat kohtalaista tai vahvaa CAIX:n immunovärjäytymistä. Munasarjakasvainten lisäksi tutkimme erilaisia kohtukasvaimia. CAIX:n esiintyminen normaalilla kohdun limakalvolla ja hyväntaatuississa myoomissa oli yleensä heikkoa tai satunnaista, mutta kohdunrungon syövää CAIX:n ilmentyminen oli voimakasta (P<0.005). CAII ja CAXII entsyyymien esiintymistä ei todettu myoomissa. Myymien sekat kohtusarkoomien vertailussa tuli selvä tilastollinen merkitsevyys kaikkien kolmen isoentsyymin osalta (P<0.001). Lynchin oireyhtymää sairastavien potilaiden suolissyöpänäytteissä CAIX ilmentyi voimakkaana, mutta vähäisempänä kohdunrungon syövää. CAII:n esiintyminen oli merkittävästi voimakkampi Lynchin oireyhtymää sairastavien potilaiden kohdunrungon syövää verrattuna satunnaiseen kohdunrungon syöpään ja värjäytyminen vahvistui siirryttäessä kohdun limakalvon liikakasvusta kohti pahanlaatuisia muotoja (P<0.001).

CAII:n voimakas esiintyminen kohdun limakalvolla tukee aikaisempaa teoriaa, jonka mukaan CAII:lla on tärkeä merkitys hedelmällisyystä. Munuaisyövää CAIX:ää tutkitaan runsaasti mahdollisena hoidon kohdemolekyyninä. Tutkimuksemme osoitti, että CAIX entsyyymi esiintyy runsaasti kohdunrungon syövää, munasarjan musinoottisessa cystadenokarsinoomassa ja useissa kohtusarkoomissa, joissa se voisi toimia mahdollisena terapeuttisena ja diagnostisena kohdeproteiinina. CAII:n esiintyminen voisi ennustaa kohdun limakalvon liikakasvun muuttumista pahanlaatuisen suuntaan Lynchin oireyhtymää sairastavilla potilaille ja se voisi tutkimuksemme perustella toimia merkkiproteiinina ennakoinaan profylaktisen kohdun poiston ajankohtaa.
1 INTRODUCTION

Tumor cells initially develop in vascularized and normoxic areas, but as a result of massive expansion some areas can become hypoxic (Hanahan and Weinberg 2011). Adaptation to hypoxia is critical for tumor cell survival, progression, and metastasis. Cancer cells within many solid tumors produce lactic acid because of inefficient vascular clearing and glucose metabolism, resulting in an acidic microenvironment within the tumor tissue. Extracellular acidosis can potentially change the intracellular pH, and only a minor change in pH can disturb various biological functions. To survive this hypoxic stress, tumor cells activate a transcription factor, the hypoxia-inducible factor (HIF), which rapidly regulates the expression of a wide range of target genes, which in turn induce the tumor cell survival responses (Brahimi-Horn et al. 2007). These target genes encode several membrane-bound enzymes, such as carbonic anhydrases. One main function of the tumor-associated CAs in cancer cells is to contribute to extracellular acidification and maintain the alkaline intracellular pH, conditions that promote the tumor cell growth and invasion (Chiche et al. 2009, McDonald et al. 2012).

CAIX is a hypoxia-inducible enzyme, associated with tumor cell growth, survival and invasiveness. It is overexpressed in many tumors and can be used as a marker for hypoxia that is often associated with a poor prognosis (Potter and Harris 2004). Its typical expression pattern on cell surfaces makes it a potential target for antibody therapy, and CA inhibitors are also likely to be useful clinically, particularly if administered in combination with conventional chemotherapy. There is also an increasing interest in using soluble CAIX present in plasma for clinical cancer detection and prognostic evaluation (McDonald et al. 2012).

Endometrial cancer (EC) typically has a good prognosis (Fujimoto et al. 2009), yet some patients still show the recurrent disease after completing therapy. Molecular markers could be helpful to select patients who are at higher risk for developing a recurrent disease, and the markers could also help in planning a more tailored therapy. In Lynch syndrome (LS) women, EC is a common cancer, with a cumulative lifetime risk of 27–71% compared with 3% in the general population (Koornstra et al. 2009). To prevent EC these women with the mismatch repair defects (MMR) gene mutation are advised to regular examinations starting at an age of 30 or 35 at an interval ranging 1–3 years. Still, some ECs have been detected less than a year after the doctor’s visit.
(Auranen and Joutsiniemi 2011). Thus, the challenge is to estimate the right time for prophylactic hysterectomy for these LS women.

Ovarian cancer (OC) patients have the lowest survival among all female genital tract cancers and the survival for advanced cancer is very low (Klint et al. 2010). The most common ovarian cancer subtype, high grade ovarian serous carcinoma (HGSC), even though the incidence is low, is found in 80% at advanced stage of the disease (Prat 2012). Platinum-based chemotherapy is still the golden standard treatment in OC, but many mucinous adenocarcinomas are less responsive to this treatment (Lalwani et al. 2011). Therefore, new OC treatment models are constantly under investigation.

Since were little was known about CA expression in gynecological cancers, this study was designed to include several tumor categories and three different isoenzymes, namely the tumor-related CAII, CAIX and CAXII. We also obtained new information about the expression of these isoenzymes in the normal endometrium.
2 REVIEW OF THE LITERATURE

2.1 Carbonic anhydrases

In the late 1920s, Henriques performed experiments with hemolyzed blood and serum and determined that the carbon dioxide release from the blood was so high that the reaction must contain a catalyst (Henriques 1928). The enzyme responsible for this reaction was isolated and partially purified for the first time in 1932 by Meldrum and Roughton and was given the name carbonic anhydrase (CA) (Meldrum and Roughton 1932). A few years later, the first studies were published pertaining to the biochemical properties of CA (Roughton and Booth 1946a, b). In the first histochemical staining, CA activity was found in the erythrocytes, pancreas, renal tubules, placenta, gastric mucosa and epididymis, and soon CA activity was also found in the rabbit uterus and the fallopian tubes of ewes (Kurata 1953, Lutwak-Mann 1954, Lutwak-Mann and Averill 1954). Nowadays, we know that the CA family in mammals consists of 16 isoenzymes of which 13 are active and 3 isoforms lack catalytic activity (Lehtonen et al. 2004, Supuran 2008, Tashian et al. 2000, Thiry et al. 2008). CAXV, found most recently, is expressed in several animal species but not in humans and chimpanzees (Hilvo et al. 2005). The mammalian CA isoenzymes belong to the α-CA family. The CA proteins are not limited only to mammals. α-CAs or α-CA-like proteins are also found in the vertebrates, plants, algae, bacteria, viruses and even in the cytoplasm of green plants (Hewett-Emmett 2000). In addition to the α-CA family there are other CA enzyme families, such as β-, γ- and δ. β-CAs are present in mono- and dicotyledonous plants, bacteria, algae, fungi and some invertebrate animal species (Syrranen et al. 2010). The γ-CAs are found in archaea and eubacteria whereas δ- and ζ-CAs have been reported in marine diatoms (Cox et al. 2000, Hewett-Emmett 2000, Xu et al. 2008).

The 12 human CA isoenzymes are located in distinct cellular compartments (Table 1). The five cytoplasmic CA isoenzymes are CAI, CAII, CAIII, CAVII and CAXIII. Mitochondria contain CA VA and CA VB; CAVI is the only secretory isoenzyme. Membrane-bound isoenzymes include CAIV, CAIX, CAXII and CAXIV (Lehtonen et al. 2004, Parkkila and Parkkila 1996, Sly and Hu 1995, Tashian 1989).
<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Subcellular localisation</th>
<th>Expression in human</th>
<th>Main references</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAVA</td>
<td>Mitochondrial</td>
<td>A: Liver B: Pancreas, stomach, kidney, salivary glands, spinal cord, heart, skeletal muscle, nasal mucosa, gastrointestinal tract epithelia</td>
<td>(Fujikawa-Adachi et al. 1999b, Tarun et al. 2003, Saarnio et al. 1999)</td>
</tr>
<tr>
<td>CAVII</td>
<td>Cytoplasmic</td>
<td>Salivary gland, nasal mucosa, placenta, cartilaginous joint, ventricular linings of the choroid plexus</td>
<td></td>
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</tbody>
</table>
CAs are zinc-containing metalloenzymes present in eukaryotes and prokaryotes, where they catalyze the conversion reaction of carbon dioxide to bicarbonate ion and proton (\( \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+ \)). As can be seen in Table 1, different CAs are widely spread in human tissues and they can participate in a variety of physiological processes. The main function of CAs is to maintain an appropriate acid-base balance in organisms, and thus they contribute to various biological functions, including \( \text{CO}_2 \) transport, regulation of pH homeostasis, bone resorption, ureagenesis, gluconeogenesis, production of body fluids like gastric and pancreatic juice and bile, metabolic processes and fertilization (Sly and Hu 1995).

The large number of physiological processes that CAs are involved in has piqued interest in CA inhibitor design for clinical applications. At the present time, there are two main classes of CA inhibitors: the metal-complexing anions and the unsubstituted sulfonamides, which also include their bioisosteres (e.g. sulfamates and sulfamides) (Supuran 2008). Sulfonamides are the most important CA inhibitors. Nowadays, there are at least 25 different drugs used clinically that have been reported to possess significant CA inhibitory properties and many of these compounds were developed years ago during the search for diuretics. CA inhibitors have been primarily used as diuretics and for treating glaucoma (Supuran 2008), epilepsy (Vullo et al. 2005) and mountain sickness (Seupaul et al. 2012). More recently these inhibitors have shown potential as anti-glaucoma agents, anti-cancer, anti-obesity and anti-infective drugs (Supuran 2008).

The activation of these enzymes has also recently raised interest. It has been found that many physiologically relevant compounds such as biogenic amines (histamine, catecholamines, and serotonin), amino acids, oligopeptides or small peptides act as activators for many human CAs. In particular, activation of human CA I and CAII has been shown to constitute a possible therapeutic treatment for Alzheimer’s disease, ageing and other conditions in which spatial learning and memory therapy need to be enhanced (Supuran 2008).

CAs participate not only in a variety of physiological processes, but also in several pathological conditions. They are functionally involved in growth and invasion of cancer cells. These interesting findings have led to the development of new cancer
treatments and biomarkers (Supuran 2008). The next chapters will describe in more
detail the most known cancer-related isoenzymes CAII, CAIX and CAXII.

2.2 Cytoplasmic carbonic anhydrase II

CAII is a high-activity isoenzyme, that is expressed in the cytosol of some cell types in
virtually every tissue or organ (Tashian 1992). It was first found in the erythrocytes,
were it is involved in the hydration of CO$_2$ (Meldrum and Roughton 1933). As a high-
activity isozyme and due to its wide expression, it has been proposed to affect several
fundamental physiological processes.

2.2.1 Carbonic anhydrase II in normal tissues

CAII was originally discovered in the erythrocytes, where it catalyzes the reversible hy-
dration of CO$_2$ in the peripheral tissues and the reverse reaction in the lungs (Swenson
2000). In red blood cells CA activity is required for efficient Cl$^-$/HCO$_3^-$ exchange by
the anion exchanger and CAII has been found to bind directly to this anion exchanger
(Vince and Reithmeier 1998). CAII also directly interacts with the Na$^+$/H$^+$ exchanger,
which can influence pH regulation in the cells (Li et al. 2002).

CAII was widely found in various epithelia throughout the human alimentary tract. Ex-
pression of CAII has been observed in the parotid and submandibular glands, where
it is thought to generate bicarbonate for saliva (Parkkila et al. 1990). In the esophagus,
CAII is expressed in the stratified squamous epithelium, where it participates in
bicarbonate production and thus protects the mucosa against acidic gastric reflux.
It also participates in membrane transport events during active cell growth and the
elimination of CO$_2$ and metabolites (Christie et al. 1997, Ogawa et al. 1993, Parkkila et
al. 1994). The gastric mucosa secrets high concentrations of bicarbonate that originate
from surface epithelial cells and protons that originate from parietal cells. Therefore,
the role of CAII in the stomach is to regulate the acidity of gastric juice. The parallel
mucus and bicarbonate secretion from the epithelial cells forms a protective surface
against acidity on the gastric and duodenal mucosa (Parkkila et al. 1994). In the colon,
CAII is thought to participate in electroneutral reabsorption of NaCl (Lönnherholm et
al. 1985, Parkkila and Parkkila 1996). CAII has been detected in the epithelial cells
of the hepatic bile ducts and gallbladders, as well as in the hepatocytes. The presence
of active CAII in the gallbladder may be related to acidification and concentration of
the bile and the decreased expression may be associated with the formation of calcified
gallstones (Juvonen et al. 1994, Parkkila et al. 1996). In the pancreas, CAII is located in the epithelial duct cells and plays a role in the secretion of bicarbonate into pancreatic juice (Kumpulainen and Jalovaara 1981, Spicer et al. 1989).

The nasal mucosa contains almost all catalytically active CA isoenzymes, and CAII, together with CAXII and CAVB, is among the most highly expressed isoenzymes. The role of CAII in the nasal mucosa is not yet specified but it may play a role in the maintenance of pH homeostasis in the nasal epithelium and electrolyte transport across the epithelial cells (Tarun et al. 2003).

CAII is expressed in the osteoclasts were CAII participates in pH regulation (Väänänen 1984). It is considered essential for bone resorption and osteoclast differentiation. It has also been suggested that parathyroid hormone activates CAII in certain bone cells where it might facilitate the resorptive processes of bones (Sly et al. 1983).

In the brain, CAII has been demonstrated in several cell types and participates in the formation of cerebrospinal fluid (Kumpulainen and Korhonen 1982, Kumpulainen 1983).

CAII has been found in several reproductive tissues, such as the placenta and fetal membranes, where it was suggested to facilitate the diffusion of CO₂ across the placental membrane by promoting rapid production and consumption of bicarbonate (Muhlhauser et al. 1994). The expression of CAII in the placenta and a variety of fetal tissues very early in intrauterine life suggests that CAII plays an important role in the process of implantation and feto-placental development (Ali Akbar et al. 1998). CAII is expressed in human semen, the epithelia of the seminal vesicle, and the ampulla of the ductus deferens and distal ductus deferens (Kaunisto et al. 1990). It is thought to participate in the secretion of bicarbonate into the seminal plasma and regulate sperm motility and pH in the seminal plasma. In the male rat reproductive tract, CAII was detected in the epithelial cells of the lateral and dorsal prostate, the epithelial cells of the seminal vesicle and the coagulating glands (Härkönen and Väänänen 1988). It was suggested that here CAII is under testosterone regulation and that it is involved in bicarbonate production, particularly in the lateral prostate.

CAII is widely expressed in renal tissue, where its role in physiological urine acidification is evident (Parkkila 2000). This fact has been demonstrated in patients with a genetic deficiency of CAII. They suffer from renal tubular acidosis, confirming that CAII plays an important role in proximal and also in distal renal tubules. These patients furthermore suffer from osteopetrosis and cerebral calcification (Sly et al. 1983). Interestingly, CAII-deficient mice also showed a slowly progressive calcification of arterioles in several organs (Spicer et al. 1989) as well as defective bicarbonate secretion in the duodenum (Leppilampi et al. 2005b).
As explained above, CAII is widely expressed in almost all normal organs where it participates in important physiological processes.

2.2.2 Carbonic anhydrase II in neoplastic tissues

Even though CAII is expressed in a wide variety of normal cells, it is also considered to be one of the tumor-associated CAs. CAII is up-regulated in some brain tumors, hematological malignancies, lung cancer, colorectal cancer, pancreatic cancer and gastrointestinal stromal tumors (GISTs) (Bekku et al. 2000, Chiang et al. 2002, Haapasalo et al. 2007, Leppilampi et al. 2002, Parkkila et al. 1995b, Parkkila et al. 1995a, Parkkila et al. 2010). In a study of pancreatic carcinoma, the expression of CAII from the ductal cells did not correlate with the malignancy of the tumor (Parkkila et al. 1995b). This result suggested that CAII has no or only limited value in pancreatic cancer diagnostics. However, it was later found that CAII expression was higher in patients who had a more differentiated pancreatic ductal adenocarcinoma with a better prognosis (Sheng et al. 2013). Therefore, CAII could potentially play some role as a clinical biomarker in predicting the prognosis of patients with pancreatic cancer. The vast majority of GISTs (95%) showed immunohistochemically positive CAII staining and the enzyme was overexpressed in most cases; it was quite selective to this mesenchymal tumor type (Parkkila et al. 2010). Strong CAII staining predicted a better prognosis and therefore, CAII can be considered a promising biomarker in GIST tumor diagnostics. In rectal cancer, higher CAII expression was associated with a better prognosis (Bekku et al. 2000).

In contrast, high CAII expression was correlated with poorer survival and a higher malignancy grade in malignant astrocytomas. In brain tumors, such as astrocytomas and oligodendrogliomas, CAII was found to be located in the capillary endothelium. Endothelial staining in the astrocytomas was associated with highly neovascularized grade IV tumors (Haapasalo et al. 2007). In medulloblastomas and primitive neuroectodermal tumors, CAII staining was found in the neovessel endothelium and the tumor cells, but the expression did not correlate with patients’ survival rates or with the grade of the tumors (Nordfors et al. 2010). Furthermore, in the study of Yoshiura et al. (2005) CAII was found in the tumor vessels of melanoma, esophageal, renal and lung cancer but not in the normal vessel endothelium. CAII expression in the in vitro angiogenesis model showed that CAII was significantly up-regulated in acidic and hypoxic conditions. These findings suggest that CAII is a tumor vessel endothelium-associated antigen. However, not all tumors had a CAII-positive tumor vessel endothelium, suggesting that CAII expression in the tumor endothelium may depend
on the acidity or hypoxia within the tumor. Expression may be one factor contributing to the survival and adaption of proliferating and hypoxic cancer cells.

2.3 Membrane-bound carbonic anhydrases IX and XII

CAIX was the first membrane-bound CA identified as a cancer-associated isoenzyme. It was first reported in renal cell carcinomas in 1986, and the novel monoclonal antibody against CAIX was named G250 (Oosterwijk et al. 1986). In 1992, Pastorekova et al. (1992) found a new membrane-bound protein that was expressed in mammary tumor cells; this protein was given the name MN. The corresponding gene was cloned two years later (Pastorek et al. 1994). In 1993, MN protein was discovered in human ovarian, endometrial and cervical carcinomas, but not in the corresponding normal organs (Zavada et al. 1993). This protein was found to belong to the CA enzyme family and named MN/CAIX, because it represented the ninth mammalian CA in chronological order (Opavsky et al. 1996). In 2000, Oosterwijk’s group (Grabmaier et al. 2000) finally confirmed G250 to be CAIX. CAIX has recently been the most intensively studied isoenzyme, because it was shown to be an interesting cellular biomarker of hypoxic malignancies (Grabmaier et al. 2000, Liao et al. 1994, Olive et al. 2001, Wykoff et al. 2000b). CAIX is a 54/58 kDa protein that forms a dimer linked by disulfide bonds and has a unique proteoglycan-like region located at the N-terminus, which is unique to CAIX among all other CAs. It has also a central catalytic CA domain, transmembrane region and a short intracellular tail (Alterio et al. 2009, Hilvo et al. 2008).

In 1998, Türeci’s group first cloned the human CA12 gene and characterized the second transmembrane enzyme, CAXII. High expression of CAXII was reported in renal cell cancer, and it was soon found that the von Hippel-Lindau (VHL) tumor suppressor gene regulates the expression (Ivanov et al. 1998, Tureci et al. 1998). CAXII as a 39 kDa transmembrane protein shows significant structural homology with CAIX, but lacks the proteoglycan domain (Potter and Harris 2003). Figure 1 shows a schematic representation of the structure of CAIX and CAXII.
2.3.1 Carbonic anhydrase IX in normal tissues

CAIX is expressed in a few normal tissues, particularly in the gastrointestinal tract. CAIX has been detected in the normal gastric and intestinal and biliary mucosa (Pastorekova et al. 1997). When CAIX expression was investigated in greater detail, it was found in the enterocytes of human duodenum, jejunum, ileum and proximal colon (Saarnio et al. 1998). The positive immunoreactions gradually decreased toward the more distal parts of the human gut. CAIX-staining was found to be more positive in the proliferating cryptal epithelium, mainly in the basolateral plasma membranes, than in the upper part of the mucosa. In the esophagus, only the basal cells of squamous epithelium showed weak CAIX expression, but the expression increased in dysplastic cells (Turner et al. 1997). This phenomenon is typical for CAIX expression: it is reasonably limited in normal adult tissues, but the expression increases with cell dysplasia. In the gastrointestinal tract, CAIX is likely involved in the maintenance of tissue integrity and the regulation of basolateral ion transport; it may play a role in the proliferation and differentiation of intestinal epithelial cells. Therefore, CAIX may be a useful marker of cell proliferation (Pastorekova et al. 1997, Saarnio et al. 1998). The expression of CAIX in normal gastrointestinal tract is induced by cellular acidity and the requirement for proton transport (Liao et al. 2009). Targeted disruption of the CA9 gene in the mice resulted only in mild phenotypic changes, most prominently gastric
25
Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome

...expression of CAIX in the normal female reproductive tract, gynecological tumors and Lynch syndrome has been observed in the gastrointestinal tract (Gut et al. 2002). Gastric acid secretion was still observed, indicating that the other gastric CA isoenzymes are efficient enough to maintain normal pH balance.

CAIX has also shown weak immunoreactivity in the basolateral plasma membrane of epithelial cells in the male excrurrent ducts (Karhuma et al. 2001a). This reaction pattern suggests that CAIX participates in ion transport and concentration processes of testicular fluids.

In human fetal tissues, CAIX expression has been found as early as 4-8 weeks in the embryonic period in primitive mesenchyme, cells involved in chondrogenesis, all epithelial cells lining the body cavity, and the ependymal cells of the central nervous system (Liao et al. 2009). The expression of CAIX progressively diminishes after 29–30 weeks of gestation and after an age of one year CAIX expression is similar to that of normal adult tissue. Evidently, when hypoxia in the developing organs decreases during embryogenesis, the expression of CAIX also decreases. Post-natal persistent CAIX expression has been reported in a number of locations, including the mesothelial cells, the flat surface epithelium of the gonads, rete testis, rete ovarii, tubule recti, the hydatids of Morgagni, appendix of the testis and efferent ductules, hair follicles, the gallbladder, bile and pancreatic ducts, surface epithelium and glands of the stomach, pyloric and Brunner’s glands, crypt cells of the duodenum and the more distal small intestine. In the nervous system, CAIX was limited to the ventricular lining cells and the choroid plexus. Some parts of the placenta and cartilaginous tissues from the joint space showed also variable degrees of CAIX expression (Ivanov et al. 2001).

Overall, CAIX expression in normal tissues appears to be tightly regulated based on cell origin, cell differentiation status, ion transport and cellular oxygen content (Liao et al. 2009). However, there are only limited data available on the regulation of CAIX expression in normal tissues (Kaluz et al. 2009). Human CAIX mRNA has been studied by microarray techniques and the results from a large dataset show that it is most highly expressed in the stomach, reticulocytes, testis, small intestine and breast (www.medisapiens.com).

2.3.2 Carbonic anhydrase IX in neoplastic tissues

Limited expression of CAIX in normal tissue and its overexpression in various solid tumors makes this isoenzyme an interesting research subject. One characteristic of CAIX is, that it is most abundant in tumors that originate from CAIX-negative tissues (Ivanov et al. 2001, Zavada et al. 1993). Tumors that originate from tissues that normally express high levels of CAIX (for example, the gastric mucosa) showed a decline
in CAIX staining (Leppilampi et al. 2003). In another study related to gastric cancer, CAIX staining was lost in most cases of cancer, however the patients whose cancers abundantly expressed CAIX had shorter post-operative survival times compared with tumors with weak or no CAIX expression (Chen et al. 2005). Further analyses of this subgroup of gastric cancer cells showed that retained CAIX expression was located in the cancer cells at the invasion front. It is assumed that loss of CAIX expression is an early event in gastric carcinogenesis and that overexpression of CAIX at the invasion front of these cancer cells may give these cells a growth advantage by enhancing their proliferation and invasive growth.

The overexpression of CAIX was first noted in ccRCC, where decreased levels of CAIX expression indicated poor prognoses (Bui et al. 2003, Genega et al. 2010, Oosterwijk et al. 1986). Thereafter, several studies have established the relationship between CAIX and patient prognosis. Upregulation of CAIX is associated with a poor prognosis in carcinomas of the lung (Kim et al. 2005), rectum (Korkeila et al. 2009), breast (Tan et al. 2009), bladder (Hoskin et al. 2003, Klatte et al. 2009), and oral cavity. The same phenomenon has been reported in soft tissue sarcomas (Maseide et al. 2004) as well as thyroid (Burrows et al. 2010), esophageal (Birner et al. 2011) and head and neck tumors (Koukourakis et al. 2006) and astrocytomas (Haapasalo et al. 2006, Nordfors et al. 2010). CAIX has been associated with tumor cell necrosis and higher malignancy grade, and strong CAIX expression has remained an independent predictor for diseasespecific survival in many tumor categories (Korkeila et al. 2009, Tan et al. 2009, Hoskin et al. 2003, Birner et al. 2011, Koukourakis et al. 2006, Nordfors et al. 2010).

In the normal bladder, CAIX is absent in the urothelial cells but is expressed in the highest layer of the urothelium in non-invasive, low-grade bladder cancer. There is a decrease in CAIX expression when the tumor invasion level decreases (Klatte et al. 2008, Ord et al. 2007). Hyrsl et al. (2009) detected 70% of transitional cell carcinomas in urinary tract patients when Western blots were used to identify a soluble form of CAIX from the urine. Two earlier undiagnosed cases with soluble CAIX in the urine developed transitional cell carcinomas within six months. This result suggested that the evaluation of CAIX might be a useful adjunct to diagnostic cytology in low-grade bladder cancer. Notably, these tumors are usually difficult to diagnose and the analysis could be utilized for example to distinguish between the benign papillary clusters and low-grade papillary tumors (Klatte et al. 2009). Urquidi et al. (2012) confirmed the previous findings; CAIX levels were significantly elevated in the urine samples from bladder cancer patients compared to controls, but of the three biomarkers (CAIX, vascular endothelial growth factor and angiogenin) tested, CAIX was the least promising (sensitivity 58% and specificity 90%). High plasma levels of CAIX represent an independent prognostic biomarker in patients with non-small cell lung cancer (Kim
Therefore, identifying these patients at high risk for recurrence would enable the development of a tailored treatment strategy for certain patients. As an example, one selection criterion for interleukin-2 treatment in renal cancer could be the high CAIX expression in the tumor. Since the best response and highest 5-year survival rates have been found in patients with high CAIX expression, interleukin-2 should be offered as a first-line therapy to patients with high CAIX expression in the tumor (Atkins et al. 2005, Bui et al. 2003). This was later confirmed by de Martino et al. (2009). They reported that the tumor tissues with high CAIX expression had a greater interleukin-2 response rate (37%) than the tissues with low CAIX expression (8%).

Several studies have demonstrated that high levels of CAIX expression are associated with a greater risk of lymph node metastasis (Choschzick et al. 2010, Lee et al. 2007b, Woelber et al. 2011). Furthermore, in cervical cancer the metastasis-free survival time is associated with CAIX levels (Kim et al. 2006, Lee et al. 2007b, Loncaster et al. 2001, Woelber et al. 2011). A large study of 3630 human breast cancers provided evidence of CAIX being vital for cancer growth and metastasis in hypoxic breast tumors (Lou et al. 2011). CAIX was also an independent poor prognostic biomarker for distant metastasis and survival. CAIX was highly expressed in basal breast cancer, a disease with an aggressive metastatic potential that is quite difficult to treat (Lou et al. 2011, Tan et al. 2009). However, in a breast cancer study involving 945 high-risk premenopausal and postmenopausal women, CAIX was an independent prognostic factor only in a subgroup of postmenopausal women with 1-3 positive nodes and a positive hormone receptor status (Kyndi et al. 2008). The results of the Medisapiens project show high CAIX mRNA expression in renal cancer, mesothelioma, mucinous ovarian cancer, bladder transitional cell cancer and cervical cell cancer (www.medisapiens.com).

In addition to the diagnostics of primary tumors, CAIX has been evaluated as a possible biomarker to detect lymph node and distant metastases. Intratumoral injection of a CAIX-specific monoclonal antibody conjugated to fluorescent dye (i.e., CA9Ab-680) marked the primary breast tumor and the tumor cells in the nearby lymph nodes and was detectable with non-invasive fluorescent imaging (Tafreshi et al. 2012). There is already an ongoing Phase 3 clinical trial where radioactive iodine-labeled cG250/CAIX monoclonal antibody has been used to visualize renal cell carcinoma tumors, as well as metastases in positron emission tomography (PET) (Stillebroer et al. 2010).

CAIX is tightly regulated in hypoxia (Beasley et al. 2001, Olive et al. 2001) and hypoxia in turn leads to chemoresistance. CAIX increases the intracellular pH and the low extracellular pH in tumor cells. The abnormal pH homeostasis in the tissue contributes to the fact that many anti-cancer drugs are only weakly taken into the tumor
cells. This fact leads to reduced cytotoxicity of the anti-cancer drug. Therefore, reducing the extracellular acidity of these tumors could lead to a better chemotherapeutic response (Raghunand and Gillies 2001). Consequently, the inhibition of CAIX in these hypoxic tumors that do not respond to classical chemotherapy or radiotherapy may be useful. There are many studies of CA inhibitors in progress and some promising sulfonamide derivatives have already shown significant anti-tumoral effects (Supuran 2008).

