PROGNOSTIC SIGNIFICANCE OF CYCLOOXYGENASE-2 AND ASSOCIATED MOLECULES IN GASTRIC CANCER

Johanna Mrena

Academic dissertation
To be presented for public examination with the permission of the Medical Faculty of the University of Helsinki, in Auditorium 2, Meilahti Hospital, Haartmaninkatu 4, Helsinki on 4 February, 2011, at 12 noon.
To my family
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARE</td>
<td>adenine- and uridine-rich elements</td>
</tr>
<tr>
<td>babA</td>
<td>blood-group antigen-binding gene A (H. pylori)</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cagA</td>
<td>cytotoxin-associated gene A (H. pylori)</td>
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<tr>
<td>CDK1, CDK2</td>
<td>cyclin-dependent kinases</td>
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<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>D1-4</td>
<td>extent of lymph node dissection in gastric cancer surgery</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGC</td>
<td>early gastric cancer</td>
</tr>
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<td>EMR</td>
<td>endoscopic mucosal resection</td>
</tr>
<tr>
<td>ESD</td>
<td>endoscopic submucosal dissection</td>
</tr>
<tr>
<td>EUS</td>
<td>endoscopic ultrasound</td>
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<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>HNPCC</td>
<td>hereditary non-polyposis colon cancer</td>
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<td>HuR</td>
<td>human antigen R</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin gamma</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>JGCA</td>
<td>Japanese Gastric Cancer Association</td>
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<tr>
<td>LUS</td>
<td>laparoscopic ultrasound</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MSI</td>
<td>microsatellite instability</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAI</td>
<td>pathogenicity island</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PET-CT</td>
<td>positron emission computed tomography</td>
</tr>
<tr>
<td>R0-2</td>
<td>extent of residual tumor in oncosurgery</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SPF</td>
<td>S-phase fraction</td>
</tr>
<tr>
<td>TFF-1</td>
<td>trefoil factor 1</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarray</td>
</tr>
<tr>
<td>TMK-1</td>
<td>gastric cancer cell line</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TNM</td>
<td>tumor, node, metastasis</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethyl rhodamine iso-thiocyanate</td>
</tr>
<tr>
<td>UICC</td>
<td>Union Internationale Contre le Cancer</td>
</tr>
<tr>
<td>vacA</td>
<td>vacuolating cytotoxin gene A (H. pylori)</td>
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2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by their Roman numerals:


*Equal last authorship.

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3. ABSTRACT

**Background and aims:** Low stage and curative surgery are established factors for improved survival in gastric cancer. However, not all low-stage patients have a good prognosis. Cyclooxygenase-2 (COX-2) is known to associate with reduced survival in several cancers, and has been shown to play an important role in gastric carcinogenesis. Since new and better prognostic markers are needed for gastric cancer, we studied the prognostic significance of COX-2 and of markers that associate with COX-2 expression. We also studied markers reflecting proliferation and apoptosis, and evaluated their association with COX-2. Our purpose was to construct an accurate prognostic model by combining tissue markers and clinicopathological factors.

**Materials and methods:** Of 342 consecutive patients who underwent surgery for gastric cancer at Meilahti Hospital, Helsinki University Central Hospital, 337 were included in this study. Low stages I to II were represented by 141 (42%) patients, and high stages III to IV by 196 (58%). Curative surgery was performed on 176 (52%) patients. Survival data were obtained from the national registers. Slides from archive tissue blocks were prepared for immunohistochemistry by use of COX-2, human antigen R (HuR), cyclin A, matrix metalloproteinases 2 and 9 (MMP-2, MMP-9), and Ki-67 antibodies. Immunostainings were scored by microscopy, and scores were entered into a database. Associations of tumor markers with clinicopathological factors were calculated, as well as associations with p53, p21, and results of flow cytometry from earlier studies. Survival analysis was performed by the Kaplan-Meier method, and Cox multivariate models were reconstructed. Cell culture experiments were performed to explore the effect of small interfering (si)RNA of HuR on COX-2 expression in a TMK-1 gastric cancer cell line.

**Results:** Overall 5-year survival was 35.1%. **Study I** showed that COX-2 was an independent prognostic factor, and that the prognostic impact of COX-2 was more pronounced in low-stage patients. Cytoplasmic HuR expression also associated with reduced survival in gastric cancer patients in a non-independent manner. Cell culture experiments showed that HuR can regulate COX-2 expression in TMK-1 cells *in vitro*, with an association also between COX-2 and HuR tissue expression in a clinical material. In **Study II**, cyclin A was an independent prognostic factor and was associated with HuR expression in the gastric cancer material. The results of **Study III** showed that epithelial MMP-2 associated with survival in univariate, but not in multivariate analysis. However, MMP-9 showed no prognostic value. MMP-2 expression was associated with COX-2 expression. In **Study IV**, the prognostic power of COX-2 was compared with that of all tested markers associated with survival in Studies I to III, as well as with p21, p53, and flow cytometry results. COX-2 and p53 were independent prognostic factors, and COX-2 expression was associated with that of p53 and Ki-67 and also with aneuploidy.

**Conclusions:** COX-2 is an independent prognostic factor in gastric cancer, and its prognostic power emerges especially in low stage cancer. COX-2 is regulated by HuR, and is associated with factors reflecting invasion, proliferation, and apoptosis. In an extended multivariate model, COX-2 retained its position as an independent prognosticator. COX-2 can be considered a promising new prognostic marker in gastric cancer.
The incidence of gastric cancer has been declining during recent decades in the Western world, in contrast to other gastrointestinal malignancies such as colorectal and pancreatic cancers. It has been postulated that improved socioeconomic conditions play a crucial role in this favorable course (Parkin et al. 2005). In gastric cancer, discovery of \textit{H. pylori}, understanding of its role in gastric carcinogenesis, and the impact of microbial eradication are significant steps in the attempt to restrict cancer-related morbidity and mortality (Helicobacter and Cancer Collaborative Group 2001). However, gastric cancer is an insidious disease, since it is almost asymptomatic at an early stage. The 5-year prognosis for all gastric cancer patients is only 20 to 25%, but among those who receive surgical treatment in time, 5-year survival is 50% or more (Hundahl 2006). The prognosis of early gastric cancer is excellent, 80 to 90% (Itoh et al. 1989). To this end, the most important prognostic factors in gastric cancer are stage and radical surgery, including removal of the tumor and adjacent lymph nodes. The role of adjuvant and neoadjuvant treatments is under discussion (Catalano et al. 2005).

It is poorly known why patients with gastric cancers of a similar stage, treated similarly show differing survival patterns. Prognostic biomarkers available at the time of diagnosis, or at surgery, may associate with clinical outcome, i.e. recurrence or death, irrespective of treatment. Biomarkers may also serve as predictive markers identifying responders or non-responders to cytotoxic agents and thus guide selection of treatment (Fareed et al. 2009). Until recently, no tissue tumor markers in clinical use could estimate survival in gastric cancer, so more accurate prognostic indicators are necessary. Cyclooxygenase-2 (COX-2), a key enzyme in prostaglandin synthesis and a primary target of non-steroidal anti-inflammatory drugs (NSAIDs), is known to associate with gastrointestinal tumor formation, and especially with gastric carcinogenesis (Dubois et al. 1998). COX-2 level is associated with the prognosis of several cancers (Saukkonen et al. 2003). The aim of this study was to evaluate whether COX-2 alone or with associated molecules could serve as novel prognostic marker in gastric cancer.
5. REVIEW OF THE LITERATURE

5.1. Epidemiology and incidence

Gastric cancer is one of the most common malignancies globally, the world’s second leading cause of cancer mortality. Its incidence shows a marked geographical variation, having declined in the industrialized nations during recent decades, but still remaining high in China, Japan, South America, and Eastern Europe (Engeland et al. 1995, Black et al. 1997). This cancer is twice as frequent in men as in women with incidence tending to rise progressively with age, reaching its peak between the ages of 50 and 70 (Crew et al. 2004). In Finland, fewer than 700 new gastric cancers are diagnosed per year, 10/100 000 in men and 5/100 000 in women, making it the fifth most common malignancy leading to death among men and the sixth in women. Of gastric malignancies, 90% are carcinomas, most of the rest are lymphomas and gastrointestinal stromal tumors (Finnish Cancer Registry 2008, www.cancer.fi).

5.2. Classification

Of the several classification systems for gastric cancer, the classic categorization comprises intestinal and diffuse types by Laurén; this is based on the histological structure of gastric carcinoma. Laurén’s classification is the one most utilized globally. Intestinal-type carcinoma forms structures resembling glands of the gastric mucosa, whereas in the diffuse type, malignant cells tend to invade the gastric wall individually. Most exophytic and ulcerative tumors represent the intestinal type. The growth pattern of the diffuse type is described as “linitis plastic,” heterogeneous thickening of the gastric wall with no clear tumor borders (Lauren 1965).

The World Health Organization (WHO) categorizes gastric adenocarcinomas as papillary, tubular, mucinous, signet cell, and undifferentiated. Furthermore, differentiation according to the WHO is classified as good, moderate, or poor. A number of other classification systems also describe macroscopic appearance, growth pattern or differentiation (Sarbia et al. 2004). Gastric cancer may be located in the cardia (25%), corpus (25-35%), antrum and pylorus (50-60%), or entire stomach. Carcinomas of the esophagogastric junction differ from other gastric carcinomas in etiopathological origin, biological behavior, and treatment (Ito et al. 2004, Sarbia et al. 2004, Siewert et al. 2006).

5.3. Etiopathogenesis of gastric cancer

5.3.1. *H. pylori* infection and gastritis

*Helicobacter pylori*, a gram-negative bacillus colonizing the gastric mucosa, was characterized and reported first in 1983 by Marshall and Warren, who were awarded the Nobel Prize in 2005 (Marshall et al. 1984, Pincock 2005). Infection is associated with an increased risk for peptic ulcer disease. Moreover, a number of epidemiological and prospective studies have shown that *H. pylori* infection is a risk factor for gastric cancer. *H. pylori* has been classified as a type I carcinogen in
humans. Cancer risk is believed to be associated with differences in *H. pylori* strains and host inflammatory responses (Crew et al. 2004). Two main factors determine the virulence of *H. pylori*: CagA and VacA. The cytotoxin-associated gene A (*CagA*) is a marker of the 40-kb region of a chromosomal DNA *cag* pathogenicity island (*cag* PAI) (Terry et al. 2005, Correa et al. 2006). *H. pylori* with *cagA*-positive strains has been associated with more severe inflammation and with increased risk for severe atrophic gastritis and gastric carcinoma (Crew et al. 2004, Correa et al. 2006). The *vacA* gene encoding vacuolating cytotoxin A is present in almost all *H. pylori* strains, but their virulence depends on the *vacA* polymorphism. VacA causes increased cellular permeability and induces apoptosis. Other important factors affecting *H. pylori* virulence are adhesins, blood-group antigen-binding proteins, especially BabA encoded by the corresponding gene *babA2*. Oxidative damage caused by *H. pylori* may be the ultimate mechanism inducing carcinogenesis (Correa et al. 2006).

Acute gastritis is a transient condition, whereas chronic gastritis is an inflammatory state of the gastric mucosa that may include structural alterations of the glandular compartment (Rugge et al. 2005). Chronic gastritis may progress to atrophic gastritis, and further, to intestinal metaplasia, dysplasia, and intestinal adenocarcinoma (Correa et al. 2006). Recently it has been suggested that three possible subpathways exist in a stepwise model: intestinal metaplasia – adenoma – carcinoma; intestinal metaplasia – carcinoma; and *de novo* carcinoma (Tahara 2004). Patients with severe atrophic gastritis develop pernicious anemia with complete loss of the normal glands of the gastric mucosa, with development of dysplasia, with anchlorhydria, and with hypersecretion of gastrin, all increasing the risk for gastric cancer. Classification of gastritis is based on location (A: corpus; B: antrum; C: pangastritis) or etiology (Table 1).

**Table 1.** Classification of gastritis according to the Sydney classification. Modified from Gastroenterologia, 2000. With permission from Duodecim.

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
<th>Etiology or co-morbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute gastritis</td>
<td>Acute phlegmonic gastritis</td>
<td>Bacterial infection, sepsis</td>
</tr>
<tr>
<td></td>
<td>Acute hemorrhagic erosive</td>
<td>Shock, alcohol, NSAID</td>
</tr>
<tr>
<td></td>
<td>gastritis</td>
<td></td>
</tr>
<tr>
<td>Chronic non-atrophic</td>
<td>Helicobacter-induced gastritis</td>
<td><em>H. pylori, H. heilmannii</em></td>
</tr>
<tr>
<td>gastritis</td>
<td>Non-spesific chronic gastritis</td>
<td>?</td>
</tr>
<tr>
<td>Chronic atrophic</td>
<td>Helicobacter-induced gastritis</td>
<td><em>H. pylori, H. heilmannii</em></td>
</tr>
<tr>
<td>gastritis</td>
<td>atrophic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autoimmune gastritis</td>
<td>Idiopathic, <em>H. pylori</em></td>
</tr>
<tr>
<td>Specific types of gastritis</td>
<td>Chemical gastritis</td>
<td>NSAID, duodenogastric reflux, idiopathic</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte gastritis</td>
<td>*H. pylori, celiac disease, idiopathic</td>
</tr>
<tr>
<td></td>
<td>Granulomatous gastritis</td>
<td>Crohn’s disease, sarcoidosis, tuberculosis, idiopathic</td>
</tr>
<tr>
<td></td>
<td>Eosinophilic castritis</td>
<td>Idiopathic, allergic</td>
</tr>
<tr>
<td></td>
<td>Focal gastritis</td>
<td>Idiopathic, Crohn’s disease</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>Bacterial (other than <em>H. pylori</em>), viral, fungal</td>
</tr>
</tbody>
</table>
5.3.2. Molecular pathobiology

Gastric carcinogenesis is a multistep process characterized by accumulation of genetic and epigenetic abnormalities. Host-related genetic polymorphism is an important endogenous factor that modulates the risk for cancer development. Carcinogenic evolution includes activation of oncogenes; overexpression of growth factors, of receptors, and of matrix metalloproteinases; and inactivation of tumor suppressor genes, DNA-repair genes, and cell-adhesion molecules; and abnormalities of cell cycle regulators. Alterations may occur in the encoding sequences, or in epigenetic areas regulating gene expression (Yasui et al. 2006).

