The Search for a Novel Diagnostic Marker or Therapeutic Target for Gastrointestinal Cancer

Dissertation

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Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation mit dem Thema

"*The Search for a Novel Diagnostic Marker or Therapeutic Target for Gastrointestinal Cancer*"

selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Quellen und Hilfsmittel vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 25.01.2006

Stacy Carl-M^{c}Grath

Zusammenfassung

Die Suche nach einem neuen diagnostischen Marker
oder einem therapeutischen Ziel für gastrointestinale Karzinome

Karzinome der Leber und des Magens gehören zu den häufigsten Krebsarten des Gastrointestinaltraktes und haben eine schlechte Prognose, da sie meist erst im fortgeschrittenen Stadium diagnostiziert werden und die therapeutischen Möglichkeiten dann eingeschränkt sind. Um die hohe Sterblichkeitsrate hepatogastrointestinaler Karzinome zu verbessern, muss die frühzeitige Diagnostik und die Therapie verbessert werden. Ziel der vorliegenden Dissertation war es, neue Marker und neue therapeutische Ziele zu finden, die die Diagnose und Behandlung hepatogastrointestinaler Karzinome verbessern können. Dazu wurden zwei unterschiedliche Vorgehensweisen gewählt.

Zunächst wurde mit Hilfe des *Differential Display* nach differentiell exprimierten Genen gesucht. Bei dem Vergleich eines hepatozellulären Karzinoms (HCC) gegen korrespondierendes tumorfreies Lebergewebe wurde GSDML identifiziert und als ein neues Mitglied der tumor-assoziierten GSDMDC Proteinfamilie eingeordnet. GSDML wird sowohl in Tumorgewebe als auch in nicht-läsionalem Gewebe exprimiert, wobei sich die Expressionsprofile der GSDML Splicingvarianten im Tumor und Nichttumor offenbar voneinander unterscheiden. Interessant ist, das GSDML auch in Tumoren des Magens und Dickdarms nachweisbar war und möglicherweise für die Tumorzellbiologie wichtig ist. Weiterführende Untersuchungen sollten sich insbesondere der Primärstruktur des Proteins und dessen biologischer Bedeutung zuwenden.

Im zweiten Ansatz wurde die potentielle Bedeutung multifunktionaler Membranproteine in der Biologie gastrointestinaler Tumore untersucht. Dabei waren NEP (CD10), APN (CD13), DPIV (CD26), ACE (CD143), ADAM 9, ADAM 12 und ADAM 15 Gegenstand der hier vorgestellten Untersuchungen. Für alle diese Ektopeptidasen konnte bereits bei anderen Tumoren eine tumorbiologische Bedeutung bestätigt werden. Tumore der Leber und des Magens waren allerdings bislang noch nicht systematisch untersucht worden.

In HCC fand sich u.a., dass sich die Expression von NEP umgekehrt proportional zur Proliferation und Differenzierung verhält. NEP war in HCC herunter reguliert und ließ zunächst vermuten, dass das Tumorwachstum gehemmt wird. Anschließende Zellkulturexperimente konnten zwar bestätigen, dass NEP-Aktivität die Proliferation beeinflusst, aber nicht in der erwarteten Weise, da es die Proliferation *in vitro* sogar anregte. Auch APN, DPIV und ACE regten die Tumorzellproliferation *in vitro* an, waren jedoch im

Gegensatz zu NEP im Tumorgewebe hochreguliert. Weiterführende Untersuchungen haben dann zeigen können, dass NEP, APN und ACE als Marker für die klinisch-pathologische Diagnostik hepatozellulärer Tumore (HCC und FNH) geeignet sind und somit ein wichtiges Studienziel erreicht werden konnte.

Auch im Magenkarzinomen ließen sich alle untersuchten Ektopeptidasen vermehrt nachweisen, und zwar sowohl auf mRNA- als auch auf Proteinebene. In-vitro-Experimente erbrachten weiterhin Hinweise, dass eine Hemmung von NEP, APN und ACE die Proliferation von Magenkarzinomzellen verlangsamt. Da bereits Inhibitoren einiger dieser Ektopeptidasen für die Behandlung von Hypertonie eingesetzt werden, bietet sich die Möglichkeit, die gleichen Medikamente auch für die Behandlung des Magenkarzinoms zu testen, womit ein weiteres Studienziel erreicht werden konnte.