2.3.2.1 Regulation of CAIX in cancer cells

The overexpression of CAIX was first reported in clear-cell renal carcinomas (ccRCC) (Ivanov et al. 1998, Tureci et al. 1998). The major genetic hallmark of ccRCC is mutations, either familial or sporadic, in the VHL tumor suppressor gene. Familial mutations cause VHL syndrome where frequently occurring tumors include ccRCC, pheochromocytomas and hemangioblastomas (Gnarra et al. 1994, Wykoff et al. 2000b). In normoxic tissues, prolyl-hydroxylases (PHDs) hydroxylate proline residues of hypoxia inducible factor (HIF-1α). The VHL protein binds to the hydroxylated HIF-1α and ubiquitinates it, which in turn leads to the degradation of HIF-1α in proteasomes. This process inactivates the HIF target genes. A loss of or an inactivating mutation in VHL gene, like in ccRCC, results in a hypoxic phenotype; this condition also occurs under normoxia. HIF-1α is stabilized constitutively, resulting in the up-regulation of hypoxia-inducible genes. Under hypoxia instead, HIF-1α is not hydroxylated, because in hypoxic conditions the PHDs are inactive. This process causes stabilization and accumulation of HIF-1α in the cytoplasm and translocation into the nucleus, were it dimerizes with the constitutively expressed HIF-1β subunit and forms an active transcription factor HIF. This active transcription factor binds to the hypoxia-responsive element (HRE) and activates transcription of hypoxia-regulated target genes, whose products facilitate the cell adaption to hypoxia. Hypoxia-responsive proteins include a number of factors that are included, for example, in angiogenesis, anaerobic metabolism, regulation of cell cycle, proliferation, apoptosis and control of intracellular pH (Pastorekova et al. 2008). The VHL/HIF-pathway is illustrated in Figure 2.

The master regulator of CAIX is the HIF pathway and hypoxia is the most important stimulator behind the expression of this isoenzyme (Kaluz et al. 2009, Loncaster et al. 2001, Wykoff et al. 2000a). In the majority of carcinomas CAIX expression is restricted to perinecrotic areas and/or to the hypoxic regions (Giatromanolaki et al. 2001, Wykoff et al. 2000a). There can still be regions in tumors, were CAIX is expressed under mild hypoxia. Additionally, other factors may influence the transcription of CA9.
high cell density under mild hypoxia has been shown to induce CAIX expression. This regulation needs only minimal HIF-1 activity, but is associated with an increase in phosphatidylinositol 3-kinase activity (PI3-K) (Kaluz et al. 2002). Normal p53 downregulates CAIX and therefore mutations of the TP53 gene can lead to increased CAIX expression, which might explain the CAIX upregulation observed in some brain tumors (Proescholdt et al. 2005). Some microenvironmental conditions, such as acidosis and glucose deprivation, may also regulate CA9. In the normal digestive tract, a low extracellular pH level may be a factor responsible for the physiological expression of CAIX. While hypoxia and HIF activation are the key regulating factors for CAIX expression, additional factors – together with HIF, are required under mild hypoxia (Kaluz et al. 2009).

Figure 2. Schematic illustration of the VHL/HIF-pathway. The mechanism is explained in detail in the text (modified from Pastorekova et al. 2008).

(HIF) hypoxia inducible factor, (HRE) hypoxia-responsive element, (VHL) von Hippel-Lindau protein, (PHD) prolyl-4-hydroxylases
2.3.2.2 Function of CAIX in cancer cells

CAIX enhances in many ways the conditions of tumor cells to survive and proliferate. CAIX contributes to extracellular acidification and maintains the physiological intracellular pH. These conditions preserve and promote the tumor cell survival in an acidic environment (Chiche et al. 2009, Svastova et al. 2004). The silencing of CA9 and CA12 in xenograft tumors leads to an 85% reduction in tumor volume, whereas silencing of only CA9 leads to a 40% reduction (Chiche et al. 2009). The location of the active site of CAIX on the extracellular surface of the plasma membrane places it in a strategic site for contributing to the acidification of the tumor microenvironment. In hypoxia bicarbonate transported into the cytoplasm buffers the intracellular pH, while protons remain in the extracellular space. Therefore, CAIX helps to maintain the slightly alkaline intracellular pH, which favors tumor growth while the increasingly acidic extracellular space facilitates tumor cell invasiveness (McDonald et al. 2012). Expression of CAIX in tumors may indicate the presence of hypoxic cells with more aggressive behavior and treatment resistance due to hypoxia-induced cellular changes (Stubbs et al. 2000, Thiry et al. 2006). These changes can lead to the expression of proteins involved in angiogenesis, apoptosis inhibition and disruption of cell-cell adhesion. These consequences are linked to the expression of CAIX (Giatromanolaki et al. 2001). In addition to the role of CAIX in pH regulation, CAIX has been also linked to cell adhesion and proliferation. The formation of complexes between E-cadherin and β-catenin is essential for the cell-adhesive function. The loss or destabilization of E-cadherin is linked to tumor invasion. Svastova et al. (2003) demonstrated that CAIX has the potential to modulate cell-cell adhesion by destabilizing of E-cadherin links to the cytoskeleton via interactions with β-catenin. This feature may contribute to increased tumor aggressiveness and metastases. Zavada et al. (2000) demonstrated that purified CAIX enhances cell adhesion, spreading and survival in vitro. Some studies have demonstrated a correlation between CAIX upregulation and proliferation. In brain tumors, expression of the proliferation marker Ki67 showes significantly correlation with CAIX (Proescholdt et al. 2005). The upregulation of CAIX may assist the cell in maintaining a proliferative state despite tissue hypoxia, which normally would cause growth arrest (Hockel and Vaupel 2001). The versatile functions of CAIX in cancer cells are shown in table 2.
Table 2. Functions of CAIX in cancers cells and its benefit for the cancer cell

<table>
<thead>
<tr>
<th>Function of CAIX in cancer cells</th>
<th>Benefit for cancer cells</th>
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<tr>
<td>pH regulation</td>
<td>growth and invasion</td>
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<tr>
<td>cell adhesion</td>
<td>increased tumor aggressiveness and metastases</td>
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<tr>
<td>cell proliferation</td>
<td>maintaining a proliferative state</td>
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2.3.3 Carbonic anhydrase XII in normal tissues

CAXII was originally demonstrated in the normal kidney and intestine (Kivela et al. 2000a, Parkkila et al. 2000b). In the renal cortex, CAXII expression was located in the epithelial cells of the distal convoluted tubules, the thick ascending limb of the loop of Henle and collecting ducts (Parkkila et al. 2000b). In the medulla, CAXII was found in the epithelial cells of the collecting ducts. Based on its localization, CAXII may be one of the key enzymes in renal physiology, particularly in regulating of ion and water transport. High levels of CAXII expression have been found already during the embryonic period between 7 and 12 weeks of gestation, particularly in the tissues that are involved in secretion or pH regulation. The expression of CAXII in fetal tissues is retained throughout adult life in certain tissues or cells, such as taste buds, acinar cells of the pancreas and salivary glands, renal tubules, epithelium of the large intestine, parietal cells of the stomach and the choroid plexus (Liao et al. 2009). CAXII is absent from the small intestine, but is expressed in all segments of the normal large intestine. In the colon, CAXII was present in the surface epithelial cells and was most prevalent in the surface epithelial cuff region. CAXII is located on the cell exterior, beneath the basolateral plasma membrane of gut enterocytes and thus may participate in NaCl reabsorption (Kivela et al. 2000a). In male epididymal ducts, CAXII was highly expressed in the epithelial cells, where it may affect luminal acidification. CAXII was also present also in the basolateral membranes of the efferent ducts, where it may be also involved in transepithelial ion transport and water absorption. Distinct co-localization of a water channel protein with CAXII observed in the efferent ducts was consistent with the role of basolateral CA in water reabsorption (Karhumaa et al. 2001a). CAXII staining has been also observed in the basal cells of the respiratory mucosa, squamous mucosal lining the oral cavity, mesothelium, esophagus and epithelium of the breast (Ivanov et al. 2001, Liao et al. 2009). Constitutive expression of CAXII in the normal breast epithelium and in benign hyperplasia may indicate that CAXII plays a role in the control of pH in normal breast tissue (Wykoff et al. 2001). CAXII is one of the key isoenzymes in the developing eye. After birth, CAXII expression in the epithelium of the cornea, lens and ciliary body decreases and in the adult eye only the ciliary
epithelium contains CAXII (Liao et al. 2003). In the ciliary body, CAXII may be involved in aqueous humour production. Overexpression of CAXII was found in non-pigmented ciliary epithelial cells of glaucoma patients. This result makes CAXII a potential therapy target for certain types of glaucoma. CAXII mRNA shows the highest expression levels in the kidney, colorectal tissue, pancreas, vulva/vagina, uterus and urinary bladder (www.medisapiens.com).

2.3.4 Carbonic anhydrase XII in neoplastic tissues

The observations of Türeci et al. (1998) of high CAXII levels in renal cell cancer, have been confirmed in later studies (Ivanov et al. 2001, Parkkila et al. 2000b). The immunohistochemical (IHC) staining intensity was moderate to strong in all renal tumor categories, except for Wilms’s tumor and angiomyolipoma; it was strongest in chromophobe renal cell carcinoma and in oncocytic tumors (Ivanov et al. 2001, Parkkila et al. 2000b). Strong CAXII expression has been detected in non-small cell lung carcinoma, where high CAXII levels could serve as biomarkers for good a prognosis (Ilie et al. 2011, Ivanov et al. 2001). High CAXII was associated with lower-grade tumors, and better overall and disease-specific survival. CAXII was significantly more expressed in squamous cell carcinoma. CAXII expression seems to be higher in well- and moderately differentiated squamous cell carcinomas and in smaller size tumors (Kobayashi et al. 2012). Several other clinical studies have shown a relationship between high CAXII expression in tumors and a favorable prognosis. In breast cancers, CAXII is associated with a lower histological grade, a lower relapse rate, positive estrogen status and the absence of necrosis and calcification (Watson et al. 2003, Wykoff et al. 2001).

On the other hand, CAXII has been linked to poor prognosis, particularly for brain tumors such as astrocytomas and medulloblastomas, and in primary oral squamous cell carcinoma (Haapasalo et al. 2008, Nordfors et al. 2010, Chien et al. 2012). CAXII is also overexpressed in several other primary brain tumors, especially in glioblastomas, as well as in metastatic brain tumors (Proescholdt et al. 2005). This finding makes CAXII an interesting target for adjuvant brain tumor therapy. In colorectal tumors, CAXII staining was slightly overexpressed compared with the normal tissue (Kivela et al. 2000a). In adenomas, the positive immunoreaction clearly increased with the grade of dysplasia. Based on cDNA microarray analyses, CAXII mRNA is most highly expressed in renal, bladder, breast, laryngopharynx, oral and ovarian clear cell cancers (www.medisapiens.com).
2.3.4.1 Regulation and function of CAXII in cancer cells

Even though CAXII is upregulated in VHL-defective renal tumors and induced in hypoxia, it is less dependent on HIF regulation than CAIX (Tureci et al. 1998). This fact supports the finding that HIF-1α and CAXII are not co-localized in tumors (Liao et al. 2009). Observations that CAXII was found focally upregulated in areas of hypoxia in some tumors support the statement that CA12 is a hypoxia-regulated gene, even the functional HRE has not yet been reported (Ivanov et al. 2001). In addition to hypoxia, CAXII expression may also be related to the cell origin of the relevant tissue and its functional status (Liao et al. 2009). It is likely that the dominant factors in CAXII regulation are related to tumor differentiation, as CAXII is observed to be highly expressed in well-differentiated carcinomas and only weakly expressed in poorly differentiated carcinomas (Watson et al. 2003, Wykoff et al. 2001). In addition, it has been shown that estrogen receptor (ER) upregulates CA12 gene expression in breast cancer cells (Barnett et al. 2008). This regulation of the CA12 gene by the ER may account for the coexpression of positive ER and CAXII, which has been observed in breast tumors. Likewise CAIX, also CAXII enhances the conditions of tumor cells to survive and proliferate. CAXII has shown a significant correlation with a proliferation marker, Ki67, in brain tumors (Proescholdt et al 2005). The observations of Ulmasov et al. (2000) suggested a role for CAXII in CO₂ and HCO₃⁻ homeostasis in cells where it is normally expressed. It may also be involved in the acidification of cancer cell microenvironment, which in turn might augment tumor invasiveness.

2.4 Carbonic anhydrases in normal gynecological organs

The very first observations of CA activity in the reproductive organs were made in rabbit and ewe uteruses and fallopian tubes in 1954 (Lutwak-Mann and Averill 1954, Lutwak-Mann 1954). The first studies related to CA activity in the human reproductive tract were conducted in the late 1960s (Korhonen et al. 1966, McIntosh and Lutwak-Mann 1967). Friedley and Rosen histochemically showed in 1975 that human endometrial epithelial cells, the epithelium and smooth muscle of fallopian tube, surface epithelium of the ovary, and the granulosa cells of maturing or mature follicles contain CA activity, but the isoenzyme responsible for the activity in these organs still remained undefined (Friedley and Rosen 1975). It was concluded that the presence of CAs throughout the female reproductive tract suggests their importance in maintaining the physiological pH in the female tract to promote fertilization. Karhumaa et al. (2000) identified the isozyme responsible for the CA activity in human endometrium. They showed,
according to IHC and Northern and Western blotting, that CAXII is present in the basolateral plasma membrane of the epithelial cells of human endometrium. The immunoreaction was strongest in the deep parts of the glands and was somewhat stronger in the proliferative cells compared with the secretory-phase cells. CAI and CAII showed immunoreactions in the erythrocytes and the endothelium of the blood vessels. In the cervix, only some occasional epithelial cells showed weak positive staining. This basolateral location of CAXII in the endometrial glands suggests that the enzyme participates in the regulation of both the luminal and mural pH homeostasis in the human uterus. These findings also support the previous view that CA activity in normal endometrium may play a role in the reproductive functions of the uterus by contributing to bicarbonate production at this site. Ivanov et al. (2001) later confirmed CAXII expression in the human endometrial glands, while these authors also showed that CAIX remained completely negative. In fact, CAIX expression in endometrial cells seems to vary according to menstrual cycle (Punyadeera et al. 2006). CAIX expression has been shown to be highest in human endometrium biopsies from the cycle days 2 through 8, after which CAIX expression rapidly decreases and completely disappears around cycle days 9–10 with no expression at the cycle days 11–15. Normal human myometrium seems to be negative for CAIX (Mayer et al. 2008).

Bicarbonate plays a major role in a series of reproductive processes, including sperm motility, hyperactivation, capacitation and acrosome reaction (Okamura et al. 1985). In the mouse endometrium, an enhanced production of bicarbonate was noted at the same time as CAI and CAXII were upregulated at estrus, a time immediately prior to ovulation (He et al. 2010). These findings suggest that estrogen can upregulate CAII and CAXII expression with a corresponding increase in both bicarbonate concentration and endometrial surface pH. Interestingly, loss of CAII in mice resulted in the absence of endometrial glands (Spicer et al. 1989). This finding led to another study where neonatal mice were treated with acetazolamide, a CA inhibitor, and findings in the uterus were compared (Hu and Spencer 2005). Acetazolamide decreased both the CA activity in the uterus and the number of endometrial glands without any effects on the stroma or myometrium. As a result, CAs, particularly CAII, may play a functional role in endometrial gland development. To date there have been no reports on the possible effects of CAXII deficiency in endometrium. Because of the high CAXII expression in the endometrial cells, one could predict some phenotypic changes in humans or mice with genetic deficiency of functional CAXII enzyme. In 1994, Liao and coworkers found weak CAIX expression in focal areas, restricted mainly to the cervical reserve cells (Liao et al. 1994). This finding was later confirmed and CAIX was found to be highly expressed in the human cervix during the 26th–40th weeks of gestation, but 12 months post-natally CAIX is rare or restricted to reserve cells of the glands (Liao et al.
Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome

Instead, CAXII was found in adults in the squamous mucosa cells lining the cervix and vagina.

In the ovary, CAIX is diffusely expressed in surface coelomic epithelium cells, primordial follicle and rete ovarii, whereas CAXII was focally found in the surface coelomic epithelium and in the remnants of mesonephric ducts of the ovary (Ivanov et al. 2001, Liao et al. 2009). The epithelium of human fallopian tube has remained negative for CAIX (Liao et al. 2009), whereas both the rat oviduct and endometrium express the enzyme (Gillies et al. 2011).

2.5 Carbonic anhydrases in gynecological tumors

CAIX is the most intensively studied CA isoenzyme among various gynecological tumors, particularly so for cervical tumors. There has been little information about CA expression in other gynecological tumors. Ivanov and coworkers used IHC to examine CAIX and CAXII in various cancers. These authors presented the expression of CAIX and CAXII in cervical, ovarian and endometrial carcinomas for the first time. CAIX protein was expressed in 99% of cervical carcinomas and 68% of these tumors showed diffuse or strong staining, whereas CAXII was expressed only in 33% of these carcinomas and positive staining was focal or weak. In *in situ* cervical adenocarcinoma, CAIX staining was found in 100% of the studied cases and diffuse staining was reported in 90% of samples. In endometrial carcinoma, CAXII staining was diffuse in 50% of cases and CAIX staining was diffuse in 30%. In endometrial hyperplasia, both CAIX and CAXII staining was evident in all cases studied. The CAIX expression was diffuse only in 14% of cases, whereas CAXII staining was found to be diffuse in 67% of cases. All of the studied cystadenoma cases expressed CAIX and CAXII. Diffuse CAIX staining was found in 67% of cases, whereas CAXII staining in these tumors was weak or focal. All types of epithelial ovarian carcinomas also expressed CAIX and CAXII but to a lesser extent. Sex-cord tumors showed only focal CAIX staining. The germ cell tumors expressed CAXII only weakly, and some focal CAIX-positive staining was also reported. (Ivanov et al. 2001)

The abundant expression of CAIX in cervical carcinomas has led to several additional studies. Stanbridge’s group, in collaboration with Pastoreks, was the first to postulate that CAIX could be a good diagnostic biomarker in cervical dysplasia. CAIX expression was restricted in normal cervix only to rare reserve cells, but both the number of positive cells and the staining intensity increased in hyperplastic specimens, particularly in the concurrent presence of carcinoma *in situ*, adenoma *in situ* or carcinoma (Liao et al. 1994, Liao and Stanbridge 1996). If malignant progression
did not occur, CAIX expression was lost in the differentiating reserve cells. The strong CAIX reactivity was lower in CIN I than in CIN II. Thus, CAIX was proposed to be an indicator of lesion progression and might potentially play the role of a biomarker in the clinical management of CIN lesions.

CAXII and CAIX have shown different staining patterns in gynecological tumors. While CAXII was expressed frequently in the normal cervix as well as in low-grade CIN lesions, the expression decreased as the tumor grade increased; the expression was lowest in invasive tumors (Lee et al. 2007b). CAXII expression was also marginally associated with a higher disease-free survival rates (Yoo et al. 2010).

As in many other tumors, there is a strong association between tumor hypoxia and poor clinical outcome in cervical carcinomas independent of the treatment method (Hockel et al. 1999). This fact has led to the hypothesis that CAIX could not only serve as an endogenous hypoxia marker but also as a prognostic marker in cervical cancers. There seems to be a correlation between CAIX expression and the exogenous hypoxia marker pimonidazole in cervical cancers (Jankovic et al. 2006, Olive et al. 2001). These findings have been challenged by some studies that could not reproduce this correlation and instead found well-oxygenated tumors exhibiting strong CAIX staining (Hedley et al. 2003, Mayer et al. 2006). Although one study showed no association between CAIX and clinical outcome in cervical carcinoma (Hedley et al. 2003), most studies have demonstrated that high CAIX expression is associated with a worse survival for cervical carcinoma (Kirkpatrick et al. 2008, Lee et al. 2007b, Liao et al. 2009, Loncaster et al. 2001). CAIX expression in cervical cancer is strongly related to enhanced metastatic potential, whereas CAXII expression seems to correlate with a lower risk of metastasis or has no relationship to metastasis (Kim et al. 2006, Lee et al. 2007b). This strong correlation between CAIX expression and metastases might serve as an important indicator for identifying patients who require more aggressive systemic therapy.

There is very little information available on the value of CA immunostaining for predicting the possible recurrence of carcinoma. One investigation of recurrent endometrial carcinoma included 59 primary endometrioid endometrial tumors immunohistochemically stained for CAIX. The mean percentage of positive CAIX expression was 8.5% in primary tumors of patients with recurrence and 12.5% in primary tumors without recurrence. These results indicated that CAIX expression may not be an independent predictive marker for recurrent endometrioid carcinoma (Pijnenborg et al. 2007).

There are only a few studies pertaining to CAIX in vulvar cancer. CAIX expression was found to be negative in the normal vulvar basal and prickle cells. CAIX staining increased in intraepithelial neoplasia (VIN) and further in vulvar squamous cell
carcinoma (VSCC) (Li et al. 2012). However, there was no more significant difference in CAIX staining between VIN and VSCC. The authors stated that, CAIX could serve as a marker of malignant progression in VSCC. Serum CAIX concentrations have been found to be higher in patients with high intratumoral CAIX expression; elevated pre-operative serum values were also associated with a poor prognosis (Kock et al. 2011). Therefore, serum CAIX might serve as a useful marker to stratify patients for adjuvant therapy in primary vulvar cancer. In contrast, two IHC studies could not find any correlation between the CAIX expression and metastases or disease-free survival in VSCC (Stone et al. 2005, van de Nieuwenhof et al. 2010).

There has only been one IHC study related to CAIX expression in leiomyosarcomas (LMS). Positive CAIX expression was observed in 78% of the LMS tumors, whereas all benign leiomyoma (LM) samples were negative for CAIX. (Mayer et al. 2008).

2.6 Epithelial tumors of the uterine corpus

2.6.1 Endometrial cancer

EC is the sixth most commonly diagnosed cancer among women worldwide, with an estimated 288,387 new cases in 2008 and a standardized incidence rate of 8.2 per 100,000 women (Weiderpass and Labreche 2012). Histologically, most ECs are adenocarcinomas either of endometrioid type with or without squamous differentiation, or mucinous, serous or clear-cell carcinomas (Shiozawa and Konishi 2006). Rare histological types are squamous, small cell and undifferentiated carcinomas. Endometrial hyperplasias are defined as abnormal proliferative lesions of endometrial glands. Hyperplasias are classified as simple without atypia, complex hyperplasia without atypia and atypical simple or complex hyperplasia. Only atypical hyperplasia is regarded as a direct precursor for endometrial EC. The majority (70–80%) of ECs represent endometrioid adenocarcinoma of low histological grade, designated as type I carcinoma (O’Hara and Bell 2012). These tumors are estrogen-related, as they seem to arise from unopposed estrogen stimulation, which, after long-lasting exposure, leads to endometrial hyperplasia and can increase the chance of developing an atypical hyperplasia and eventually type I EC. In a study of 560 individuals with endometrial hyperplasias, 52% of patients with atypical hyperplasia progressed into type I EC (Horn et al. 2004). The overall risk of EC in complex (atypical) hyperplasia is 3.5% (43.8%). Epidemiological risk factors include obesity, polycystic ovary syndrome, persistent exposure to estrogens, nulliparity, early age at menarche, late age at menopause and diabetes (O’Hara and Bell 2012, Shiozawa and Konishi 2006). If diagnosed early, the prognosis for type I EC is
favorable, with 5-year survival rates in stage I (II) of 98% (94%) (Fujimoto et al. 2009). Instead, type II EC occurs typically in older postmenopausal women and is not related to estrogen exposure. The prognosis is poorer than in the type I disease (Horn et al. 2007). These tumors typically have a higher grade and various histologies, particularly serous and clear-cell carcinomas that arise from atrophic endometrium.

Type I EC is characterized by a variety of somatic alterations, such as microsatellite instability (MSI), alteration in the phosphatidylinositol 3 kinase pathway (PI3K), mutations in Kirsten rat sarcoma viral oncogene homolog (KRAS), catenin (cadherin-associated protein) beta1 (CTNNB1) or fibroblast growth factor receptor 2 (FGFR2), and dysregulation in AT-rich interactive domain 1A gene (ARID1A) are other contributing factors (The cancer genome atlas research network 2013). On the other hand, TP53 mutation is the most striking genetic alteration in about 90% of serous type II EC carcinomas, whereas PTEN and MSI play a minor role. The typical gene alterations of type I and II EC are shown in figure 3.

The most frequently altered biochemical pathway in EC is the PI3K-PTEN-AKT signal transduction pathway, which regulates numerous biological processes including cellular proliferation, growth, differentiation, survival, migration and metabolism (O’Hara and Bell 2012). More than 80% of EC tumors have one or more alterations in the PI3K pathway. Phosphatase and tensin homolog (PTEN) is a major negative regulator of the
PI3K pathway and its inactivation leads to a persistent active PI3K pathway (Mutter 2001). Loss of or altered PTEN expression results in apoptotic escape and aberrant cell growth (Bansal et al. 2009, Okuda et al. 2010). PTEN is the most commonly altered gene in type I EC (table 3) and it is commonly observed in endometrial hyperplasias with or without atypia, suggesting that the loss of PTEN function is an early event in type I endometrial transformation (Slomovitz and Coleman 2012, Zhou et al. 2002). Thus, in the endometrium, PTEN functions as a gatekeeper for initiation of carcinogenesis. The hormonal environment is one systemic factor that modulates the physiological demand for the PTEN protein (Mutter et al. 2000, Mutter 2001). In abnormal hormonal conditions, such as protracted estrogen exposure, a mutation in PTEN could initiate the genesis of pre-cancerous lesions. PTEN mutations are believed to precede MMR in the progression of sporadic ECs (Zhou et al. 2002). PTEN mutations occur in 60–86% of MSI-positive endometrioid EC cases, but only in 24–35% of MSI-negative tumors (Okuda et al. 2010).

Somatic mutations in KRAS have been described in EC and it seems that these mutations are also an early event, since they have been described in atypical hyperplasias (Enomoto et al. 1991, Mutter et al. 1999). Endometrial hyperplasia loses its ability for DNA mismatch repair prior to acquiring a mutation in KRAS and thus MSI appears to precede KRAS mutations in the progression of EC (Cohn et al. 2001).

CTNNB1 encodes β-catenin, which is known to form a complex with E-cadherin on the cell membrane; the complex is essential for cell differentiation and the maintenance of normal tissue architecture. A β-catenin mutation leads to cytoplasmic and nuclear β-catenin accumulation and constitutive target gene activity (Bansal et al. 2009, Okuda et al. 2010). This nuclear accumulation is seen in endometrioid lesions, as well as in hyperplasias, suggesting that β-catenin plays a role in the early development of EC tumorigenesis. CTNNB1 mutations have been found to be significantly more common in patients younger than age 60 and these mutations have been associated with a low tumor grade (Byron et al. 2012). There is, however, no correlation between β-catenin mutations and MSI, KRAS or PTEN mutations (Okuda et al. 2010).

MSI is a result of unrepaired errors that arise during DNA replication. Typical of this phenotype is a high frequency of mutations at sites with short nucleotide repeats (microsatellites) within the genome (O’Hara and Bell 2012). An aberration in one of the MMR genes, the normal function of which is to preserve genetic stability, prevents accurate repair of the base mismatches produced during DNA replication, leading to an altered length of the DNA chain, a phenomenon called MSI. MSI can lead to an increased frequency of errors in target genes involved in carcinogenesis and result in malignant transformation of the cell. In sporadic EC, MSI positivity reflects this increased mutation rate resulting from somatic alterations in these MMR genes. In EC
tumors, the germline defects in DNA MMR genes mainly involve $MLH1$ and it has been shown that the mechanism silencing the $MLH1$ gene is promoter hypermethylation. Hypermethylation of $MLH1$ has been found in 40.4–91% of sporadic EC samples (Banno et al. 2004, Esteller et al. 1999). As $MLH1$ promoter hypermethylation has also been documented in 3% of complex hyperplasia cases and in 33% of atypical endometrial hyperplasia cases, it may be an early event in the pathogenesis of EC (Esteller et al. 1999). The loss of DNA mismatch repair function is proposed to lead to mutations of other genes such as $PTEN$ and $KRAS$ (Bansal et al. 2009).

Table 3. The frequency rate of MSI and the 10 most common genomic and proteomic aberrations among endometrioid EC (O’Hara and Bell 2012)

<table>
<thead>
<tr>
<th>Tumor characteristic</th>
<th>Frequency (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN mutation</td>
<td>26–79</td>
</tr>
<tr>
<td>ERBB2 amplification</td>
<td>1–63</td>
</tr>
<tr>
<td>E-Cadherin negative expression</td>
<td>5–53</td>
</tr>
<tr>
<td>PIK3CA mutation</td>
<td>20–52</td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>10–50</td>
</tr>
<tr>
<td>CTNNB1 mutation</td>
<td>2–45</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>8–43</td>
</tr>
<tr>
<td>PIK3R1 mutation</td>
<td>21–43</td>
</tr>
<tr>
<td>ARID1A mutation</td>
<td>40</td>
</tr>
<tr>
<td>p16 positive expression</td>
<td>5–38</td>
</tr>
<tr>
<td>MSI+</td>
<td>20–23</td>
</tr>
</tbody>
</table>

2.7  Mesenchymal tumors

2.7.1  Smooth muscle tumors

2.7.1.1  Leiomyoma uteri

Sporadic LMs are the most common benign pelvic tumors in women, occurring in 20–40% of women in their reproductive years (Wallach and Vlahos 2004). In a systematic histological examination of hysterectomy specimens, the prevalence of LMs has been found to be as high as 77% (Cramer and Patel 1990). LMs originate from smooth-muscle cells of the myometrium. In some cases, LMs arise from the smooth-muscle cells of uterine blood vessels (Wallach and Vlahos 2004). They are usually
slow-growing, related to genetic predisposition and derived from single-cell clones generated independently without invasive and metastatic features. They may be solitary or multiple and can be located within the myometrium, externally extending to the serosa, internally impinging to the uterine cavity or be pedunculated.