Cytogenetic changes such as polysomy, chromosomal translocation, inversions or deletions can occur in most gastric carcinomas, although whether they are the cause or consequence is unknown (Stock et al. 2005). However, altered chromosomal regions may contain tumor suppressor genes or oncogenes crucial for gastric carcinogenesis (Correa et al. 2006).

Gene amplification, deletions, and mutations in coding or regulatory regions lead to altered gene expression. Mutations in tumor suppressor genes such as CDH1 and p53, and in activating oncogenes such as K-ras and beta-catenin are frequently observable in gastric carcinoma (Shang et al. 2005, Stock et al. 2005). Beta-catenin forms an E-cadherin complex with alpha- and gamma-catenin, one essential for cell-cell communication and cell adhesion. Germline mutations of E-cadherin are associated with hereditary gastric carcinogenesis of the diffuse type (Shang et al. 2005). Trefoil peptide TFF1 is expressed in normal epithelium of the stomach, protecting the gastric mucosa from injuries such as those caused by NSAIDs (Saukkonen et al. 2003). Expression of TFF1 is absent in half of all human gastric carcinomas, and animal models have shown that TFF1 deletion enhances tumor formation (Saukkonen et al. 2003, Shang et al. 2005). Gene polymorphism in the interleukin gene family of cytokines, especially the IL-1beta-promoter polymorphism, is suggested to associate with susceptibility to gastric carcinogenesis through alteration of the host inflammatory response (Schneider et al. 2008). H. pylori-induced gastritis causes an influx of polymorphonuclear leukocytes, leading to secretion of interleukins (IL-1beta, IL-6, IL-8, IL-18, and TNF-alfa), and thus to an inflammatory response in the gastric mucosa (Shang et al. 2005). Genetic variation in the COX molecules may play a role in cancer formation (Menter et al. 2010). A meta-analysis showed that three potentially functional COX-2 polymorphisms were significantly associated with increased risk for digestive system cancers (Dong et al. 2010).

Approximately 1 to 3% of cancers arise as a result of inherited gastric cancer predisposition syndromes (Fitzgerald et al. 2004). Certain clinical conditions are associated with elevated risk for gastric cancer. It is suggested that patients with blood group A are at a 16 to 20% elevated risk for gastric cancer, and the risk is diminished in patients with blood type O. However, blood group O has also been associated with elevated risk for gastric cancer in those under 50 (Yaghoobi et al. 2004). A defective DNA repair system is reflected by microsatellite instability (MSI). Mutations in the mismatch repair genes hMLH1 and hMSH2b are associated with hereditary non-polyposis colorectal cancer (HNPCC), an autosomal dominant cancer syndrome characterized by colon cancer appearing at a young age and incidence of various extracolonic tumors (Stock et al. 2005). Patients with HNPCC have a cumulative risk of up to 19% for developing gastric cancer with an early onset, with gastric cancer, usually of the intestinal type, being one of the extracolonic manifestations of HNPCC (Gylling et al. 2007). In contrast, familial adenomatous polyposis (FAP) is associated with gastric polyposis, but no evidence has appeared that FAP is directly associated with gastric cancer, although dysplastic duodenal adenomas and even duodenal cancer may occur (Al-Sukhni et al. 2008).
5.3.3. Gastric polyps and mucosal dysplasia

Gastric polyps may develop as a result of epithelial or stromal cell hyperplasia, inflammation, ectopia, or neoplastic alteration. Hyperplastic polyps, the most common, comprising 75% of all gastric polyps, are non-neoplastic but often associated with atrophic gastritis, or other transient condition associated with mucosal regeneration. Risk for gastric cancer equals that of atrophic gastritis. However, malignant transformation of sporadic hyperplastic polyps is rare (Odze et al. 2009). Hamartomatous cystic polyps, found in 1 to 2% of the population, are benign in nature and require no surveillance.

Adenomas account for up to 10% of all gastric polyps. They are mostly flat in shape and solitary in appearance, and often occur concomitantly with chronic gastritis and intestinal metaplasia. Of gastric adenomas, 4 to 40% may progress to cancer (Sarbia et al. 2004), and the cancer risk is related to lesion size, being particularly high for those more than 2 cm in diameter. Microscopically, they are predominantly of intestinal type, seldom of gastric type, and are classified according to principles mentioned below (Odze et al. 2009). Adenomas that appear in the normal gastric mucosa may be associated with juvenile or familial adenomatous polyposis syndrome.

Dysplasia is regarded as a precancerous lesion of the stomach. Gastric dysplasia can present as a polypoid, flat, ulcerated, or cancer-like lesion. The classification of mucosal dysplasia is heterogeneous, and efforts have been made to create a uniform nomenclature. Variance is especially great between Japanese and western classifications. The severity of dysplasia has been assessed with a two-grade (Ming et al. 1984) or three-grade classification (Morson et al. 1980). The Padova International Classification, based on the Vienna classification of gastrointestinal epithelial neoplasia, is a consensus-based nomenclature including five categories: Negative for dysplasia includes subgroups of normal mucosa, reactive foveolar hyperplasia, and intestinal metaplasia, all with increased cancer risk, and thus recommended for surveillance. Indefinite for dysplasia is reserved for those cases in which it is impossible for a pathologist to decide whether the lesion includes neoplastic or non-neoplastic cells; new biopsies are recommended. Non-invasive neoplasia refers to phenotypically neoplastic epithelium confined to glandular structures inside the basement membrane. It is divided into low-grade and high-grade subcategories, and the latter equals carcinoma in situ. Suspicious for invasive gastric cancer represents an irreversible neoplastic lesion, but invasion cannot be clearly demonstrated. Additional biopsies or mucosal resection is recommended. Invasive carcinoma is self-defined and an indication for surgical treatment, when feasible (Schlemper et al. 2000, Rugge et al. 2005).

5.3.4. Other risk factors

A fraction of ulcers turn out to be cancer by underlying malignancy. A persistent ulcer with no response to treatment should be biopsied. One typical morphological feature for a malignant ulcer is cancerous tissue down to the muscularis mucosa at the ulcer’s margin but not at the base (Shang et al. 2005).

Previous partial gastrectomy is a double-fold risk factor for gastric cancer. The risk for developing gastric cancer increases 15 to 20 years after the initial surgery. Refluxing intestinal fluid causes gastritis, and bile salts in contact with the gastric mucosa is considered to act as a carcinogen (Shang et al. 2005).
Exposure to tobacco smoke and pollution, and also alcohol intake are associated with gastric cancer risk. Dietary factors such as salt and nitrite raise the risk, whereas fresh fruit and vegetables play a protective role. Genetic polymorphisms of metabolic enzymes like cytochrome P450, epoxide hydrolase, N-acetyltransferase, and glutathione S-transferase are responsible for variations in ability to metabolize and detoxify detrimental or carcinogenic agents. Thus, equal exposure does not raise the risk for cancer in all individuals (Shang et al. 2005).

Ménétrier’s disease is a rare form of acquired gastropathy associated with risk for gastric cancer. It is characterized by giant folds in the gastric body, foveolar hyperplasia, and markedly decreased oxyntic glands. This condition causes abdominal pain, nausea, vomiting, and peripheral swelling secondary to hypoalbuminemia caused by protein loss across the gastric mucosa. Diagnosis is set by endoscopy and full-thickness mucosal biopsies. Ménétrier’s disease may be confused with gastric polyps or polyposis syndromes. Cetuximab is shown to be an effective pharmacological treatment, but the only definitive treatment is total gastrectomy (Rich et al. 2010).

5.4. Prevention

Screening endoscopy for gastric cancer is justified among patients living in areas of high incidence like Asia, patients with hereditary predisposition syndrome, ones with chronic atrophic gastritis, or ones with previous gastric resection. Screening and eradication of H. pylori, consumption of fresh vegetables and fruit and daily aspirin use seem to reduce risk for gastric cancer (Rothwell 2010, Sasako et al. 2010). Gene mapping and evaluation of genetic polymorphism may prove useful as well (Dong et al. 2010).

5.5. Diagnosis

5.5.1. Symptoms and signs

Early diagnosis of gastric cancer, crucial for successful surgical treatment, is difficult, since clinical manifestations at the beginning of the disease may remain silent. At this point, symptoms are usually mild and unspecific, such as mild upper gastrointestinal distress, flatulence, abdominal fullness prematurely after meals, and excessive belching. Striking symptoms such as vomiting, dysphagia, fatigue, weight loss, gastrointestinal bleeding, and abdominal mass are signs of advanced cancer and obstruction. Manifestations of metastatic disease may be abdominal pain, liver enlargement, ascites, jaundice, or palpable lymph nodes in the left axilla or in the left supraclavicular area (Virchow's node). Ovarian metastases (Krukenberg tumor) and a palpable pelvic mass (Blumer's rectal shelf) are due to peritoneal spread of the disease. Occasionally, patients with advanced cancer may have paraneoplastic conditions such as acanthosis nigricans, dermatomyositis, and inappropriate intravascular coagulation leading to arterial and venous thrombi (Trousseau's sign) (Catalano et al. 2005).
5.5.2. *Endoscopy*

The fundamental examination in gastric cancer diagnostics is esophagogastroduodenoscopy. It allows direct visualization and biopsies of suspicious lesions and thus has replaced barium swallow investigation almost entirely. Diagnostic sensitivity of endoscopy with biopsies is 98%, whereas a barium swallow x-ray has a false negative rate up to 50%. As much as 5% of lesions appearing benign have been histologically confirmed as malignant. The diffuse type of gastric cancer (“limitis plastica”) is difficult to diagnose regardless of the technique used (Catalano et al. 2005). Chromoendoscopy, high-definition endoscopy, image-enhanced endoscopy including autofluorescence, and narrow band imaging with magnifying endoscopy have all improved diagnostic accuracy in detecting small and superficial gastric lesions (Kaise et al. 2010). At present, confocal laser endomicroscopy provides the most powerful magnification, and with fluorescein contrast, helps in differential diagnosis between gastric intestinal metaplasia and gastric cancer *in vivo* (Li et al. 2010).

5.5.3. *Preoperative staging*

When gastric cancer has been diagnosed, preoperative staging is mandatory for proper treatment selection. Computed tomography (CT) of the thorax and the whole abdomen is recommended to evaluate the local extent of the tumor and to diagnose distant disease (Davies et al. 1997, Paramo et al. 1999). Diagnostic accuracy for tumor infiltration increases with progression of the disease. Sensitivity of CT has been 23 to 56% in early-stage gastric cancer, whereas in cases of advanced disease, sensitivity can reach 92 to 95% (Halvorsen et al. 1996, Tschmelitsch et al. 2000). Estimation of lymph node involvement by CT is inaccurate. Lymph nodes over 10 mm are considered pathological, but differentiation between inflammatory and metastatic nodes is impossible (Fukuya et al. 1995, Rossi et al. 1997). Detection of hepatic or other metastases by CT depends on the bulk of the mass, and in practice, CT may fail to discover hepatic or peritoneal metastasis smaller than 1 cm in diameter. It has been shown that endoscopic ultrasound (EUS) reveals tumor infiltration and lymph node metastasis with an accuracy of 67 to 92% (Pollack et al. 1996). Most metastatic lymph nodes close to the gastric wall can be visualized by EUS, but its ability to detect more distant or very small metastatic nodes is limited (Botet et al. 1991). EUS, playing a central role in assessing invasion depth in the gastric wall and selecting treatment options, overall, offers more detailed information in preoperative staging than does CT (Okada et al. 2010).

Magnetic resonance imaging (MRI) does not improve accuracy in staging compared with that of other modalities, but it may prove useful in detection and determination of liver lesions (Tschmelitsch et al. 2000). The role of positron emission computed tomography (PET-CT) is still unclear. It has been suggested that PET-CT is insufficiently sensitive to determine lymph node status and does not improve accuracy in detecting peritoneal metastasis compared with CT. (Kim et al. 2010). However, PET-CT has advantages in detecting other distant metastasis and metachronous malignancies, and FDG PET-CT, based on the enhanced sugar metabolism in neoplastic and inflammatory tissues, is suggested to be a superior post-therapy surveillance modality for the diagnosis of recurrent gastric cancer (Gupta et al. 1996, Bilici et al. 2010, Sun et al. 2010).