Auch ADAMs beeinflussen die Proliferation von Magenkarzinomzellen. Obwohl die ADAMs beim Magenkrebs hochreguliert sind, ist ihr Expressionsprofil nicht tumor-spezifisch genug, um in der Diagnostik oder als therapeutisches Ziel eingesetzt werden zu können.

Die funktionelle Bedeutung der Ektopeptidasen für die Tumorbiologie wurde dann für das ACE, bzw. das lokale Angiotensin-System detaillierter untersucht. ACE spaltet Ang I in Ang II, das an die G-Protein-gekoppelten Rezeptoren AT1 und AT2 bindet. Nachdem die differentielle Expression von ACE in Magenkarzinomen nachgewiesen worden war, wurde in Anlehnung an die hier gemachten Beobachtungen gezeigt, dass der ACE-Genpolymorphismus beim Magenkarzinom mit der lokalen und lymphogenen Tumorausbreitung korreliert (Ebert et al 2005, Röcken et al 2005). Eine tumorbiologische Relevanz des ACE war somit belegt. Eigene Untersuchungen fanden außerdem eine auffällige Expression von ACE in der Nachbarschaft von Lymphfollikeln und es wurde die Hypothese aufgestellt und in daraufhin durchgeführten Zellkulturexperimenten belegt, dass die Expression des ACE durch Zytokine reguliert wird. Auch die immunhistologisch nachweisbare Expression des AT1 korreliert signifikant mit der Anzahl der Lymphknotenmetastasen bei Magenkarzinompatienten (Röcken et al, eingereicht). Umfangreiche In-vitro-Experimente an Magenkarzinomzelllinien belegten dann, dass alle Mitglieder des lokalen Angiotensin-Systems (ACE, AT1 und AT2) von Magenkarzinomzelllinien exprimiert werden und deren Inhibition, bzw. Antagonisierung die Tumorzellinvasion im Matrigel-Assay signifikant hemmen kann. So hat die systematische Untersuchung der Ektopeptidasen dazu geführt, dass das lokale Angiotensin-System als potentielles prognostisches und therapeutisches Target zur Behandlung des Magenkarzinoms identifiziert wurde. Hier laufen bereits weiterführende Studien.

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1 GASTROINTESTINAL CARCINOMAS: EPIDEMIOLOGY AND PATHOGENESIS

Cancer annually accounts for 12 percent of total deaths worldwide, and in industrialised countries, 25 percent of people die of cancer each year. The increase in tobacco consumption, the earlier exposure to work-related carcinogens, and the adoption of increasingly unhealthy diets and lifestyles means that the incidence and mortality rates are continually increasing. Regional variations, such as geographical location and economic/industrial status, contribute to the wide variation in cancer rates between countries.

Although lung and breast cancers are the most common single cancers in men and women, respectively, cancers of the gastrointestinal tract, including oesophageal, stomach, liver, colon, and pancreas cancers, are responsible for approximately 3 million new cases and over 2 million deaths each year (Hamilton&Aaltonen 2000), making them the most frequent cancers worldwide. Cancers of the stomach and the liver are among the most frequent gastrointestinal tract cancers, irrespective of gender- or regional-specific variations. However, incidence of these cancers, compared with non-gastrointestinal cancers, varies according to the geographical location, which is mainly due to regional variations in diet and lifestyle, as well as localised bacterial or viral infections.

Bacterial and viral infections leading to chronic inflammation play a major role in both stomach and liver carcinogenesis. A history of infection and chronic inflammation is strongly associated with the risk of stomach and liver cancers and their precursor lesions (Hamilton&Aaltonen 2000). A range of gastrointestinal cancers arise from inflammation and are preceded by a lengthy precancerous process, developing via multiple sequential steps. Substance- or disease-related chronic inflammation or oxidative stress results in the initiation of continual regenerative processes, where the replacement of lost and injured cells through cell division offers an opportunity for the accumulation of genetic damage (Orlando 2002). Characteristic for the progression to invasive cancer, the appearance of abnormal cells (dysplasia) is followed by the development of preneoplastic lesions, and then, finally, the emergence of a malignant carcinoma (Raza 2000).