LMs are estrogen-dependent tumors; they decrease in size during menopause and under hypostrogenic conditions (Ciarmela et al. 2011). Concurrent with the growth of a LM there are also a variety of growth factors, their receptors and related proteins. A hereditary predisposition to LMs is suggested, since LMs were found 2.2 times more frequently among first-degree relatives in families with two or more verified LM cases (Vikhlyaeva et al. 1995). A rare syndrome of hereditary LM and renal cell cancer has been revealed; this disease is an autosomal dominant disorder characterized by smooth-muscle tumors of the skin and uterus and renal cancer (Lehtonen et al. 2006). The gene that causes predisposition to the disease has been identified as fumarate hydrase. These patients have a significantly higher risk for developing several benign tumors, such as LMs, but also a 71-fold increased risk for LMs. It seems that LMs are driven by stress arising from oncogenic activation (MED12 mutation) or severe metabolic aberrations, such as those arising from fumarate hydrase deficiency. LMs may also be caused by specific chromosomal changes (Mehine et al. 2013). These chromosomal changes commonly result from interconnected complex chromosomal aberrations resembling chromothripsis.

Malignant transformation of LMs is extremely rare and it is believed that LMSs arise de novo and may be unrelated to benign LMs (Wallach and Vlahos 2004). It has been estimated that a risk of < 0.1% exists for the progression of LMs to LMSs (Hodge and Morton 2007). Typical of benign LMs, they lack coagulative tumor cell necrosis and significant atypia but may have any mitotic index (Kempson and Hendrickson 2000). LMs are called mitotically active when there is neither coagulative tumor cell necrosis nor significant atypia, but the mitotic index can range up to >10 mf/10 hpf. These LMs behave in a benign fashion even if the mitotic index extends to 20 mf/10 hpf. In atypical LMs focal moderate- to -severe cytologic atypia can be found without tumor cell necrosis and a mitotic index less than or equal to 10 mf/10 hpf. When atypia is found to be diffuse in these tumors with less than 10 mf/10 hpf and no cell necrosis, the tumor can be called atypical LM and is characterized by a low risk of recurrence. If the tumor shows tumor cell necrosis, absent to mild atypia and a mitotic index less than 10 mf/10 hpf, the tumor can be characterized as a smooth-muscle tumor with a low malignant potential (Ip and Cheung 2011).

Furthermore, the growth of some benign LMs can be confused with a malignant behavior and challenge the diagnosis. These very rare cases are shown in table 4.
Table 4. Rare subtypes of leiomyoma (Brown 2008, Kempson and Hendrickson 2000)

- Parasitic leiomyoma
- Benign metastasising leiomyomatosis
- Intravascular leiomyoma
- Lipoleiomyoma
- Neurilemmoma-like
- Epitheloid or myxoid differentiation

2.7.1.2 Smooth muscle tumors of unknown malignant potential

Some LMs cannot be reliably diagnosed as being either benign or malignant based on current diagnostic criteria. These LMs that show focal or extensive necrosis can be difficult to differentiate from tumor necrosis. Thus, the term “smooth-muscle tumors of uncertain malignant potential” (STUMP) has been coined to describe those tumors that may show increased mitotic activity and atypia, but where tumor necrosis is undetectable, or other aspects of differentiation increase suspicion of malignancy. Therefore, when a tumor shows any unusual combinations of histologic features that do not satisfy the criteria for LMs, a diagnosis of STUMP is appropriate. Since the definition of STUMP varies in different studies, it may be difficult to interpret the recurrence rate, but cases report recurrence ranges 0% to 26.7%. In most studies, recurrence is rare and delayed, with a median survival of at least 63.9 months. Management of these tumors is the same as that of LMs, but follow-up is recommended (Gadducci et al. 2008)

2.7.1.3 Leiomyosarcoma

LMS is defined as a malignant neoplasm composed of cells with smooth-muscle differentiation (Trope et al. 2012). LMSs exhibit marked cellular atypia, a high mitotic index and tumor cell necrosis. LMS is the most common pure mesenchymal uterine tumor (Brown 2008). According to a large Norwegian study of 1042 uterine sarcomas (US), the most common sarcoma is LMS which accounts for 41% of all cases (Nordal and Thoresen 1997). The median age in a study of 1396 women with LMS was 52 years (range: 18–92 years) (Kapp et al. 2008). LMSs are usually solitary intramural masses and mimic LM clinically. The tumor is often a large irregular mass containing hemorrhage and necrosis (Tirumani et al. 2013). The symptoms of LMS are very much the same as those of LM; pelvic or abdominal pain, vaginal bleeding and a growing uterine mass (Leibsohn et al. 1990). LMSs are usually diagnosed unexpectedly, discovered incidentally on histopathology review following hysterectomies for supposedly benign LM. In a study of Parker et al. (1994) the reported incidence for US among patients
operated on LMs was as low as 0.23%. An accurate pre-operative diagnosis of USs can be problematic depending on the US subtype. Pre-operative endometrial curettage sampling correctly predicted histologic subtype in 66% of US cases and pipele sampling in 61% of US cases (Bansal et al. 2008). Endometrial sampling reliably predicted the correct histologic diagnosis in 66.7% of patients with LMSs. Transvaginal ultrasond is the standard imaging technique, but magnetic resonance imaging (MRI) of the pelvis optimizes image evaluation of the invasion into adjacent structures of the pelvis and myometrial involvement (Tirumani et al. 2013). In a study by Umesaki et al. (2001) five women with US underwent positron emission tomography (PET), MRI and power Doppler ultrasound, with positive findings of 100%, 80% and 40% of cases, respectively. PET can aid in pre-operative work up by detecting metastatic lesions, but the main role of PET is in post-therapy surveillance to detect metastasis or local recurrence (Tirumani et al. 2013). In rare tumors like US, technical imaging like PET and MRI is still expensive and time-consuming. Therefore, there is still a need for effective and inexpensive pre-operative diagnostic tools.

The overall 5-year survival rate for patients with stage I (IV) LMS varies in different studies between 51% and 65% (0% and 28%) (Abeler et al. 2009, Kapp et al. 2008, Nordal and Thoresen 1997). The reported risk of recurrence varies from 45% to 73% (Wu et al. 2011). The most important prognostic factors associated with significantly poorer survival rates in different studies are lack of primary surgical treatment, disease stage and grade, age, race, increasing parity, mitotic count and tumor size (Abeler et al. 2009, Kapp et al. 2008, Koivisto-Korander et al. 2008).

2.7.2 Endometrial stromal neoplasias

2.7.2.1 Endometrial stromal nodule

These rare tumors are composed of cells reminiscent of proliferative-phase endometrial stromal cells. They can occur at any age and are mostly incidental findings in a hysterectomy specimen. The tumors are usually solitary, round, not encapsulated and non-infiltrating. They can locate in the endometrium, where they are polypoid, however they may be intramyometrial or subserosal. No vascular invasion can be found and the sometimes seen finger-like projections into the myometrium do not exceed 3 mm. Endometrial stromal nodules have an excellent prognosis and patients can be cured by hysterectomy. (D’Angelo and Prat 2010.)
2.7.2.2 Endometrial stromal sarcoma, low grade

Endometrial stromal sarcoma (ESS) arises typically from the cells that resemble the stromal cells of the proliferative endometrium (D’Angelo and Prat 2010). ESS is usually a low-grade sarcoma with low mitotic activity. Typical of ESS are the irregular, nodular, worm-like growths infiltrating into the endometrium, myometrium and the parametrial veins and lymphatics. Necrosis is rarely seen. ESSs are very rare malignant tumors comprising, about 0.2% of all uterine malignances. Compared with other uterine malignancies, ESS affects younger women with a mean age of 40–58 years. ESS typically has a favorable prognosis and long-term survival, but has a propensity for late metastases and about 37–60% eventually recur after a long time (Gadducci et al. 2008). The 5-year survival rate in stage I ESS is 85%, whereas in stage IV the 5-year survival rate is 37% (Nordal and Thoresen 1997). In a Finnish study of US patients, the 5-year survival rate was found to be 65% in cases of ESS (Koivisto-Korander et al. 2008). The pathogenesis is unknown, but ESS is associated with unopposed estrogen, polycystic ovarian disease and tamoxifen therapy (Tirumani et al. 2013, Wu et al. 2011). These malignancies seem to be hormone sensitive. In a study of 21 ESS patients, estrogen receptor expression was found in 71% of tumors and progesterone receptor expression was found in 95% of tumors (Reich et al. 2000). This finding may have implications for hormone therapy in the management of these tumors. The prognosis of ESS is related to age, mitotic index, tumor cell necrosis, grade, cellular atypia, parity and tumor size (Abeler et al. 2009, Koivisto-Korander et al. 2008, Trope et al. 2012).

2.7.2.3 Undifferentiated endometrial sarcoma

The highly mitotically active form of ESS, previously called high-grade endometrial stromal sarcoma, is now termed as undifferentiated endometrial sarcoma (Gadducci et al. 2008). These tumors exhibit myometrial invasion, severe nuclear pleomorphism, high mitotic activity (10–50 mf/10 hpf) and tumor cell necrosis, and they lack smooth muscle or endometrial differentiation (D’Angelo and Prat 2010). Undifferentiated endometrial sarcomas have very poor prognosis, with 5-year survival rates ranging from less than 25–55% (Gadducci et al. 2008).
2.8  Mixed epithelial and mesenchymal tumors

2.8.1  Carcinosarcoma (or Mixed Müllerian Mesodermal Tumor)

Carcinosarcoma, previously called Mixed Müllerian Mesodermal Tumor (MMMT), represents 4% of malignancies of the uterine corpus (Wu et al. 2011). MMMT is usually defined by a biphasic mixture of epithelial malignancy, usually endometrioid adenocarcinoma with a malignant stromal component. It is divided into homologous (in which the sarcomatous component is made of endometrial, fibrous and/or smooth-muscle tissues found in the uterus) or heterologous (when it is made of tissues outside the uterus like bone, cartilage and/or skeletal muscle) classes. MMMTs appear as bulky, polypoid masses that can prolapse through the cervical canal and are associated with obesity, estrogen use and tamoxifen therapy. In contrast to typical sarcomas, dissemination occurs mainly through lymphatic channels, as other sarcoma types spread mainly hematogenously (Tirumani et al. 2013, Brown 2008). Today, MMMTs are regarded as a subtype of endometrial carcinoma (Trope et al. 2012). Although MMMTs have an epithelial origin, they have a more aggressive tumor behavior than high-risk epithelial carcinomas, mainly due to a higher incidence of pulmonary metastases after primary surgery (Amant et al. 2005). The peak incidence of MMMT is 5–10 years later than in of LMS or ESS, being about 65–79 years (Nordal and Thoresen 1997). Painful postmenopausal vaginal bleeding is the most frequent symptom, occurring in more than 85% of these women (Wu et al. 2011). The 5-year survival rate in cases of MMMT has been reported to be 49% (Koivisto-Korander et al. 2008). The 5-year survival rate decreased in another study from 62% in stage I to 17% in stage IV (Nordal and Thoresen 1997). The most important prognostic factor is the tumor stage; the clinical outcome is poor when the tumor has extended beyond the uterus. Some authors have reported that the presence of heterologous component is associated with a poorer prognosis compared with the homologous component, but other studies have failed to detect any difference. Lymph-node metastases and a higher grade of sarcoma are associated with a poor prognosis according to Gadduci et al. (2011). Two mutation profiles have been associated with carcinosarcoma, an endometrioid profile (ARID1A, PTEN, PIK3CA, KRAS) and a serous mutation profile (TP53) (McConechy et al. 2012). This fact suggests a dualistic molecular evolution of MMMT with an endometrioid-like or a serous-like mutation pattern.
2.9 Lynch syndrome-related cancers

Lynch syndrome (LS), also called hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominantly inherited disorder that is characterized by an early onset of colorectal cancer (CRC) and is associated with extracolonic cancers of the endometrium, ovary, small bowel, stomach, renal pelvis, ureter, brain and skin (Lynch and Smyrk 1996, Watson et al. 2008). The diagnosis of LS is usually based on family history and clinical criteria, such as the Amsterdam II criteria or Bethesda guidelines (Vasen et al. 1999, Umar et al. 2004). LS is the result of a germline mutation in a class of genes involved in DNA MMR, including $MSH2$, $MLH1$, $MSH6$ and $PMS2$ (Vasen et al. 1999).

2.9.1 Lynch syndrome related endometrial cancer

Whereas the cumulative lifetime risk for EC in the general population is 3%, for women with a genetic predisposition to LS the cumulative lifetime risk for EC is 27–71% (Koornstra et al. 2009). In many of these women EC is the first or sentinel cancer to develop. The incidence of LS in EC varies in different studies between 0.5 and 2% (Banno et al. 2004, Goodfellow et al. 2003, Hampel et al. 2006, Ollikainen et al. 2005). In a study of Obermaier et al. (2010), the 10-year cumulative risk of EC following colorectal cancer in the LS group was 23.4%, compared with a corresponding risk of 1.6% for the non-LS group. Affected patients are at risk for multiple synchronous and metachronous tumors (Aarnio et al. 1999). The lifetime risk of LS patients for ovarian cancer is 12% and 25% of the ovarian cancer cases are reported to be synchronous with EC (Schmeler et al. 2006). The only known cause for tumors in LS patients is an inherited mutation in DNA MMR genes mainly including $MLH1$ (Bronner et al. 1994) and $MSH2$ (Fishel et al. 1993) and families with excess EC also $MSH6$ (Cederquist et al. 2004, Wijnen et al. 1999). Mutations in $MSH3$ are also observed (Taylor et al. 2006). In a minority of cases, mutations in $PMS1$ and $PMS2$ have also been found (Vasen et al. 1999). In a retrospective cohort study of 112 LS-associated colorectal carcinoma patients who had subsequeent EC, the majority of the mutations occurred in either the $MLH1$ gene (50%) or the $MSH2$ gene (41%) (Obermair et al. 2010). $MSH6$ mutations were observed in 9.1% in the LS group, compared with 2.2% in the non-LS group. On the other hand, in a study of Broaddus et al. (2006), a group of 50 LS women with diagnosed with EC, most had $MSH2$ mutations (n=47) and only few were $MLH1$ mutation carriers (n=3). Germ-line $MSH6$ mutations appear to be associated with later age of onset of malignancy than $MLH1$ and $MSH2$ mutations.
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and MSH6 appears to confer a particular risk for EC (Cederquist et al. 2004, Wagner et al. 2001, Wijnen et al. 1999). Women with MSH6 mutations were noted to have two-fold greater risk for EC compared with cohorts with mutations in MLH1 and MSH2 (Hendriks et al. 2004). One cohort study of MSH6 mutations in the EC samples of LS women revealed that 71% of these patients were older than 50 years (Goodfellow et al. 2003). Taylor et al. (2006) provided evidence that MLH3 mutation also plays a role in EC. In their study, they noted that both germline and somatic mutations in MLH3 could lead to EC tumorigenesis. It seems that abnormality in the MMR may be an early event of endometrial carcinogenesis in LS patients. Decreased MMR protein expression has been detected as early as 12 years before EC, and complex hyperplasia without atypia and complex hyperplasia with atypia were found to be equally important as precursor lesions in these patients (Nieminen et al. 2009).

PTEN alterations also occur at high frequency in LS-related ECs. Distinct from sporadic ECs, PTEN mutations are particularly frameshift mutations that arise in individuals with LS, a consequence of profound DNA MMR deficiency (Zhou et al. 2002). Zhou and coworkers postulated that LS ECs arise because of germline mutations in one of the MMR genes, followed by somatic inactivation of other MMR genes. Because of this profound MMR deficiency, subsequent somatic mutations occur in other target genes, such as PTEN. In contrast, in sporadic ECs, somatic PTEN mutations are one of the earliest events that may precede MMR deficiency. In sporadic ECs and in LS related ECs β-catenin proteins, which are normally located in the plasma membranes, increase in amount in the cytoplasm with subsequent nuclear translocation and accumulation. This fact was evident in a study of Kariola et al. (2005) as they observed abnormal β-catenin nuclear expression in 53% of LS EC samples. It seems that PTEN alterations are also the most common changes in LS-related ECs, whereas PIK3CA mutations play a minor role and the effects of KRAS mutations are equal in LS-related and sporadic ECs. Table 5 lists the differences in mutation profiles between EC tumors presenting germline MMR gene mutation and tumors with no MMR gene mutation.
Table 5. Proportion of endometrial tumors with alterations in PTEN, MSI, PIK3CA and KRAS (Ollikainen et al. 2007)

<table>
<thead>
<tr>
<th>Tumor characteristic</th>
<th>MMR gene mutation absent</th>
<th>MMR gene mutation present</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN expression reduced</td>
<td>50%</td>
<td>70%</td>
</tr>
<tr>
<td>MSI +</td>
<td>23%</td>
<td>63%</td>
</tr>
<tr>
<td>PIK3CA mutation</td>
<td>7%</td>
<td>12%</td>
</tr>
<tr>
<td>PIK3CA amplification</td>
<td>24%</td>
<td>5%</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>10%</td>
<td>10%</td>
</tr>
</tbody>
</table>

There are several ways to improve the identification of LS. Low body mass index, an age under 50 years and a positive family history have all been identified as risk factors in EC patients who might benefit from LS screening. The fact that MSH6 mutations carriers tend to develop EC later than MLH1 or MSH2 mutation carriers, does unfortunately not justify an age of 50 for referring patients for genetic consultations (Hampel et al. 2006). In a prospective LS screening study, 562 women with EC had a mean age of 54 years at the time of diagnosis of LS. If these authors had used 50 years as the screening cut off, 69% of patients would have been missed. Therefore, the revised guidelines for the clinical management of LS recommend immunohistochemistry of the four MMR proteins or MSI analysis for all women with EC under 70 years (Vasen et al. 2013). There are some limitations in primary MSI testing, because positive MSI will not discriminate between MSI presence as a result of epigenetic silencing of MLH1 or a loss of function of the MRR protein. It also seems obvious that MSI has a lower predictive value in MSH6 mutation carriers, because more of these tumors may be microsatellite stable. In a study of 543 EC patients, MSI was identified in 22% of cases and 94% of these MSI cases were due to methylation of MLH1 (Hampel et al. 2006). Of the MSI-positive cases 75% demonstrated a lack of MLH1 expression by IHC. The recommend immunohistochemistry tests should be accompanied by methods that identify MLH1 promotor methylation (Vasen et al. 2013). Some pathological factors associated with EC have been evaluated with respect to their ability to predict the presence or absence of LS. The typical features for LS-related EC are listed in Table 6.
Broaddus et al. (2006) found the trend that LS patients have more non-endometrioid tumors compared with sporadic EC patients. While all non-endometrioid tumors (14%) were in the MSH2 group, non-endometrioid tumors were extremely rare in the sporadic, MSI-positive, MLH1 methylated group. Their data indicate a genotype-phenotype relation in which MSI resulting from MLH1 methylation is associated with endometrioid tumors and MSH2 mutation can result in a more variable histological spectrum of EC. The prognosis of LS-related EC varies in different studies and there are data of LS-related EC having a better (Wang et al. 2009), equal (Boks et al. 2002, Carcangiu et al. 2010) or poorer (van den Bos et al. 2004) prognosis as compared with sporadic EC.

2.9.2 Lynch syndrome-related colorectal cancer

LS accounts for 2–4% of all CRCs and the lifetime risk of CRC is estimated to be 50–80% (Jasperson et al. 2010). CRCs and polyps arise in LS patients at a younger age, and a more proximal location is more common compared to sporadic CRCs. The mean age at CRC diagnosis is 43–46 years in MSH2 or MLH1 mutation carriers vs. 51–57 years in MSH6 mutation carriers (Peltomäki 2005). Mutations of MSH2 and MLH1 account for up to 90% of LS cases. MSH6 mutation carriers have a slightly lower CRC risk compared to MSH2 and MLH1 carriers. These cancers are histologically poorly differentiated, mucinous and have large numbers of tumor-infiltrating lymphocytes (Jasperson et al. 2010). Regular colonoscopy leads to reduction of CRC-related mortality. Therefore, the recommended interval for mutation carriers is 1–2 years and
should be started at the age of 20–25 (Vasen 2013). Also longterm aspirin use has been shown to reduce CRC incidence in LS patients.

2.10 Epithelial ovarian tumors

Epithelial ovarian tumors can be divided into benign tumors, borderline ovarian tumors (BOTs) or OC (Lalwani et al. 2010). BOTs comprise up to 14% of all epithelial ovarian malignancies (Auranen et al. 1996). These tumors are relatively uncommon with an incidence of 1.8 per 100000 women-years in Finland. Ovarian and fallopian tube cancer constitute the eighth most common cancer type among women worldwide with a standardized incidence rate of 6.3 per 100,000 women (Weiderpass and Labreche 2012). In a large Nordic female cancer survival study, OC was found to have the lowest survival rates among genital cancers (Klint et al. 2010). The relative survival rate during the years 1999–2003 in Finland was 44%. Standard first-line treatment, surgery and six courses of platinum-based chemotherapy results in complete clinical remission in up to 75% of cases, but 75% of the responders will relapse within a median time of 18–28 months (Mei et al. 2013).

Symptoms of ovarian tumors are non-specific, including diffuse abdominal complaints, changes in bowel habits, newly occurred meteorism and unexplained weight loss and massive abdominal swelling (Lalwani et al. 2011). Therefore, 70% of OCs are not diagnosed until the cancer has reached an advanced stage (stages IIB–IV) (Heintz et al. 2006). The 5-year survival rates in these cases are less than 40% while a diagnosis at earlier stage (stage I–IIA) yields a survival rate of 80%. Transvaginal ultrasound is the diagnostic tool of choice for determining whether ovarian tumors are benign or malignant. For differential diagnosis between OC and gastrointestinal tumors, computed tomography or MRI may be useful (Kinkel et al. 2005). However, in advanced OC, both of these procedures tend to underestimate peritoneal and mesenteric carcinomatosis and surgery is still the most important procedure for staging OC (Salani et al. 2008). Serum biomarkers, such as CA125 and HE4, are aimed at detecting OC at an earlier stage. The highest true CA125 positive results have been seen in patients with serous and metastatic tumor types (Vranes et al. 2012). CA125 is the most used marker to distinguish malignant from benign ovarian tumors and it is commonly used in the follow-up of patients after treatment, monitoring disease response to chemotherapy and for estimating prognosis. Unlike CA125, HE4 does not increase in ovarian endometrioma or any other types of endometriosis (Huhtinen et al. 2009) and it is also less frequently elevated in other benign conditions (Moore et al. 2012b) or in pregnancy (Moore et al. 2012a). HE4 levels increase with patient age and
the median serum levels are higher in postmenopausal women compared with their premenopausal counterparts (Lowe et al. 2008, Moore et al. 2012a). Therefore, HE4 gives some added value, particularly when used for testing premenopausal women.

2.10.1 Borderline ovarian tumors

BOTs are histologically characterized as epithelial tumors with a stratified growth pattern. The main difference from ovarian carcinoma is the absence of stromal invasion and typically the age of presentation of BOTs is approximately 20 years earlier than that of ovarian carcinoma (Lalwani et al. 2010). The overall survival of BOTs, even with peritoneal spread, is better compared with that of carcinoma. The 5-year and 10-year survival rates for stage I, II and III disease are 99% and 97%, 98% and 90%, and 96% and 88%, respectively. Like OCs, BOTs can also spread to the peritoneum and rarely to the lymph nodes. Peritoneal disease from BOTs is referred to as implants and these implants can be non-invasive (about 85% of implants) or invasive. Residual disease is an important prognostic factor and therefore operative treatment with salpingo-oophorectomy with or without hysterectomy and removal of all the peritoneal implants is the standard surgical procedure. BOTs are classified on the basis of histopathology and histogenesis into serous, mucinous, endometrioid, clear cell and transitional (Brenner) subtypes (Morice et al. 2012).

2.10.1.1 Serous borderline ovarian tumors

Serous BOTs (SBOT) are the most common BOTs, accounting for 65% of all BOTs (Lalwani et al. 2010). They are slow-growing neoplasms and the mean age range of presentation is 34–40 years. SBOTs are divided into two subtypes: the typical subtype (90%) and the micropapillary subtype (10%). Most SBOTs are thought to arise from serous cystadenoma or adenofibroma and some of them can progress to invasive low-grade serous carcinoma (LGSC). Micropapillary tumors, have more ovarian surface involvement, a higher frequency of advanced stage and are more often bilateral compared to the typical subtype (Burks et al. 1996, Eichhorn et al. 1999). Micropapillary tumors also more often have invasive implants, and this form of SBOT seems to have more often an unfavorable outcome than the typical SBOT (Eichhorn et al. 1999, Kurman et al. 2005). However, micropapillary tumors that are in stage I or accompanied by noninvasive peritoneal implants, do not have a worse prognosis than the typical subtype.
Up to 50% of SBOTs are characterized by \textit{BRAF} mutations and \textit{KRAS} mutations are found in more than a third of SBOTs (Lalwani et al. 2010). Invasive or non-invasive peritoneal implants may occur in 35% of cases and lymph node involvement may be seen in 27% of SBOTs. The prognostic factors for invasive recurrence in SBOT include invasive implants, peritoneal residual disease, micropapillary patterns and stromal microinvasion (Morice et al. 2012). Furthermore, surgical procedures, such as intraoperative tumor rupture or incomplete staging are important predictive factors for tumor recurrence (Ewald-Riegler et al. 2012). Fertility-conserving surgery seems to be more related to recurrences than bilateral salpingo-oophorectomy (Avril et al. 2012, Cadron et al. 2007).

2.10.1.2 Mucinous borderline ovarian tumors

Mucinous BOTs (MBOTs) comprise about 32% of all BOTs (Song et al. 2012). MBOTs, compared with SBOTs, are often larger in size (mean diameter: 15.6 cm) and therefore patients have more often symptoms such as a palpable abdominal mass or pain. These tumors are divided into two histological subtypes: the intestinal (90%) and the Müllerian (10%) histotypes (Lalwani et al. 2010). Characteristic features of the intestinal type include more frequent unilateral localization of the tumor and large multilocular cystic lesions that are associated with pseudomyxoma peritonei. About 90% of intestinal MBOTs are unilateral and, if the tumor is bilateral, metastatic tumors of the gastrointestinal sites, appendix, pancreas or endocervix should be excluded (Hart 2005). Other features favoring an ovarian primary origin include a large size (> 12 cm), a smooth outer surface, no surface or hilar involvement, and no extensive necrosis or vascular invasion. Extensive intra-abdominal spread is rarely found in MBOTs (Leen and Singh 2012). Extra-ovarian spread is found seldom, only in about 2% of patients (Morice et al. 2012). The Müllerian subtype is bilateral in up to 40% of patients and coexists with ipsilateral ovarian or pelvic endometriosis in 20–30% of patients. Like SBOTs, the Müllerian subtype of MBOTs is also associated with invasive or non-invasive peritoneal implants. The precursor lesions are mucinous cystadenomas, and there are sometimes endometriotic cysts in the Müllerian subtype (Hart 2005). MBOTs can stepwise progress from intraepithelial carcinomas to invasive mucinous carcinomas. Mutations in the \textit{KRAS} genes occur in more than 60% of MBOTs (Fauvet et al. 2011). This high frequency of \textit{KRAS} mutations has also been found in several mucinous cystadenomas and carcinomas. Like SBOTs, MBOTs are found to react more aggressively in pregnant patients with a higher stage of an intraepithelial carcinoma or microinvasion (21%). MBOTs are typically found in stage I, where the 5-year survival
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Rates are excellent: 99–100% (Hart 2005). Pure high-stage MBOTs are uncommon as they tend to contain invasive carcinomas with infiltrative patterns and can be fatal. However, the rate of progression to carcinoma is found to be less than 2% (Zanetta et al. 2001).

2.10.2 Epithelial ovarian carcinoma

Epithelial ovarian carcinoma has several histological subtypes that show characteristic cytogenetic features, molecular signatures, oncologic signaling pathways and clinical-biological behaviors (Lalwani et al. 2011). These tumors account for 90% of all OC cases, and they are divided on the basis of pathogenetic mechanism into two categories. Type I carcinomas, commonly arise from genetically stable precursor lesions, such as from BOTs. They have a relatively indolent clinical course, grow slowly, have a low mitotic index, are found at early-stage disease and have an overall good prognosis. The histological types of type I carcinomas are low-grade serous, endometrioid, clear-cell and mucinous. The type II carcinomas, in contrast, have a more aggressive behavior. These tumors are thought to originate from the adnexal epithelium, grow rapidly with a high mitotic index, often demonstrate chromosomal instability, and are diagnosed at an advanced stage and thus have a poorer prognosis. Type II carcinomas include high-grade serous and endometrioid types, malignant mixed mesodermal tumors and undifferentiated carcinomas (Kurman and Shih 2008). Inherited OC accounts for 5–10% of all OCs (Tagliaferri et al. 2009). Germline mutations of BRCA1 and BRCA2 tumor suppressor genes are responsible for 90–95% of the hereditary OCs. These patients have a 20–50% life time risk of developing OC. BRCA-associated OCs are typically high-grade serous carcinomas (HGSCs) but, compared with non-mutation carriers, BRCA survival rates are better (Ben David et al. 2002). LS patients have a lifetime risk of 10–12% for developing OC (Prat et al. 2005).