Despite these methods to rule out metastatic disease, false-negative results can occur in up to 23% of cases (Burke et al. 1997, Roder et al. 1998). Laparoscopy offers an accurate method to study peritoneal surfaces, the liver, and lymph nodes for metastases, to inspect the tumor directly, and to assess stomach movement for tumor infiltration. Laparoscopic findings may lead to revision of the
treatment plan, but still consensus is lacking whether it should be performed as an individual procedure or as part of definitive surgical treatment. However, laparoscopy is recommended for all those patients with advanced disease to exclude peritoneal metastases (Stell et al. 1996, Burke et al. 1997). The limitations of laparoscopy are the lack of tactile sensation and the restricted visual field in the peritoneal cavity, which can be compensated for partly with laparoscopic ultrasound (LUS) (Conlon et al. 1996). A combination of pretherapeutic EUS and LUS, followed by surgery, has predicted resectability and was comparable with TNM stage in estimating survival; it proved at least as good as CT-based staging (Mortensen et al. 2010).

In selection of treatment modality, in addition to thorough preoperative staging, evaluation of exercise tolerance, co-morbidity, and nutritional status are essential (McCulloch 2002). It is mandatory that the surgeon is part of the multidisciplinary team (Van Cutsem et al. 2008). APACHE (Acute Physiology and Chronic Health Evaluation) II and POSSUM (Physiological and Operative Severity Score for the enUmeration of Morbidity and Mortality) with its variants are models to assess postoperative morbidity and mortality. Physiological and operative severity scores are entered into a program and the risk is calculated. The revised P-POSSUM is suggested to be the most accurate, but it still overpredicts mortality (Dutta et al. 2010).

5.6. Treatment

5.6.1. Surgery

The only curative treatment for gastric adenocarcinoma is surgical resection (Sasako 2003, Catalano et al. 2005, Archie et al. 2006, Van Cutsem et al. 2008). The primary tumor can be removed by total gastrectomy, esophagogastrectomy, or distal gastrectomy with appropriate reconstructions. Total gastrectomy should be performed for patients with large tumors of the posterior wall of the stomach or when treating diffuse types of gastric cancer. Esophagogastrectomy is the treatment of choice for carcinomas of the cardia, fundus or lesser curvature. Distal subtotal gastrectomy is feasible in antral or prepyloric carcinomas of intestinal type (Lee et al. 1997).

Assessment of the radicality of the operation is based on the surgeon’s peroperative evaluation and the pathologist’s microscopic measurement. If no residual tumor is left, the operation is classified as R0. R1 stands for microscopic residual tumor or inappropriate margins, and R2 for macroscopic residual (Lee et al. 1997). In the Dutch trial, reasons for designating resection R1 rather than R0, were peritoneal fluid containing tumor cells, resection-line involvement, or distant lymph node involvement in positions 12 to 16 (Songun et al. 2010). A minimum of 6 cm of apparently normal stomach should be included in the specimen to ensure a radical operation. Margins should be confirmed with frozen sections during the operation, at least in diffuse carcinomas. In microscopic examination, 30% of patients had positive margins if the macroscopic normal tissue margin was estimated as 2 cm, and 10% in those with a 4 to 6 cm grossly normal margin (Lee et al. 1997).

In addition to removal of the primary tumor, radical surgery involves lymph node dissection. Nomenclature of the surgical procedures D1 to D4 is based on extent of lymphadenectomy because of the anatomical location of the lymph nodes (Fig. 1, Table 3) (Shimada 2004). The extent of lymph node dissection has remained controversial. A non-randomized study by Siewert et al showed that D2 dissection improves survival only in patients at stages II and IIIA (Siewert et al. 1998). The results of the prospective randomized Dutch trial with 711 patients showed that morbidity and mortality were significantly more frequent in the D2 than in the D1 group, and after
11 years of follow-up, overall survival rates were a respective 35% and 30% with no statistically significant difference. Only patients with N2 nodal involvement according to TNM classification may benefit from D2 dissection. After 15 years of follow-up, D2 lymphadenectomy was associated with lower locoregional recurrence and lower gastric cancer-related death rates than D1 surgery. (Bonenkamp et al. 1995, Bonenkamp et al. 1999, Hartgrink et al. 2004, Songun et al. 2010). In a prospective MRC study, 400 of 737 patients were randomized to D1 and D2 groups, and no difference emerged in overall survival. In multivariate analysis, clinical stages II and III, male sex, old age, and removal of the spleen and pancreas were independent prognostic factors for poor survival. One suggestion, however, is that D2 dissection without pancreatico-splenectomy may be better than D1 dissection (Cuschieri et al. 1999). A meta-analysis of 19 studies performed separately for randomized and non-randomized studies showed no survival benefit for D2 dissection, although it was suggested that intermediate-stage groups may benefit from D2 dissection. Excess operative mortality was associated with pancreatico-splenectomy, low case volume, and lack of specialist training (McCulloch et al. 2005). In one single-center randomized study with 221 patients, D1 dissection, when compared with D3 dissection, showed a significant improvement in survival in the D3 group (Wu et al. 2006). In contrast, the results of a Japanese multi-center study with 523 patients showed no difference in survival between D2 dissection and D2 dissection combined with para-aortic nodal dissection (Sasako et al. 2008). The European Organisation for Research and Treatment of Cancer (EORTC) recommendation for surgery is resection of the primary tumor with free margins and at least D1 lymph node dissection combined with removal of a minimum of 15 lymph nodes (Van Cutsem et al. 2008).

To estimate the likelihood of nodal involvement for each regional lymph node region, the Maruyama index can be calculated by a specific computer program. This program matches a given case with a data base collected from the National Cancer Center Hospital in Tokyo. This program has proven accurate in predicting nodal involvement and may be useful when planning tailored lymph node dissection, or as quality control (Kampschoer et al. 1989). Low Maruyama-Index surgery is suggested to enhance locoregional control and survival, but has no effect on occurrence of isolated distant metastases (Hundahl et al. 2007). It also may help to distinguish patients at high risk for relapse and select those who will benefit from tailored multimodality treatment (Songun et al. 2009).

Although D2 gastrectomy is the standard treatment for resectable gastric cancer, less invasive gastrectomy with limited lymphadenectomy can be performed in selected patients. Other options are modified gastric resections such as pylorus-saving gastrectomy, proximal gastrectomy, and laparoscopic gastric resection (Sano et al. 2006). Even in high-incidence countries in Asia, with their high volumes of gastric surgery, the role of laparoscopic gastric resection has been controversial. However, retrospective studies show that laparoscopic distal or total gastrectomy with lymphadenectomy is as safe as open surgery at all stages, and 5-year survival within stages I to II is comparable as well (Kodera et al. 2010). A Korean prospective, randomized phase III trial shows similar results for stage I patients, but long-time survival results are not yet available (Kim et al. 2010). Ongoing studies may change guidelines for gastric cancer surgery in the future, favoring laparoscopic surgery at least for low-stage patients.

### 5.6.2. Adjuvant therapy

Localized disease can be treated by surgery alone. However, there already exists risk for lymph node metastasis in T1 and T2 cancers (Sasako 2003, Ahmadi et al. 2008), and therefore what is necessary is systemic treatment. Furthermore, gastric cancer is often at an advanced stage at
diagnosis, making systemic treatment mandatory (Hansson et al. 2000). Still, no consensus exists as to postoperative adjuvant chemotherapy, since despite numerous randomized studies and meta-analyses, any survival benefit has proven modest (Hermans et al. 1993, Earle et al. 1999, Mari et al. 2000, Panzini et al. 2002). In one Japanese study, 529 stage II to III gastric cancer patients were randomized to undergo D2 surgery followed by adjuvant therapy with S-1, an oral fluoropyrimidine combination drug, or surgery alone. The 3-year overall survival was 80.1% in the S-1 group, and 70.1% in the surgery-only group. Locoregional control was also better in the S-1 group. That study was halted because the S-1 group had clearly better survival (Sakuramoto et al. 2007). Thus, S-1 seems promising, at least in that study population of East Asian patients.

Surgery plus postoperative chemoradiotherapy was compared with surgery alone in a large SWOG 9008/INT 0116 Phase II trial including 556 patients. The adjuvant treatment consisted of fluorouracil, leucovorin, and radiotherapy. Median overall survival was longer after complementary chemoradiation than after surgery alone (36 months vs. 27 months). However, 54% of the patients underwent less than D1 surgery, and it is possible that chemoradiation compensated for insufficient surgery (Macdonald et al. 2001).

In the MAGIC trial, 503 patients were randomized to receive either perioperative chemotherapy and surgery, or surgery alone. Chemotherapy comprised three preoperative and three postoperative cycles of epirubicin, cisplatin, and 5-fluorouracil. The perioperative chemotherapy group showed a better 5-year survival than did the surgery group (36% vs. 23%). In patients treated with perioperative chemotherapy, tumor size was significantly smaller than in the control group (Cunningham et al. 2006). Similar results have been reported in the FNLC ACCORD 07/FFCD 9703 phase III trial with 224 patients randomized to receive perioperative cisplatin and 5-fluorouracil vs. surgery alone. Their 5-year survival rates were 38% vs. 24% (Boige V 2005).

Some have suggested that the European recommendation for adjuvant treatment be platin-fluoropyrimidine-based perioperative chemotherapy 8 to 9 weeks before surgery, and 8 to 9 weeks after surgery, if tolerated. Postoperative chemoradiotherapy should be considered in patients with T3 to 4 tumors and nodal involvement, in cases for whom no preoperative chemotherapy was administered and for whom lymphadenectomy has remained suboptimal (Van Cutsem et al. 2008).

5.6.3. Palliation

Palliative treatment is indicated when gastric cancer is locally advanced or metastatic, or when R0 surgery cannot be performed. The primary goal is to relieve symptoms in order to improve quality of life, not necessarily to lengthen life. Decisions are based on comorbid conditions, performance status, extent and prognosis of the cancer, natural history of the primary and secondary symptoms, potential durability of the intervention, and quality of life and life expectancy of the patient (Cunningham et al. 2007).

Surgical options for palliative treatment are surgical resection with no mandatory lymph node dissection, gastrojejunoanostomy, and other by-pass procedures. A number of studies concerning palliative gastrectomy or gastric resection show a possible improvement in survival, if only one or two metastatic sites exist. However, quality of life is not better, although survival might be. In contrast, patients with wide metastatic spread do not benefit from gastrectomy or gastric resection (Cunningham et al. 2007). The impact of lymphadenectomy, pancreatcico-splenectomy or peritoneectomy combined with intraperitoneal chemotherapy has also remained poor (Yonemura et al. 2000, Yonemura et al. 2003). The role of surgical treatment in hepatic metastases is controversial.
due to the tendency of gastric cancer to form multiple metastases. However, it is suggested that
selected patients with solitary hepatic metastasis may benefit from hepatic resection (Roh et al.
2005).

Gastrojejunostomy, performed by laparotomy or laparoscopy, is a traditional procedure to discharge
distal gastric obstruction. However, technical difficulties and complications include delayed gastric
emptying (gastric atony due to dilation), mechanical obstruction related to the formation of a
vicious cycle, emptying into the antiperistaltic afferent limb causing dilation of the afferent limb
nausea, vomiting, anastomotic bleeding, fistula formation, and later, dumping syndrome. A self-
expanding metallic stent applied by endoscopy is the treatment of choice in proximal but also in
distal obstruction. Compared with gastrojejunostomy, it shows no difference in technical success or
survival, but clinical success is better and fewer complications arise. Thus, self-expanding stents are
recommended (Cunningham et al. 2007, Ly et al. 2010).

A number of studies report on systemic chemotherapy in patients with unresectable tumors.
Conventional cytotoxic chemotherapy can improve overall survival, quality of life, and the
symptom-free period. A combination of cytotoxic agents is better than single-agent treatment
(Wagner et al. 2006). Until recently, no combination has become accepted as the gold standard (Van
Cutsem et al. 2008). However, results of the ToGA trial (Trastuzumab for Gastric Cancer) show that
patients with inoperable locally advanced, recurrent or metastatic gastric or gastroesophageal
junction adenocarcinoma overexpressing human epidermal growth factor receptor 2 (HER2 or
ERBB2) benefit from trastuzumab, a monoclonal antibody against HER2, combined with
chemotherapy. The overall median survival was 13.8 months in the trastuzumab group (296
patients) compared with 11.1 months in the chemotherapy-only group (290 patients). Thus,
trastuzumab combined with chemotherapy is suggested to be a new standard option for advanced,
HER2-positive gastric or gastroesophageal junction cancer (Bang et al. 2010).

5.6.4. Early gastric cancer (EGC) and endoscopic surgical techniques

Early gastric cancer (EGC) is defined as adenocarcinoma confined to the mucosa or submucosa
irrespective of lymph node involvement. The prognosis for early gastric cancer is excellent, 5-year
rates being more than 90% in surgically treated patients (Sasako 2003, Nieminen et al. 2009). Depth
of invasion is associated with the risk for lymph node metastases, which have been reported in 3%
of intramucosal and in 20% of submucosal tumors (Sasako 2003, Sano et al. 2006). Endoscopic
mucosal resection (EMR) is the choice if complete removal with a clear margin of the primary
tumor is possible, and if there is practically no risk of lymph node metastasis. Larger tumors can be
removed with endoscopic submucosal dissection (ESD) on similar terms (Sano et al. 2006). For
judging of the risk for lymph node metastasis by evaluating infiltration depth and size of the lesion,
endoscopic ultrasound before these endoscopic surgical techniques is recommended, as well as
chroendoendoscopy and narrow band imaging. Endoscopic surveillance is mandatory (Sano et al.
5.7. Prognostic factors

5.7.1. Stage

The Union Internationale Contre le Cancer (UICC) classification places its emphasis on extent of disease by assessment of tumor infiltration (T), nodal metastasis (N), and distant metastasis (M). Combinations of these factors yield stages 0 to IV (Table 2). TNM classification has been modified several times. In the 5th edition (Sobin et al. 1997), nodal status is defined by the number of metastatic lymph nodes, whereas in the 4th edition, lymph node status is based on anatomical location (Hermanek et al. 1992). In the 6th edition (Sobin et al. 2002), the actual stage groups have remained as they were, but tumor invasion is divided into T2a (invasion to the muscularis propria) and T2b (invasion to the subserosa). The current 7th edition (Sobin et al. 2009), shows major changes. The Mx category has been removed, and intraperitoneal-positive cytology is considered as M1. Cut-off values for nodal-positive disease have changed. Tumor in situ (Tis) includes high grade dysplasia, T1 is subdivided into T1a and T1b, T2b is included in T3, and serosal involvement is considered as T4. These changes are considered useful in planning pre- and postoperative treatment and in estimating survival (Rausei et al. 2010).