Although surgery or combined surgery and chemo-/radiotherapy may be curative for a large proportion of tumours of the gastrointestinal tract, both stomach and liver cancers are among the top three causes of cancer-related deaths, exhibiting a persistently high mortality (10.4% and 8.8% of all cancer deaths per year, respectively). The exceedingly poor prognosis of these cancers is mostly due to presentation in an advanced stage, and the limited range of treatment options. Decreasing hepatogastrointestinal cancer mortality will require earlier diagnosis of these cancers, as well as a wider range of therapeutic alternatives.

1.1 Liver Cancer: Epidemiology, Pathology and Pathogenesis

The primary malignant epithelial tumour of the liver is the hepatocellular carcinoma (HCC), a malignant tumour derived from hepatocytes. Intrahepatic cholangiocarcinoma is composed of cells resembling those of bile ducts. Bile duct cystadenocarcinoma is a cystic tumour lined by serous or mucinus epithelium with papillary infoldings. Combined hepatocellular and cholangiocarcinoma is a rare tumour containing recognizable elements of both hepatocellular and cholangiocarcinoma and has to be differentiated from synchronously occurring HCC and cholangiocarcinoma. Hepatoblastoma is a malignant embryonal tumour with divergent patterns of differentiation. Undifferentiated carcinoma does not fit into any of the above mentioned categories.

1.1.1 Epidemiology

HCC is the fifth ranking tumour in frequency worldwide, and the third-leading cause of death due to cancer (Hamilton&Aaltonen 2000, Anthony 2001). Men are affected more commonly than women, with the global number of new cases estimated at 316,000 for males and 121,100 for females, which respectively accounts for 7.4% and 3.2% of all malignancies, excluding skin cancer (Hamilton&Aaltonen 2000). The distribution pattern of HCC varies geographically ranging from an annual incidence rate of less than 3.6 per 100,000 in North and South America, South-Central Asia, Northern Europe, Australia and New Zealand to more than 20.1 per 100,000 in Sub-Saharan, South Africa, East Asia, and Melanesia (Hamilton&Aaltonen 2000). The incidence has remained stable or decreased over time in most countries. However, in countries with low or moderate incidence, the number of HCCs has increased, which has been attributed to an increase in the incidence of Hepatitis C virus (HCV) infection (Anthony 2001). Regional age-specific incidence rates differ significantly. Generally, it can be perceived that in high risk areas, HCC occurs in young to middle aged patients (20-34 years), and in low risk areas, HCC generally affects patients aged 55-59 years of age (Hamilton&Aaltonen 2000, Anthony 2001).

1.1.2 Pathogenesis

The pathogenesis of HCC is multifactorial. Environmental, infectious, nutritional, metabolic, and endocrine factors contribute directly or indirectly to hepatocarcinogenesis. The importance of individual factors varies geographically depending on environmental and socio-economic influences. Synchronous occurrence of different risk factors, such as chronic viral hepatitis and aflatoxin burden or iron overload, in a single patient or patient population increases the risk of developing HCC.

1.1.2.1 Chronic hepatitis and liver cirrhosis

Chronic hepatitis and liver cirrhosis have been recognized as important risk factors for the development of HCC. Hepatitis is associated with liver-cell necrosis, inflammation, regeneration, and fibrosis, which may proceed to cirrhosis. Following liver cell necrosis, quiescent hepatocytes start to proliferate. The local inflammation induces oxidative stress and may cause DNA damage. Chronic hepatitis is characterized by repetitive cycles of necrosis and regeneration, which facilitate successive acquisition of genomic alterations. These may escape repair mechanisms, which ultimately leads to the development of HCC through monoclonal expansion. It has recently been shown that irregular regeneration of hepatocytes is an important factor for hepatocarcinogenesis, even in non-cirrhotic livers (Ueno et al 2001). Fibrosis disrupts the normal cell-to-cell and cell-to-extracellular matrix interaction and leads to further loss of control over cell growth. More than 80% of the patients with HCC have liver cirrhosis. However, in the West, there is a small number of cases in which HCC occurs in the absence of liver cirrhosis. This group of patients shows no male preponderance; and patients with HCC and without fibrosis are significantly younger than those with HCC and minimal fibrosis (Bralet et al 2000). However, HCC in non-cirrhotic livers is associated with similar risk factors compared with HCC occurring in cirrhotic livers, such as chronic hepatitis B and C, alcohol consumption, and haemochromatosis (Bralet et al 2000, Grando-Lemaire et al 1999).