2.10.2.1 High and low-grade ovarian serous carcinoma

Previously there used to be at least three common grading systems for ovarian serous cancer (FIGO, WHO and Silverberg’s system) (Rosen et al. 2010). However, recent advance in molecular genetics demonstrated that ovarian serous carcinoma may develop along two distinct pathways which lead to the low-grade and the high-grade subtypes. The most common histological subtype is HGSC accounting for over 50% of all ovarian carcinomas (Lalwani et al. 2011). HGSCs appear as complex cystic-solid or
entirely solid masses in imaging and 84% of these tumors are bilateral. The mean age at presentation is 55–65 years (Vang et al. 2009). With most patients (80%), the disease is found at an advanced stage with widespread peritoneal or lymphatic metastases (Prat 2012). HGSC cells, in contrast with LGSC cells, show nuclear pleomorphism, and the median mitotic index is found to be 38 mfs/10 hpfs (range: 14–137 mf/10 hpf) (Malpica et al. 2004). The prognosis is poor, with a 5-year survival rate for stage I HGSC 9–34% (Vang et al. 2009). Up to 80% of HGSCs show initial response to platinum-based chemotherapy, but about 70% may demonstrate recurrence (Lalwani et al. 2011).

LGSCs account for about 10% of all OCs. These tumors appear often as bilateral (74%–77%) large cystic neoplasms with septa and solid components inside the cysts (Lalwani et al. 2011). The mean ages at presentation are 45–57 years, slightly younger than individuals with HGSCs (Vang et al. 2009). LGSCs are relatively slow-growing and usually follow a relatively indolent course (Prat 2012). They frequently have a non-invasive SBOT component and most likely represent the progression of SBOTs beyond microinvasion. Typical microscopy finding includes small papillae of tumor cells exhibiting uniform nuclei and hyalinized stroma that often contains psammoma bodies. LGSCs, compared with HGSC, have only infrequent mitotic figures (mean 4 mfs/10 hpfs) and uniform nuclei (Malpica et al. 2004). The 5-year survival rate for stage I LGSC is 40–56% (Vang et al. 2009).

The precursors of LGSCs are still thought to be ovarian inclusion cysts that develop from ovarian surface epithelium and then undergo tubal metaplasia. LGSCs are purported to develop through a slow stepwise process from cystadenoma/adenofibroma to typical SBOT and finally to invasive LGSCs. Another theory is, that LGSCs are of tubal origin. Because of a disruption of the ovarian surface epithelium due to ovulation or inflammation, benign tubal mucosa may be implanted on the ovary and become entrapped within the ovarian cortex. This hypothesis is supported by the finding, that normal fallopian tube-like cells can be found in some inclusion cysts. According to the papillary tubal hyperplasia pathway theory, chronic inflammation induces proliferation of the tubal mucosa, which progresses to papillary tubal hyperplasia. The resultant epithelial clusters and papillae then shed and implant on the ovary and peritoneum and form peritoneal implants and SBOTs. These lesions may further develop to non-invasive micropapillary serous carcinomas and eventually to invasive LGSCs (Vang et al. 2013). LGSCs are genetically relatively stable and typically display a variety of mutations related to KRAS or BRAF; mutations related to TP53 are rare (Li et al. 2012). KRAS and BRAF mutations have been also detected in BOTs, indicating that they are early events in the carcinogenesis of LGSCs. KRAS mutations have been found in 35% and BRAF mutations in 30% of LGSCs (Singer et al. 2003). Mutations in ERBB2 have been detected in 9% of LGSCs (Li et al. 2012). Thus, mutations of KRAS, BRAF and
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ERBB2 are found in more than 60% of LGSCs. In about 10% or fewer cases, LGSCs can develop into HGSCs upon acquiring a TP53 mutation.

Traditional theories of the pathogenesis of serous ovarian tumors propose that damage to the ovarian surface epithelium occurring at the time of ovulation and cortical inclusion cysts are etiologically important (Kurman and Shih 2011). During the last few years, studies have strongly implied that the majority of ovarian and peritoneal HGSCs originate from the fallopian tube. Implantation of malignant cells in the ovary that derive from tubal origin lead to a tumor mass that gives the impression of a tumor originating from the ovary. Tubal intra-epithelial carcinoma (TIC) has been found to be associated with HGSC not only in BRCA mutation carriers, but also in 63% and in 49% of women with consecutive sporadic peritoneal and ovarian HGSC, respectively (Kindelberger et al. 2007). A high frequency of TP53 mutations has been found in both TIC and synchronous ovarian/peritoneal HGSCs (Carlson et al. 2008, Kindelberger et al. 2007, Kuhn et al. 2012). Significantly higher p53 and γ-H2AX nuclear staining has been found in BRCA1/2-mutation-positive tubal epithelium compared with controls, but this finding was not detected in ovarian epithelium (Staff et al. 2014). Moreover, the gene expression profiles of tubal and ovarian serous carcinoma are similar (Tone et al. 2008) and the expression patterns of ovarian serous carcinoma are more similar to those of normal tubal mucosa compared with normal ovarian epithelium (Marquez et al. 2005). These findings support the theory that HGSC may originate from exfoliated fimbrial cells to the ovary. HGSCs may also develop from ovarian cortical inclusion cysts, derived from implanted fimbrial tubal epithelium (Kurman and Shih 2011).

The principal molecular events involving HGSCs involve the TP53, BRCA1 and BRCA2 genes (Li et al. 2012). Mutations in TP53 have been found in 96% of cases and BRCA1/2 alterations have been found in 22% of tumors, based on a combination of germline and somatic mutations (The cancer genome atlas research network 2011). Like TP53 mutations, BRCA inactivations seem to be a consistent genetic alteration in HGSCs (Bowtell 2010). TP53 mutation is an early event in the genesis of HGSC, leading to TIC in the distal fallopian tube. BRCA loss occurs after TP53 mutations, leading to chromosomal instability and a widespread number of copy changes. The subsequent mutations facilitate tumor progression.

2.10.2.2 Mucinous carcinoma

Mucinous neoplasms account for 10–15% of all ovarian tumors, but primary mucinous ovarian carcinoma (MOC) is rare, accounting for 3–5% of all epithelial ovarian tumors (Lalwani et al. 2011). MOC is often unilateral, appear as large (>13 cm), multilocular
cystic-solid tumors. Metastatic gastrointestinal primary tumors can mimic primary ovarian MOC. However, metastases are typically smaller, bilateral and associated with the presence of pseudomyxoma peritonei. Most patients with MOC are 40–50 years old at the time of diagnosis. MOC is diagnosed mostly at stage I (80%) and therefore have a good prognosis after treatment (>95% survival rate). Compared with HGSC, MOC is less responsive to platinum-based chemotherapy.

MOC appear often as a heterogeneous tumor (Prat 2012). Benign-appearing components, borderline, noninvasive and invasive components are found within an individual tumor, confirming the typical development type of MOC from benign to borderline and eventually to carcinoma. MOC do not display Müllerian phenotype, like serous, endometrioid or clear-cell tumors (Seidman et al. 2010). Instead, mucinous tumors are associated with Walthard cell nests, a benign transitional-type epithelium, frequently found in the tubal-peritoneal junction close to the paraovarian and peritubal locations. These tumors may arise from transitional metaplasia in this tuba-peritoneal junction area. A subset of gastrointestinal-type MOC may arise from ovarian mature cystic teratomas (Vang et al. 2007). MOC can be divided into expansile and infiltrative types (Lee and Scully 2000). The expansile type lacks obvious stromal invasion, while the infiltrative type shows evident stromal invasion and has poorer prognosis. In studies of MOC with extra-ovarian spread, almost all also exhibited infiltrative invasion in the ovary and 94% were fatal. Instead, the risk for recurrence in stage I tumors is small, about 5.8%. The risk increases slowly but progressively in stage II and III categories. MOC is frequently associated with KRAS mutations (The cancer genome atlas research network 2011).

2.10.2.3 Endometrioid and clear-cell ovarian carcinoma

Endometrioid ovarian carcinoma (EnOC) is the second most common OC, accounting for 10–20% of cases (Lalwani et al. 2011, Prat 2012). EnOCs appear as large, mostly unilateral, complex, multisepate cystic neoplasms affecting women in peri- or postmenopausal age. These tumors are associated with ipsilateral ovarian or pelvic endometriosis in up to 42% of cases. Fifteen to 20% of cases are associated with concurrent endometrial carcinoma. EnOCs and clear-cell carcinomas are the most common types encountered in patients with LS (Gras et al. 2001). EnOCs are classified as low- or high-grade tumors, depending on their pathogenetic pathway (Geyer et al. 2009). Five-year survival rates for patients with stage I–IV tumors are 78%, 63%, 24% and 6%, respectively (Lalwani et al. 2011).
Ovarian clear-cell carcinomas (OCCCs) account for about 10% of ovarian carcinomas (Prat 2012). The mean age at presentation is 55 years. These tumors are typically composed of clear cells, growing in a solid, tubular or papillary pattern, with hobnail cells lining the tubules and cysts. The mostly unilateral, large 3–20 cm tumor mass, is typically detected at earlier stages. OCCCs particularly are associated with an increased prevalence of thromboembolic disease in up to 40% of patients (del Carmen et al. 2012). About 40% of OCCCs are found at stage I, when the 5-year survival rate is 69%, the 5-year survival rates for stages III and IV are 14% and 4%, respectively (Lalwani et al. 2011). Even if the diagnoses were made in early stages, OCCCs have a poor prognosis, because these tumors are chemoresistant (del Carmen et al. 2012). OCCCs express significantly higher levels of HIF-1α than other histological OC types and its expression is independent of VHL, suggesting that tumor hypoxia plays a role in the chemoresistance of OCCCs (Lee et al. 2007a).

The precursor lesions for low-grade EnOC are borderline endometrioid tumors and atypical ovarian endometriotic tissue (Kurman and Shih 2008). The ectopic endometrium in women with endometriosis exhibits intrinsic molecular abnormalities, including activation of oncogenic pathways. These changes possibly permit the endometrial tissue to implant, survive and invade on the ovarian surface. OCCCs are also derived from endometriosis in a step-wise progression from adenofibromas and BOTs to OCCCs (Higashiura et al. 2012). This adenofibroma-OCCC sequence develops from non-cystic endometriosis and is associated with adenofibromatous components. Another form arises from ovarian endometriomas and is not associated with an adenofibromatous background, but these two pathways can overlap. Endometriosis-associated OCCC has a high frequency of PIK3CA and ARID1A mutations (del Carmen et al 2012). ARID1A mutations have been found in 46% of OCCCs and in 30% of EnOCs (Wiegand et al. 2010). It seems that ARID1A mutations are an early event in the transformation of endometriosis into cancer, since ARID1A mutations can be seen in atypical but also in benign endometriosis adjacent to OCCC (Wiegand et al. 2010, Xiao et al. 2012). The HNF gene is found to be upregulated in reactive or atypical endometriosis as well as in OCCCs associated with endometriosis (Kato et al. 2006). The overexpression of HNF-1β might enable endometriotic epithelium to acquire resistance to apoptosis and subsequently to develop OCCCs. Mutations of KRAS, BRAF and TP53 are present in low frequency in some OCCCs (Kurman and Shih 2008). Low-grade EnOCs are associated with mutations of KRAS, PTEN and MSI, while overexpression of p53 is seen exclusively in patients with de novo development of high-grade EnOCs with a poor prognosis (Geyer et al. 2009). KRAS and BRAF mutations are reported in about 10% of EnOCs and PTEN mutations are reported in 20–46% of EnOCs. Mutations of β-catenin have been detected in over 60% of EnOC cases and also in endometrioid
borderline tumors (Kurman and Shih 2008). Table 7 summarises the typical clinical and molecular features of all five epithelial OC types.

Table 7. Clinical and molecular features of the five different epithelial OC types: high-grade serous carcinoma (HGSC), low-grade serous carcinoma (LGSC), mucinous OC (MOC), endometrioid OC (EnOC) and ovarian clear-cell carcinoma (OCCC), tubal intra-epithelial carcinoma (TIC), serous borderline ovarian tumor (SBOT), mucinous borderline ovarian tumor (MBOT) (adapted from Prat 2012)

<table>
<thead>
<tr>
<th></th>
<th>HGSC</th>
<th>LGSC</th>
<th>MOC</th>
<th>EnOC</th>
<th>OCCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor lesions</td>
<td>TIC</td>
<td>SBOT, papillary tubal hyperplasia</td>
<td>Cystadenoma/MBOT</td>
<td>Atypical endometriosis</td>
<td>Atypical endometriosis</td>
</tr>
<tr>
<td>Pattern of spread</td>
<td>Early transcoelomic</td>
<td>Transcoelomic</td>
<td>Usually confined to ovary</td>
<td>Usually confined to pelvis</td>
<td>Usually confined to pelvis</td>
</tr>
<tr>
<td>Molecular abnormalities</td>
<td>BRCA, TP53</td>
<td>BRAF, KRAS</td>
<td>KRAS, HIF2</td>
<td>PTEN, ARID1A</td>
<td>HNF1, ARID1A</td>
</tr>
<tr>
<td>Chemosensitivity</td>
<td>High</td>
<td>Intermediate</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Poor</td>
<td>Intermediate</td>
<td>Favorable–Poor</td>
<td>Favorable</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>
3 AIMS OF THE STUDY

The present study was undertaken to evaluate the expression profiles of CAII, CAIX and CAXII in different gynecological tumors. The specific aims were:

1. To investigate the expression of CAII, CAIX and CAXII in the normal mouse uterus, ovary and placenta. (I)
2. To investigate CAIX and CAXII expression in different ovarian tumors. (II)
3. To investigate CAII, CAIX and CAXII expression in the normal human endometrium and uterine tumors. (IV)
4. To investigate CAII, CAIX and CAXII expression in colorectal and endometrial tumors and endometrial hyperplasias in patients with LS. (III, V)
4 MATERIALS AND METHODS

4.1 Tissue samples and collection

4.1.1 Tissue samples for mouse tissue analyses (I)

Two adult Balb/c mice were sacrificed by CO$_2$ asphyxiation followed by decapitation. Uterus, ovary, and placenta samples were collected from both animals. The samples were immersion-fixed overnight in Carnoy’s fluid (ethanol, chloroform and acetic acid (6:3:1). Then, the specimens were treated with absolute ethanol for 30 min, with a 1:1 mixture of ethanol and chloroform for 15 min, and finally with chloroform for 30 min. Paraffin embedding was performed in a vacuum oven for 2 h at +58°C. Paraffin wax was purchased from Fluka Chemie GmbH (Buchs, Switzerland). To collect the placenta sample, a mouse was sacrificed at 9 days of pregnancy. For Western blotting, the uterus, kidney and colon were removed and rapidly frozen in liquid nitrogen. The tissue samples for Western blotting were homogenized with HEPES buffer. The total protein concentration was determined after homogenization using BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. The study protocols were approved by the Animal Care Committee of Tampere University.

4.1.2 Tissue samples for human tissues (II, III, IV, V)

All specimens for IHC were collected during surgery, fixed in formaldehyde and embedded in paraffin. The tissue samples from epithelial ovarian tumors (study II) were obtained during operations carried out at Oulu University Hospital between 1994 and 2002.

For study III the specimens were collected in Jyväskylä Central Hospital between 1979 and 2004. The samples included in the IHC staining were not the same tumors that were studied using microarray analyses. The colorectal tumor samples are presented in table 8. 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
Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome

rectosigmoid colon (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 were at stage B, 16 were at stage C, and 3 were at stage D. The microarray data were produced in an earlier gene expression study for colorectal cancer (Kruhoffer et al. 2005). We reanalyzed the obtained data for CA mRNA expression levels. In total, 113 colorectal specimens were screened for CA mRNA expression levels (table 10). The study was approved by the Ethics Committee of Jyväskylä Central Hospital.

The tissue samples for study IV were obtained during surgical operations carried out at Tampere University Hospital between 1990–2009. Our material consisted of samples that were retrospectively and randomly collected from consecutive patients with uterine tumors. All EC samples were endometrial adenocarcinomas. Of the MMMTs, there were three heterologous and two homologous cases.

The samples from MMR gene mutation carriers were retrospectively collected in 1996–2005 at two participating hospitals (Jyväskylä Central Hospital and Helsinki University Hospital). The samples were either endometrial aspiration biopsies (Pipelle) or hysterectomy specimens. The samples of study V were based on female MMR gene mutation carriers enrolled in a previous clinical surveillance study for EC (Renkonen-Sinisalo et al. 2007). In study V, we used the specimens of these mutation carriers who developed endometrial hyperplasia or EC. Most of them had MLH1 germ-line mutations (Nieminen et al. 2009). The sporadic EC samples were from the same patients used in our previous study IV.

All studies were approved by the local Ethics Committees. The tumor samples are presented in table 8.
Table 8. Tissue samples from studies II–V.

<table>
<thead>
<tr>
<th>IHC Samples/N</th>
<th>Study II</th>
<th>Study III</th>
<th>Study IV</th>
<th>Study V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBOT/3</td>
<td>MSS/43</td>
<td>normal endometrium/9</td>
<td>sporadic EC/30 (CAII, CAIX)</td>
</tr>
<tr>
<td></td>
<td>SBOT/4</td>
<td>MSI/18</td>
<td>EC/30 (CAII, CAIX)</td>
<td>sporadic EC/27 (CAXII)</td>
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<tr>
<td></td>
<td>OCCC/4</td>
<td>LS/16</td>
<td>EC/27 (CAXII)</td>
<td>SH/8</td>
</tr>
<tr>
<td></td>
<td>MOC/7</td>
<td></td>
<td>LM/33</td>
<td>CH/9</td>
</tr>
<tr>
<td></td>
<td>EnOC/14</td>
<td></td>
<td>ESS/10</td>
<td>CAH/17 (CAII)</td>
</tr>
<tr>
<td></td>
<td>OSCC/34</td>
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<td>MMT/10</td>
<td>CAH/19 (CAIX, CAXII)</td>
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<td></td>
<td></td>
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<td>LMS10</td>
<td>LS EC/9 (CAII)</td>
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<td></td>
<td>LS EC/10 (CAIX, CAXII)</td>
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<table>
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<tr>
<th>Gradus/N</th>
<th>OSCC:</th>
<th>ESS:</th>
<th>EC:</th>
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<tr>
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<td>low/29</td>
<td>1/12</td>
</tr>
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<td></td>
<td>2/13</td>
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<table>
<thead>
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<th>normal/17</th>
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<tr>
<td></td>
<td>MSS/56</td>
<td></td>
</tr>
</tbody>
</table>

4.2 Immunohistochemistry

4.2.1 Immunohistochemistry of study I

In the mouse tissues, the localization of CAIX and CAXII was examined by the immunoperoxidase method. Antibodies against CAII and CAXIII were used as positive controls for the immunostaining. All experiments were performed in duplicate and included control staining with nonimmune normal rabbit serum (NRS). NRS was obtained from a rabbit that was later immunized against mouse CAXIII. The tissue samples fixed in Carnoy’s fluid and embedded in paraffin, were cut in 5 μm sections and placed on microscope slides.

The peroxidase-anti-peroxidase complex method included the following steps:
1. Pretreatment of the sections with undiluted cow colostral whey (Biotop, Oulu, Finland) for 40 min and rinsing in phosphate-buffered saline (PBS).
2. Incubation for 1h with the primary antiserum (anti-mouse CAII, CAIX or CAXII) or NRS diluted in a ratio of 1:100 in PBS containing 1% bovine serum albumin (BSA) (BSA-PBS solution).
3. Washing at room temperature, three times for 10 min in PBS.
4. Treatment with undiluted cow colostral whey (40 min).
5. Incubation for 1 h with swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) diluted in a ratio of 1:100 in 1% BSA-PBS.
6. Washing three times for 10 min in PBS.
7. Incubation for 30 min with peroxidase-anti-peroxidase rabbit conjugate (Dakopatts) diluted in a ratio of 1:500 in PBS.
8. Washing four times for 5 min in PBS.
9. Incubation for 2 min with 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution (6 mg DAB in 10 ml PBS plus 3.3 μl H2O2) as chromogen.

All of the incubations and washings were carried out at room temperature. The sections were finally mounted in Neo-Mount (Merck, Darmstadt, Germany).

4.2.2 Immunohistochemistry of studies II, III, IV and V

The rabbit anti-human CAII serum was characterized earlier (Parkkila et al. 1993), and the monoclonal antibody M75, recognizing the N-terminal domain of human CAIX, was also described previously (Pastorekova et al. 1992). The rabbit anti-human CAXII antiserum to the secretory form of CAXII was produced in professor William Sly’s laboratory at Saint Louis University and characterized in earlier studies (Karhumaa et al. 2000). All of these antibodies had been previously utilized in numerous studies and were specific for each carbonic anhydrase. NRS was used as a control. Five μm sections were processed for immunoperoxidase staining, which was performed using automated Lab Vision Autostainer 480 (LabVision Corporation, Fremont, CA, USA). Automated immunostaining was performed using an automated Power Vision+™ Polyhorseradish peroxidase (HRP) IHC kit reagents (ImmunoVision Technologies Co., Hillsborough, CA, USA).

Immunostaining was performed according to the following protocol:
1. Rinsing in wash buffer.
2. Treatment in 3% H2O2 in ddH2O for 5 min and rinsing in wash buffer.
4. Incubation with the primary antibody or NRS diluted in a ratio of 1:1000 (anti-CAIX) or 1:2000 (anti-CAII, anti-CAXII and NRS) in Universal IHC Blocking/Diluent for 30 min.
5. Rinsing in wash buffer for 3 x 5 min.
6. Incubation in Poly HRP-conjugated anti-mouse or anti-rabbit IgG for 30 min and rinsing in wash buffer for 3 x 5 min.
7. Incubation in 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution (one drop DAB solution A and one drop DAB solution B) with 1 ml ddH2O for 6 min.
8. Rinsing with ddH2O.
9. CuSO4 treatment for 5 min to enhance the signal.
10. Rinsing with ddH2O.
All procedures were carried out at room temperature. After the immunostaining, the sections were counterstained with haematoxylin, mounted in Entellan Neu (Merck, Darmstadt, Germany).

4.3 Western blot in study I

The specificities of the antibodies were tested by Western blotting in study I. The samples containing 50 μg of protein from mouse uterus, kidney and colon were analyzed by SDS-PAGE under reducing conditions according to Laemmli et al. (1970). All of the reagents and the protein standard (BenchMark™ Prestained Protein Ladder) for SDS-PAGE were purchased from Invitrogen (Carlsbad, CA, USA), except the sample buffer, which was obtained from Sigma (St. Louis, MO, USA). Electrophoresis (200 V for 40 min) was conducted in a Novex Xcell II mini cell electrophoresis unit (Invitrogen) with a 10% Bis-Tris gel (Invitrogen). The separated proteins were transferred electrophoretically from the gel to a polyvinylidene fluoride membrane (Invitrogen) in a Novex Xcell II blot module (Invitrogen). The transfer buffer (NuPAGE Transfer Buffer™) was purchased from Invitrogen. The blot module was filled with the transfer buffer until the gel/membrane assembly was covered. The outer buffer chamber was filled with 650 ml of deionized water. The protein transfer was performed using a constant voltage of 36 V for 1 h and 20 min. After the transblotting, the sample lines were detected by ECL Western blotting detection reagents and analysis system (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s instructions. All of the steps were carried out at room temperature. The Western blotting experiments were performed in triplicate to confirm the reproducibility of the results.

4.4 Evaluation of CA immunoreactivity (I–V)

A Zeiss Axioskop 40 microscope was used for the IHC evaluation of CAs. The intensity (INT) of the staining was scored on a scale of 0–3 by the joint analysis of two investigators as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction. The extent (EXT) 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 showed no evidence of specific immunostaining. The staining index (SI) was calculated using the following formula: SI = √(EXT x INT). This method was derived from a previous study on gastric tumors (Leppilampi et al. 2003) and it was used in our studies III–V. In studies I and II, we
scored only the intensity of immunoreactions on a scale of absent-strong (study I) or 0 (no reaction), 1 (weak reaction), 2 (moderate reaction), 3 (strong reaction) (study II). In study III, the tissue sections immunostained for CAIX were photographed with x100 magnification and subjected to digital image analysis. The staining extent was analyzed with analySIS software (Soft Imaging SystemGmbH). Two rectangular regions were scored from each section using color threshold values of 242 (red and green colors) and 153 (blue color). Each analyzed region (1,300 x 1,000 μm) included a representative tumor sample. The obtained relative area value indicated the mean percentage of stained area within the analyzed regions.

Table 9. Evaluation of immunoreactivity in studies I–V

<table>
<thead>
<tr>
<th>Study</th>
<th>Evaluation scores for CAs</th>
<th>CA isoenzymes evaluated</th>
<th>Authors involved in evaluation</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>absent–strong</td>
<td>II, IX, XII, XIII</td>
<td>PH, SP</td>
</tr>
<tr>
<td>II</td>
<td>0–3</td>
<td>IX, XII</td>
<td>PH, HH, SP</td>
</tr>
<tr>
<td>III</td>
<td>√(EXT x INT) 0-3</td>
<td>II, IX, XII</td>
<td>SP, PH, AN</td>
</tr>
<tr>
<td>IV</td>
<td>√(EXT x INT) 0-3</td>
<td>II, IX, XII</td>
<td>PH, SP</td>
</tr>
<tr>
<td>V</td>
<td>√(EXT x INT) 0-3</td>
<td>II, IX, XII</td>
<td>PH, SP</td>
</tr>
</tbody>
</table>

4.5 mRNA preparation and analysis in study III

The preparation of the Human Genome U133A GeneChip arrays (Affymetrix, Inc.), patient material and hybridization methods used on the expression arrays were described in Kruhoffer at al. (2005). Student’s t test for unequal variances was used for the probe sets of interest (CAs and HIF) to analyze the gene expression among the MSI, MSS, and LS groups.

4.6 Statistical methods

Statistical analyses of the results for studies II, IV and V were performed using SPSS for Windows software (SPSS Inc., Chicago IL, USA). Kaplan-Meier survival analysis and log-rank tests were used to compare the distribution of the lifetime data of the patients after diagnosis and surgery in study II.

In study III, one-way ANOVA and Bonferroni’s t-test were used to evaluate the significance of differences in protein expression among the LS, MSI, and MSS groups.
Mann-Whitney- and Kruskal-Wallis-tests were used to determine the P-values in studies IV and V.
5 RESULTS

5.1 Carbonic anhydrases in normal reproductive tissues

5.1.1 Immunohistochemical analyses (I,IV)

The IHC study of CAs in mouse reproductive organs found that CAII was located in the surface epithelial cells of the endometrium. Surprisingly the immunoreaction was most intensive at the plasma membrane (study I, Figure 1). No significant staining was observed in the mouse ovary. In the mouse placenta, a positive reaction was located in both the endothelium of the blood vessels and erythrocytes. There was also some reaction in the amniotic epithelium but other parts of the placenta remained negative. Only a weak reaction of CAIX was found in the mouse endometrium, and the staining was located in the surface and glandular epithelial cells. No CAIX immunostaining was found in the mouse ovary, nor did we observe a signal in the placenta. The surface epithelial cells of the mouse endometrium clearly expressed CAXII, but the staining was distinctly stronger in the deep endometrial glands. CAXII was the only CA that showed a faint reaction in the mouse ovary. Occasionally some positive CAXII staining was seen in the corpus luteum. In the mouse placenta, CAXII was highly expressed in the decidual glands, while the amniotic epithelium remained negative.

In the human endometrium the CAII was located in the cytosol of epithelial cells. The SI ranged from 0 to 2.5 and the median SI was 1.0 (study IV, Figure 1A). The human endometrium also showed weak CAIX staining in the glandular epithelial cells, with a more intensive signal seen during the proliferative phase (study IV, Figure 1B). The strongest reaction for CAXII was associated with the basolateral plasma membrane of the epithelial cells. The SI ranged from 2.0 to 3.0 and the median SI was 2.45 (study IV, Figure 1C). The IHC reaction was particularly strong when the endometrium was in its proliferative phase.
5.1.2 Western blot analysis of CAII, CAIX and CAXII (I)

To evaluate the specificity of the immunoreactions, we performed Western blotting for the mouse uterine proteins. Mouse kidney and colon samples were used as positive control tissues. The results are shown in Figure 5 of study I. All tissue specimens showed a strong CAII-positive signal with a predicted molecular mass of 30 kDa. CAIX showed a weak positive 51 kDa band for the uterine proteins. The strongest signal for CAIX was seen in the colon. The signal for CAXII, with a molecular mass of 46 kDa, was weaker in the uterus compared with the colon and kidney. The anti-mouse CAXII serum cross-reacted with a 30 kDa polypeptide in Western blotting. This result may be due to a cross-reaction with CAI, since the colon and erythrocytes contain high levels of CAI. A previous study with the same anti-CAXII and anti-CAII antibodies in gastric mucosa, indicated that anti-CAXII serum does not recognize CAII (Halmi et al. 2004).