A Japanese staging system has been developing since 1962. Although T, N, and M categories are used in this system as well, the most striking difference is that N refers to the anatomical location of the lymph nodes and not the number of nodal metastases (Fig 1, Table 3). Furthermore, the presence of peritoneal or hepatic metastases is classified as a separate factor affecting stage. The Japanese staging system can be converted to the UICC system, but the opposite is not possible. Thus, the UICC TNM system is primarily a prognostic tool, whereas the Japanese system, in addition to prognostics, is surgery oriented (Ikeguchi et al. 2004, Sayegh et al. 2004).
Table 2. TNM stages by UICC, 4th and 7th edition. With permission from Wiley.

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**Primary tumor (T)**

- **Tx**: Primary tumor cannot be assessed
- **T0**: No evidence of primary tumor
- **Tis**: Carcinoma in situ
- **T1**: Tumor invades lamina propria or submucosa
- **T2**: Tumor invades muscularis propria or subserosa
- **T3**: Tumor penetrates the serosa without invasion of adjacent structures
- **T4**: Tumor invades adjacent structures (not including duodenum, esophagus)

**Regional lymph nodes (N)**

- **Nx**: Regional lymph nodes cannot be assessed
- **N0**: No regional lymph node metastases
- **N1**: Metastases in perigastric lymph nodes within 3 cm of the edge of the primary tumor
- **N2**: Metastases in perigastric lymph nodes more than 3 cm from the edge of the primary tumor or in lymph nodes along the left gastric, common hepatic, splenic or celiac arteries

**Distant metastases (M)**

- **Mx**: Presence of distant metastases cannot be assessed
- **M0**: No distant metastases
- **M1**: Distant metastases

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**Primary tumor (T)**

- **Tx**: Primary tumor cannot be assessed
- **T0**: No evidence of primary tumor
- **Tis**: Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria, high grade dysplasia (T1a), or submucosa (T1b)
- **T1**: Tumor invades lamina propria, muscularis mucosae (T1a), or submucosa (T1b)
- **T2**: Tumor invades muscularis propria
- **T3**: Tumor invades subserosa
- **T4**: Tumor perforates serosa (T4a) or invades adjacent structures (T4b)

**Regional lymph nodes (N)**

- **Nx**: Regional lymph nodes cannot be assessed
- **N0**: No regional lymph node metastases
- **N1**: Metastases in 1 to 2 regional lymph nodes
- **N2**: Metastases in 3 to 6 regional lymph nodes
- **N3**: Metastases in 7 or more regional lymph nodes (N3a: 7 to 15, N3b: 16 or more)

**Distant metastases (M)**

- **M0**: No distant metastases
- **M1**: Distant metastases Distant metastasis includes peritoneal seeding, positive peritoneal cytology, and omental tumor not part of continuous extension
Figure 1. Regional lymph nodes in gastric cancer according to JGCA guidelines (Sayegh et al. 2004). With permission from Springer.
Table 3. Nodal stations and D1-4 dissections according to JGCA (Modified from Sayegh et al. 2004). With permission from Springer.

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In several studies, tumor infiltration into the gastric wall and the presence of lymph node metastases have proven the most important prognostic factors (Kim et al. 1992, Adachi et al. 2000, Kooby et al. 2003, Fotia et al. 2004, Abbas et al. 2005, Llanos et al. 2005). Studies show that by both the TNM and Japanese classification systems, the number and location of metastases are both significant prognostic factors (Kim et al. 1992, Adachi et al. 2000, Fotia et al. 2004). Some authors claim that the number of metastatic lymph nodes is more relevant than their distance from the primary tumor (de Manzoni et al. 1999).

5.7.2. Histology

The intestinal subtype of gastric cancer according to the Laurén classification represents a differentiated cancer with a tendency to form glands. In contrast, the diffuse subtype corresponds to undifferentiated cancer with extensive submucosal growth and early metastases (Lauren 1965). The diffuse subtype has been associated with poor outcome, but non-independent of TNM stage (Lauren 1965, Archie et al. 2006).

5.7.4. Tumor location

Carcinomas located in the upper part of the stomach have a worse prognosis than do distal ones (Ito et al. 2004). However, classification and nomenclature of the tumors situated in the esophagogastric junction are heterogenic, and thus show much variation in treatment modalities (Sievert et al. 2006). One anatomic-topographic classification divides junctional tumors into I (tumors arising from the distal esophagus and infiltrating the junction from above), II (true cardia carcinoma), and III (subcardial carcinoma infiltrating the junction from below). Between these groups, histopathological origin, lymph node spread, and surgical approach all vary (Sievert et al. 2006).
5.8. Tumor markers

Tumor markers are agents expressed by the tumor itself, or are reactive products detected from bodily fluids or tissues. Tumor formation and carcinogenesis is a multistep process involving control of cell growth and proliferation, susceptibility to apoptosis, angiogenesis, cell adhesion, and migration. Factors associated with these steps are mapped by study of genetic alterations, mRNA expression, or protein expression. In the carcinogenesis cascade are factors not only expressed in carcinomas but also associated with clinical outcome (Fareed et al. 2009).

For gastric cancer, no standard tumor markers are yet in clinical use for estimating prognosis. Serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are known to associate with lymph node metastasis and tumor invasion in gastric cancer patients, but their roles as prognostic factors are inconsistent (Ishigami et al. 2001, Louhimo et al. 2004, Mihmanli et al. 2004, Dilege et al. 2010, Tsirlis et al. 2010). HER2 overexpression is recognized as a frequent molecular abnormality in gastric cancer and known to associate with advanced stage and poor survival (Gravalos et al. 2008). Furthermore, HER2 is a therapeutic target of trastuzumab (Bang et al. 2010). However, when HER2 is detected in endoscopic biopsies, false negative results occur because of a prominent intratumoral heterogeneity of HER2 expression in gastric cancer (Grabsch et al. 2010).

5.8.1. Cyclooxygenase-2

Cyclooxygenase (COX) is the rate-limiting enzyme in the synthesis involved in turning arachidonic acid to prostaglandins, and is the best-known target of aspirin and other NSAIDs (Dubois et al. 1998, Gupta et al. 2001). The two isoenzymes COX-1 and COX-2 have a 60% resemblance in amino acid structure and similar enzymatic activity. However, a difference exists in expression and regulation of COX-1 and COX-2. COX-1 is considered to be a housekeeping enzyme and is detectable in almost all tissues, excluding red blood cells. It controls physiological functions such as synthesis of prostanoids that protect the gastric mucosa, and synthesis of thromboxane, a platelet-produced protein associated with thrombocyte aggregation. It also takes part in vasodilatation of the kidney. In contrast, COX-2, an inducible enzyme associated with inflammation, reproduction, and carcinogenesis, is activated by growth factors, hormones, cytokines, and tumor promoters (Dubois et al. 1998, Saukkonen et al. 2003).

A direct link may exist between COX-2 and carcinogenesis. In mice, intestinal polyp formation is suppressed by pharmacologic inhibition and by genetic deletion of COX-2 (Chulada et al. 2000, Oshima et al. 2004). The selective COX-2 inhibitor celecoxib reduces tumor formation in a trefoil factor 1–deficient mouse tumor model (Saukkonen et al. 2003), and reduces chemically induced carcinogenesis in the rat (Hu et al. 2004). Secondly, transgenic expression of COX-2 and microsomal prostaglandin E synthase has induced hyperplastic tumor formation in the mouse stomach (Oshima et al. 2004). The mechanisms supporting COX-2-mediated tumor enhancement are inhibition of apoptosis (Tsuji et al. 1995), stimulation of angiogenesis (Tsuji et al. 1998, Masferrer et al. 2000), and an increase in tumor invasion and metastatic potential (Tsuji et al. 1997, Kakiuchi et al. 2002, Niki et al. 2002). In humans, COX-2 expression is detectable in gastric cancer tissue (Ristimäki et al. 1997), and it is already present in noninvasive gastric dysplasias (Lim et al. 2000, Saukkonen et al. 2001, van Rees et al. 2002). Thus, it seems likely that COX-2 plays a role in early gastric carcinogenesis.
Chronic atrophic gastritis caused by *H. pylori* activates growth factors and cytokines, and also stimulates gastrin secretion from the G-cells of the gastric mucosa, leading to elevated COX-2 expression (Konturek et al. 2009). CagA-positive *H. pylori* infection enhances COX-2 expression in human gastric cancer (Guo et al. 2003), and in gastric cancer cells 3 to 6 hours after *H. pylori* exposure, COX-2 mRNA levels were already elevated (Iwamoto et al. 2008). *H. pylori* thus plays a central role in induction of COX-2 synthesis during gastric carcinogenesis.

COX-2 expression is associated with the clinical outcome of several cancers, like breast (Ristimäki et al. 2002), colorectal (Sheehan et al. 1999), esophageal (Buskens et al. 2002), ovarian (Erkinheimo et al. 2003), and pancreatic cancers (Juuti et al. 2006). In gastric cancer, COX-2 is connected with clinicopathological factors and survival (Chen et al. 2001, Shi et al. 2003, Okano et al. 2004, Tatsuguchi et al. 2004). Epidemiologic studies have suggested that aspirin reduces mortality from digestive tract malignancies, including gastric cancer (Thun et al. 1993, Schreinemachers et al. 1994, Zaridze et al. 1999, Abnet et al. 2009). As a result, COX-2 is considered a potential therapeutic target in solid cancers. There is an increased risk for gastrointestinal hemorrhage associated with regular use of NSAIDs. Coxibs are suggested to be safer in this setting. Recently it has emerged that cardiovascular toxicity, including cardiac and thromboembolic events, is associated with both of these. However, the risk levels vary, and it is possible to recognize high-risk patients by screening for genetic polymorphism (Menter et al. 2010).

5.8.2. HuR

HuR is a RNA-binding protein originally identified in *Drosophila melanogaster* (Campos et al. 1985). This member of the embryonic lethal abnormal vision (ELAV)/Hu protein family (Brennan et al. 2001) serves as a shuttling protein between nucleus and cytoplasm and stabilizes mRNA containing unstable adenine- and uridine-rich elements (ARE). During translocation from nucleus to cytoplasm, HuR is bound to these labile transcripts and prevents them from degrading, providing efficient translation of the target protein. HuR can also regulate translation (Gallouzi et al. 2001).

It has been suggested that HuR is an important regulator of cellular proliferation and plays a central role in all steps of carcinogenesis. HuR is associated with many cellular mRNAs, affecting a diverse set of cellular functions such as cell cycle progression, DNA replication, transcription, splicing, development, and morphogenesis (Brennan et al. 2001). Recent studies show that HuR positively regulates expression of growth factors, proliferative, and proto-oncogenic factors silencing growth inhibitory signals, leading to enhanced cell division. HuR may promote invasion and metastasis via matrix metalloproteinases, and inhibit apoptosis by up-regulating anti-apoptotic proteins. HuR may also play a role in promoting angiogenesis by enhancing expression of vascular growth factors, and in reducing immunologic recognition of tumors by induction of immunosuppressive cytokines (Lopez de Silanes et al. 2005). For example, COX-2 mRNA contains AREs and is regulated by HuR (Dixon et al. 2001, Lopez de Silanes et al. 2003, Sengupta et al. 2003). Furthermore, in synchronous colorectal carcinoma cells, HuR directly binds mRNAs encoding cyclins A and B1 (Wang et al. 2000). In gastric cancer, HuR expression is associated with advanced tumors, and cell proliferation increases with HuR overexpression, but is reduced by HuR knockdown (Kang et al. 2008).

HuR is associated with clinicopathological factors and prognosis in several solid cancers (Erkinheimo et al. 2003, Denkert et al. 2004, Heinonen et al. 2005, Yoo et al. 2009). An association between HuR and COX-2 has been reported at least in breast (Denkert et al. 2004), ovarian (Erkinheimo et al. 2003), uterine cervical (Lim et al. 2007), colorectal (Denkert et al. 2004, Lim et al. 2009), prostate (Niesporek et al. 2008), laryngeal (Cho et al. 2007) and salivary (Cho et al. 2007)
cancers, as well as in mesothelioma (Stoppoloni et al. 2008). These studies suggest that HuR expression may help in differentiating borderline tumors from malignancy, may predict early relapse, and may be associated with poor prognosis. It has also been suggested that HuR modulates the efficacy of gemcitabine in pancreatic cancer through enhancing its enzymatic metabolism by up-regulating deoxycytidine kinase. Thus, HuR expression may indicate the non-responders to this chemotherapeutic agent (Costantino et al. 2009). In vitro studies show that inhibition of HuR represses cell growth (Guo et al. 2009). Tissues with high cell proliferation and turnover express the highest levels of HuR, and genetic deletion of HuR in a mouse model affects first those tissues (Ghosh et al. 2009, Katsanou et al. 2009). These data suggest that silencing of HuR may serve as a future therapeutic option in cancer treatment.