1.1.2.2 Hepatitis B Virus (HBV)

Epidemiological studies have convincingly demonstrated that chronic infection with HBV is a major risk factor for the development of HCC. In fact, chronic infection with HBV, HCV, or both is the most common cause of HCC worldwide (Kao&Chen 2000). Integration of HBV DNA into cellular DNA of HCC and chronic hepatitis is frequently encountered and is considered to be a hallmark of HBV-related hepatocarcinogenesis (Rabe et al 2001). Integration of viral DNA is generally random and not directed. However, a trend has been recognized that HBV DNA shows high levels of integration into chromosomes 11 and 17. Viral DNA insertion occurs during liver-cell proliferation, secondary to necrosis/apoptosis of adjacent hepatocytes, and may induce chromosomal rearrangements, including deletions and translocations. The genome of human HBV does not contain oncogenes and it probably exerts its effect on hepatocarcinogenesis through *trans*-activation or *trans*-repression of cellular genes or factors by HBV-related gene products. The X gene and its transcript (HBx) are encoded by the integrated HBV DNA. HBx activates a wide variety of cellular and viral genes, including genes controlling cell growth and apoptosis. HBx acts in the nucleus, but the

majority is localized in the cytoplasm. HBx induces late G1 cell-cycle arrest, followed by induction of apoptosis. Mutations of the HBx-gene have been identified in HCCs and abolished its growth suppressive and apoptotic effects. Thus, the putative pleiotropic effect of HBx is further modified by naturally-occurring mutations of the HBx-gene.

Liver cell transformation may also be influenced by truncated forms of the PreS2/S envelope protein and spliced HBV transcript coding for the new HBV spliced protein. Insertion of viral DNA into a cellular host gene with subsequent modification (*cis*-activation) of its expression, i.e. insertional mutagenesis, has been described to occur in HCCs related to chronic HBV-infection. However, this generally appears to be rare and does not account for the majority of HBV-associated HCCs.

Integration of viral DNA may also be responsible for hepatocarcinogenesis in HBsAg-negative patients. However, the number of DNA-copies in this particular patient population is commonly low and contributing factors, such as concurrent infection with HCV or alcoholic liver disease, may be involved. Thus, absence of HBV DNA in serum and HBsAg-negativity do not rule out HBV-infection being a contributory factor of hepatocarcinogenesis in a particular patient.

1.1.2.3 Hepatitis C Virus (HCV)

HCV is a single positive stranded RNA-virus of approximately 9,500 nucleotides without transcriptase activity. At present, there is no evidence that viral RNA is integrated into the host genome, and in this respect, hepatocarcinogenesis related to chronic infection with HCV differs from HBV. Chronic hepatitis on its own is a major risk factor for HCC (see Section 1.1.2.1) and is found in about 60-80% of the cases with HCV and in less than 10% of the cases with HBV. Approximately 20% of HCV carriers develop HCC, while the incidence of HCC in HBV carriers is about 5%. Patients suffering from chronic hepatitis C have a 2.7-fold increased risk of HCC compared with patients suffering from chronic hepatitis B (Colombo 1999). The inflammation in chronic viral hepatitis is related to clearance of hepatocytes by cytotoxic T cells (CTL) and mononuclear cells. During inflammation, hepatocytes may be exposed to genotoxic agents, such as oxygen radicals, perforin and granzyme secreted by inflammatory cells, leading to DNA damage. Thus, induction of chronic hepatitis is one major driving force of hepatocarcinogenesis related to infection with HCV. However, this effect is indirect and unspecific.

A direct effect of HCV on hepatocarcinogenesis may be mediated by viral proteins, which influence regulation of cell growth and apoptosis. A single open-reading-frame of the viral genome encodes a polyprotein of approximately 3000 amino acid residues (Ray&Ray