5.2 Carbonic anhydrases in gynecological tumors

5.2.1 CAIX and CAXII expression in ovarian tumors (II)

The strongest immunostaining for CAIX was found in the MOCs, where strong staining was found in 57% (4/7) of cases. The remaining 43% (3/7) of cases showed a moderate reaction. In MBOTs strong or moderate staining was found in 33% (1/3) and 67%, 2/3 of cases, respectively. Variation in the staining intensity was higher in OSCCs, where about 12% (4/34) showed strong expression. In SBOT 75% (3/4) of cases had moderate staining and 25% (1/4) showed a weak intensity of CAIX staining. Some of the EnOC and OCCC tumors were completely negative. The lowest staining was seen in clear cell tumors, where 50% (2/4) were negative and 50% (2/4) showed only weak reactions (study II, Figure 1). In OSCCs and EnOCs the highest expression of CAIX was particularly found in areas adjacent to necrosis.

CAIX immunostaining in OSCCs was analyzed in groups that were defined according to the grade of differentiation. There were no significant differences among the groups, although most grade I tumors showed weak immunoreactivity and the staining intensity was slightly stronger with more cases of strong reactions in high grade (grades II and III) tumors (study II, Figure 4). Because the category of OSCCs included the highest number of cases and the tumors showed distinct variations in SI, it was possible to explore whether CAIX has any prognostic value in this disease category. The patients were grouped into two categories depending on the CAIX staining intensity.
Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome

(weak and moderate/strong). The cancer patients with weak CAIX expression had a slightly, although insignificantly better survival rate compared with the moderate/strong group (P=0.14) (study II, Figure 3).

The staining intensity for CAXII was lower in every tumor category, compared with CAIX, except for the OCCCs. All OCCCs showed weak immunostaining and none were completely negative, whereas all other categories additionally showed some negative cases. Most MOCs had moderate CAXII staining (study II, Figure 5). In contrast with the CAIX immunostaining results, CAXII-positive staining was not concentrated to perinecrotic areas.

5.2.2 CAII, CAIX and CAXII expression in uterine tumors (IV)

IHC analyses of CAII and CAIX showed that these isoenzymes were upregulated in ECs. The median SI values for CAII and CAIX were 1.41 and 2.0, respectively (study IV, Figure 1A and 1B). CAIX was more strongly expressed in ECs compared with normal endometrium (P<0.005). In carcinoma specimens, CAIX expression was typically stronger in areas close to necrosis and was confined to the plasma membranes. CAII staining was prominent in the endothelium of the blood capillaries, where about 30% of cases showed a strong signal. Interestingly, CAXII was significantly less expressed in ECs than in normal endometrium (P<0.004). The carcinoma samples also showed more variation in SI (SI 0–3.0), and the median SI was 1.73 (study IV, Figure 1C). CAXII showed diffuse reactions within the tumor without any clear association with hypoxic areas.

All LM specimens remained negative for CAXII. Although the LM stroma remained negative for CAII, there was some CAII-positive staining in the endothelium of the blood capillaries. The staining intensity varied from negative to strongly positive, and most samples showed weak CAII-positivity. Most LMs also remained negative for CAIX, but 5 of the 33 specimens showed clear positive staining. The SI values of these CAIX-positive LMs ranged from 1.4 to 2.5 (study IV, Figure 4). In most cases of the five CAIX-positive LMs the staining was focal and there was no necrosis. The sizes of the positive tumors varied from 1–7 cm. All patients were alive 15 years after the operation with no recurrences of tumors. Therefore, there was no common factor to explain the CAIX-positivity in these five LMs.

The expression of CAII, CAIX, and CAXII was also investigated in LMSs, stromal sarcomas, and in MMMTs. The SI varied significantly among different tumor types. CAII expression was negative in the tumor cells in several cases, but the endothelium of blood capillaries was strongly immunopositive. Strong positive staining in the
endothelium was seen in 80% of MMMTs, whereas 20% were negative. About 70% of stromal sarcoma specimens showed strong staining in the endothelium and the remaining 30% showed weak CAII immunoreactivity (study IV, Figure 6). The median SI for CAII in stromal sarcoma and MMMTs was 1.4 compared with 0.5 in LMS. CAIX showed the highest immunoreactivity in LMSs (median SI 1.7) and MMMTs (median SI 1.7). It was typical of CAIX to show strong positivity in some focal areas, and there was a significant amount of intratumoral variation in the distribution of staining. The cells around the neovessels often remained negative for CAIX, whereas more hypoxic cells farther from the vessels showed strong CAIX staining. The CAXII staining in the sarcoma specimens was predominantly widely spread but weak. The median SI for LMS was 1.2, with corresponding values of 0 and 1.0 for stromal sarcoma and MMMT, respectively.

There was a significant difference in the staining reactivity for CAII, CAIX and CAXII between LMs and USs (P<0.001, P<0.002 and, P<0.001, respectively).

5.2.3 CAII, CAIX and CAXII expression in Lynch syndrome

5.2.3.1 Immunohistochemistry (III, V)

Because LS is characterized by an early onset of colorectal cancer and is associated with extracolonic cancers (of which EC is the most common), we sought to study first the expression of CAs in different groups of colorectal cancer, including LS-associated cancer, MSS and MSI sporadic carcinomas. Then, we extended our studies to the expression of CAs in LS-associated EC and hyperplasia.

The IHC analyses showed that colorectal tumor cells generally expressed low levels of CAII. The CAII SI was highest in MSI, followed by LS and MSS. The differences in CAII SI values were significant (P=0.002) between the different groups (study III, Figure 5). There was no difference among the groups, when classified according to Dukes classification or grade of differentiation. The endothelial CAII staining showed slightly increased staining when the dysplasia grade increased in the colorectal cancer samples, but otherwise the endothelial staining did not show any correlation with any subgroup (study III, Figures 3 and 4). In EC, CAII staining was significantly stronger in LS cancers compared with sporadic EC (P<0.0129). The CAII SI increased in LS when the hyperplasia degree developed into EC. CAII expression was low in SH but the expression increased when moved to CAH and the expression was highest in EC (P<0.001) (study V, Figure 1).
In colorectal cancer, CAIX immunostaining was moderate or high in most tumor samples and only few samples showed weak staining or none at all. The CAIX staining was highest in LS tumors compared with MSI and MSS (P=0.05) (study III, Figure 2). In the LS tumors, the mean positively stained area included about 28% of the area, whereas in MSS and MSI the corresponding fractions were 12% and 14%, respectively. When the SI was compared between LS and MSS or MSI, the values differed significantly (P=0.003 and P=0.04, respectively). These results indicate that CAIX is clearly overexpressed in LS colorectal tumors (study III, figure 5). In EC the CAIX expression was likewise highest in the LS group (median SI 2.45). However, when compared with sporadic EC (median SI 2.00) the difference was not statistically significant (P<0.45) (study V, Figure 2). In colon cancer, the grade of dysplasia did not show any correlation with CAIX SI. In the endometrial samples, there was a notable difference in SI values between hyperplasias and LS ECs (P<0.02).

The SI for CAXII was highest in MSI and lowest in MSS colorectal tumors (study III, Figure 2). When MSI or MSS tumors were compared with LS colorectal tumors, there was no significant difference in SI values, nor did we find a significant difference in SIs among the various tumor grades or stages. In EC, there was no significant (P<0.724) difference in CAXII staining between LS and sporadic tumors. In contrast, a significant difference was found in SI values between the hyperplasias and LS ECs (P<0.002) (study V, Figure 3). CAXII is usually highly expressed in the normal endometrium, but interestingly, in the LS-associated simple and complex hyperplasias, the median SI was 0.

5.2.3.2 mRNA analysis of CAs (III)

Microarray analyses were performed to study CA mRNA expression levels in 113 colorectal specimens. The specimens were screened for the expression of most CA isoforms (study III, Table 1). The highest signals were detected in normal colon for CAI, CAXII, and CAIV, and these isoenzymes were significantly downregulated in all forms of colorectal cancer. Instead, most CAs (CAIII, CAVA, CAVB, CAVI, CAX, and CAXI) showed no significant difference between the normal colorectal mucosa and any form of colorectal cancer. When compared with the normal colorectal mucosa, CAII and CAXII were downregulated in both LS and sporadic carcinomas. Even though CAII and CAXII mRNA expression was higher in LS than sporadic tumors, the difference was not statistically significant. CAIX was the only isoform that was significantly up-regulated in cancer specimens and showed the highest overexpression...
in LS-related tumors. Table 10 lists the relative mRNA expression levels of CAII, CAIX and CAXII in different carcinomas.

Table 10. The relative mRNA expression levels of CAII, CAIX and CAXII in the normal colon, sporadic colorectal carcinomas, and colorectal carcinomas associated with LS, from study III

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<tr>
<th>Gene symbol</th>
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<th>CA9</th>
<th>CA12</th>
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<tbody>
<tr>
<td>Probe set ID</td>
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<td>205199_at</td>
<td>210735_s-at</td>
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<td>3.17</td>
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<tr>
<td>Mean Lynch</td>
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<td>1.38</td>
<td>1.21</td>
<td>1.33</td>
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<tr>
<td>Mean sporadic</td>
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<td>1.93</td>
<td>1.02</td>
<td>0.98</td>
<td>0.99</td>
<td>1.03</td>
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P, Lynch vs sporadic

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<td>0.014</td>
<td>0.100</td>
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<td>0.959</td>
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<td>P, Lynch vs normal</td>
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6 DISCUSSION

6.1 Carbonic anhydrases II, IX and XII in normal gynecological organs

After the first observations of CAs in rabbit and ewe uterus in 1954 (Lutwak-Mann, Lutwak-Mann and Averill 1954), we presently know more about CA expression and the role that these enzymes play in the female genital tract. Previous studies have shown CA activity in the endometrium of several species (Falk and Hodgen 1972, Friedley and Rosen 1975). Our findings, that human endometrial glands weakly express CAII and abundantly express CAXII, are in agreement with other studies (Karhumaa et al. 2000). CAII expression is clearly stronger in the mouse than in human endometrium (Ge and Spicer 1988, Lehtonen et al. 2004).

The finding of CAII expression in spermatozoa, seminal plasma, decidual tissues, placental tissues and a variety of fetal tissues suggests that this enzyme plays an important role in the processes of fertilization, implantation and feto-placental development (Ali Akbar et al. 1998). In the female genital tract, the endometrial and oviductal epithelia may produce an alkaline environment for maintaining sperm motility (Karhumaa et al. 2000). Altered CAII expression has been reported in the decidualized mouse uterine tissues depending on the number of days after mating (Chiang et al. 2004). IHC results showed strong CAII expression in the decidualized cells in the pre- and postimplantation stages. In the pre-implantation stage, the alkaline environment in the genital tract is important for the sperm motility, and the high CA activity contributes to the critical alkalinization. On the other hand, CA expression was significantly reduced at the implantation stage, a stage characterized by the early development of blastocysts. Downregulation of cytosolic CAs leads to an acidic environment in the endometrium, and thus CAs may be functionally involved in the pH-dependent blastocyst implantation.

In the mouse endometrium, estrogen likely upregulates CAII and CAXII expression with a corresponding increase in bicarbonate transport and endometrial surface pH (Gholami et al. 2013, He et al. 2010). In cultured mouse endometrial cells, CAII and CAXII were upregulated at estrus, the time immediately prior to ovulation, and the uterine luminal pH was more alkaline than at diestrus (He et al. 2010).
is a constant cooperation with these CAs under the influence of both estrogen and progesterone to maintain the appropriate pH balance in the uterine lumen at different stages of the menstrual cycle (Gholami et al. 2013). Besides mediating pH, CA isoenzymes are also involved in the regulation of uterine fluid volume and electrolyte concentration changes, which may indirectly occur via other membrane transporters. Very few studies of these isoenzymes in different stages of the human menstrual cycle have been reported. So far, we only know that CAXII is abundantly expressed in the normal human endometrium and that the expression of CAII and CAIX is very low. Our findings and previous studies (Karhuma et al. 2000) together suggest that CAXII expression is most intense in the basolateral plasma membrane of the epithelial cells and that the most intense expression occurs during the proliferative phase in human endometrial cells. CAXII may be functionally coupled to the bicarbonate transporter system and is likely regulated by steroid hormones. CAXII may be the main isoenzyme involved in implantation, development and maintenance of pregnancy in humans.

CAIX is rarely seen in normal genital tract cells. According to our results CAIX is not expressed in the mouse ovary and other studies have also shown that the normal human ovary is negative for CAIX (Woelber et al. 2010). We found that the normal endometrium contains only faint reactivity for CAIX. In another study, CAIX and HIF1α expressions were found to be highest in human endometrium biopsies from cycle days 2–8, the signals rapidly decreased thereafter, and CAIX expression completely disappeared around cycle days 9–10 with no expression at cycle days 11–15 (Punyadeera et al. 2006). During menstruation the endometrium is under ischemia, which may explain the high CAIX expression when the angiogenic response is aimed at repairing the damaged vessels in the functional layer. This hypothesis is in line with the finding that HIF-1α, the key regulator of CAIX, is strongly expressed in the early-phase proliferative endometrium, where new blood vessels are formed and the endothelium is regenerated (Sivridis et al. 2002). In the late proliferative and secretory phases, the period when the vascular supply results in reoxygenation of the tissue, a progressive reduction of HIF-1α is noted. The enhanced expression of CAIX during the initial cycle days is a result of hypoxia, which promotes vascular growth. Together with angiogenic factors CAIX might be involved in remodeling and repair of the endometrium (Punyadeera et al. 2006). This statement agrees with our finding that CAIX is expressed more intensively in the glandular epithelial cells during the proliferating phase. Furthermore, this assertion is consistent with the findings of Horree et al. (2007), who detected no CAIX expression in the inactive endometrium. Studies with knockout mice have suggested that CAIX is not necessary for reproductive functions, as no abnormalities have been reported in the reproductive capacity of CAIX-deficient mice (Leppilampi et al. 2005a). Taken together, our results suggest that CAXII is the most abundant CA
in the endometrium known thus far. It would be interesting to study the reproductive capacity of CAXII knockout mice if such a model was available.

6.2 Carbonic anhydrases II, IX and XII in gynecological tumors

Our finding of CAIX expression in EC was in line with several previous studies. CAIX has been found in 76–92% of endometrial carcinoma cases (Horree et al. 2007, Seeber et al. 2010). CAIX staining was mostly in perinecrotic areas (Horree et al. 2007, Seeber et al. 2010), where it was significantly associated with HIF-1α expression (Seeber et al. 2010). In line with these findings we also observed high CAIX expression in hypoxic areas close to necrosis. Even though some normal endometrial cells stained for CAIX, the expression in endometrial carcinoma was significantly stronger (P<0.005). Our normal endometrial samples were taken in the proliferating or secretory phases. Because the inactive endometrium does not express CAIX (Horree et al. 2007) the difference in immunoreactivity of the normal endometrium and cancer would have been more significant if the samples had been taken only in the secretory phase or from an inactive endometrium. Increased levels of CAIX have already been observed in hyperplasia, which seems to correlate with the early events of carcinogenesis of endometrial tissue (Horree et al. 2007, Yunokawa et al. 2007). CAIX expression levels have been found to be significantly higher in postmenopausal rather than premenopausal subjects, suggesting the existence of cross-talk between hypoxia and estrogen signaling (Yunokawa et al. 2007). Also in some breast cancer studies, high CAIX expression has been associated with negative hormone receptor status (Chia et al. 2001, Schutze et al. 2013).

The upregulation of CAIX indicates the presence of hypoxia in various types of cancer and is related to a poor prognosis (Beasley et al. 2001, Giatromanolaki et al. 2001, Loncaster et al. 2001), but unfortunately there have been no studies related to CAIX expression profile and patient prognosis in EC. Importantly, tumor cell necrosis (Scholten et al. 2004) and overexpression of HIF-1α indicate a poor prognosis (Sivridis et al. 2002). It seems that hypoxia-driven perinecrotic HIF-1α expression is related to a poor prognosis particularly in low-stage EC (Seeber et al. 2010). Since, there was a significant correlation between CAIX and HIF-1α in these hypoxic areas, it would be of interest to study if the strong CAIX expression in these tumors could also predict a poor prognosis. Notably, another study found that CAIX could not be used to discriminate between patients with or without a risk of endometrial carcinoma recurrence (Pijnenborg et al. 2007). This fact might suggest that CAIX may not be an ideal prognostic marker in this tumor category. Nevertheless, the high CAIX expression in EC makes it an interesting potential target for EC therapy.
The overexpression of CAXII has been found in several cancers (Ivanov et al. 2001). Therefore, it was interesting to observe in our study that CAXII was significantly less expressed in endometrial adenocarcinoma than in normal endometrium (P<0.004). A similar trend was seen in a previous study, where 67% of endometrial hyperplasias showed diffuse CAXII staining, while only 50% of ECs stained for CAXII (Ivanov et al. 2001). CAXII expression is often high in normal tissues with active water transport (colon, kidney, choroid plexus), suggesting important roles in normal physiology (Potter and Harris 2003). The role of CAXII in the endometrium may be also related more to normal functions, such as fertilization and regulation of the bicarbonate concentration in the uterus.

The finding of CAII being widely expressed in the tumor vessel endothelium (Yoshiura et al. 2005) led us to study its expression in gynecological tumors. Of all studied sarcoma specimens at least 70% showed some endothelial CAII staining. Interestingly, over 80% of benign LM samples showed weak to strong CAII staining in the vessel endothelium. In an in vitro angiogenesis model study, CAII was significantly up-regulated in normal human vein endothelial cells, suggesting that CAII is required in normal angiogenesis (Yoshiura et al. 2005). CAII seems to play a critical role in tumor angiogenesis. This suggestion is in line with the finding that CAII is present in the neovessels of astrocytic tumors (Nordfors et al. 2010). Although the exact role of CAII in the endothelium has not been established, it may play a role in the regulation of intracellular pH in proliferating and differentiating endothelial cells (Kida et al. 2006). Previous findings have suggested that CAII could be a potential target antigen for dendritic cell therapy in melanoma (Yoshiura et al. 2005). Since most stromal sarcomas and MMMTs strongly express CAII in the endothelial cells, similar approaches could be beneficial to patients with these tumors.

Our findings of CAIX expression in LMs are in line with previous observations (Mayer et al. 2008). Most of our LM samples were negative for CAIX, but 5 out of 33 showed positive staining. There was no common factor in these LMs to explain the positive CAIX expression. There must be another regulator of CAIX in these tumors besides the hypoxia. Even though Mayer et al. (2008) observed severe hypoxia among LMs, the IHC of HIF-1α and CAIX in these LMs remained negative. Instead, stronger CAIX staining was observed in US, where 78% showed immunoreactivity, a finding that is in line with our results. It will be important to find novel biomarkers to distinguish between LM and aggressive growing sarcoma, because they clinically resemble each other and the sarcoma diagnosis is often available only after the operation. Because all three CAs were negative in different sarcomas, they may be used together as histopathological biomarkers to differentiate between LM and LMS. Notably, CAII
could also represent a potential biomarker for differential diagnostics of LMS and GISTs, the latter tumor being strongly positive in almost all cases (Parkkila et al. 2010).

The finding of CAIX expression in several cases of mesenchymal tumors makes CAIX also a possible target for sarcoma therapy. Because CAIX has been associated with a poor prognosis in sarcoma patients (Boeuf et al. 2010, Maseide et al. 2004), it would be interesting to study if there is any correlation with outcome in gynecological sarcomas. Both the high propensity for metastases and the high risk for recurrences are typical features for uterine sarcomas. If CAIX can be used as a marker of tumors that have a high risk for developing early metastases, it may be possible to develop a more personalized therapy plan for high-risk patients.

In a previous study on epithelial OC, CAIX expression was diffuse, while CAXII staining was weak/absent or focal (Ivanov et al. 2001). Our IHC results demonstrated strong CAIX expression in 57% of MOCs and in 33% of MBOTs. Our results of high CAIX expression in MOC were later confirmed by two different research groups (Choschzick et al. 2011, Kim et al. 2013) Both IHC studies showed higher CAIX expression in MOCs (45.5% and 79.2%) than in EnOCs (27.7% and 63.3%). Most of our EnOC samples (about 60%) stained moderately for CAIX. In our study, variation in SI was higher in serous cystadenomas, where about 12% showed strong CAIX signal. Other tumors exhibited lower expression and about 50% of OCCCs were completely negative for CAIX. These findings are in line with the results from more recent studies (Choschzick et al. 2011, Kim et al. 2012). These results show that CAIX is frequently, but at variable levels, expressed in OC. Epithelial OCs are a group of different subtypes of tumors and these subtypes involve quite a few different molecular pathways (Kurman and Shih 2008). The most important mechanism for CAIX upregulation is hypoxia, which is strongest in areas of tumor necrosis in large mucinous filled cysts. However, under normoxia or mild hypoxia, CAIX can be upregulated by a high cell density via the PI3K and MAPK pathways (Kaluz et al. 2002, Kopacek et al. 2005). This activation of oncogenes or inactivation of tumor suppressor genes leads to HIF-1α accumulation in the tumor cells, even in normoxia. Instead, down-regulation of CAIX is induced by p53 tumor suppressor (Kaluz et al. 2009). All of these results suggest that CAIX can be upregulated in OC through different molecular mechanisms, which can be one reason for the variable staining in different subtypes. In a study of HIF-1α in different OC types, significantly increased HIF-1α staining was observed in OCCCs compared with the other histological types (Lee et al. 2007a). In our study, CAIX staining was negative in 50% of OCCCs. This finding also confirms that the regulation of CAIX is not straightforward; other conditions, such as transient hypoxia, cell density, low extracellular pH or glucose deprivation may also effect CAIX expression in these tumors (Kaluz et al. 2009). Jankovic et al. (2006) affirmed the possibility that the
tumors expressing high levels of CAIX but low amounts of HIF-1α are less likely to progress. Thus, the finding of less aggressive mucinous cancers, expressing high CAIX but low HIF-1α, is in line with previous observations.

Because the category of serous cystadenocarcinomas showed distinct variation in the staining intensity and included the largest number of patients, we were able to explore the prognostic value of CAIX in this disease. We found a trend of a lower survival rate in patients who had moderate or strong CAIX staining compared with patients with weak CAIX staining. Because our ovarian cancer material was collected before 2002 when the grading system was different, we cannot show the prognostic value of CAIX in high and low grade serous ovarian cancer. Instead, other investigators have found a correlation between high CAIX expression and poor clinical outcome (Choschzick et al. 2011). In this study, in all ovarian tumor categories, the prognosis of stage I tumors with high CAIX seemed to be worse than for advanced tumors without CAIX overexpression.

E-cadherin, present in 96% of ovarian tumors, is essential for the formation of cell-cell contacts and the stabilization of tissue architecture (Sarrio et al. 2006). The loss of its function promotes tumor invasion and metastasis in various cancers. It has been shown, that CAIX has the capacity to modulate E-cadherin-mediated cell adhesion via interactions with β-catenin in a cell culture (Svastova et al. 2003). Overexpression of CAIX in these cells reduced the binding of E-cadherin to β-catenin and this mechanism led to reduced cell adhesion capacity. This de-adhesion capacity and interaction with β-catenin, may be another mechanism by which CAIX increases tumor aggressiveness. It remains to be examined, if this hypothesis can explain the poor outcome of the CAIX-positive, stage I ovarian tumors compared with the CAIX-negative tumors.

Compared with CAIX, the SI for CAXII was lower in every tumor category except for OCCCs. In contrast to CAIX staining, all OCCCs showed weak positive CAXII staining. Similar to CAIX, the highest CAXII expression was observed in MOC where over 80% showed moderate SI. CAXII was not adjacent to necrosis. Hypoxia seems to upregulate CAXII in some cell lines, although a hypoxia responsive element has never been reported for the CA12 gene. Differences in the immunolocalization of CAIX and CAXII in ovarian tumors raise a question whether the CA12 is a real HIF-target gene. In a breast cancer study, CA12 was one of the genes whose expression was most highly correlated with ERα (Barnett et al. 2008). The authors concluded that the CA12 gene is under a primary transcriptional up-regulation by the estrogen-occupied estrogen receptor, and that this regulation in breast cancer cells is mediated by ER action through a distal enhancer. Additional studies are needed to investigate, if this hypothesis can also explain the regulation mechanism for CAXII in OC.
Since LS is associated with an early and high risk of colorectal cancer and in women with EC, we expanded our investigations to include specimens from colorectal and EC of LS patients.

The upregulation of CAIX was most evident in the LS group compared with sporadic colorectal cancers, at both the mRNA and protein levels. In colorectal tumor cells, CAII was generally very weakly expressed and CAXII staining did not significantly differ between the colorectal cancer groups. In EC, the IHC staining for CAIX was slightly higher in LS cases. Despite the strong CAIX staining in the LS group, the difference was not statistically significant (P<0.45). Likewise, the difference was not statistically significant for CAXII (P<0.72). The difference between the sporadic and LS EC tumors might have reached a statistically significant result if the sample sizes had been larger. Significantly stronger CAII staining was seen in LS-associated EC compared with sporadic EC (P<0.012).

CAIX is known to regulate the acid-base balance and acidify the extracellular milieu of the tumor cells. CAIX thereby creates an optimal microenvironment for tumor spread and growth (Chen et al. 2005, Robertson et al. 2004). The relation between CAIX and hypoxia is known and CAIX is often located in perinecrotic tumor areas (Potter and Harris 2004). The unique tissue environment of a tumor, which is characterized by hypoxia, low pH and nutrient deprivation, is a major cause of the genetic instability seen in cancers (Yuan et al. 2000). The high expression of CAIX in most LS-associated colon cancers and ECs may increase the risk of further genetic alterations. This fact may be in line with the finding, that LS-associated ECs show more frequent alterations in other genes as well, particularly PTEN mutations (Ollikainen et al. 2007). The role of CAII in cancer cells, is still poorly understood, but CAII may possibly play the same role in EC as CAIX and CAXII do, i.e, modulating the intracellular pH balance and promoting extracellular acidification.

EC of MSH2 mutation carriers is associated with non-endometrioid histotypes, such as clear-cell cancer, histotypes with a more aggressive clinical course (Broaddus et al. 2006). In our study, primary histotypes were endometrial adenocarcinomas; most of our LS-associated EC tumors were from MLH1 mutation carriers. Therefore, it remains to be studied how CAII, CAIX or CAXII are expressed in MLH6 or MSH2 tumors or in different histological subtypes. As CAIX was moderately expressed in several endometrial tumor categories it cannot be recommended as a specific biomarker in LS-associated EC. Its presence in EC and in colorectal cancer suggests, however, that it could serve as a potential therapeutic target in these LS-associated tumors.
We also investigated CAII, CAIX and CAXII expression in different LS-associated endometrial hyperplasias. The expression levels of CAIX were equal in CH and in CAH, and the staining did not significantly increase when moving from CAH to EC (P< 0.094). Previous findings of Nieminen and coworkers suggest that CH and CAH are equally important as precursor lesions of LS patient malignancies and that molecular changes are detectable in endometrial tissue many years before the emergence of EC (Nieminen et al. 2009). Thus, our results showing equal expression of CAIX in CH and CAH indirectly support the findings of Nieminen and coworkers that CH and CAH are equally important precursors of EC in LS patients.

The results from study IV showed that CAXII is highly expressed in the normal endometrium. Interestingly, CAXII was very weakly expressed in SH and CH of LS patients (median SI: 0.00 in both categories), even thought there was a wide variation. Because no data exist related to CAXII expression in LS normal endometrium, it would be of interest to study if there are differences in CAXII expression in the normal endometrium of mutation carriers before the hyperplasia changes occur in the endometrium. In LS-associated endometrial tissues, molecular changes are noted even 12 years before the diagnosis of EC, and 7% of normal endometrium specimens show decreased MMR protein expression (Nieminen et al. 2009). Thus, it could be hypothesized that during the development of endometrial hyperplasia the normal function of the endometrium declines and the expression of CAXII, already playing an important role in the normal endometrial function, may also decrease (an early sign of the carcinogenic process). Since CAXII expression in LS-related EC remained lower than in the normal endometrium, CAXII cannot be recommended as a marker for LS-associated EC.

The normal endometrium expresses only low levels of CAII. In contrast, EC of LS patients’ contains moderate to high levels of CAII. Since EC is the most common gynecological cancer and is the sentinel event for many LS women, MMR mutation carriers are advised to have regular endometrium screenings (Auranen and Joutsiniemi 2011). In order to detect pre-malignant lesions, these endometrial samplings should be taken every year, starting at the age of 30 or 35. Because it is difficult to determine the right time for prophylactic hysterectomy in LS patients, novel biomarkers to predict the right operation time would be helpful. As the upregulation of CAII was significantly higher in the EC of LS patients and CAII seemed to differentiate hyperplasias from carcinoma, CAII may serve as a novel indicator of malignant transformation of the endometrium in these women.
7 SUMMARY AND CONCLUSIONS

1) Among the analyzed isoenzymes CAXII is the most widely expressed form in the mouse and human endometrium. The high expression of CAXII in the normal endometrium, supports the theory of CAXII being important for normal reproductive functions. In EC, CAXII expression is reduced compared with that of the normal endometrium. CAIX overexpression is evident in most ECs, whereas only sporadic CAIX expression is present in the normal human and mouse endometrium. High expression of CAIX in ECs suggests that it could represent a potential target for cancer therapy, but further studies are needed.