5.8.3. Cyclin A

Cyclin A, belonging to the cyclin protein superfamily, can activate two cyclin-dependent kinases, CDK1 and CDK2. Levels of cyclin A accumulate progressively throughout the interphase, and it disappears rapidly at the end of mitosis. Currently, the phosphorylated cyclin A-CDK complex is suggested to play an important role in initiation of DNA replication in the S phase. The precise function of cyclin A in mitosis is unclear, but it may prevent other cyclins from degradation. Overexpression of cyclin A and dysregulation of CDK-cyclin complexes both promote tumor cell growth, which can be facilitated by phosphorylation of oncoproteins and tumor suppressors (Sherr 1996, Yam et al. 2002).

Two known isoforms of cyclin A are cyclin A1 and cyclin A2. Cyclin A1 is the embryonal form of the protein, its role is limited to male meiosis (Wolgemuth et al. 2004), but it is also expressed in normal human testis and brain tissues, and in myeloid leukemia cell lines (Yang et al. 1997). In contrast, cyclin A2 is present in proliferating somatic cells, and the cyclin A2 gene is lethal if disrupted (Murphy et al. 1997). Since cyclin A2 forms a complex with CDK1 and CDK2, and associates with carcinogenesis - whereas expression of cyclin A1 is restricted to specific tissues and occasions - cyclin A2, in particular, may be relevant in solid cancers (Yasmeen et al. 2003, Wolgemuth 2008).

Overexpression of cyclin A is associated with clinicopathological characteristics, proliferation rate, and poor prognosis in several cancers (Yam et al. 2002). Some have shown that low expression of cyclin A is, however, associated with poor survival (Bondi et al. 2005). In clinical gastric cancer, the role of cyclin A is ambiguous (Brien et al. 1998). In Barrett’s esophagus surveillance, cyclin A has been correlated with cancer risk (Lao-Sirieix et al. 2007), and in breast cancer, cyclin A has predicted recurrence and poor prognosis (Bukholm et al. 2001, Aaltonen et al. 2006, Li et al. 2010). In vitro studies suggest that expression of cyclin A in cancer cell lines is associated with cytotoxic response to 5-FU and doxorubicin (Volm et al. 1997, Kawashima et al. 2004), and patients with soft tissue sarcomas or head and neck squamous cell carcinomas have shown a better response to chemotherapy (Huhtanen et al. 1999, Rodriguez-Pinilla et al. 2004). Thus, cyclin A is considered a promising predictive and prognostic marker.

5.8.4. MMP-2 and MMP-9

Controlled degradation of the extracellular matrix (ECM) is a central feature in numerous biological processes such as embryonic development, tissue remodeling, and tissue repair (Ala-aho et al. 2005). In cancer, it is a critical part of growth, invasion, angiogenesis, and metastasis, and is
provided by metalloproteinases (MMPs). These zinc-dependent proteolytic enzymes cooperate and activate each other. A basic component of basal membranes and ECM, type IV collagen, is dissolved by gelatinases A (MMP-2) and B (MMP-9) (Curran et al. 1999), creating favorable conditions for tumor growth and angiogenesis (Nelson et al. 2000). MMP-2, in particular, is considered to play an important role in the first steps of tumor invasion (Tryggvason et al. 1993).

In various solid cancers, MMPs are associated with grade, stage, and survival (Turpeenniemi-Hujanen 2005). In gastric cancer, MMP-2 and MMP-9 have been associated with tumor invasion and metastasis as well as with poor prognosis (Grigioni et al. 1994, Schwartz 1996, Allgayer et al. 1998, Torii et al. 1998). Cell culture experiments show that COX-2 promotes production of MMP-2, MMP-9, and trypsins, suggesting that activation of MMPs is one of the downstream effects of COX-2 expression, and that invasion and metastasis caused by COX-2 are mediated by MMPs (Dohadwala et al. 2002, Nyberg et al. 2002). COX-2 expression is associated with MMP-2 expression in breast (Sivula et al. 2005), thyroid (Siironen et al. 2004), and renal cell carcinomas (Miyata et al. 2003), and also in gastric cancer (Dicken et al. 2006).

5.8.5. p53

Tumor suppressor gene p53 and its corresponding protein play a central role of signaling in cell-cycle arrest and apoptosis. p53 consists of four domains, each of which has a specific function: activation of transcription factors, recognition of specific DNA sequences, telomerization, and recognition of damaged DNA. In the cell cycle, p53 regulates the transition from G0 to G1 (Levine 1997). Increased expression of p53 induces cell cycle arrest, preventing replication of damaged DNA, and it activates DNA repair enzymes (Kuerbitz et al. 1992). Furthermore, p53 can activate apoptosis (Yonish-Rouach et al. 1991).

The loss of normal p53 function due to mutation in the p53 gene induced by chemicals, radiation, or viruses, is crucial in carcinogenesis (Steele et al. 2005). Damaged DNA repair mechanisms of p53 lead to accumulation of genetic disorders and to gross chromosomal changes, leading to tumor formation and malignant transformation (Levine 1997). Mutated p53 protein tends to accumulate in nuclei and may inhibit normal p53 function (Baas et al. 1994, Blagosklonny 2002). Being more stable than wild type p53, the mutated form of p53 is detected by immunohistochemistry (Finlay et al. 1988) and is present in several forms of cancer (Hollstein et al. 1991). In gastric cancer, p53 may associate with poor prognosis (Victorzon et al. 1996, Lee et al. 2003, Fondevila et al. 2004, Wiksten et al. 2008).

In mouse embryonal fibroblasts, wild type p53 has suppressed COX-2 mRNA and protein levels, whereas the mutated form has not (Subbaramaiah et al. 1999). In gastric cancer, nuclear accumulation of p53 is associated with COX-2 expression (Kawabe et al. 2002, Shun et al. 2003), suggesting that loss of p53 function is associated with COX-2 expression, and that p53 is one of the factors regulating COX-2 expression (Leung et al. 2001).

5.8.6. p21

The cyclin-dependent kinase inhibitor p21 plays a central role in cell-cycle control. Induction of p21 leads predominantly to cell-cycle arrest, whereas its repression may have diverse consequences (Gartel et al. 2005). As a transcriptional target of p53, p21 is an important factor in mediating growth arrest after DNA damage, for instance that caused by doxorubicin or irradiation (el-Deiry et
al. 1993, 1994). Thus, p21 is a tumor suppressor, as supported by the data from a mouse model showing spontaneous tumor formation in p21 knock-out mice (Martin-Caballero et al. 2001). Several oncogenes repress p21, which results in enhanced tumorigenesis, but p21 is also an inhibitor of apoptosis, and with this function, lack of p21 may have an anticancer effect as well (Gartel et al. 2002). p21 is produced by normal gastric mucosa, but not by gastric cancer tissue (Xie et al. 2004). The prognostic role of p21 in gastric cancer is unclear, since both expression and lack of p21 have been associated with good prognosis (Okuyama et al. 2002, Al-Moundhri et al. 2005, Gamboa-Dominguez et al. 2007).

5.8.7. Ki-67

Ki-67 is a nuclear and nucleolar protein, the phosphorylation of which during mitosis is associated with condensation of the chromosomes and separation of sister chromatides (Kausch et al. 2003). It is expressed in all phases of the cell cycle except G0, indicating the proportion of proliferating cells, and thus reflecting rapid proliferation and growth potential of the tumor (Endl et al. 2000). In gastric cancer, high Ki-67 expression has shown no prognostic value (Kunisaki et al. 2001, Oshima et al. 2005, Joo et al. 2006), but a low Ki-67 proliferation index was associated with poor outcome (Lee et al. 2010). Overexpression of Ki-67 in esophageal cancer has predicted complete endoscopic response after chemoradiotherapy (Ressiot et al. 2008). In gastrointestinal stromal tumors, Ki-67 immunohistochemistry serves in detection of mitotic cells, the proportion of which determines the grade of malignancy and associates with prognosis (Neves et al. 2009). A high Ki-67 index is associated with poor prognosis in gastrointestinal neuroendocrine carcinomas (Boo et al. 2007, Hentic et al. 2010). In breast cancer, because of its prognostic and predictive value, Ki-67 is suggested for inclusion in routine biological markers (Yerushalmi et al. 2010).

5.8.8. DNA ploidy and S-phase fraction

Originally, flow cytometry was useful in investigating blood cells. More recently, cultured cells and cell lines, bacteria, sperm, and plankton have been analyzed, but also smaller particles such as viruses, nuclei, chromosomes, and DNA fragments (Givan 2011). Under normal conditions, human autosomal cells contain 46 chromosomes and are called diploid. Any divergence from this is aneuploidy, indicating an abnormal amount of DNA. Flow cytometry measures DNA contents of the cancer cells with a laser light of a specific wave length. The DNA, stained with a fluorescent dye and scanned with a laser beam, emits the light at an altered wave length, a signal detected and digitally analyzed. One of the parameters detected is the percentage of cells under DNA synthesis (S-phase fraction, SPF) (Hedley et al. 1983)(Baba et al. 2002). These data on cellular and molecular characteristics of various cancers can be applied in clinical use; for instance, aneuploidy is a marker for poor prognosis in gastric cancer (Korenaga et al. 1988, Victorzon et al. 1996).
6. AIMS OF THE STUDY

The purpose of the study was to determine the prognostic role of COX-2 in an unselected gastric cancer series, and, in particular, to find a combination of novel immunohistochemical biomarkers to estimate outcome, and then to evaluate their association with clinicopathological parameters.

The specific aims were to discover:

- The prognostic role of COX-2 in gastric cancer.
- The regulation of COX-2 expression by the mRNA stability factor HuR and to learn whether HuR has prognostic significance in gastric cancer *per se*.
- The prognostic role of cyclin A in gastric cancer and to find whether any association exists between cyclin A and HuR.
- The possible downstream pathways of COX-2: whether MMP-2 or MMP-9 have prognostic significance in gastric cancer, and whether any association exists between COX-2 and MMPs.
- Any association between COX-2 and factors reflecting apoptosis and proliferation, such as p53, p21, Ki-67 or flow cytometry, and to reconstruct an extended multivariate model for more accurate estimation of prognosis by combining several tumor markers.
7. PATIENTS AND METHODS

7.1. Patients (I-IV)

The study included 342 consecutive patients who underwent surgery for histologically verified gastric adenocarcinoma at the Department of Surgery, Meilahti Hospital, Helsinki University Central Hospital, between 1983 and 1999. Approval of the study came from the local ethics committee. Diagnosis and staging was performed from specimens according to the UICC classification (TNM 1992, 4th edition, 2nd revision). Five patients were excluded due to incomplete information: missing clinical data (one patient), unclear cause of death (one), missing follow-up (two) and lacking archive block (one). A total of 337 patients were included. Patients were: 141 (42%) low stage (I to II) and 196 (58%) high stage (III to IV). Lymph-node metastases were evident in 184 (55%) and distant metastases in 93 (28%). Median age was 66 years (range 30-87) among the 163 women and 174 men. Surgery for cure (total or partial gastrectomy with lymph node dissection) was performed in 176 (52%), and 143 (43%) underwent a palliative procedure (partial gastrectomy, bypass, or laparotomy only). Extended lymphadenectomy (D2-4) was performed for 34 (10%) patients. None received neoadjuvant therapy, but 32 patients received postoperative adjuvant therapy: 28 chemotherapy, 2 radiotherapy, and 2 received both. Of these patients, 28 represented stages III to IV. Survival data and cause of death were obtained until October 2007 from patient records, the Finnish Cancer Registry, and the Population Register Centre of Finland. Their clinicopathological characteristics are described in Table 7. Median follow-up time was 12.7 years (range 4.7-20.8). During follow-up, 210 (64%) patients died of gastric cancer.

7.2. Tissue microarray (TMA)(IV)

Representative tumor regions in routinely fixed paraffin-embedded samples were defined from H&E-stained sections and marked. Paraffin-embedded donor tissue blocks were sampled with 0.6-mm punchers using a tissue microarray instrument (Manuel Tissue Arrayer 1, Beecher Instruments Inc., Silver Spring, MD, USA). Three cores were cut from each donor block for the tissue microarray blocks. From the tumor samples available, 6 tissue array blocks were prepared, each containing 80 to 180 tumor samples (Kononen et al. 1998). Sections of 4 µm were cut and processed for immunohistochemistry.