2001). This polyprotein is cleaved by viral and host proteases into putative structural and non-structural proteins. Among the processed HCV polyprotein, the core protein of 191 amino acids is a central component of the virion and is necessary for nucleocapsid formation. Viral proteins were shown to interact with various cellular proteins, including 14-3-3 protein, apolipoprotein AII, TNF-receptor, lymphotoxin-β receptor, DEAD domain of RNA helicase, nuclear ribonucleoprotein, PKR, p53 and SNARE-like protein (Ray&Ray 2001, Shimotohno 2000). The core protein of HCV exerts an anti-apoptotic effect by activating NF-κB mainly in the perinuclear region (Shimotohno 2000). Apoptosis in chronic hepatitis is induced by CTLs. CTLs express Fas-ligand, which binds to Fas on hepatocytes and induces apoptosis. HCV-infected hepatocytes may escape CTL-induced and Fas-mediated apoptosis. However, the core protein may also be able to generate a pro-apoptotic effect by binding to the TNF-receptor and the lymhotoxin-β receptor. Further studies are necessary to clarify these contradicting observations (Ray&Ray 2001, Shimotohno 2000). Core protein binds to p53 and promotes cell growth. It appears to be able to modify the activity of genes and/or proteins involved in cell cycle control, cell proliferation, differentiation and apoptosis (such as cyclin E, p21(waf1), Rb1, TGF-β, FADD, insulin-like growth factor 2, Elk1, and many others) (Ray&Ray 2001). Core protein and v-Has-ras gene co-operatively transform BALB/3T3 A31-I-1 cells. Other viral proteins may also be involved in hepatocarcinogenesis: truncates of viral NS3 protease were able to transform murine fibroblasts. In summary, infection with HCV may mediate hepatocarcinogenesis via chronic hepatitis and the range of interactions between viral proteins and host genes and proteins. This modulation of cell growth, differentiation, apoptosis, and immune function is currently an active field of investigations (Ray&Ray 2001, Shimotohno 2000).

1.1.2.4 Chemicals

Epidemiologic and experimental evidence indicates that exposure to chemicals also contributes to the development of HCC. Hepatotoxic chemicals may be divided into genotoxic and non-genotoxic. Genotoxic chemicals directly interact with DNA, forming covalent adducts, and induce genetic changes upon cell replication. Non-genotoxic chemicals stimulate tumour formation by altering kinetics of cell proliferation, cell death and cell differentiation through a variety of epigenetic pathways. Aflatoxin B_1 (AFB), high dose oral contraceptives, azathioprine, vinyl chloride, polychlorinated biphenyls, arsenic, and alcohol contribute to hepatocarcinogenesis in humans (Bannasch&Zerban 1997, Wogan 2000).

AFB is the best studied human hepatocarcinogen. Aflatoxins are toxic metabolites of certain spoilage molds that contaminate food and feed crops. AFB is genotoxic and forms

adducts with proteins and DNA. Exposure to AFB is associated with a specific mutation in codon 249 of the p53 gene leading to an exchange G:C to T:A (Wogan 2000). The incidence of HCC correlates highly significantly and directly with AFB intake. In third-world countries with high levels of AFB exposure, AFB is more significant than HBV infection for hepatocarcinogenesis, as many more individuals are exposed to AFB than to HBV infection (Wogan 2000). However, in individuals with both AFB exposure and HBV infection the relative risk for developing HCC is 59.4, as compared for AFB (relative risk 3.4) or HBV (relative risk 7.3) alone. These values demonstrate that coinciding risk factors may multiply the risk of developing HCC manifold.

1.1.2.5 Metabolic disorders

HCC occurs regularly in patients suffering from inherited metabolic disorders, including glycogen storage disease type 1, α1-antitrypsin deficiency (AAT), porphyria cutanea tarda, and haemochromatosis. Cirrhosis may be the main predisposing risk factor for liver carcinoma in patients with severe AAT-deficiency, and, even in heterozygous PiZ-carriers, cirrhosis seems to bear a higher risk for hepatocarcinogenesis. However, liver cancer may occur in patients suffering from AAT-deficiency even in the absence of cirrhosis and is probably not related to a lack of anti-protease activity, but rather to intracellular aggregation of AAT (Zhou et al 2000).

The relative risk for the development of HCC in haemochromatosis has been calculated as being greater than 200 and is also not only related to cirrhosis (Hamilton&Aaltonen 2000, Fracanzani et al 2001). The risk of HCC after adjustment for alcohol abuse, smoking and family history of cancer was 1.9 in patients with haemochromatosis (Fracanzani et al 2001). The intracellular accumulation of iron that occurs due to haemochromatosis may cause oxidative stress and damage to DNA (Deugnier&Turlin 2001). Non-inherited iron overload is also considered to be a contributing factor for hepatocarcinogenesis (Deugnier&Turlin 2001).