2) CAIX is completely absent and CAXII is only weakly present in the mouse ovary. Ovarian tumors are often positive for CAIX and CAXII. In particularly, most MBOTs and MOCs are moderately or strongly positive for CAIX. Because CAIX staining is most evident in hypoxic regions of different ovarian tumors, it could serve as a histopathological marker protein for hypoxia in these tumors. The wide expression of CAIX and CAXII in ovarian tumors suggests that these isoenzymes could serve as potential targets for OC therapy.

3) In LS-related tumors, CAIX is upregulated in colorectal cancer. The expression of CAIX is lower in LS-related EC compared to sporadic EC. CAIX could also serve as a therapeutic target in LS-related colorectal cancer and EC. CAII expression is significantly stronger in LS-related EC than in sporadic cancer, and the staining increases from simple hyperplasia to more malignant forms. Thus, CAII could be linked to malignant transformation of the endometrium in LS patients and may be used as a biomarker to predict the most appropriate time for prophylactic hysterectomy.
This study was carried out at the Departments of Obstetrics and Gynecology, Tampere University Hospital and the Divisions of Anatomy and Pathology of the School of Medicine, University of Tampere. A thesis is not a result of the work of an individual and therefore, I would like to warmly thank all of the following people, who helped and supported this thesis.

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Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome

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Finally, I thank my husband Petri and my lovely children Onni and Oiva for being my best teachers and reminding me of the most important things in life.

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Tampere, August 2014

Piritta Hynninen


Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome


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Expression of carbonic anhydrases II, IX and XII in the normal female reproductive tract, gynecological tumors and Lynch syndrome.
Abstract
Background: Carbonic anhydrase (CA) classically catalyses the reversible hydration of dissolved CO₂ to form bicarbonate ions and protons. The twelve active CA isozymes are thought to regulate a variety of cellular functions including several processes in the reproductive systems.

Methods: The present study was designed to investigate the expression of transmembrane CAs, CA IX and XII, in the mouse uterus, ovary and placenta. The expression of CA IX and XII was examined by immunoperoxidase staining method and western blotting. CA II and XIII served as positive controls since they are known to be present in the mouse reproductive tract.

Results: The data of our study indicated that CA XII is expressed in the mouse endometrium. Only very faint signal was observed in the corpus luteum of the ovary and the placenta remained mainly negative. CA IX showed weak reaction in the endometrial epithelium, while it was completely absent in the ovary and placenta.

Conclusion: The conservation of CA XII expression in both mouse and human endometrium suggests a role for this isozyme in reproductive physiology.
form being present in saliva and milk [7]. The cluster of membrane-bound CAs includes four isozymes: CA IV, IX, XII, and XIV [8-11]. The other members of the CA gene family (CA VIII, X and XI) are inactive isoforms whose functions have not yet been described [3,12,13].

It has been previously suggested that CAs may play important roles in the uterine endometrium by maintaining the appropriate pH balance through the catalysis of the production of bicarbonate ions [14]. Indeed, the role of bicarbonate in fertilization has been demonstrated in a number of previous studies. It is functionally involved in some key processes such as sperm cell capacitation and regulation of sperm motility [15-17]. Similarly, CAs may have several functions also in the placenta. They can be active in intermediary metabolism and provide ions for exchange in transepithelial movement of ions and fluid [18].

CA activity has been studied in pig, horse, cow, mink, rat and human placentas, and the results show considerable heterogeneity among different species [18]. Previous immunohistochemical studies have shown evidence for expression of CA II but not CA I or III in the bovine placenta [19]. Both CA I and II are expressed in the human syncytiotrophoblasts [20-22] and, especially CA II, in the fetal villous endothelium of mature placenta [22]. CA IV-positive staining has been reported in the mouse placenta by Rosen and coauthors [23]. Their data showed strong CA IV immunoreactivity in the mouse trophoblasts and endodermal layer of the yolk sac. In the mouse genital tract, CA I, II and III have been reported by Ge and Spicer [24]. These isozymes were reported to be present in the theca interna cells in the mouse ovary, and CA I was found in the zona pellucida and cytoplasmic foci in follicular granulosa cells. In the mouse oviductal epithelium, CA II expression showed distinct variation. The reaction was absent in the infundibulum, whereas the ampulla and isthmus showed positive staining. CA XIII is the newest member of the CA enzyme family, which has been described in the mouse and human endometrium along with several other positive tissues [4]. As a cytosolic isozyme it may be one of the major proteins regulating the pH and bicarbonate homeostasis not only in the endometrial cells but also in the lumen of the uterus. These mechanisms are complex due to the presence of several isozymes, however, and may greatly differ between species. For example, the human endometrium contains CA II only in the capillaries, whereas this high activity isozyme is abundantly expressed in the epithelial cells of the mouse endometrium [4,24].

CA IX is expressed at the basolateral plasma membrane of the human, rat and mouse epithelial cells [25,26]. In a recent extensive study, Ivanov et al [27] analyzed a number of normal human tissues for the expression of CA IX. Among reproductive organs, they reported positive signal for CA IX mRNA and protein in the efferent ducts, rete testis, and rete ovarii.

Human CA XII is expressed in several organs including colon, kidney, and pancreas [28-30]. In the human female reproductive tract, CA XII has been shown both in the glandular and surface epithelium of the endometrium, while it was only occasionally present in the cervix [14]. Ivanov et al [27] further confirmed CA XII expression in the glandular epithelium during the proliferative phase.

In this report we studied the expression of CA II, IX, XII and XIII in mouse female genital organs including uterus, ovary and placenta. The studies were specially focused on CA IX and XII, which have been designated as tumor-associated isozymes [9,10]. In addition to some normal tissues, both isozymes are overexpressed in several carcinomas such as renal and colorectal cancers [9,27,31,32]. A previous study has also demonstrated CA IX and XII expression in a number of neoplasias derived from the female reproductive tract [27]. However, there have been no previous studies on these isozymes in the female murine reproductive organs. The conservation of CA XII expression in both mouse and human endometrium shown in the present paper suggests a role for this isozyme in reproductive physiology.

Materials and methods

Antibodies

In the present study, we used the following antibodies which have been produced and characterized earlier: rabbit anti-mouse CA II [4], rabbit anti-mouse CA IX [26], rabbit anti-mouse CA XII [33], and rabbit anti-mouse CA XIII [4].

Collection of tissue samples

Two adult Balb/c mice were sacrificed by CO₂ asphyxiation followed by decapitation. Uterus, ovary and placenta samples were collected from both animals. The samples were immersion-fixed overnight in Carnoy's fluid (ethanol, chloroform and acetic acid (6:3:1)). Then the specimens were treated with absolute ethanol for 30 min, with 1:1 mixture of ethanol and chloroform for 15 min, and finally with chloroform for 30 min. Paraffin embedding was performed in a vacuum oven for 2 h at +58°C. Paraffin wax was purchased from Fluka Chemie GmbH (Buchs, Switzerland). To collect a placenta sample, a mouse was sacrificed at 9 days of pregnancy. The ninth day was chosen since it represents the middle gestational phase. It is also the time when the most critical steps of organogenesis occur in mouse. For western blotting, uterus, kidney and colon were removed and rapidly frozen in liquid nitrogen. The tissue samples for western blot were...
homogenized with HEPES buffer. Total protein concentration was determined after homogenization using BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer’s instructions. The study protocols were approved by the Animal Care Committee of Tampere University.

**Immunohistochemistry**

In the mouse tissues, the localization of CA IX and XII was examined by immunoperoxidase method. Antibodies against CA II and XIII were used as positive controls for the immunostaining. All experiments were performed in duplicate and included control staining with non-immune normal rabbit serum (NRS). NRS was obtained from a rabbit that was later immunized against mouse CA XIII. The tissue samples fixed in Carnoy’s fluid and embedded in paraffin were cut at 5 μm sections and placed on microscope slides. The peroxidase-anti-peroxidase complex method included the following steps: a) pretreatment of the sections with undiluted cow colostral whey (Biotop, Oulu, Finland) for 40 min and rinsing in phosphate-buffered saline (PBS); b) incubation for 1 h with the primary antiserum (anti-mouse CA II, CA IX, CA XII or CA XIII) or NRS diluted 1:100 in PBS containing 1% bovine serum albumin (BSA) (BSA-PBS solution); c) treatment with undiluted cow colostral whey (40 min); d) incubation for 1 h with swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) diluted 1:100 in 1% BSA-PBS; e) incubation for 30 min with peroxidase-anti-peroxidase rabbit conjugate (Dakopatts) diluted 1:500 in PBS; f) incubation for 2 min with 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution (6 mg DAB in 10 ml PBS plus 3.3 µl H2O2) as chromogen. The sections were washed three times for 10 min in PBS after incubation steps b and d and four times for 5 min after incubation step e. All of the incubations and washings were carried out at room temperature. The sections were finally mounted in Neo-Mount (Merck, Darmstadt, Germany). The stained sections were examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany).

**Western blot**

The samples containing 50 µg of protein from mouse uterus, kidney and colon were analyzed by SDS-PAGE under reducing conditions [34]. All of the reagents and the protein standard (BenchMark™ Prestained Protein Ladder) for SDS-PAGE were purchased from Invitrogen (Carlsbad, CA) except Laemmli sample buffer that was obtained from Sigma (St. Louis, MO). Electrophoresis (200 V for 40 min) was performed in a Novex Xcell II mini cell electrophoresis unit (Invitrogen) with a 10% Bis-Tris gel (Invitrogen). The separated proteins were transferred electrophoretically from the gel to a polyvinylidene fluoride (PVDF) membrane (Invitrogen) in a Novex Xcell II blot module (Invitrogen). The transfer buffer (NuPAGE Transfer Buffer™) was purchased from Invitrogen. The blot module was filled with the transfer buffer until the gel/membrane assembly was covered. The outer buffer chamber was filled with 650 ml deionized water. The protein transfer was performed using a constant voltage of 36 V for 1 h 20 min. After the transblotting, the sample lines were detected by ECL western blotting detection reagents and analysis system (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s instructions. First, the sample lines were incubated with TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3 % Tween 20) containing 10 % cow colostral whey for 25 min and then the first antibodies diluted 1:2000 (anti-CA II, anti-CA IX, anti-CA XII, NRS) or 1:1000 (anti-CA XIII) in TBST buffer for 1 h. The PVDF membranes were washed five times for 5 min with TBST buffer and incubated for 1 h with peroxidase-linked ECL Anti-Rabbit IgG (Amersham Biosciences) diluted 1:25 000 in TBST buffer. After washing four times 5 min in TBST buffer, the polypeptides were visualized by a chemiluminescence substrate (ECL detection reagents 1 + 2, Amersham Biosciences). Kodak™ Biomax™ MS-1 films (Amersham Biosciences) were exposed to the chemiluminescence for 5 min (CA IX and XII) or 1 min (CA II and CA XIII). All the steps were carried out at room temperature. The western blotting experiments were performed in triplicate to confirm the reproducibility of the results.

**Results**

**Immunohistochemistry**

All the studied CA isozymes showed positive immunostaining in the epithelial cells of the mouse endometrium (Fig. 1). CA II and XII showed a somewhat reciprocal distribution pattern in that CA II was confined to the surface epithelial cells (Fig. 1C), while CA XII was more intensely stained in the deep endometrial glands (Fig. 1A). It is noteworthy, however, that CA XII was clearly expressed also in the surface epithelial cells, but the staining intensity was weaker compared to the glands. As expected, the strongest reaction for CA XII was associated with the basolateral plasma membrane, and unexpectedly, also CA II immunoreaction was most intense at the plasma membrane. CA IX and XIII showed weak reactions in both surface and glandular epithelia (Fig. 1B,1D). The control immunostaining with NRS was negative (Fig. 4A).

In the ovary, immunoreactions for different CA isozymes were negligible (Fig. 2). In fact, only CA XII showed occasional positive cells in the corpus luteum (Fig. 2A). No staining for these isozymes was observed in the developing follicles. No immunoreaction was obtained with NRS (Fig. 4B)
In the 9-days-old mouse placenta, the immunostaining reactions for CA isozymes remained quite weak or absent (Fig. 3). CA II was located to the endothelium of the placenta blood vessels and erythrocytes (Fig. 3E), and it was also present in the amnionic epithelium (Fig. 3F). The amnionic epithelium showed no or very weak staining for CA XII, whereas the decidual glands were strongly labeled (Fig. 3B). The control staining again showed no positive signal (Fig. 4C).

**Western blot**
Western blotting was performed for the mouse uterine protein to evaluate the specificity of the immunoreactions. Mouse kidney and colon samples were used as
positive control tissues, since they are known to express CA II, XII and XIII [4,33,35], and the colon contains CA IX [36]. CA II and XIII were positive in all tissue specimens (Fig. 5). Both CA IX and XII showed weak positive reactions in the mouse uterus. The molecular weights for these isozymes were 51 and 46 kDa, respectively. Based on the western blotting the expression of CA XII was weaker in the uterus than in the colon or kidney. On the other hand, CA IX showed the strongest signal in the colon. It is notable that anti-mouse CA XII serum cross-reacted with 30 kDa polypeptide in the western blotting. Previous immunostaining of gastric mucosa with the same anti-CA XII and anti-CA II antibodies has clearly indicated that anti-CA XII serum does not recognize CA II which has a molecular mass of 30 kDa in western blot [35]. Even though gastric epithelial cells contain high levels of CA II, no immunoreaction was obtained with anti-CA XII serum in those cells. Furthermore, no staining has been obtained by anti-CA XII antibody in the red cells which contain high levels of CA I and II, nor in the brain which expresses high levels of CA II and XIII (data not shown).

Discussion

This study describes the expression of CA II, IX, XII and XIII in mouse female genital organs including uterus, ovary and placenta. CA II showed a very limited distribution pattern in the mouse placenta, being present only in the erythrocytes, endothelium of the blood vessels and amnionic epithelium. In previous studies, CA II has been detected by immunohistochemistry in the human villous syncytiotrophoblasts and in varying amounts in fetal villous endothelium [21,22]. Using a histochemical staining method, Ridderstråle et al [18] showed in several species that the highest CA activity located in the maternal capillaries, and the membrane-bound CA activity varied among different species. To date, CA IV is the only membrane-bound CA isozyme which has been detected in the mouse placenta [23]. In our study, CA IX and XII were not found in the mouse placental tissue except that CA XII showed a very weak reaction in the amnionic epithelium. Concluding from the results of the present and previous studies, CA I and II appear to represent the enzyme forms that are most relevant for the placental function [22], while CA IX and XII may play a role in other reproductive organs such as the male excurrent duct and female uterus [14,37].

It is known that CA activity facilitates transport of CO₂ across biological membranes by converting it to bicarbonate and hydrogen ions. These ions are then translocated across the plasma membrane through specific carrier proteins in a coordinated manner. It is of considerable interest that CA isozymes II and IV have been recently described to form active metabolon systems with ion exchanger proteins such as anion exchanger isoform 1

Figure 4
Control immunostaining of mouse uterus, ovary and placenta with normal rabbit serum. No immunoreaction is seen. Original magnifications: × 400.
Figure 2
Immunolocalization of CA XII (A,B), CA IX (C,D), CA II (E,F), and CA XIII (G,H) in the mouse corpus luteum (A,C,E,G) and follicle (B,D,F,H). Only faint positive reaction for CA XII can be seen in occasional cells of the corpus luteum that is indicated in the insert of the panel A (arrows). Original magnifications: × 200 (A,C,E,G), × 400 (B,D,F,H).
Figure 3
Immunohistochemical staining of CA XII (A,B), CA IX (C,D), CA II (E,F), and CA XIII (G,H) in the mouse placenta (A,C,E,G) and amnionic epithelium (B,D,F,H). CA II is located in the endothelium of the blood vessels and erythrocytes (arrows in the panel E). It is also expressed in the amnionic epithelium (arrows in the panel F). Insert of the panel B shows that CA XII is highly expressed in the decidual glands, while the amnionic epithelium is negative. DE = Decidua, P = placenta. Original magnifications: × 400.
(AE1) and Na+/H+-exchanger isoform 1 (NHE1) [38-40]. Even though these associations have not yet been described in the placental tissue, it is possible that such metabolons play a role in facilitating placental ion transport processes.

Previous studies have shown CA activity in the endometrium of several mammalian species [41,42]. Until now the only established isozymes in the human endometrium are CA XII [14] and CA XIII [4]. Interestingly, the high activity isoenzyme, CA II, is not expressed in the human endometrial epithelium [4]. In the present study, all the examined CA isozymes – including CA II – showed positive immunostaining in the epithelial cells of the mouse endometrium. To our knowledge, there are only a few examples of clear species-specific difference in CA expression. These include e.g. CA XII expression in the kidney (human principal cells versus mouse intercalated cells) [33,43] and CA XIII in the human and mouse testis [4]. What would be the physiological consequence of such variation between different species? Of course, there are marked differences in human and rodent reproductive physiology. Mouse is characterized by tremendous reproductive potential. Females generally have 5–10 litters per year if conditions are suitable. Gestation period is 19–21 days. Litters consist of 3–12 (generally 5 or 6) offspring, and the mice reach sexual maturity at 5–7 weeks. Even though our observations do not provide any clues whether CA expression could contribute to some of the described characteristics, these differences may have fundamental physiological effects that should be addressed in future investigations.

In the present study, CA XII and II showed more intense staining in the surface endometrial epithelia than CA IX and XIII. CA XII was more intensely stained in the deeper endometrial glands, while CA II was confined to the surface epithelial cells. Interestingly, CA II showed positive immunoreaction not only in the cytoplasmic compartment but also at the plasma membrane of the cells that is quite surprising for a cytosolic enzyme. The same phenomenon is detectable in some other tissues including the human gallbladder [25] and gut [44]. The cell membrane reactivity may reflect a possible physical association between CA II and ion transport proteins, which has been demonstrated in cell cultures [38-40]. When CA XII was

![Figure 5](http://www.rbej.com/content/2/1/73)

**Figure 5**
Western blotting of total homogenate from mouse uterus, kidney and colon for CA II, IX, XII and XIII. Normal non-immune rabbit serum (NRS) was used instead of the first antibodies as a negative control. Both CA IX and XII show weak positive reactions for the uterine proteins (arrowheads). The molecular weights for these isozymes are 51 and 46 kDa, respectively. The signal for CA XII is weaker in the uterus than in the colon or kidney. Note that anti-CA XII serum cross-reacts with a 30-kDa polypeptide. This cross-reaction is evident only in western blotting conditions as pointed out in the Results section. CA IX shows the strongest signal in the colon. CA II and XIII are positive in all tissue specimens.
first discovered in the normal human endometrium, it was suggested to play a role in reproductive functions [14]. In the endometrium, pH and ion balance has to be tightly regulated to ensure normal fertilization. For example, the bicarbonate concentration has been implicated in the regulation of sperm motility, capacitation, and acrosome reaction [15,17,45]. One major regulatory pathway includes a bicarbonate-sensitive adenylate cyclase that is present in the plasma membrane of the sperm cell [46]. In the female genital tract, the endometrial and oviductal epithelium may produce an alkaline – bicarbonate rich – environment for maintaining the sperm motility. This suggestion is in agreement with the observations by Guerin et al. [47], that the sperm motility is improved by co-culture of human spermatozoa with either endometrial or oviductal epithelial cells.

In the future studies, it will be important to investigate whether the hormonal status regulates the expression of different CA isozymes – particularly CA XII – in the endometrium. Another interesting line of investigations would be to analyze the fertilization capacity of CA XII knockout mice as soon as they become available. One could hypothesize that endometrial CA isozymes are important factors, contributing to the appropriate bicarbonate concentration and pH balance in the cervical and endometrial mucus needed for normal fertilization process. Based on our recent studies, CA IX-deficient mice showed no apparent phenotypic changes linked to fertility [26]. Even more interesting from this point of view is that CA XII may be an important isozyme present in the endometrium, and therefore, CA XII knockout mice will be very attractive targets for reproductive physiological studies.

Conclusions

The present paper demonstrates for the first time the expression of transmembrane carbonic anhydrase isozymes IX and XII in the female murine reproductive tract. The data indicates that the endometrial epithelium is a prominent site for CA XII expression. The conservation of CA XII expression in the endometrium of different species (mouse and human) suggests a role for this isozyme in reproductive physiology.

Authors’ contributions

All authors participated in the design of the study. PH, JL, and SP collected the tissue samples. PH, JL, ET, PK and SP drafted the manuscript. PH, JL and SP performed the western blotting. SPas, JP, AW and WSS provided the antibodies. PH, JL and SP participated in the immunohistochemical staining. All authors read and approved the final manuscript.

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Expression of transmembrane carbonic anhydrases IX and XII in ovarian tumours

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Expression of transmembrane carbonic anhydrases IX and XII in ovarian tumours

Aims: Carbonic anhydrase (CA) isozymes IX and XII have been suggested to play a role in oncogenic processes. The aim of the present study was to investigate CA IX and XII expression in patients with ovarian tumours.

Methods and results: A series of ovarian tumours was immunostained for CA IX and XII and the results were correlated with histopathological and clinical parameters. Most cases of borderline mucinous cystadenomas, mucinous cystadenocarcinomas and serous cystadenocarcinomas were moderately or strongly positive for CA IX. In malignant tumours, the staining was most prominent in hypoxic regions. Expression of CA XII was detected in all tumour categories, although the mean staining intensity was weaker than for CA IX in all groups except for clear cell carcinomas.

Conclusions: The wide expression of CA IX and XII in ovarian tumours suggests that these isozymes could represent potential targets in ovarian cancer therapy. The expression pattern of CA IX suggests that it could also serve as a useful histopathological marker protein for hypoxia in malignant ovarian tumours.

Keywords: carbonic anhydrase, hypoxia, ovarian cancer

Abbreviations: CA, carbonic anhydrase; HIF, hypoxia-inducible factor; HRP, horseradish peroxidase; IHC, immunohistochemistry; VHL, von Hippel-Lindau

Introduction

Carbonic anhydrases (CAs) play a central role in various physiological processes by catalysing the interconversion of carbon dioxide and bicarbonate. At present, 13 isozymes, differing in their tissue distribution and enzymatic activity, have been identified in mammals.1 Among these enzymes, CA IX and XII are unique with respect to their association with various cancers.

CA IX enzyme has been shown to consist of a signal peptide, a proteoglycan-related sequence, a CA domain, a transmembrane segment and a short intracellular tail.2 It is a 459 amino acid glycoprotein of 54 and 58 kDa mass expressed at the basolateral plasma membrane of epithelial cells and, in some cases, also in the nucleus.3 CA IX was initially discovered in HeLa cells and its participation in oncogenesis was suggested by the facts that its expression correlated with the...
tumorigenicity of HeLa–fibroblast hybrids and its expression was also detected in various carcinomas but not in corresponding normal tissues. On the other hand, carcinomas originating from CA IX+ tissues, such as gastric and biliary mucosa, have typically shown reduced levels of CA IX expression. Experiments with cultured cells and tumour specimens have indicated that CA IX expression is regulated by von Hippel-Lindau (VHL) protein and hypoxia-mediated pathway.

CA XII was cloned and characterized by two groups almost simultaneously. The 354-amino acid CA XII polypeptide contains a signal sequence, a CA domain, an additional short extracellular segment, a transmembrane segment and a C-terminal cytoplasmic tail. When expressed in transformed African green monkey kidney fibroblast cells (COS) and Chinese hamster ovary cells, the cDNA produced a 43–45-kDa enzymatically active membrane-bound protein. Like CA IX, CA XII has been detected in some normal tissues and is overexpressed in certain cancers and tumour cell lines. It is also involved in VHL-mediated carcinogenesis.

Although CA IX and XII have been identified as cancer-associated CAs, their functions in cancer cells are still poorly understood. Both isoforms are catalytically active enzymes, suggesting a role in pH regulation in the tumour cell microenvironment. Indeed, CA IX can contribute to acidification of the extracellular milieu of hypoxic cells. Previous studies have also suggested roles for CA IX in cell proliferation and intercellular communication. It has been shown to participate in cell adhesion via its proteoglycan-related domain.

The present study was designed to investigate CA IX and XII expression in ovarian cancer. Overall, the prognosis of ovarian cancer remains poor, with a 53% 5-year survival rate. For example, in the USA approximately 16 000 women die every year from ovarian cancer. Because of its high prevalence and mortality all attempts should be made to clarify the pathogenic mechanisms involved and to develop improved detection methods and treatment.

**Materials and methods**

**Preparation of tissue samples**

The tissue samples from epithelial ovarian tumours were obtained, together with routine histopathological specimens, during surgical operations carried out at Oulu University Hospital. The study was approved by the Research Ethics Committee of Oulu University Hospital. Cases included four borderline serous cystadenomas, three borderline mucinous cystadenomas, 34 serous cystadenocarcinomas, seven mucinous cystadenocarcinomas, 14 endometrioid adenocarcinomas and four clear cell adenocarcinomas. The samples were fixed in 4% formaldehyde, embedded in paraffin and sectioned at 5 μm. Routine histology was carried out after haematoxylin and eosin (H&E) staining of the sections.

**Antibodies and immunohistochemistry**

The monoclonal antibody M75, recognizing the N-terminal domain of human CA IX, has been described previously. The rabbit antihuman CA XII antiserum to the secretory form of CA XII has been characterized by Karhumaa et al. Normal rabbit serum was used as a control. Immunohistochemistry (IHC) was performed using an automated Laboratory Vision Autostainer 480 (ImmunoVision Technologies Co., Brisbane, CA, USA) and Power Vision+/C212 Poly horseradish peroxidase (HRP) IHC Kit reagents (ImmunoVision Technologies). Immunostaining was performed according to the following protocol: (i) rinsing in wash buffer; (ii) treatment in 3% H₂O₂ in ddH₂O for 5 min and rinsing in wash buffer; (iii) blocking with Universal IHC Blocking/Diluent for 30 min and rinsing in wash buffer. The monoclonal antibody M75, recognizing the N-terminal domain of human CA IX, has been described previously. The rabbit antihuman CA XII antiserum to the secretory form of CA XII has been characterized by Karhumaa et al. Normal rabbit serum was used as a control. Immunohistochemistry (IHC) was performed using an automated Laboratory Vision Autostainer 480 (ImmunoVision Technologies Co., Brisbane, CA, USA) and Power Vision+/C212 Poly horseradish peroxidase (HRP) IHC Kit reagents (ImmunoVision Technologies). Immunostaining was performed according to the following protocol: (i) rinsing in wash buffer; (ii) treatment in 3% H₂O₂ in ddH₂O for 5 min and rinsing in wash buffer; (iii) blocking with Universal IHC Blocking/Diluent for 30 min and rinsing in wash buffer.

**Figure 1.** Intensity of carbonic anhydrase (CA) IX immunostaining in different types of ovarian tumours. The strongest reactions were observed in the borderline mucinous cystadenomas, mucinous cystadenocarcinomas and serous cystadenocarcinomas.

in wash buffer; (iv) incubation with the primary antibody or normal rabbit serum diluted 1:1000 (anti-CA IX) or 1:2000 (anti-CA XII and normal rabbit serum) in Universal IHC Blocking/Diluent for 30 min; (v) rinsing in wash buffer for 3 × 5 min; (vi) incubation in Poly HRP-conjugated antimouse or antirabbit IgG for 30 min and rinsing in wash buffer for 3 × 5 min; (vii) incubation in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (one drop DAB solution A and one drop DAB solution B) with 1 ml ddH2O for 6 min; (viii) rinsing with ddH2O; (ix) CuSO4 treatment for 5 min to enhance the signal; and (x) rinsing with ddH2O. All procedures were carried out at room temperature. After the immunostaining, the sections were counterstained with haematoxylin, mounted in Entellan Neu (Merck, Darmstadt, Germany) and finally examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany). Intensity of the staining was scored on a scale of 0–3 by three of the investigators (P.H., H.H. and S.Par.) as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction.

**Statistical Analysis**

Statistical analysis of the results was performed using SPSS for Windows software (SPSS Inc., Chicago, IL, USA). Kaplan–Meier survival analysis and log rank test were used to compare the distribution of the lifetime data of the patients after diagnosis and surgery.

Results

CA IX in Ovarian Tumours

The immunohistochemical results demonstrated very strong CA IX expression in some benign and malignant tumours. Figure 1 shows the expression pattern in each disease category. The strongest expression was observed in the mucinous cystadenocarcinomas and borderline mucinous cystadenomas. Strong staining was detected in 57% and 33% of cases, respectively. Variation in the staining intensity was higher in serous cystadenocarcinomas, about 12% showing a strong signal. Other tumours exhibited lower expression and 50% of clear cell adenocarcinomas were completely negative.

Figure 2 demonstrates representative examples of immunostaining in different tumour categories. Figure 2C,D show the presence of intense labelling in perinecrotic areas of a serous cystadenocarcinoma, whereas the cells in the near vicinity of neovessels exhibited lower staining intensity. This expression pattern is indicative of CA IX's association with hypoxia.8 Also in endometrioid adenocarcinomas, the highest expression was found in areas adjacent to necrosis (Figure 2F).