7.3. Immunohistochemistry (I-IV)

Formalin-fixed paraffin-embedded archive tissue blocks were freshly cut into 4-µm-thick sections onto slides and dried to fix for 12 to 24 h at 37 °C. Tissue sections were then deparaffinized in xylene and rehydrated through graded alcohol and deionized water. For the antigen retrieval, these sections were heated in a microwave oven in 0.3% citrate buffer (pH 6.0) for 4 x 5 min, and washed with phosphate-buffered saline (PBS) for 2 x 5 min, or treated with 0.5% trypsin solution for 30 min and rinsed with PBS for 3 x 5 min. Endogenous peroxidases were inactivated by 30-min incubation in methanol containing 1.6% hydrogen-peroxidase followed by a PBS wash for 3 x 5 min. To block
non-specific binding sites, the sections were treated with normal horse serum for 15 min. The sections were incubated overnight with specific antibodies (Table 3). After the overnight incubation with the primary antibody, the sections were first reacted for 30 min with biotinylated anti-mouse IgG (Vector Laboratories, Inc., CA, USA) diluted 1:200 in PBS containing 1% normal horse serum, and then for 30 min in avidin-biotinylated peroxidase complex (Vectastain ABC Kits, Vector). Between each step in the staining procedure, sections were washed with PBS for 3 x 5 min. Staining was visualized with 3-amino-9-ethyl-carbazole (A5754; Sigma Chemical Co, St. Louis, MO, USA), 0.2 g/ml in 0.5 M acetate buffer containing 0.03% hydrogen peroxide (pH 5.0) for 15 min. Subsequently the sections were washed thoroughly in tap water for 10 min, counterstained with Meyer’s hematoxylin for 15 to 60 s, washed, and mounted in aqueous mounting media (Aquamount; BDH, Poole, UK). This procedure, carried out with no specific antibody, served as a negative control. The effect of non-specific immunoreactivity was assessed as described (Saukkonen et al. 2001). All steps of the procedure were carried out at room temperature.

7.4. Scoring (I-IV)

7.4.1. COX-2

Cytoplasmic COX-2 immunoreactivity was assessed by intensity of staining and percentage of positivity area. The intensity of staining was graded from 0 to 3 (absent, weak diffuse, moderate granular, strong granular), and the area of positivity was estimated as percentage of total area of the tumor (less than 10%; between 10 and 89%; equal to or more than 90%). The actual score consisted of these two variables: score 0 (negative staining or intensity 1 under 10% area); score 1 (intensity 1 between 10 and 100% or intensity 2-3 under 10% area); score 2 (intensity 2 over 10% or intensity 3 under 90% area); score 3 (intensity 3 over 90% area). Colorectal carcinoma specimens served as positive controls as previously described (Buskens et al. 2002). Immunoreactivity was scored by two independent interpreters without preliminary knowledge of any clinical data. The final consensus score case-by-case was discussed and determined in a common session. In statistical analysis, COX-2 scores were handled in all four subgroups (0-3), or in two groups (low: 0-1, high: 2-3).

7.4.2. HuR

HuR staining was observed in the same blinded manner. Patients were initially scored into five groups according to nuclear and cytoplasmic intensity of immunoreactivity: nucleus only (1), cytoplasm only (2), nucleus more intense than cytoplasm (3), cytoplasm more intense than nucleus (4), or totally negative (0). These groups were combined, and analyses were performed between several combinations: cytoplasm-negative (0 and 1) versus cytoplasm-positive (2, 3, and 4), and nucleus-negative (0 and 2) versus nucleus-positive (1, 3, and 4).

7.4.3. Cyclin A

Cyclin A immunoreactivity was measured by the estimation of the number of tumor cells and the percentage of positive nuclei under a medium power (x20) magnifying objective in areas where staining was most intense. At least five fields were scored. A value of less than 5% was considered
negative (score 0), and one of 5% or greater considered positive (score 1). Cyclin A immunostaining was scored in a consensus manner without preliminary knowledge of clinical data.

### 7.4.4. MMP-2 and MMP-9

MMP-2 immunoreactivity was scored separately for epithelial and stromal cell expression in cancer tissue. The score was determined by the percentage of positive tumor area. Absence of immunoreactivity was scored as 0. Less than 10% positivity was scored 1, \( \geq 10\% \) but <70% was scored 2, and \( \geq 70\% \) up to 100% was scored 3. The groups were combined as MMP-2-negative (score 0) and MMP-2-positive (scores 1-3). MMP-9 immunoreactivity was detected in the cytoplasm of the tumor cells and scored identically. The scoring was performed in the blinded manner described above.

### 7.4.5. Scoring of Ki-67 from TMA sections

Three punches were taken from representative areas of tumor tissue from each patient. All dots were scored by the author and discussed in a common session with a senior pathologist. Staining pattern was nuclear. Immunostaining \( \geq 10\% \) was considered positive. All the scores were entered into an internet database, and the final score was defined from the average value. This score was utilized in statistical analysis.

### 7.4.6. P53 and p21

Staining for p53 and p21 was considered high when more than 20% of the nuclei of cancer tissue were positive (Wiksten et al. 2008).
Table 4. Characteristics of immunohistochemical staining and cut-off values for tumor markers.

<table>
<thead>
<tr>
<th>Tumor marker</th>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Amplification kit</th>
<th>Pre-treatment</th>
<th>Cut-off for analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>160112</td>
<td>Cayman Chemical, Ann Arbor, MI, USA</td>
<td>1:200</td>
<td>Vectastain</td>
<td>700W microwave</td>
<td>intensity and percentage of cytoplasmic staining</td>
</tr>
<tr>
<td>HuR</td>
<td>19F12</td>
<td>Clonegene, Hartford, CT, USA</td>
<td>1:10000</td>
<td>700W microwave</td>
<td>positive cytoplasmic staining</td>
<td></td>
</tr>
<tr>
<td>Cyclin A</td>
<td>6E6</td>
<td>Novo Castra Laboratories, Newcastle-Upon-Tyne, UK</td>
<td>1:100</td>
<td>Vectastain</td>
<td>700W microwave</td>
<td>≥5%</td>
</tr>
<tr>
<td>MMP-2</td>
<td>VC2</td>
<td>Neomarkers, Fremont, CA, USA</td>
<td>1:200</td>
<td>Vectastain</td>
<td>700W microwave</td>
<td>≥10%, (epithelial and stromal separately)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>160112</td>
<td>Neomarkers</td>
<td>1:5000</td>
<td>Vectastain</td>
<td>700W microwave</td>
<td>≥10% (cytoplasmic)</td>
</tr>
<tr>
<td>p53</td>
<td>DO7</td>
<td>Dako, Glostrup, Denmark</td>
<td>1:300</td>
<td>700W microwave</td>
<td>≥20% (nuclear)</td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>4D10</td>
<td>Novo Castra</td>
<td>1:20</td>
<td>K1500 0.5% trypsin-PBS</td>
<td>≥20% (nuclear)</td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>A0047</td>
<td>Dako</td>
<td>1:500</td>
<td>K1500 700W microwave</td>
<td>≥10% (nuclear)</td>
<td></td>
</tr>
</tbody>
</table>
7.5. Cell culture and RNA interference (I)

The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (PromoCell GmbH, Heidelberg, Germany), 2 mM L-glutamine, and antibiotics (Bio Whittaker Europe, Verviers, Belgium), and maintained in 37°C at 5% CO2 in air. The siRNA duplexes were synthesized by Dharmacon Inc. (Lafayette, CO, USA). The sequences were, for HuR: sense 5’-AAC AUG ACC CAG GAU GAG UUA dTdT-3’ and antisense 5’-UAA CUC AUC CUG GGU CAU GUU dTdT-3’, and for β-actin: sense 5’-AAU GAA GAU CAA GAU CAC UGC dTdT-3’ and antisense 5’-GCA AUG AUC UUG AUC UUC AUU dTdT-3’. The day before transfection, TMK-1 cells were trypsinized and diluted 1:20 with optiMEM 1-medium (GIBCO, Paisley, UK) supplemented with 10% FCS without antibiotics and transferred to 12-well plates, 1 ml per well, the final split ratio being 1:4. Transient transfection of siRNAs was carried out with Oligofectamine Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following manufacturer’s instructions and as described (Erkinheimo et al. 2003). Final siRNA concentrations ranged from 0.5 nM to 150 nM. The cells were lysed 78 h after the transfection in 400 μl 95°C 1x lysis buffer (60 mM Tris-HCl, pH= 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.002% bromophenol blue). The samples were heated for 4 min at 95°C, and centrifuged at room temperature at 14 000 g for 10 min; 50 μl of the sample was taken and separated by 12% SDS-PAGE. In selected experiments, interleukin-1β (IL-1β; 10 ng/ml, R&D Systems, Minneapolis, MN, USA) was added for the last 24 h of the incubation period.

7.6. Immunofluorescence (I)

For immunofluorescence experiments, TMK-1 cells were grown on coverslips in 24-well plates overnight and transfected with 50 nM HuR siRNA as described above. After 72 hours the cells were fixed with 4% PFA in PBS for 20 min at room temperature and washed once with PBS. Cells were permeabilized with 0.5% NP-40 in PBS for 5 min at room temperature and washed three times with PBS. Nonspecific binding of antibodies was blocked with 3% BSA/TBS for 10 min at room temperature. The samples were then incubated with a 1:10 000 dilution of the monoclonal HuR antibody in 3% BSA/TBS for one hour at 37°C and washed three times with PBS. The secondary antibody (1:50 dilution) TRITC-conjugated rabbit anti-mouse (DAKO A/S, Glostrup) was incubated for one hour at 37°C. After three washes with PBS, these samples were incubated for 2 minutes at room temperature with Hoechst (1:1000, Sigma). Images were obtained with a Zeiss Axiplan imaging fluorescence microscope (Carl Zeiss, Jena, Germany).

7.7. DNA flow cytometry (IV)

The method of DNA flow cytometry has been described (Victorzon et al. 1996). Of the 337 tumor samples, 313 were representative, as evaluated by this method. The DNA index (DI) was designated as 1.00 for the lowest peak, with other peaks referred to this baseline. DI value 1.20 or less was considered as diploid, others as aneuploid. The S-phase fraction (SPF) was calculated in 278 of 313 of the tumor samples as either computer-assisted or manually. If the sample contained less than 15% aneuploid cells, SPF was not assessed. A minimum of 10 000 nuclei from each specimen were analyzed. The cut-off level was set at 7.6%, which was the median SPF.
7.8. Statistical analysis (I-IV)

Associations between factors, including clinicopathological variables and immunohistochemical scores, were assessed by Chi-Squared or Fisher’s exact test in case of low expected frequencies. Survival rates were calculated by the Kaplan-Meier method, and statistical significance between the groups was analyzed by the logrank test or logrank test for trend in case of three or more ordered groups. Gastric cancer-specific survival was calculated from the date of diagnosis to death from gastric cancer or last day of follow-up. Deaths due to intercurrent causes were censored. Multivariate survival analysis was performed with the Cox proportional hazards model. A p-value <0.05 was adopted as the limit for inclusion of a covariate. All p-values are two-sided.

7.9. Agreement of whole-slide immunohistochemistry with TMA immunohistochemistry (unpublished data)

To explore the correspondence between whole-slide and TMA techniques, we chose three prognostic markers differing in staining patterns: p53 with positive nuclear immunoreactivity (positive vs. negative), HuR with cytoplasmic (positive vs. negative), and COX-2 with cytoplasmic (positive vs. negative including intensity observed). Whole slides and TMA slides were stained in parallel, and each TMA score was compared with the corresponding whole slide score. Percentage of observed agreement and percentage of agreement expected by chance alone were calculated, producing from these a kappa value indicating true agreement (Table 5).

Table 5. Classification of kappa values indicating agreement.

<table>
<thead>
<tr>
<th>Kappa value</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.20</td>
<td>Poor</td>
</tr>
<tr>
<td>0.21-0.40</td>
<td>Fair</td>
</tr>
<tr>
<td>0.41-0.60</td>
<td>Moderate</td>
</tr>
<tr>
<td>0.61-0.80</td>
<td>Good</td>
</tr>
<tr>
<td>0.81-1.00</td>
<td>Very good</td>
</tr>
</tbody>
</table>
8. RESULTS

8.1. Immunoreactivity (I-IV)

COX-2 immunoreactivity was evaluated in 321 specimens, of which 65 (20%) were negative, 80 (25%) represented score 1, 153 (48%) score 2, and 23 (7%) score 3. The groups were combined as COX-2 low (score 0-1) and COX-2 high (score 2-3), the values were 145 (45%) and 176 (55%), respectively. Positive staining was observable in the cytoplasm of the malignant cells and in the perinuclear region whenever strong positivity was observable.

For HuR immunoreactivity evaluation, 316 specimens were available. Both nuclear and cytoplasmic staining was observable. No cytoplasmic immunoreactivity appeared in 189 (60%) specimens, but positive cytoplasmic immunoreactivity appeared in 127 (40%). There were 37 (12%) negative in nuclear immunostaining, and 279 (88%) in positive. The staining intensity was not assessed.

Cyclin A immunoreactivity was investigated in 325 specimens. Of these, 146 (45%) were negative and 179 (55%) positive. The staining pattern of cyclin A is nuclear, and seldom was there any cytoplasmic immunoreactivity.

Of the 329 specimens, epithelial MMP-2 immunoreactivity was negative in 242 (74%) and positive in 87 (26%) specimens. Negative stromal MMP-2 immunoreactivity appeared in 87 (26%) specimens and positive in 242 (74%). Corresponding figures for MMP-9 immunoreactivity in 330 cases were 47 (14%) and 283 (86%).

In the 258 specimens available for Ki-67 immunohistochemistry, immunoreactivity was assessed from the nuclei; it was negative in 88 (34%) specimens and positive in 170 (66%).

Wiksten et al (2008) described the distribution of these factors in this patient material. In summary, the figures for positive immunostaining were 104 of 336 (31%) for p53 and 43 of 317 (14%) for p21. Flow cytometry showed aneuploidy in 83 of 306 (27%), and high S-phase fraction in 137 of 278 (49%) specimens.