1.1.3 Pathology

The macroscopic appearance of HCC varies depending on the size of the tumour and the presence or absence of liver cirrhosis. HCC may occur as a single mass, multinodular, with many tumours scattered throughout the liver, or as a diffuse growing lesion. Multinodular HCCs in cirrhotic livers most commonly represent multicentric HCCs, whilst multinodular HCCs in non-cirrhotic liver are often related to intraheptic metastases (Tannapfel et al 2001). Multicentric HCCs are particularly common in patients with chronic

hepatitis C and tend to grow in more damaged segments of the liver (Shimada et al 2001, Ariizumi et al 2000). The colour of the cut surface may be green (due to bile production), yellow (due to fatty change), tan-brown, or grey-white. HCCs are almost always soft tumours, except for the rare fibrolamellar variant. Tumour volume doubling time ranges from 1 to 20 months (median 6 months).

HCC may be associated with invasion of the hepatic vein, bile ducts and portal vein thrombosis. The incidence of extrahepatic metastases ranges between 50% and 80% (Kojiro 1997). Haematogenic tumour spread most frequently affects the lungs (47.6%), followed by adrenal glands (8.3%), bone (5.6%), gastrointestinal tract (4.7%), gall-bladder (3.5%) and pancreas (3.0%). Lymphatic metastases are found in approximately one forth of the cases, and usually occur in hilar, peripancreatic, perigastric and periaortic nodes, and only in advanced disease at distant lymph nodes (Kojiro 1997). The current TNM-classification and stage grouping of HCCs is given in Tables 1.1 and 1.2 (Hamilton&Aaltonen 2000).

Table 1.1: Tumour-Node-Metastasis (TNM) Classification of Tumours of the Liver

Primary Tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Solitary tumour 2 cm or less in greatest dimension without vascular invasion
T2	Solitary tumour 2 cm or less in greatest dimension with vascular invasion; or multiple tumours limited to one lobe (none more than 2 cm in greatest dimension) without vascular invasion; or solitary tumour more than 2 cm in greatest dimension without vascular invasion
T3	Solitary tumour more than 2 cm in greatest dimension with vascular invasion; or multiple tumours limited to one lobe (none more than 2 cm in greatest dimension) with vascular invasion; or multiple tumours limited to one lobe (any more than 2 cm in greatest dimension) with or without vascular invasion
T4	Multiple tumours in more than one lobe; or tumour(s) involving a major ranch of the prtal or hepatic vein(s); or tumour(s) with direct invasion of adjacent organs other than gallbladder; or tumours with perforation of visceral peritoneum
Regional Lymph Nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant Metastasis (M)	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 1.2: UICC Staging based on TNM Classification for Tumours of the Liver

Stage	Tumour	Nodal	Metastasis
I	T1	N0	M0
II	T2	N0	M0
IIIA	T3	N0	M0
IIIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4	Any N	M0
IVB	Any T	Any N	M1

1.1.3.1 Histological classification

The histological appearance of HCC is variable, with different architectural and cytological patterns. The trabecular pattern is the most common architectural variant (Ishak et al 2001). The pseudo-glandular and acinar pattern is characterized by dilated bile-canaliculi-like structures, often filled with bile. In the compact variant, sinusoid-like blood spaces are inconspicuous and slit-like. The scirrhous HCC is an uncommon type and is characterized by marked fibrosis. The rare fibrolamellar HCC differs from the scirrhous type and is characterized by distinctive fibrous lamellae and polygonal cells that have an eosinophilic, coarsely granular cytoplasm. This variant differs from other HCCs in that it shows a female preponderance, usually occurs in the absence of a chronic liver disease, and patients usually present at young age (mean age 23 years) (Ishak et al 2001). Cytologically, tumour cells of HCCs may show fatty or clear cell change, Mallory hyaline bodies, globular hyaline bodies (which may be deposits of α1-antitrypsin), pale bodies, pleomorphic cells, and sarcomatous changes. Often HCCs exhibit different cytological variants within the same tumour.