Since the category of serous cystadenocarcinomas included the highest number of cases and the tumours showed distinct variation in the staining intensity from weak to strong, we wanted to explore whether CA IX had any prognostic value in this disease. The patients were grouped into two categories based on their tumour immunoreactivity: those patients with moderate or high signal intensity and those with weakly stained tumours. The results indicated that the patients in the latter category had a slightly better survival rate (Figure 3), but the difference between these categories was not statistically significant (P = 0.14), which could, in part, be due to the small number of cases.
Figure 4 demonstrates a comparison of CA IX immunostaining in serous cystadenocarcinomas grouped according to the grade of differentiation. Most grade 1 tumours showed weak immunoreactivity, while the staining intensity was slightly stronger in grade 2 and 3 tumours. However, the differences were not statistically significant.

Figure 5 shows a graphical illustration of CA XII expression in different groups of ovarian tumours. Compared with CA IX, the staining intensity for CA XII was lower in every tumour category except for the clear cell adenocarcinomas. The highest signal intensity was observed in mucinous cystadenocarcinomas. All six tumour categories are represented in Figure 6, showing examples of staining for CA XII. The induction of expression (as observed for CA IX) was not evident adjacent to necrosis (Figure 6C,E). In the group of patients with serous cystadenocarcinomas, CA XII intensity showed no significant correlation with the survival rate or grade (data not shown).

Discussion

The present study has shown that both CA IX and XII are expressed in several types of ovarian tumours. The high expression of CA IX in mucinous and serous
Figure 6. Immunohistochemistry of carbonic anhydrase XII in ovarian tumours. The immunoreactions are shown in a borderline serous cystadenoma (A), borderline mucinous cystadenoma (B), serous cystadenocarcinoma (C), mucinous cystadenocarcinoma (D), endometrioid adenocarcinoma (E) and clear cell adenocarcinoma (F). No distinct difference was observed in the distribution of the immunostaining between hypoxic and non-hypoxic areas. N, Necrosis; BV, blood vessels. Bars: 50 μm.

cystadenocarcinomas suggests that these tumour types can now be listed as candidates for CA IX-targeted therapy. A chimeric monoclonal antibody WX-G250 against CA IX is currently under evaluation for the therapy of metastatic and non-metastatic renal cancer.\textsuperscript{19,20} Interestingly, mucinous cystadenocarcinomas showed the highest expression and are considered to be highly aggressive, with a poor survival rate in patients with advanced disease.\textsuperscript{21} It is possible that CA IX could represent a valuable diagnostic marker for ovarian mucinous tumours, as is the case for pancreatic\textsuperscript{22} and colorectal\textsuperscript{23} mucinous tumours, since they all show high levels of CA IX expression.

The distribution of the immunoreactivity suggests that the oxygen level within tissue regulates CA IX expression in ovarian tumours, in that the malignant cells in the vicinity of neovessels show lower expression compared with those located adjacent to necrotic areas. CA XII, on the other hand, did not show such variation in staining intensity. This is an interesting difference since a previous study has indicated that both isozymes can be induced by hypoxia.\textsuperscript{8} Studies of the CA9 promoter have demonstrated that sequences close to the transcriptional initiation site are sufficient to convey a hypoxia-inducible response. This activity was mediated by hypoxia-inducible factor (HIF)-1\textgamma and was dependent on a consensus hypoxia-responsive element lying adjacent to the transcription initiation site. Collectively, these data indicated that CA9 gene is a target for HIF-mediated regulation both in vitro and in vivo.\textsuperscript{24} Although hypoxia also seemed to increase CA XII mRNA expression in some cell lines, a functional hypoxia responsive element has never been reported for the CA12 gene. Differences in the immunolocalization of CA IX and XII in ovarian tumours bring into question whether CA12 is really an HIF-target gene. To resolve this uncertainty, it will be important to elucidate the mechanisms regulating CA XII expression.

Disturbed cell adhesion has been implicated as a factor of great importance in the invasive capacity of malignant tumours.\textsuperscript{25} The down-regulation of cadherins or dysfunction of the cadherin–catenin complex has been noted in different types of tumours, including ovarian cancer. An important cell adhesion molecule, E-cadherin, is expressed in inclusion cysts of the normal ovary and in ovarian tumours with invasive capacity.\textsuperscript{26,27} Based on histopathological studies, early malignant changes characteristically seem to occur in ovarian surface epithelium-lined clefts and inclusion cysts,\textsuperscript{28,29} suggesting that up-regulation of E-cadherin in normal ovarian surface epithelium surrounded by stromal cells could play a role in the early events of malignant transformation. However, hypoxia attenuates the expression of E-cadherin, leading to reduction of cellular adhesion and facilitating invasiveness of ovarian cancer cells.\textsuperscript{30} Also, the cadherin-binding molecules, \textalpha- and \textbeta-catenin, are expressed in normal ovarian surface epithelium and ovarian tumours.\textsuperscript{31,32} Interestingly, previous studies have shown that CA IX associates with \textbeta-catenin.\textsuperscript{31} Based on these observations, one could hypothesize that CA IX most probably binds to the \textbeta-catenin–E-cadherin complex and/or free \textbeta-catenin in ovarian tumours. Due to mutations and/or hypoxia, the tumour cells have large pools of cytoplasmic \textbeta-catenin that is free to interact with both the cell–cell adhesion complex and the transcription factor T-cell factor/lymphoid enhancer factor (Tcf-Lef).\textsuperscript{33} The \textbeta-catenin–Tcf-Lef complex then translocates to the cell nucleus, resulting in the activation of several genes, some of which are implicated in tumour progression. Although we cannot predict the functional significance of such protein interaction in ovarian tumours at present, one might hypothesize that this complex would allow a route for CA IX to enter the cell nucleus. Previous studies have indeed shown that CA IX may occasionally reside in the nucleus.\textsuperscript{3} Accordingly, some nuclear signal was also seen in the present ovarian tumour samples (data not shown), even though the majority of CA IX labelling was located on the cell surface.

Although we have recently documented that CA IX is not expressed in the normal mouse ovary,\textsuperscript{35} the present results show that it is induced in neoplastic cells, most probably due to cellular hypoxia. It has been hypothesized that the progression of malignant tumours is mediated by hypoxia and necrosis, which initiate a chain of events leading to the acquisition of a more malignant phenotype.\textsuperscript{16,37} A malignant tumour would first undergo uncontrolled growth, eventually outstripping its blood supply, resulting in hypoxia and starvation due to lack of nutrients. The resultant necrosis would release active substances, including cytokines, peptide growth factors and cytotoxic factors and would result in a population of cells exposed to sublethal ischaemia. Ischaemia would trigger oncogenic metabolism, reduce cell adhesion, increase DNA mutations and stimulate angiogenesis. With the in-growth of neovessels and reoxygenation of the affected cells, the resultant tumour acquires a more aggressive behaviour due to multiple genomic mutations. In addition, the oncogenic metabolism of hypoxic tumour cells, which relies on anaerobic glycolysis, produces excessive lactic acid that acidifies the extracellular microenvironment and further promotes invasion of tumour cells.\textsuperscript{38} Interestingly, hypoxia activates both the level and capacity of CA IX to augment extra-
cellular acidification, suggesting that another way it can be functionally implicated in tumour progression is via modulation of tumour pH.\textsuperscript{12} Expression of an extracellular CA on the cell surface is known to enhance proton-coupled lactate secretion in astrocytes.\textsuperscript{10} Such activity in tumour cells would contribute to extracellular acidification and possibly enhance cell growth by removing the excess lactic acid produced in the cytosol of the tumour cells.\textsuperscript{40,41}

Unlike CA IX, CA XII does not contain an N-terminal proteoglycan domain and has not been linked to cell adhesion. However, it is a highly active enzyme and may also serve as a modulator of extracellular pH.\textsuperscript{9,10} In fact, \textit{in vitro} experiments indicate that inhibition of CA activity in renal carcinoma cells that express CA XII reduces their invasion capacity through matrigel.\textsuperscript{42}

Although evidence suggesting roles for CA IX and CA XII in oncogenesis have been proposed, the case is not yet definitive. The data presented here indicate that CA IX and CA XII are expressed in ovarian cancer cells and should be evaluated as potential clinically useful markers and/or target molecules.

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References


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 (HNPCC)]. 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 HNPCC 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 HNPCC tumors express high 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 HNPCC. CA IX could be a potential diagnostic and therapeutic target in HNPCC. (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 (HNPCC; 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 HNPCC is better than for the sporadic form of cancer (3, 4), although the patients with HNPCC 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 → H2CO3 → H+ + HCO3−). There are at least 13 active α-CA isoforms 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...
Carbonic anhydrase isozymes II, IX, and XII in uterine tumors

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Histopathological diagnostics of gynecological malignancies continues to be challenging despite the well established criteria. For example, the morphological distinction of uterine leiomyosarcoma from certain variants of benign leiomyoma can be difficult. Herein, we investigated the expression of Carbonic anhydrase (CA) II, IX, and XII in the normal endometrium, leiomyomas, uterine sarcomas, and endometrial adenocarcinomas using immunohistochemistry. These isozymes are considered promising diagnostic markers and therapeutic targets. The normal endometrium showed high CA XII expression, whereas the signals were lower in endometrial adenocarcinoma (p < 0.004). Only sporadic CA IX staining was found in the normal endometrium, whereas the enzyme was overexpressed in most cases of endometrial adenocarcinoma (p < 0.005). CA II expression was slightly weaker in the normal endometrium than that in the adenocarcinomas (p < 0.008). Positive immunostaining reactions for CAs were observed in the uterine sarcomas, whereas all leiomyomas were negative for CA II and XII. A comparison between leiomyomas and sarcomas showed statistically significant differences for all studied isozymes (p < 0.001). Our study shows that CA isozymes could together serve as histopathological biomarkers for differential diagnosis between uterine leiomyosarcoma and leiomyoma. In addition to being found in leiomyosarcomas, CA II and IX were overexpressed in endometrial adenocarcinoma, where they might regulate the pH of the tumor microenvironment.

Key words: Adenocarcinoma; carbonic anhydrase; immunohistochemistry; mesenchymal tumor; sarcoma; uterine cancer.

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Endometrial carcinoma is a very common gynecological malignancy type in the Western world (1, 2). The National Cancer Institute estimated 40 470 new cases in the USA in 2011 (http://www.cancer.gov). Even though the 5-year survival rates for all the stages are all about 80%, an estimated 9000 women in Europe die from endometrial cancer each year (3). In contrast to endometrial carcinoma, uterine leiomyosarcoma is rare (incidence: 0.64 per 100 000 women), accounting for 0.2–6% of all smooth muscle tumors of the uterus (4). Independent of the stage, these tumors are aggressive and have
a high recurrence rate as well as metastatic capacity. Uterine leiomyomas are the most common solid benign pelvic tumors in women. A systematic histological examination of hysterectomy specimens has shown a prevalence of uterine leiomyomas as high as 77% (5). In another study, it has also been estimated that 30–50% of women have leiomyomas. The risk for the progression of a benign uterine leiomyoma to uterine sarcoma has been estimated to be <0.1% (6).

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide in the reaction \( \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \). The main function of CAs is to maintain an appropriate acid–base balance in organisms, and thus they participate in various biological processes, including \( \text{CO}_2 \) transport, the regulation of pH homeostasis, bone resorption, ureagenesis, gluconeogenesis, the production of body fluids, and fertilization (7–9). The CA family consists of 13 active isozymes in mammals, 12 of which are expressed in humans (10). CA IX and CA XII are membrane-bound, tumor-related members of the CA family, and their expression is induced by hypoxia. They are both catalytically active, suggesting a role in the pH regulation of the tumor cell microenvironment. CAs have recently become a target for intensive research of tumor invasion and carcinogenesis, and previous studies have also suggested roles for CA IX in cell proliferation and adhesion (11–14).

The membrane-bound CA IX is expressed in a limited number of normal tissues, such as the stomach, gallbladder, ileum, colon, liver, and pancreas (15), whereas it is highly overexpressed in human epithelial tumors, such as carcinomas of the uterine cervix, breast, ovary, lung, and kidney, all of which are tissues that have low or no endogenous CA IX expression (14, 16–22). Interestingly, the tumors originating from normally CA IX-positive tissues, such as the gastric mucosa, show lowered expression of CA IX (23). The presence of CA IX usually indicates a poor prognosis for the patient, with the exception of renal cancer (17, 19–21, 24). CA IX clearly colocalizes with regions of tumor hypoxia, and its expression is also induced in tumor cells when cultured under hypoxia (14, 25, 26). Due to its hypoxic regulation, CA IX has been considered a potential biomarker not only for differential diagnostics and prognosis but also for determination of tumor hypoxia (27, 28). There are a few previous studies on the expression of CA IX in gynecological cancer. Yunokawa et al. (29) recently reported higher CA IX expression in endometrial hyperplasia and endometrial carcinoma than in the normal endometrium. Overexpression of CA IX is well known in cervical cancer (19), and recently, Liao et al. (30) demonstrated that tumor hypoxia measured by immunohistochemical expression of CA IX is an independent prognostic factor for survival in high-risk, early-stage cervical cancer.

CA XII is expressed in the basolateral plasma membrane of the normal endometrial epithelium, suggesting that the enzyme plays a role in reproductive physiology (31, 32). This isozyme is also found in the epithelial cells of the efferent ducts, kidney, colon, and mammary gland (14, 33–35). The overexpression of this enzyme has been reported in several cancers (14, 22), and it correlates with a favorable prognosis in invasive breast cancer (36). In renal cell carcinomas, it shows a slight correlation with the histological grade (34). In colorectal tumors, CA XII is more widespread compared to the normal mucosa, and the expression increases with the grade of dysplasia (35). The other tumor categories in which CA XII is overexpressed include e.g. ovarian, renal, and brain tumors (21, 34, 37, 38). As with CA IX, CA XII expression has shown a correlation with patient prognosis in some tumor categories (37).

Carbonic anhydrase II is a cytosolic enzyme that is highly expressed in most organs and contributes to several important physiological processes (39). The few studies on CA II in tumors indicate that in most cases malignant cells contain no or only low levels of CA II (40, 41). Yoshiura et al. (42) recently demonstrated CA II expression in the tumor vessel endothelium of melanoma, esophageal, renal, and lung cancer, and later it was also found in the neovessels of malignant astrocytic gliomas (43). It has been shown that cell culture conditions reminiscent of a cancer cell microenvironment induce CA II expression in endothelial cells in vitro.

For a clinician, it is difficult to distinguish a benign leiomyoma from an aggressive uterine sarcoma. Tumor size or speed of tumor growth does not lead to a conclusive differential
diagnosis between leiomyoma and leiomyosarcoma, and therefore the leiomyosarcoma diagnosis is often a negative surprise not only to the patient but also to the operating gynecologist. These unsatisfactory conditions have promoted research for novel biomarkers and targets for the prevention and/or therapy of these tumors. Our findings on the expression of CA IX and CA XII in the normal endometrium, endometrial adenocarcinoma, uterine sarcoma, and leiomyoma, and the demonstration of CA II in the endothelium of these tumor tissues, brings new knowledge on the distribution of these enzymes in gynecological tumors.

MATERIALS AND METHODS

Preparation of tissue samples

The tissue samples from endometrial adenocarcinoma, sarcoma, myoma, and normal endometrium were obtained together with routine histopathological specimens during surgical operations carried out at Tampere University Hospital. Our material consisted of samples which were retrospectively and randomly collected from consecutive patients with uterine tumors. The protocol was approved by the Research Ethics Committee of the Tampere University Hospital. Our study included 30 adenocarcinoma (in CA XII 27), 10 leiomyosarcomas, 10 stromal sarcomas, 5 mixed Müllerian tumors, 33 leiomyomas, and 9 normal endometrium specimens. Of the stromal sarcomas there were five high grade and four low grade tumors, and the data in one tumor were missing. Of the adenocarcinoma tumors 12 were of gradus I, 14 of gradus II, 3 of gradus III, while the data were not available in one case. Of the mixed Müllerian tumors there were three heterologous and two homologous cases. These samples were fixed in 4% formaldehyde, embedded in paraffin, and sectioned at 5 μm. Routine histology was carried out after hematoxylin and eosin staining of the sections.

Antibodies and immunohistochemistry

The monoclonal antibody M75, recognizing the N-terminal domain of human CA IX, has been described previously (44). The rabbit anti-human CA XII serum to the secretory form of CA XII has been characterized by Karhumaa et al. (31). The rabbit anti-human CA II serum has been previously utilized in numerous studies and have been shown to be specific for each isozyme. Normal rabbit serum was used for the control staining. Five micrometer 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 2000 + Poly-horseradish peroxidase (HRP) Immunohistochemistry kit (ImmunoVision Technologies Co., Hillsborough, CA, USA) reagents and included the following steps: (i) rinsing in wash buffer; (ii) treatment in 3% H₂O₂ in ddH₂O for 5 min and rinsing with wash buffer; (iii) blocking with cow colostrum diluted 1:2 in Tris-buffered saline containing 0.05% Tween-20 for 30 min and rinsing in wash buffer; (iv) 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; (v) rinsing in wash buffer three times for 5 min; (vi) incubation in poly-HRP-conjugated anti-rabbit/mouse IgG for 30 min and rinsing in wash buffer three times for 5 min; (vii) incubation in 3,3' diaminobenzidine tetrahydrochloride (DAB) solution (one drop of DAB solution A and one drop of DAB solution B in 1 mL of ddH₂O) for 6 min; (viii) CuSO₄ treatment for 5 min to enhance the signal; and (ix) rinsing with ddH₂O. 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 (INT) of the staining was scored on a scale of 0–3 by two of the investigators (P. Hynninen and S. Parkkila) as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; and 3, strong reaction. The extent (EXT) of 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. The staining index (SI) was calculated using the following formula: \( \sqrt{\text{EXT} \times \text{INT}} \). This method was derived from a previous study on gastric tumors (23).

Statistical analysis

Statistical analyses of the results were performed using SPSS for Windows software (SPSS Inc., Chicago IL, USA) in Tampere University Hospital. Mann–Whitney and Kruskal–Wallis tests were used to determine the p-values.

RESULTS

Expression of carbonic anhydrases in normal endometrium and endometrial adenocarcinoma

Carbonic anhydrase II is a cytosolic enzyme that is mainly absent or very weakly expressed
in the normal endometrial epithelium. Our results showed that CA II immunostaining was little higher in endometrial adenocarcinoma (p < 0.008). Figure 1A shows that the median SI in normal endometrium was 1.0, whereas it was 1.41 in adenocarcinoma specimens. Figure 2A demonstrates a representative image of negative CA II staining in the normal endometrium (proliferative phase). In contrast, a grade-2 adenocarcinoma specimen shows a clear positive signal for CA II in the malignant cells (Fig. 2C).

Normal endometrium contains only faint reactivity for CA IX (Figs 1B and 3A). As predicted, this ‘cancer-associated’ enzyme was more strongly expressed in endometrial adenocarcinoma specimens (p < 0.005). Figure 3C shows strong expression for CA IX in the plasma membrane of malignant cells in a grade-1 adenocarcinoma sample. The median SI values in the normal endometrium and adenocarcinoma specimens were 1.0 and 2.0, respectively (Fig. 1B). In carcinoma specimens, CA IX was often more strongly expressed in hypoxic areas close to necrosis.

High expression of CA XII has been previously demonstrated in the normal human and mouse endometrium (31, 32). The present results interestingly showed that CA XII is less expressed in endometrial adenocarcinoma than in normal endometrium (p < 0.004) (Fig. 1C). The median SI in the normal endometrium was 2.45, whereas it was only 1.73 in adenocarcinomas. CA XII staining was highest in the basolateral plasma membrane of epithelial cells (Fig. 5A). Whereas CA XII was constantly found in all normal specimens, the endometrial adenocarcinoma samples showed more variation in the staining intensity. Figure 5C shows that the immunostaining was quite strong in small areas typically located in close proximity to necrotic regions.

Carbonic anhydrases in mesenchymal tumors
Carbonic anhydrase expression was also studied in a series of mesenchymal tumors including uterine leiomyomas, leiomyosarcomas, stromal sarcomas, and mixed Müllerian tumors. It was notable that no CA II immunostaining was observed in leiomyomas, whereas the other tumor categories showed some positivity (p < 0.001). The median SI in leiomyosarcomas was 0.5, compared to 1.4 in stromal sarcomas and in mixed Müllerian tumors (Fig. 4A).

![Fig. 1. A boxplot illustration of carbonic anhydrase (CA) II (A), CA IX (B), and CA XII (C) staining in normal endometrium and endometrial adenocarcinoma samples. (A) In the normal endometrium specimens, the staining index (SI) of CA II ranged from 0 to 2.5 and the median SI was 1.0. In adenocarcinomas, the SI values ranged from 0 to 3 and the median SI was 1.41 (p < 0.008). (B) In the normal endometrium samples, the SI of CA IX ranged from 0 to 2.5 and the median SI was 1.0. In adenocarcinomas, the SI ranged from 0 to 3 and the median SI was 2.0 (p < 0.005). (C) In the normal endometrium samples, the SI of CA XII ranged from 2.0 to 3.0 and the median SI was 2.45. In the adenocarcinoma samples, the SI ranged from 0 to 3 and the median SI was 1.73 (p < 0.004).](image-url)
and D–F demonstrates some examples of mesenchymal tumors immunostained for CA II. In several cases, the tumor cells remained negative, but the capillary endothelium was strongly immunopositive.

Carbonic anhydrase IX showed higher immunoreactivity in malignant tumors even though there was a lot of intratumoral variation in the distribution of staining (Fig. 4B). Some focal areas were strongly positive, which might reflect poor oxygenation of the cells and stabilization of (Hypoxia-inducible factor) HIF-\(\alpha\) in these regions. The median SI values in leiomyosarcomas, stromal sarcomas, and mixed Müllerian tumors were 1.7, 0.5, and 1.7, respectively. Interestingly, 5 out of 33 specimens showed positive staining for CA IX, although most leiomyoma samples were completely negative. The SI values of these five positive leiomyoma specimens were: 2.5, 1.7, 1.7, 1.4, and 1.4. A more detailed histopathological analysis revealed no other exceptional features in these specimens, nor did we find any recurrences in the follow-up of these patients. Figure 3B shows a negative leiomyoma specimen, whereas leiomyosarcoma (Fig. 3D), stromal sarcoma

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**Fig. 2.** Immunohistochemical staining of carbonic anhydrase (CA) II in the normal uterus and uterine tumors. No staining is present in normal endometrium of the proliferative phase (A). A myoma specimen shows positive signal only in the blood vessel endothelium and erythrocytes (B). In (C), CA II is demonstrated in a sample of grade-2 adenocarcinoma, where the malignant epithelial cells show strong positive immunostaining. The tumor stroma in leiomyosarcoma (D), stromal sarcoma (E), and mixed Müllerian tumor (F) show different staining intensities. In the leiomyosarcoma – CA II-positive reaction can be seen in the blood capillaries. High-intensity capillary reactions are shown in the stromal sarcoma. The malignant cells of mixed Müllerian tumor are strongly positive. Original magnifications: 400x.
(Fig. 3E), and mixed Müllerian tumor (Fig. 3F) show some positive immunostaining. In Fig. 3D and E, CA IX staining is absent in the perivascular region, whereas the cells farther off the neovessels show strong immunostaining, indicating the association of CA IX with hypoxia (25).

Carbonic anhydrase XII showed significant differences in the staining reactivity between the leiomyomas and sarcomas (p < 0.001). No immunostaining was observed in leiomyomas, whereas the other tumor categories showed mainly weak reactivity (Fig. 4C). In leiomyosarcomas, the median SI was 1.2, and in mixed Müllerian tumors it was 1.0. In stromal sarcomas, the median SI remained as 0. Figure 5B shows a typical negative staining result in a leiomyoma specimen. A weak immunoreaction is present in the leiomyosarcoma (Fig. 5D). Also the specimens of stromal sarcoma (Fig. 5E) and mixed Müllerian tumor (Fig. 5F) show some weak reaction.

**CA II immunoreactions in tumor endothelium**

Because previous studies have shown that CA II is present in the endothelium of neovessels of
different tumors, we wanted to investigate whether or not it is expressed in the endothelial cells of gynecological tumors. The results clearly show that CA II-positivity is commonly seen in these tumors. Figure 6 demonstrates that the highest endothelial immunoreactions for CA II were detected in mixed Müllerian tumors and stromal sarcomas. Notable, none of the tumor categories remained completely negative. The endothelial expression of CA II was not limited to malignant tumors. In fact, several benign leiomyomas showed positive reactions, even though the tumor stroma remained negative (Fig. 2B).

**DISCUSSION**

Carbonic anhydrases have been described to associate with various cancers, where they contribute to the regulation of the pH homeostasis of the tumor cell microenvironment (22). CA IX has shown the strongest association with certain tumors, suggesting that it is a strong candidate protein as a therapeutic target and as a diagnostic marker (16, 46, 47). It has been shown to be induced by tumor hypoxia via the HIF-1α-regulated pathway (14, 48). In normoxic cells, HIF-1α is normally targeted to proteosomal degradation due to ubiquitinylation by von Hippel-Lindau (VHL) tumor suppressor protein. Patients with VHL-mutations develop tumors, especially renal clear cell carcinomas, which strongly overexpress CA IX because of the defective function of VHL tumor suppressor protein (22, 49). In patients with normal VHL alleles, CA IX expression is typically up regulated in hypoxic tumors with poor prognosis. CA II and CA XII represent the other CA isozymes that are highly

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**Fig. 4.** A boxplot illustration of carbonic anhydrase (CA) II (A), CA IX (B), and CA XII (C) immunostaining in leiomyoma and uterine sarcoma specimens. (A) There was no reaction for CA II in leiomyomas. In leiomyosarcoma samples, the staining index (SI) ranged from 0 to 3.0 and the median SI was 0.50. In stromal sarcomas, the SI values ranged from 0 to 2.5 and the median SI was 1.4. In mixed Müllerian tumors, the SI ranged from 0 to 3.0 and the median SI was 1.4. There was a significant difference in the staining reactivity for CA II between the leiomyomas and uterine sarcomas (p < 0.001). (B) A boxplot illustration of CA IX staining in leiomyomas and uterine sarcomas (p < 0.002). Five leiomyoma specimens showed positive CA IX immunostaining, while the remaining 27 samples were negative. In leioyosarcomas, the SI ranged from 0 to 3.0 and the median SI was 1.7. In stromal sarcomas, the SI ranged from 0 to 2.5 and the median SI was 0.5. In mixed Müllerian tumors, the SI ranged from 0 to 2.5 and the median SI was 1.7. (C) A boxplot illustration of CA XII staining in uterine leiomyoma and sarcoma specimens (p < 0.001). All leiomyoma samples remained negative. In leiomyosarcoma samples, the SI ranged from 0 to 2.5 and the median SI was 1.2. In stromal sarcomas, the SI ranged from 0 to 1.7 and the median SI was 0. In mixed Müllerian tumors, the SI ranged from 0 to 1.4 and the median SI was 1.0.
expressed in certain cancers such as renal carcinoma (CA XII) (34), brain tumors (CA II and CA XII) (37, 43), and colorectal carcinoma (CA XII) (35). There are also reports showing that the expression levels of CA II and CA XII seem to correlate with patient prognosis in some tumor categories (37, 43).

In the present study, we investigated the expression of CA II, CA IX, and CA XII in a series of gynecological malignancies including adenocarcinomas and mesenchymal tumors. Our specimens involved a total of 33 leiomyomas, all of which were negative for CA II and CA XII. Our group recently investigated CA II expression in a series of mesenchymal tumors mainly originating from the gastrointestinal tract (50). CA II was found to be a sensitive and quite specific marker for gastrointestinal stromal tumors. In the previous study, three of 35 leiomyomas showed some positive reaction for CA II. Based on the previous and present results, there is no doubt that CA II is absent in most leiomyomas.

Previously, Mayer et al. (51) showed that 17 leiomyomas were all negative for CA IX. Interestingly, the present 33 leiomyoma specimens included five cases that were found to be positive for CA IX. The staining was focal in four
specimens, while one showed a diffuse staining pattern. Our first hypothesis was that the CA IX-positive specimens were probably hypoxic tumors because the induction of HIF-1α and its target genes like CA IX is perceived as being a physiological reaction to hypoxia (52). However, we found no necrosis in any of the five CA IX-positive leiomyomas, suggesting that hypoxia was not a significant factor for the CA IX overexpression in these tumors. The ages of the patients with CA IX-positive tumors differed from 44 to 68 years, and the tumor sizes ranged from 1 to 7 cm. HIF-1 expression has been reported in the leiomyomas of patients with a rare syndrome of hereditary leiomyoma and renal cell cancer (53). These rare patients have a 71-fold risk for developing the leiomyoma into a leiomyosarcoma (54). In our study, all the patients with CA IX-positive leiomyomas were alive at 15 years after the operation and had no other tumors or recurrences. Even though these cases were interesting, we discovered no common factor to explain the CA IX-positivity.