8.2. Associations of tumor markers (I-IV)

8.2.1. Clinicopathological characteristics

Associations of tumor markers with clinicopathological factors are presented in Table 6. COX-2 and cyclin A were associated with several factors, indicating an advanced pattern of the disease. Cytoplasmic HuR and epithelial MMP-2 showed moderate correlation with these factors, with no or little correlation for nuclear HuR, stromal MMP-2, MMP-9 or Ki-67.
Table 6. Association of tumor markers with clinicopathological characteristics, and proportion of positive stained-specimens.

<table>
<thead>
<tr>
<th>Tumor marker</th>
<th>Positive/ all (%)</th>
<th>Age</th>
<th>Gend er</th>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Tumor location</th>
<th>Laurén</th>
<th>Tumor size</th>
<th>Intent of surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>176/321 (55)</td>
<td>NS</td>
<td>*</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>** (prox)</td>
<td>*</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>HuR cyt</td>
<td>127/316 (40)</td>
<td>*</td>
<td>**</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
<td>** (prox)</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HuR nuc</td>
<td>279/316 (88)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
<td>(dist)</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>179/325 (55)</td>
<td>NS</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>NS</td>
<td>*</td>
<td>(prox)</td>
<td>**</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>MMP-2 epith</td>
<td>87/329 (26)</td>
<td>NS</td>
<td>*</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>MMP-2 strom</td>
<td>242/329 (74)</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>** (diff)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-9</td>
<td>47/330 (14)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ki-67</td>
<td>170/258 (66)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*p<0.05 and **p<0.01 indicating positive correlation, NS = not significant, cyt = cytoplasmic immunostaining, nuc = nuclear immunostaining, epith = epithelial immunostaining, strom = stromal immunostaining, prox = proximal 1/3 of stomach, dist = distal 2/3 of stomach, int = intestinal, diff = diffuse

8.2.2. Associations between markers

We found high COX-2 expression to be associated with epithelial MMP-2 (p<0.0001), Ki-67 (p=0.013), and p53 (p<0.0001) expression, with an association also between COX-2 and diploid DNA pattern (p<0.0001), and high SPF (p<0.0001). We could show no association between COX-2 and stromal MMP-2 (p=0.70), MMP-9 (p=0.75), or p21 (p=0.33).

Cytoplasmic HuR expression correlated positively with COX-2 (p<0.0001). There was also a significant association between cytoplasmic HuR and cyclin A expression (p<0.001). No associations emerged between nuclear HuR and COX-2 nor between nuclear HuR and cyclin A (0.29<p≤1.0).

8.3. Survival analysis (I-IV)

8.3.1 Survival

The overall 2-year survival was 49.5% (range 44.0-54.8%, CI 95%) and 5-year survival 35.1% (range 29.8-40.4, CI 95%). Median survival was 1.98 years (range 1.42-2.53, CI 95%) (Fig 2). Univariate analysis of the clinicopathological characteristics is shown in Table 6. All but gender and
Laurén classification were significant. Associations of tumor markers and flow cytometry with survival are described in Table 8.

**Figure 2.** Overall survival of gastric cancer in 337 patients.
Table 7. Univariate analysis of the relationship between preoperative characteristics and survival in 337 patients with gastric cancer.

<table>
<thead>
<tr>
<th>Clinicopathological variable</th>
<th>Patients</th>
<th>Cumulative 5-year survival %</th>
<th>$\chi^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;66 years</td>
<td>165</td>
<td>44</td>
<td>10.37</td>
<td>0.0013</td>
</tr>
<tr>
<td>≥66 years</td>
<td>172</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>163</td>
<td>38</td>
<td>1.42</td>
<td>0.23</td>
</tr>
<tr>
<td>Male</td>
<td>174</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TNM stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IA</td>
<td>52</td>
<td>96</td>
<td>224.83</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stage IB</td>
<td>48</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>41</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>67</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>29</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>100</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Penetration depth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa (T1)</td>
<td>26</td>
<td>96</td>
<td>130.26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Submucosa (T1)</td>
<td>33</td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscularis propria (T2)</td>
<td>48</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subserosa (T2)</td>
<td>12</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serosa (T3)</td>
<td>155</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjacent structures (T4)</td>
<td>63</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastases</strong></td>
<td>(N=336)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>152</td>
<td>67</td>
<td>178.96</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N1</td>
<td>95</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>89</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Distant metastases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>244</td>
<td>47</td>
<td>137.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>M1</td>
<td>93</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor location</strong> (N=335)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper 1/3</td>
<td>69</td>
<td>22</td>
<td>31.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Middle 1/3</td>
<td>114</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower 1/3</td>
<td>126</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stump</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Laurén classification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal type</td>
<td>142</td>
<td>34</td>
<td>0.01</td>
<td>0.96</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>195</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor size median</strong> (N=319)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5 cm</td>
<td>185</td>
<td>53</td>
<td>67.17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>12</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Resectability</strong> (N=319)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intent to cure</td>
<td>176</td>
<td>61</td>
<td>195.64</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-curative</td>
<td>143</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Corresponding p-values were calculated with the log-rank or log-rank test for trend. Abbreviation: 95% CI = 95% confidence interval.
Table 8. Kaplan-Meier analysis of tumor markers in gastric cancer patients.

<table>
<thead>
<tr>
<th>Marker</th>
<th>5-year survival</th>
<th>( \chi^2 )</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COX-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>53</td>
<td>53.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>high</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HuR cytoplasmic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>40</td>
<td>8.30</td>
<td>0.004</td>
</tr>
<tr>
<td>positive</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HuR nuclear</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>28</td>
<td>0.06</td>
<td>0.81</td>
</tr>
<tr>
<td>positive</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cyclin A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>45</td>
<td>16.80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>positive</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MMP-2 epithelial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>37</td>
<td>7.05</td>
<td>0.008</td>
</tr>
<tr>
<td>positive</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MMP-2 stromal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>40</td>
<td>1.55</td>
<td>0.21</td>
</tr>
<tr>
<td>positive</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>35</td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>positive</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ki-67</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>32</td>
<td>0.113</td>
<td>0.737</td>
</tr>
<tr>
<td>positive</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p53</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>40</td>
<td>10.30</td>
<td>0.001</td>
</tr>
<tr>
<td>high</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p21</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>33</td>
<td>7.46</td>
<td>0.006</td>
</tr>
<tr>
<td>high</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DNA ploidy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diploid</td>
<td>39</td>
<td>29.89</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>aneuploid</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-phase fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>46</td>
<td>24.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>high</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

8.3.2. COX-2(I,IV)

In univariate analysis, COX-2 expression was associated with reduced survival in the whole patient material \( p<0.0001, \chi^2=52.66 \) (Fig. 3). Since it is suggested that intestinal and diffuse type carcinomas have diverse backgrounds of etiopathogenesis, we sought any difference in COX-2 expression and survival among these entities. High COX-2 expression was associated with unfavorable outcome in both groups (Fig 4 and 5, unpublished data).
Figure 3. COX-2 scores and survival in gastric cancer.

Figure 4. COX-2 expression in intestinal cancer.
We also studied whether COX-2 expression could provide additional prognostic information in non-advanced disease. The results show that COX-2 is associated with poor outcome in patients with tumoral penetration of T1-2, but not T3-4. Similarly, COX-2 is associated with poor survival in patients with N0 or M0 disease, but not in patients with N1-2 or M1. According to flow cytometry results, COX-2 is correlated with reduced survival in patients with a diploid tumoral DNA pattern, but not with the aneuploid subgroup.

8.3.3. HuR (I)

Cytoplasmic HuR expression is associated with poor prognosis ($p=0.004$, $\chi^2=8.30$). Presence of nuclear HuR immunoreactivity did not correlate with survival ($p=0.81$). In subgroup analysis, cytoplasmic HuR expression was associated with poor prognosis in patients with the intestinal type of gastric cancer, but not in those with the diffuse (Fig. 6, unpublished data).
8.3.4. Cyclin A (II)

Positive cyclin A expression was associated with reduced survival (p<0.0001, $\chi^2=16.80$). In subgroup analysis, cyclin A was associated with poor survival in diffuse type cancers (n=184, p<0.0001, $\chi^2=16.0$), but no statistically significant association appeared for intestinal cancers (n=141, p=0.065, $\chi^2=3.4$).

8.3.5. MMP-2 and MMP-9(III)

Of the MMPs tested, epithelial MMP-2 expression showed prognostic value in univariate analysis (p=0.008, $\chi^2=7.05$). Neither stromal MMP-2 nor MMP-9 immunoreactivity was statistically significant in univariate analysis (p≥0.21).

8.3.6. Ki-67

In our material Ki-67 was not a prognostic marker (p=0.737, $\chi^2=0.113$).

8.3.7. Multivariate analysis (I-IV)

In each study, separate multivariate models were calculated. All the models showed stage and intent of surgery inevitably to be independent prognostic factors.

Study I showed that COX-2 was an independent prognostic factor. To obtain more detailed information about COX-2 in early stages of gastric cancer, separate models were created for stage groups I to II and III to IV. In both models, COX-2 remained independent, as did stage and intent of surgery. The prognostic power of COX-2 was more pronounced in stages I and II than III to IV (hazard ratio 2.54, CI95% 1.32-4.86; vs. 1.72, CI95% 1.22-2.45). Neither of the HuR
immunostaining patterns showed an independent prognostic value. In *Study II*, cyclin A expression was an independent prognostic factor for poor survival. In concordance with *Study I* findings, cytoplasmic HuR was non-independent. *Study III* showed that neither MMP was an independent prognostic factor, but again, COX-2 was. In *Study IV*, we reconstructed an extended multivariate model by including all markers showing prognostic significance in Studies I to III, and moreover, including the factors indicating apoptosis and proliferation known to associate with survival in this material (Wiksten et al. 2008). The biomarkers remaining independent were COX-2 and p53.

8.4. Cell culture experiments (I)

To test the hypothesis that HuR regulates COX-2 expression in gastric carcinoma cells, we treated TMK-1 cells with HuR siRNA molecules. This treatment suppressed HuR expression as detected by immunofluorescence and immunoblotting. Furthermore, we observed a concentration-dependent suppression of COX-2 protein expression after the HuR siRNA treatment. β-Actin siRNA lacked any effect on HuR or COX-2 expression. These results indicate that HuR can regulate COX-2 expression in gastric cancer cells.

8.5. Agreement of whole slide with TMA immunohistochemistry (unpublished data)

We found that the percentage of agreement for p53 whole-slide and TMA immunohistochemistry was 88%, the percentage agreement expected by chance alone was 60%, and the kappa value was 0.71 (good). Corresponding figures for COX-2 were 79%, 75%, and 0.14 (poor), and for HuR 79%, 50%, and 0.58 (moderate), respectively. Nuclear appearance of p53 expression and percentage of positive nuclei is more unambiguous than is cytoplasmic expression and intensity of HuR and COX-2, and moreover, COX-2 has more heterogenic immunostaining than does HuR, which may in part explain these results.
9. DISCUSSION

9.1. Patient material and survival

Rates for survival of gastric cancer range from 20 to 30%. In this series, 5-year overall survival was 35.1%, higher than average in Western countries (D’Souza M et al. 2009, Klint et al. 2010). All stages were represented, emphasis being on the advanced stages III to IV comprising 58% of patients, and the proportion of non-curatively operated patients, as high as 42%. Thus, this series can be considered as consecutive and unselected, although collected from over a time period of 17 years (1983-1999).

9.2. TNM staging

The TNM classification system has been revised several times during this period. Diagnostic, operative and postoperative strategies for gastric cancer have changed until recently. Hence, the latest of TNM classification (7th edition, 2009) cannot be applied retrospectively to this material. As a compromise, we have used TNM 1992 (4th edition, 2nd revision).

9.3. Surgery and adjuvant therapy

D2 lymph node dissection was adopted in the 1990’s at Helsinki University Central Hospital, and even D3-4 dissections were performed. Only 34 (10%) patients underwent D2-4 dissection; consequently, the effect of extent of lymphadenectomy on survival cannot be estimated reliably. Furthermore, according to Western trials, the superiority of D2 dissection over D1 is controversial (Cuschieri et al. 1999, McCulloch et al. 2005, Songun et al. 2010). However, 176 (52%) patients were operated upon with curative intent (R0 resection), and for those, outcome was very good: their 5-year survival rate was 61%. This highlights the role of meticulous R0 surgery for the prognosis of gastric cancer patients.

None of the patients received neoadjuvant therapy, but 33 (10%) received postoperative adjuvant therapy: chemotherapy (29 patients), radiotherapy (2) or both (2); all but 4 patients represented stages III to IV, and only 5 patients receiving postoperative treatments were operated on non-curatively. The effect of these treatments on survival cannot be estimated in this series on a larger scale. According to present European recommendations, perioperative chemotherapy is the most feasible, providing survival benefit for patients with carcinoma of T3 or higher (Van Cutsem et al. 2008).
9.4. Tumor markers

9.4.1. COX-2

We showed that COX-2 expression is associated with old age, male gender, intestinal subtype, proximal location, large tumor size, and advanced stage. These results are in line with previous findings (Murata et al. 1999, Yamamoto et al. 1999, Chen et al. 2001, Lee et al. 2001, Leung et al. 2001, Joo et al. 2002, Joo et al. 2003, Li et al. 2003, Shi et al. 2003, Yu et al. 2003, Okano et al. 2004). Association of COX-2 with survival has been controversial, since some studies show no such link (Sung et al. 2000, Leung et al. 2001, Rajnakova et al. 2001, Joo et al. 2002). A non-independent association of COX-2 with survival has been reported in patients with advanced stage (Chen et al. 2001, Okano et al. 2004, Tatsuguchi et al. 2004), as well as an independent association in a selected group of low-stage patients (Shi et al. 2003). However, we were the first to report that COX-2 expression is an independent prognostic factor in a consecutive patient series with gastric cancer (Study I).

Some have postulated that COX-2 plays an important role, especially in early gastric carcinogenesis, since in rodent models genetic deletion or pharmacological inhibition of COX-2 led to reduced tumor formation and gastric carcinogenesis (Oshima et al. 1996, Chulada et al. 2000, Saukkonen et al. 2003, Hu et al. 2004). Furthermore, transgenic expression of COX-2 induces tumor formation in mice (Oshima et al. 2004). In humans, COX-2 is expressed even in noninvasive dysplasias. Several clinical studies have demonstrated that COX-2 expression is associated with aggressive and advanced disease forms (Murata et al. 1999, Yamamoto et al. 1999, Chen et al. 2001, Lee et al. 2001, Leung et al. 2001, Joo et al. 2002, Joo et al. 2003, Li et al. 2003, Shi et al. 2003, Yu et al. 2003, Okano et al. 2004). To this end, we had a special interest in COX-2 expression in low-stage disease, in whether it could identify those patients with non-advanced, operable, but potentially aggressive cancer. We found that low-stage patients with high tumoral COX-2 expression were at higher risk for gastric cancer-related death than were high-stage patients. COX-2 remained an independent prognostic factor in both groups. Knowledge of patients’ preoperative COX-2 status could help clinicians to target adjuvant treatment.

However, effects of cytotoxic agents on COX-2 expression are poorly known. One report shows that after 5-fluorouracil and leucovorin treatment followed by surgery, lack of COX-2 expression was an independent prognostic factor for poor survival (Wu et al. 2009). Moreover, hypermethylation of the COX-2 promoter region down-regulates COX-2 expression (Song et al. 2001). In a clinical retrospective study, hypermethylation of the COX-2 promoter region, caused by preoperative treatment with fluorouracil, doxorubicin, and methotrexate, was inversely associated with COX-2 expression, and served as an independent factor for good prognosis of gastric cancer patients (de Maat et al. 2007).

COX-2 as a target of chemoprevention is a point of interest (Macdonald 2006). The selective COX-2 inhibitor celecoxib has proven useful in patients with familial adenomatous polyposis (FAP) in reducing polyp burden, inspiring hope that other precancerous and even malignant lesions of the gastrointestinal tract can respond to these drugs (Cooper et al. 2010). Increased cardiovascular morbidity associated with celecoxib and other NSAIDs has, however, lessened enthusiasm around pharmacological COX-2 inhibition (Menter et al. 2010). Genetic silencing of COX-2 with small interfering RNA (siRNA) has reduced levels of COX-2 and prostaglandin E in a gastric cancer cell line (MKN45), and siRNA treatment has had a more potent antiproliferative effect on gastric cancer cells than has the specific high-dose COX-2 inhibitor NS398. Furthermore, COX-2 siRNA also has reduced tumor growth in nude mice and enhanced the apoptotic reaction of MKN45 cells to...
cisplatin therapy (Chan et al. 2007).

In conclusion, COX-2 expression is a marker for poor prognosis in gastric cancer at all stages, but especially in low-stage patients.

9.4.2. HuR

Cytoplasmic HuR expression was associated with old age, male gender, high stage, distant metastases, proximal location of the tumor, intestinal subtype, and non-curative surgery. Nuclear HuR expression correlated with old age, and unlike cytoplasmic HuR, with distal tumor location and diffuse subtype. Cytoplasmic HuR expression was associated with reduced survival, but in a non-independent manner. In a study by Kang et al, high levels of HuR detected by polymerase chain reaction were associated with advanced tumors, but not with grade, histological subtype, age or sex. HuR transcript levels correlated with cytoplasmic HuR protein expression, detected by immunohistochemistry, which in turn associated with stage and grade (Kang et al. 2008). The role of cytoplasmic staining is more pronounced, which is in line with the function of HuR as a shuttling protein between nucleus and cytoplasm (Brennan et al. 2001). In other cancers, like ovarian (Erkinheimo et al. 2003, Denkert et al. 2004), breast (Denkert et al. 2004, Heinonen et al. 2005), uterine (Lim et al. 2007), and colorectal (Yoo et al. 2009), HuR was associated with clinicopathological characteristics or survival or both. Our results are similar.

In TMK-1 gastric cancer cells treated with HuR siRNA, HuR expression was suppressed, and furthermore, a concentration-dependent suppression of COX-2 was apparent. These in vitro results agree with findings on colon (Dixon et al. 2001, Lopez de Silanes et al. 2003), breast (Sengupta et al. 2003) and ovarian (Erkinheimo et al. 2003) cancer cells, and indicate that HuR can regulate COX-2 expression. We report that cytoplasmic HuR expression is associated with COX-2 expression in clinical gastric cancer. Similar results have appeared (Milne et al. 2006) in other cancers (Erkinheimo et al. 2003, Denkert et al. 2004, Denkert et al. 2004, Lim et al. 2007, Yoo et al. 2009).

9.4.3. Cyclin A

Study II assessed the role of cyclin A in gastric cancer. We found that cyclin A was associated with old age, high stage, proximal location of the tumor, intestinal subtype, and non-curative surgery. Overexpression of cyclin A was an independent prognostic factor for poor survival. Until now, few studies have been published about cyclin A in a clinical gastric cancer series, with no correlation with survival found (Brien et al. 1998). In esophageal squamous cell carcinoma, cyclin A immunoreactivity was associated with tumor progression and poor outcome (Furihata et al. 1996), and in colorectal cancer, cyclin A overexpression was a prognostic factor (Handa et al. 1999, Bahnassy et al. 2004, Nozoe et al. 2004). The prognostic role of cyclin A is arguable, since some studies show that reduced cyclin A expression is a marker for poor prognosis in colon cancer (Li et al. 2002, Bondi et al. 2005). In epidermoid anal carcinoma, after preoperative chemoradiotherapy high cyclin A expression is associated with better prognosis (Nilsson et al. 2006). This may reflect a sensitivity towards radiotherapy, and a percentage of synchronous cells. However, the majority of studies show that, in solid cancers, overexpression of cyclin A is associated with poor outcome (Furihata et al. 1997, Volm et al. 1997, Chao et al. 1998, Dobashi et al. 1998, Molendini et al. 1998, Aaltomaa et al. 1999, Huuhtanen et al. 1999, Wołowiec et al. 1999, Zhai et al. 1999, Noguchi et al. 2000, Bukholm et al. 2001). Our results are in line with those reports.
Reduction of HuR expression leads to decreased levels of cyclin A mRNAs in colorectal cells (Wang et al. 2000), and HuR knockdown reduced expression of cell-cycle related proteins like cyclin A in oral cancer cells (Kakuguchi et al. 2010). No reports cover the association of cyclin A with HuR in clinical cancer, but we discovered that most of the specimens showing high cytoplasmic HuR expression also showed high cyclin A expression. These results are in accordance with cell culture experiments suggesting that cyclin A expression is, in part, controlled by HuR.

9.4.4. MMP-2 and MMP-9

Study III assessed the significance of MMP-2 and MMP-9 in gastric cancer. Epithelial MMP-2 expression was associated with male sex, high stage, advanced penetration depth, and non-curative surgery, but stromal MMP-2 with advanced stage, diffuse subtype, and non-curative surgery. MMP-9 expression correlated only with intestinal subtype. No correlation of MMPs with lymph node metastasis emerged (Kabashima et al. 2000, Monig et al. 2001). In gastric cancer, MMP-2 and MMP-9 are associated with clininopathological features and survival (Grigioni et al. 1994, Sier et al. 1996, Allgayer et al. 1998, Allgayer et al. 1998, Murray et al. 1998). In our study, only epithelial MMP-2 immunoreactivity showed prognostic value, and only in univariate analysis, which is in line with results of Alakus et al (Alakus et al. 2008). MMP-2 and MMP-9 have proven to be independent prognostic factors in gastric cancer based on zymography (Sier et al. 1996) or enzyme-linked immunosorbent assay (Kubben et al. 2006). Semiquantitative reverse transcriptase (RT)-PCR data showed no significant correlation with clinicopathological parameters (Shim et al. 2007). In one study, high MMP-9 expression was an independent prognostic factor for poor survival (Zhao et al. 2009). The inconsistency between these studies may be explained by these different methods. Zymography and immunosorbent assay measure enzyme activity, which is impossible to assess by expression only. Based on Study III, we concluded that epithelial MMP-2 expression is more relevant than stromal MMP-2 as a prognostic factor.

High epithelial MMP-2 expression was associated with high COX-2 expression, but stromal MMP-2 or MMP-9 expression was not. Our Study III reported a positive correlation between COX-2 and MMP-2 in clinical gastric cancer, an association also reported in renal cell (Miyata et al. 2003), thyroid (Siironen et al. 2004), and breast (Sivula et al. 2005) cancers. These clinical data, combined with in vitro data showing that elevated COX-2 levels lead to raised MMP-2 expression, (Tsujii et al. 1997, Callejas et al. 2001, Church et al. 2003, Symowicz et al. 2005, Wu et al. 2005, Zhang et al. 2009), suggest that COX-2-enhanced tumor growth and infiltration is, in part, mediated by MMP-2.

9.4.5. The role of COX-2 compared with that of HuR, cyclin A, MMP-2, p53, p21, Ki-67, DNA ploidy, and SPF

Study IV showed high COX-2 expression to be associated with tumoral aneuploidy and high S-phase fraction. In the human esophageal cancer cell line TE-13, NS-398, a COX-2 selective inhibitor, reduced proliferation and raised the percentage of apoptotic cells in a dose-dependent manner. These changes correlated with reduced COX-2 mRNA and protein expression (Liu et al. 2009). Our results are in line with these in vitro data. We also reported that, in a subgroup of patients with diploid tumors (patients with better prognosis), COX-2 expression was associated with survival, but in a subgroup of aneuploid tumors, was not. These data are in line with our previous results suggesting that COX-2 expression may reveal those patients with apparently curable gastric cancer who eventually suffer a more unfavorable outcome than expected.
Because Ki-67 is known to associate with tumor proliferation and poor survival in some cancers (Endl et al. 2000), its epithelial immunoreactivity was therefore analyzed in our series. Although widely used as a proliferation marker, it showed no correlation between Ki-67 expression and TNM stage (Xu et al. 1999). Nor could we demonstrate any association with survival, which is in concordance with other findings (Kunisaki et al. 2001, Oshima et al. 2005, Joo et al. 2006). One report shows that low Ki-67 proliferation index is associated with poor prognosis (Lee et al. 2010). However, we do show Ki-67 expression to be correlated with COX-2 expression. Joo et al reported that a Ki-67 labeling index is associated with COX-2 expression (Joo et al. 2006), and Zhang et al demonstrated that concomitant downregulation of COX-2 and Akt1 lead to downregulation of Ki-67 (Zhang et al. 2009). These data suggest that Ki-67 is associated with COX-2, and that COX-2 may lead to an increased number of Ki-67-positive cells.

Study IV shows that COX-2 expression has a positive correlation with p53 expression, as also described by other authors (Leung et al. 2001, Kawabe et al. 2002, Shun et al. 2003, Milne et al. 2006). In one cell culture experiment, wild-type p53 suppressed COX-2 expression, whereas mutated p53 did not (Subbaramaiah et al. 1999), suggesting that loss of p53 function, as indicated by p53 expression, enhances COX-2 expression and thus enhances proliferation. In contrast, we could demonstrate no association between COX-2 and p21. An extended multivariate analysis showed COX-2 and p53 to be independent prognostic factors for poor survival alongside high TNM stage and non-curative surgery.

9.5. Future prospects

These results confirm that COX-2 expression provides valuable clinical information as an independent, consistent prognostic factor. These data should encourage further prospective, clinical trials with an updated D2 series, aiming at clinical use of COX-2. These studies should include a critical evaluation of preoperative biopsies and surgical specimens concerning COX-2 expression, and evaluation of any effect of neo-adjuvant chemotherapy on COX-2 expression. It might be justified to direct adjuvant therapy toward those low-stage, curatively operated patients with high tumoral COX-2 reactivity, because of their elevated risk for cancer-related death. With this background, it would be reasonable to include COX-2 inhibitors in clinical pharmacological trials to test them as a part of combination chemotherapy in the future.
10. CONCLUSIONS

- COX-2 is an independent prognostic factor in gastric cancer, and its prognostic role is more pronounced in low-stage patients.

- COX-2 expression is associated with cytoplasmic HuR expression, and down-regulation of HuR expression leads to reduced COX-2 expression in gastric cancer cells, suggesting that COX-2 expression is regulated by HuR. Cytoplasmic HuR expression is associated with gastric cancer survival in a non-independent manner, but nuclear HuR expression shows no prognostic value.

- Cyclin A is an independent prognostic factor in gastric cancer, and in our gastric cancer material, high cyclin A expression is associated with cytoplasmic HuR expression.

- Epithelial MMP-2 expression is a non-independent prognostic factor in gastric cancer, and is associated with high COX-2 expression. Neither stromal MMP-2 nor cytoplasmic MMP-9 has any association with survival or with COX-2 levels.

- In gastric cancer, COX-2 expression is associated with aneuploidy, S-phase fraction, and Ki-67 expression. COX-2 expression is also associated p53 expression.

- In an extended multivariate model with eight prognostic markers and clinicopathological factors, COX-2 expression is an independent prognostic factor alongside with p53, stage, and intent of surgery.
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