Positive staining of CA II, CA IX, and CA XII was detected in many cases of sarcomas. To our knowledge, there are only two studies in which CA IX expression has been evaluated in very small cohorts of leiomyosarcomas (51). Our study confirmed the earlier positive results of CA IX expression in leiomyosarcoma and also added new data on the expression levels of CA IX in stromal sarcomas and mixed Müllerian tumors. In addition to the results for CA IX, our findings showed that CA II and CA XII are often weakly or moderately expressed in these mesenchymal tumors. It would be important to find novel biomarkers for sarcomas because they are aggressively growing tumors and respond poorly to treatments. Among these tumors all isozymes showed variable staining results, suggesting that they have only limited value in sarcoma diagnostics if used alone. In addition to being a promising immunohistochemical marker, CA IX can also be detected from serum or tissue samples using a specific immunoassay. Interestingly, Zhou et al. (55) recently showed that the tissue CA IX levels measured by enzyme-linked immunosorbent assay (ELISA) highly correlated with the CA IX levels detected by immunohistochemistry in clear cell renal cell carcinomas (CCRCC). Serum CA IX levels in CCRCC patients were significantly higher than in non-CCRCC patients and the serum CA IX level detected by ELISA correlated with the tumor size in CCRCC patients. In a vulvar cancer study, CA IX serum concentration was higher in patients with high intratumoral expression and elevated preoperative serum values were associated with unfavorable prognosis (56). There have also been some disappointing news like in the study of Woelber et al. (57) where they found no correlation between serum CA IX concentration and its intratumoral expression in patients with primary cervical cancer, even though CA IX was observed in 81.9% of the tumor specimens. In a preoperative characterization of CCRCC patients, Divgi et al. (58) could identify 15 of 16 CCRCC patients with (124)I-cG250 PET scanning and all 9 non-CCRCC patients were negative for the anti-CA IX tracer. More studies are needed to find out if it could be possible to use these kind of methods to distinguish a benign leiomyoma from an aggressive growing sarcoma.

Some CA IX expression was also observed in the normal endometrium. Punyadeera et al. (59) previously reported the highest signals for CA IX at cycle days 2–8. The expression of CA IX disappeared around cycle days 9–10, and, furthermore, no staining was found during cycle
days 11–15. We found the same tendency in the normal endometrium specimens. The more intense staining of CA IX during the proliferative phase suggests that CA IX participates in cell adhesion and proliferation processes (12, 60, 61). On the other hand, studies on knockout mice have suggested that CA IX is not necessary for reproductive function, as no abnormalities were reported in the reproductive capacity of CA IX-deficient mice (61, 62). Compared to the normal epithelium, CA IX was overexpressed in endometrial adenocarcinoma. This finding was in line with the Yunokawa et al. (29) study, where they reported increased CA IX expression in endometrial adenocarcinoma compared to the normal endometrium. Eventual activation of the HIF-1 pathway probably explains the induction of CA IX expression in these tumors. The high expression of CA IX in several adenocarcinoma specimens suggests that it could represent a potential target for cancer therapy.

Our results confirmed the findings of Karhumaa et al. (31), showing high CA XII expression in the normal endometrium. High levels of CA XII in the normal endometrium suggest that it may play a role in reproductive functions by regulating the luminal/epithelial/mural ion and pH homeostasis in the uterus. A new observation was the reduced expression of CA XII in the endometrial adenocarcinoma specimens when compared to the normal endometrium. This finding is of interest because the overexpression of CA XII has been reported in several types of cancer (14). Analogously with our results, CA XII expression is slightly reduced in colon carcinoma, where the immunostaining is typically more widely spread than in normal mucosa but where the peak signal intensity remains lower (63). In the endometrial carcinomas, we observed a similar staining pattern for CA XII, showing diffuse reactions within the tumor without any clear association with hypoxic regions.

In our study, adenocarcinoma recurrences were detected only in four patients after the operation. The mean CA IX and CA XII SI values were 2.75 and 1.88, respectively. Even though we did not find any significant difference in CA immunoreactivity between different tumor stages, further studies are still warranted to investigate if these isozymes, especially CA IX, have some prognostic value in the histopathological diagnosis of endometrial adenocarcinoma.

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(CA IV, CA IX, CA XII, CA XIV, and CA XV) forms. The discovery that some of the CA isozymes (CA II, CA IX, and CA XII) are implicated in tumorigenesis has ignited a completely new era in CA research during the past decade (7, 9–12). Colorectal cancer was one of the first tumor types wherein CA expression was explored in detail (13–15). It has been shown that CA II is down-regulated and CA IX is up-regulated in the tumor tissue compared with the normal intestinal epithelium (14, 16, 17). CA XII levels remained quite constant during the colorectal tumor progression, although the distribution of the enzyme became more widespread, in contrast to its confined expression in the normal mucosa (15). Tumor-associated CAs may participate in oncogenesis by acidifying the extracellular milieu adjacent to cancer cells. This acidification would create a microenvironment within the tumor that would be conducive to cancer growth and spread (18, 19). Although the exact mechanisms are largely unknown, acidification has been linked to various events of tumor progression such as defective intercellular adhesion, increased invasion, and up-regulation of growth factors and matrix metalloproteinases. Interestingly, acidification seems to contribute to the increase in mutation rate and reduction in DNA repair (20)—both being cardinal features of genetic instability. It is plausible to predict that CAs, as the major pH-regulating enzymes, are involved in modulating pH in tumor microenvironments. This hypothesis was strongly supported by recent observations that CA IX contributes to the acidification process in cells that are cultured under hypoxic conditions (21).

The aim of this study was to obtain a comprehensive view of CA expression in different groups of colorectal cancer including microsatellite stable (MSS) and microsatellite unstable (MSI) sporadic carcinomas and HNPCC. In the first part of the study, we analyzed mRNA expression levels of multiple CA isozymes in colorectal cancers using an expression microarray technology. Thereafter, we studied CA II, CA IX, and CA XII expression in MSS, MSI, and HNPCC colorectal tumors by immunohistochemistry. To the best of our knowledge, this is the first study on CA expression in hereditary colorectal cancer and the first correlation of CAs to the acidification process in cells that are cultured under hypoxic conditions (21).

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 detectable levels in majority (>50%) of the samples, which were removed from further analyses (22).

Tumor Samples for Immunohistochemistry. Tumor samples were collected together with routine histopathologic specimens taken during surgical operations in Jyväskylä Central Hospital between 1979 and 2004. 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 rectosigmoidum (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 formaldehde, 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 isoforms were 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
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 \((\text{INT} \times \text{EXT}; \text{ref. 26})\). 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, sporadic carcinomas, and normal tissue.

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

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

<table>
<thead>
<tr>
<th>Probe set ID</th>
<th>Gene symbol</th>
<th>Mean normal</th>
<th>Mean HNPCC</th>
<th>Mean sporadic</th>
<th>(P_A), HNPCC vs sporadic</th>
<th>(P_A), HNPCC vs normal</th>
<th>(P_A), sporadic vs normal</th>
<th>(P_A), MSI sporadic vs normal</th>
<th>(P_A), MSS sporadic vs normal</th>
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<tr>
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<td>CA1</td>
<td>43.27</td>
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<td>13.11</td>
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<td>0.000*</td>
<td>0.000*</td>
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<td>0.000*</td>
</tr>
<tr>
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<td>CA3</td>
<td>1.07</td>
<td>0.69</td>
<td>0.68</td>
<td>0.835</td>
<td>0.144</td>
<td>0.108</td>
<td>0.136</td>
<td>0.120</td>
</tr>
<tr>
<td>206209_s_at</td>
<td>CA4</td>
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<td>3.56</td>
<td>4.23</td>
<td>0.624</td>
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<td>0.001*</td>
<td>0.001*</td>
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<tr>
<td>206208_s_at</td>
<td>CA5</td>
<td>84.79</td>
<td>4.41</td>
<td>5.45</td>
<td>0.540</td>
<td>0.001*</td>
<td>0.001*</td>
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<td>0.315</td>
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<td>0.846</td>
<td>0.838</td>
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<tr>
<td>210822_s_at</td>
<td>CA5C</td>
<td>1.59</td>
<td>1.20</td>
<td>1.15</td>
<td>0.709</td>
<td>0.293</td>
<td>0.166</td>
<td>0.840</td>
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<td>206873_s_at</td>
<td>CA6</td>
<td>1.64</td>
<td>1.28</td>
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<td>0.358</td>
<td>0.629</td>
<td>0.211</td>
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<tr>
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<td>0.001*</td>
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<td>1.02</td>
<td>0.100</td>
<td>0.009*</td>
<td>0.003  (^{*})</td>
<td>0.009 (^{*})</td>
<td>0.002 (^{*})</td>
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<td>0.000*</td>
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<td>3.56</td>
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<td>204508_s_at</td>
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<td>1.99</td>
<td>1.00</td>
<td>1.03</td>
<td>0.959</td>
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<td>1.29</td>
<td>1.01</td>
<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>
</tr>
</tbody>
</table>

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

\( ^* \)0.01 < \( p \) ≤ 0.001.

\( ^{1*} \)0.01 < \( p \) ≤ 0.05.

\( ^{2*} \)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 ~28% of the tumor (Fig. 5). In MSS and MSI tumors, the corresponding values were ~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.

Figure 1. Immunohistochemical demonstration of CA II (left column), CA IX (middle column), and CA XII (right column) in three cases of HNPCC. Case A shows different expression of CA II, CA IX, and CA XII in both normal ($N$) and cancerous ($T$) tissues. In the normal colon, CA II and CA XII are localized to the superficial part of the mucosa (arrowheads), and CA IX is present only in the crypt cells. Endothelial staining for CA II is shown in the first image of case A (arrows). Case B shows weak staining for CA II and CA XII and strong staining for CA IX. Case C shows moderate staining for CA II and CA XII and strong staining for CA IX.

Figure 2. Mean staining indices of CA II, CA IX, and CA XII in different subtypes of colorectal cancer. CA II, $P = 0.002$; CA IX, $P = 0.05$; CA XII, $P = 0.037$. }

Cancer Epidemiology, Biomarkers & Prevention

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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 probe 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 “carcioembryonic antigens,” which are expressed normally only in embryonic life but induced postnatally when differentiated cells expressing them undergo malignant transformation and dedifferentiate. In that sense, CA IX behaves in the opposite fashion in parallel with the carcioembryonic 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 may 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


CARBONIC ANHYDRASES II, IX AND XII IN LYNCH SYNDROME PATIENTS WITH ENDOMETRIAL HYPERPLASIA OR CARCINOMA

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported
ABSTRACT

Objective. The purpose of this study was to analyze the expression of carbonic anhydrase (CA) II, CAIX and CA XII in endometrial hyperplasias and endometrial carcinoma (EC) of Lynch syndrome (LS) patients, and compare the expression pattern between LS and sporadic EC. Design. Retrospective study. Setting. Finland 1996-2005. Sample. The material included tumor specimens from LS patients with simplex hyperplasia (n=8), complex hyperplasia (n=9), atypical complex hyperplasia ((n=19 CAIX and XII), n=17 CAII)) and EC (n=10 CAIX and XII), n=9 CAII)). There were 27 (CAXII) and 30 (CAII and CAIX) sporadic EC samples. Methods. The specimens were immunohistochemically stained to study the levels of CAII, CAIX and CAXII expression. Main outcome measures. Expression of CAII, CAIX and CAXII in sporadic EC and in endometrial hyperplasias and in EC of LS patients. Results. All CAs were up-regulated in the EC samples, as compared to the simplex hyperplasia. CAIX expression was only slightly higher in the LS-associated ECs than in the sporadic ECs. CAII upregulation was significantly higher in the LS-associated ECs than in the sporadic ECs. Conclusions. Because of moderate expression of CAIX in the LS-associated EC, CAIX does not present a specific biomarker for LS-associated EC. Nevertheless, its presence in endometrial tumors suggests that it could serve as a potential therapeutic target. CAII could be linked to malignant transformation of endometrium, as its expression seemed to differentiate simplex and complex hyperplasias from carcinomas and the upregulation was significantly higher in the LS-associated ECs than in the sporadic ECs.

Keywords: Lynch syndrome, carbonic anhydrase, endometrial cancer, endometrial hyperplasia, immunohistochemistry

Abbreviations: LS, Lynch syndrome; HNPCC, hereditary non-polyposis colorectal cancer; EC, endometrial cancer; MMR, mismatch repair genes; HIF, hypoxia inducible factor; CA, carbonic anhydrase; SH, simplex hyperplasia; CH, complex hyperplasia; CAH, complex hyperplasia with atypia; INT, Intensity; EXT, extent; SI, staining index; VHL, Von Hippel-Lindau.
Key Message: CAIX could serve as a therapeutic target in LS-related EC. CAII expression could be linked to malignant transformation of the endometrium in LS patients and may be used as a biomarker to predict the right time for prophylactic hysterectomy.
INTRODUCTION

Lynch syndrome, LS (previously referred as hereditary non-polyposis colorectal cancer, HNPCC) is an autosomal dominant colorectal cancer syndrome with inactivation in one of the four DNA mismatch repair (MMR) genes in germ line (1). LS is characterized by an early and high (up to 70-80%) life-long risk of colorectal cancer and with increased risk of some extracolonic cancers of the endometrium, ovary, small bowel, stomach, renal pelvis, ureter, brain and skin (1,2). Of the extracolonic cancers, endometrial cancer (EC) is the most common cancer in LS women with a cumulative lifetime risk of 27-71% compared with 3% in general population (3). Tumors in LS patients are associated with mutations in MMR genes, mainly including MLH (4) and MSH2 (5). The families with excess endometrial cancer have also mutations in MSH6 gene (6).

Endometrial carcinoma is classified as type I and type II tumors. Type I tumors account for 80-85% of the ECs and are usually endometrioid or mucinous type, estrogen dependent, low-grade, localized and associated with a favorable outcome as compared to type II tumors which occur in older women, are of high grade, advanced stage, and have a worse prognosis (7). LS-associated endometrial carcinomas usually belong to the type I category. Endometrial hyperplasia is considered to precede type I tumor growth. Epidemiologic studies suggest that the risk of progression to malignancy is low (<3%) for hyperplasia without atypia, whereas atypia is clearly associated with a significant risk of carcinoma, being 8% for simple hyperplasia and 29% for complex hyperplasia (8). In contrast, it has been shown in MMR gene mutation carriers that complex hyperplasia either without or with atypia are equally important as precursor lesions of endometrial carcinoma (9). Hyperplasia in these patients may also transform to carcinoma faster than in patients without the MMR gene mutation.

The pathway from a normal cell to carcinoma is complex and affected by many factors, such as the environment and lifestyle besides the genes. Genetic instability like observed in LS patients can be promoted by cell stress factors, such as hypoxia, within the tumor microenvironment (10). To survive hypoxia the cell activates a transcription factor called hypoxia inducible factor (HIF)-1α. This factor 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 survival. One of these genes is called CA9 which encodes carbonic anhydrase (CA) IX isozyme. CAs are zinc-containing metalloenzymes that catalyse the reversible hydration of carbon dioxide in the reaction CO₂+H₂O→HCO₃⁻+H⁺. Their main function is to maintain an appropriate acid-base balance in organisms and thus, they participate in various biological processes, including CO₂ transport, regulation of pH homeostasis, bone resorption, ureagenesis, gluconeogenesis, production of body fluids and fertilization (11). Of the 13 active CA isozymes that are found in mammals CAII, IX and XII have
been implicated in tumorigenesis (12). It has been previously shown that CAIX is highly expressed in sporadic endometrial carcinoma like in several other carcinomas, and the expression seems to be very high in most cases of colorectal carcinomas in LS patients (12-14). CAXII is another isozyme that has been reported to be inducible by hypoxia (15). In sporadic endometrial carcinomas, CAXII expression is weaker than in normal endometrium where it shows very high signal in epithelial cells (14). The tumor-associated CAs may participate in tumorigenesis by promoting the acidification of the extracellular milieu adjacent to cancer cells. This acidification in the tumor microenvironment has been linked to various events in tumor progression, such as cell adhesion, increased invasion and up-regulation of growth factors and matrix metalloproteinases (16). The acidification may be also a contributing factor in tumorigenesis by promoting genetic instability, which is one of the characteristic features of LS tumors (17).

The aim of this study was to analyze the expression of CAII, IX and XII in different endometrial lesions from LS patients, including simplex hyperplasia (SH), complex hyperplasia (CH), complex hyperplasia with atypia (CAH) and endometrial carcinoma (EC). The findings of LS-associated EC samples were compared to the results obtained from sporadic EC specimens.

MATERIALS AND METHODS

Patients and samples

The samples from MMR gene mutation carriers were collected retrospectively during 1996-2005 at two participating hospitals (Jyväskylä Central Hospital and Helsinki University Hospital). The samples were either endometrial aspiration biopsy (Pipelle) or hysterectomy specimens. The patients were female MMR gene mutation carriers enrolled in clinical surveillance for endometrial carcinoma (18). All who developed endometrial hyperplasia or endometrial carcinoma were included in the study. The patient material has been described in detail previously (9). The samples of sporadic endometrial carcinoma were collected from routine histopathological specimens, at Tampere University Hospital. These patients were randomly chosen for the study. The material included eight simplex hyperplasias, nine complex hyperplasias, 17 (CAII) and 19 (CAIX and CAXII) complex hyperplasias with atypia and nine (CAII) and ten (CAIX and CAXII) endometrial carcinomas of LS patients. There were also 27 (CAXII) and 30 (CAII and CAIX) sporadic endometrial carcinoma samples. The study protocols were approved by the Ethics Committee of the Tampere University Hospital.
Antibodies and immunohistochemistry

The monoclonal antibody M75, recognizing the N-terminal domain of human CA IX, has been described previously (19). The rabbit anti-human CA XII antiserum to secretory form of CA XII has been characterized by Karhumaa et al. (20). Normal rabbit serum (NRS) was used for control staining. Immunoperoxidase staining was performed using automated Lab Vision Autostainer 480 (ImmunoVision Technologies Co., Brisbane, CA) and Power Vision+™ Poly-HRP IHC Kit (ImmunoVision Technologies, Co.) reagents. Immunostaining was performed according to the following protocol: (a) rinsing in wash buffer; (b) treatment in 3% H₂O₂ in ddH₂O for 5 min and rinsing in wash buffer; (c) blocking with Universal IHC Blocking/Diluent for 30 min and rinsing in wash buffer; (d) incubation with the primary antibody or NRS diluted 1:1000 (anti-CA IX) or 1:2000 (anti-CAXII and anti-CAII and NRS) in Universal IHC Blocking/Diluent for 30 min; (e) rinsing in wash buffer for 3 x 5 min; (f) incubation in Poly-HRP-conjugated anti-mouse or anti-rabbit IgG for 30 min and rinsing in wash buffer for 3 x 5 min; (g) incubation in DAB (3,3’–diaminobenzidine tetrahydrochloride) solution (one drop DAB solution A and one drop DAB solution B with 1 ml ) ddH₂O for 6 min; (h) rinsing with ddH₂O; (i) CuSO₄ treatment for 5 min to enhance the signal; and (j) rinsing with ddH₂O. All procedures were carried out at room temperature. After the immunostaining, the sections were counterstained with hematoxylin, mounted in Entellan Neu (Merck; Darmstadt, Germany) and examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany). Intensity (INT) of the staining was scored on a scale of 0 to 3 by two of the investigators as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction. The extent (EXT) of the staining was also scored by the same investigators 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 which had no evidence of specific immunostaining. Staining index (SI) for each sample and antigen were calculated using the following formula: \( \sqrt{\text{EXT} \times \text{INT}} \). This method was originally described by Leppilampi et al. (21).

Statistical analysis

Statistical analysis was performed using SPSS for Windows software (SPSS 11.0, Chicago, IL, USA). Mann-Whitney and Kruskal-Wallis tests were used to determine the P-values. P-values <0.05 were considered statistically significant.

RESULTS

CAII

CAII immunostaining have previously shown to be significantly higher in the sporadic endometrial adenocarcinoma than in the normal endometrium (14). The present results are in
line with the earlier findings, showing moderate CA II-positive signal in the carcinoma specimens. Figure 1 shows CA II staining in LS patients with various degrees of endometrial hyperplasia or carcinoma and in patients with sporadic endometrial carcinoma. The staining index (SI) in SH and CH ranged from 0 to 1.41. The median SI was 1.00 in both SH and CH. The median SI values were 1.41 (SI ranged between 0-2.45) in CAH and 2.00 (SI ranged between 0-3.00) in LS endometrial carcinoma specimens, respectively. This increase in more malignant forms was statistically significant (P< 0.001). In sporadic EC, the median SI was 1.41 (SI ranged between 0-3.00). The CAII staining was significantly stronger in endometrial carcinomas with LS than in sporadic cases (P<0.012). There was one outlier (SI = 0) among the LS endometrial samples. The typical cytosolic staining pattern of CAII can be seen in figure 4A. The staining becomes more diffuse and stronger in the LS EC image (Fig. 4D) compared to the hyperplasia specimens (Fig.4A-C).

CAIX

In the previous work we showed that CA IX was strongly expressed in the endometrial adenocarcinoma specimens, whereas the normal endometrium contained only faint reactivity for CA IX (14). Figure 2 shows the SI values for SH, CH, CAH, EC of LS patients and sporadic EC. The median SI in SH was 1.20 and in CH 1.73. In CAH, the SI ranged from 0 to 3.00 and the median SI was 1.73. CAIX showed strong staining in LS endometrial carcinoma samples (median SI 2.45). The median staining was slightly weaker in sporadic EC (median SI 2.0), but the difference was not statistically significant (P<0.45). There was a notable difference in SI values between hyperplasias and LS endometrial carcinoma (P<0.02). Typical immunoreaction pattern for CA IX can be seen in figure 5A-D. The immunoreaction became stronger when atypia increased in cells. The plasma membrane-associated signal is most evident in the panel D.

CA XII

Previous results interestingly showed that CA XII is less expressed in the endometrial adenocarcinoma than in the normal endometrium (14). In this study, the SI in SH ranged from 0 to 2.45 and in CH from 0 to 1.41 (Fig. 3). The median SI was 0 in both SH and CH. In the CAH specimens, median SI rose to 1.00 (SI ranged between 0 to 2.45). The median SI in sporadic EC was 1.73 (SI ranged between 0 to 3.00) and in LS-associated endometrial carcinoma 2.10 (SI ranged between 0 to 2.45) but the difference was not significant (P<0.724). In contrast, there was a significant difference in SI values between the hyperplasias and LS endometrial carcinomas (P<0.002). Some examples of CA XII immunostaining can be seen in figure 6. In this case, the SH specimen shows positive staining with a pattern characteristic for normal epithelium (Fig. 6A). The CH specimen is devoid of
immunostaining (Fig. 6B), whereas positive signals can be seen in both CAH (Fig. 6C) and LS carcinoma samples (Fig. 6D).

DISCUSSION

Our previous results on the expression of carbonic anhydrase II, IX and XII in LS and sporadic colorectal cancer led us to investigate the expression of these proteins in LS patients with different endometrial pathologies from simple hyperplasia to endometrium carcinoma. According to the previous findings in colorectal cancer, the up-regulation of CAIX expression was greatest in the LS group (13). The present data also indicated slightly higher staining reactions for CA IX in the EC specimens of LS patients than in sporadic EC specimens even though the difference did not reach statistical significance. Notably, both CAII and CAXII staining reactions were slightly stronger among the tumor specimens of mutation carriers compared to the sporadic EC. However, when LS-associated and sporadic carcinomas were compared, only CAII showed significantly higher immunostaining index in the former group. It is possible that CAIX and CAXII, showing the same tendency, would have reached statistical significance if the number of specimens was higher.

Von hippel-Lindau (VHL) disease was one of the first hereditary cancer syndromes where CAIX and CAXII overexpression was reported (16). It was found that the mutant form of VHL protein fails to polyubiquitinylate HIF-1α transcription factor under normoxic conditions and makes it possible for HIF-1α to escape the normal proteosomal degradation (22). HIF-1α binds to the promotor region of CA9 gene and induces its expression (15). Although posttranslational regulation of HIF-1α is important, there may be also transcriptional regulation which is driving upregulation of hypoxia pathway in cancers. We showed earlier that HIF-1α mRNA levels were higher in LS-associated colorectal cancer than in sporadic tumors (13). Jiang et al. (23) demonstrated that the increased level of HIF-1α mRNA could lead to higher HIF-1α protein expression, and therefore, supported the theory that the higher HIF-1 α mRNA levels could contribute to higher expression of CAIX. This would, in turn, have important consequences in cancer development. It is known that CAIX can acidify the extracellular milieu of the tumor cells and thereby create a microenvironment conducive to tumor growth and spread (24). The acidification may also represent a contributing factor promoting genetic instability, which is characteristic for LS tumors.

Nieminen et al. (9) confirmed that LS-associated cases of endometrial complex hyperplasia without atypia and complex hyperplasia with atypia are equally important as precursor lesions of endometrial carcinoma, and the molecular changes in endometrial tissues are detectable many years before the emergence of endometrial carcinoma. In our study, the expression levels of CAIX were equal in CH and CAH, and the staining index did not significantly increase when transferring from CAH to EC (P< 0.094). This indirectly supports the previous
observations of CH and CAH being equally important as precursors of endometrial carcinoma lesions in LS. Like in the previous study our material mainly consisted MLH1 mutation carriers. Therefore, it remains to be studied later how CAIX is expressed in tumors with other MMR gene mutations, such as MSH6 or MSH2.

Our previous work showed that CAXII is highly expressed in the normal endometrium where the median SI was 2.45 when evaluated by the same investigators and criteria used in the present study (14). According to our present results the CA XII staining was very weak in SH and CH (median SI 0.00 in both categories), even though the staining index varied in SH from 0 to 2.45. It actually remains unclear what explains this faint staining pattern in SH and CH. Like CAIX, CAXII staining was strongest in LS-associated EC (median SI 2.1), but the staining still remained weaker than in the normal endometrium. In contrast, CAII staining significantly increased when the atypia degree increased from CAH to EC (P< 0.034). CAII could represent a novel indicator of malignant transformation of endometrium in LS patients as its upregulation seemed to differentiate simplex and complex hyperplasias from atypical carcinomas. Even though MMR mutation carriers are advised for regular endometrium screening, it is still difficult to predict the right time for prophylactic hysterectomy. Based on our findings, a rise in CAII staining in endometrium biopsy may indicate malignant transformation and could possibly serve as an indicator for prophylactic hysterectomy in patients with LS mutations.

CA IX protein contains a large extracellular part that includes the active site of the enzyme. This feature makes CAIX accessible to antibodies raised against the extracellular part of the protein and to drugs targeted to the active site of the enzyme. A number of studies have shown promise in the development of such therapeutic agents targeting CAIX (25). CAIX is also a potential protein target for a diagnostic purpose. Although there are many ongoing studies it is too early to draw definitive conclusions on the suitability of CAIX as a serum biomarker (26-28). Future studies will show if CAIX as a circulating biomarker could have the potential to be used in conjunction with the specific targeted therapies for patient selection, monitoring and management, and thus bringing new opportunities also for LS patients.

In summary, even though CAXII staining was strong in LS-associated EC, it remained weaker than in the normal endometrium. Therefore, it cannot be recommended as a marker in endometrial cancer. Because CAIX was moderately expressed in several tumor categories, it does not present a specific biomarker for LS-associated EC. Nevertheless, its presence in endometrial tumors suggests that it could serve as a potential therapeutic target. Based on the present study CAII could be linked to the malignant transformation of the endometrium, as its upregulation seemed to differentiate simplex and complex hyperplasias from carcinomas.
Thus a rise in CAII staining in the endometrial samples could serve as an indicator for prophylactic hysterectomy in patients with Lynch syndrome.

REFERENCES


A boxplot illustration of CAII staining in simplex hyperplasia (SH), complex hyperplasia (CH), complex hyperplasia with atypia (CAH), LS endometrial carcinoma (Ca HNPCC) and sporadic endometrial carcinoma. The staining index (SI) in SH and CH ranged from 0 to 1.41. The median SI was 1.00 in both SH and CH. The median SI values were 1.4 in CAH and 2.00 in LS endometrial carcinoma, respectively. In sporadic endometrial carcinoma, the median SI was 1.41. There was one outlier (SI = 0.00) among the LS endometrial carcinoma samples.
FIGURE 2.

A boxplot illustration of CAIX staining in simplex hyperplasia (SH), complex hyperplasia (CH), complex hyperplasia with atypia (CAH), LS endometrial carcinoma (Ca HNPCC) and sporadic endometrial carcinoma. The median SI in SH was 1.20 and in CH 1.73. In CAH, the median SI was 1.73. CAIX showed strong staining in LS endometrial carcinoma samples (median SI 2.45). The median staining was slightly weaker in sporadic endometrial carcinomas (median SI 2.00).
A boxplot illustration of CAXII staining in simplex hyperplasia (SH), complex hyperplasia (CH), complex hyperplasia with atypia (CAH), LS endometrial carcinoma (Ca HNPCC) and sporadic endometrial carcinoma. The median SI was 0 in both SH and CH. In CAH median SI rose to 1.00. The median SI in sporadic endometrial carcinoma was 1.73 and in LS-associated endometrial carcinoma 2.10.
FIGURE 4.

Immunohistochemical staining of CA II in SH (A), CH (B), CAH (C) and in endometrial carcinoma of LS patient (D). CA II is a cytosolic enzyme that can be seen in the staining pattern in panels A and D. The positive staining is strong and more widely spread in the carcinoma specimen (D).
FIGURE 5.
Immunohistochemical staining of CA IX in SH (A), CH (B), CAH (C) and in LS endometrial carcinoma (D). In panel A, only weak reactions are observed in the epithelial cells. In CH (B) the staining is strong but focal. The staining reaction becomes stronger in atypical cells (C and D). The strongest immunoreactions are confined to the plasma membrane, which is clearly seen in panel D.
FIGURE 6.

Immunohistochemical staining of CA XII in SH (A), CH (B), CAH (C) and in LS-associated endometrial carcinoma (D). In panel A, SH endometrium shows a strong signal in the epithelial cells. The staining is absent in CH (B). In panel C, the positive staining is evident in atypical areas. Positive membrane-associated CA XII expression can be seen in the malignant cells in panel D.