1.1.4 Precursor Lesions

It is now generally accepted that HCC associated with cirrhosis evolves from precancerous lesions, and well differentiated HCC further progresses to a less differentiated form. Although the term *intraepithelial neoplasia* (Hamilton&Aaltonen 2000) has become a generally adopted term for lesions differing from the normal epithelium by the occurrence of cellular and architectural dysplasias, and is now widely used in many different organs and epithelial types, this does not apply for the liver (Hamilton&Aaltonen 2000, Anthony 2001, International Working Party 1995, Hytiroglou&Theise 1998) .

1.1.4.1 Dysplastic nodule (syn.: macroregenerative nodule, adenomatous hyperplasia)

A dysplastic nodule (DN) is defined as a nodular region of hepatocytes at least 1 mm in diameter with dysplasia, but without definitive histologic criteria of malignancy (International Working Party 1995). DNs are usually identified macroscopically and measure on average 15-20 mm in greatest dimension. However, smaller DNs do occur and may be distinguished by colour or texture from the surrounding liver. The DNs may or may not be surrounded by a fibrous rim and they tend to bulge on the cut surface. Attempts have been made to divide DN into *low grade* and *high grade*. Low grade DNs are usually devoid of architectural and cytological atypia and may enclose portal tracts, terminal hepatic veins and unpaired arteries, and may be similar to regenerative nodules. High grade DNs are characterized by the presence of a variable number of cellular and architectural atypias (Hamilton&Aaltonen 2000), and can be difficult to distinguish from HCC. However, it has to be kept in mind that HCC also arises without evidence of a DN, and in non-cirrhotic livers. Thus, DN is not a necessary prerequisite for the development of HCC.

1.1.4.2 Large and small cell dysplasia

Large cell dysplasia (LCD) is characterized by cellular enlargement, nuclear pleomorphism, hyperchromasia, and multinucleation of hepatocytes, which occur in groups or sometimes occupy whole cirrhotic nodules. The occurrence of LCD is associated with an increased risk of hepatocellular carcinoma in cirrhotic livers (odds ratio 3.3 - 3.8) and recognition in liver biopsy specimens may help identify patients at risk (Anthony 2001, Lee et al 1997, Borzio et al 1995, Ganne-Carrie et al 1996). However, LCD may not be a true precursor lesion of HCC, but is simply a marker of liver cell injury, predicting the development of HCC (Anthony 2001, Libbrecht et al 2005).

Small cell dysplasia (SCD) is characterized by a high nucleo-cytoplasmic ratio, cytoplasmic basophilia and a tendency to form small round foci. It has been proposed that SCD is more likely to represent a premalignant condition of hepatocytes than LCD (odds ratio 6.3) (Hamilton&Aaltonen 2000, Anthony 2001, Kondo 1997). LCD and SCD may occur in the same liver and tend to be more frequent and extensive in mixed or macronodular cirrhosis (Le Bail et al 1997).

1.1.4.3 Iron-free foci

Hepatic iron-free foci are defined as nodules of hepatocytes that are found in genetic haemochromatosis. The foci contain significantly less or no iron compared with the surrounding parenchyma (Ishak et al 2001). Iron-free foci may be single or multiple and have been shown to be preneoplastic in nature.

1.1.4.4 Adenoma

HCCs occasionally do arise in hepatocellular adenomas and it could be presumed that an adenoma-carcinoma-sequence, as it occurs in the colorectum, is also present in the liver. However, a few things have to be kept in mind. Hypothetically, adenomas, like focal nodular hyperplasia, may occur in cirrhotic livers, but it appears almost impossible to separate these lesions from regenerative nodules, DN or surrounding cirrhosis. HCCs occur in non-cirrhotic livers without evidence of a pre-existing adenoma, and liver cell adenomas preferentially effect women (Ishak et al 2001), whereas HCCs are more common in men. Thus, as yet, there is not enough epidemiological and pathological evidence to postulate an adenoma-carcinoma sequence as common trait in hepatocarcinogenesis.

1.1.5 Prognosis

The prognosis after diagnosis of HCC has improved from 20 years ago, when patients survived no longer than 1 year from diagnosis, irrespective of treatment (Okuda et al 1985). Improvements in diagnosis have resulted in 30-40% of patients in developed countries being diagnosed at initial stages (Llovet et al 2003). Curative treatments may be optimally applied during the early stages of HCC, with 5-year survival rates of 50-70% for early HCC (Arii et al 2000, Mazzafero et al 1996, Llovet et al 1999a,), and 71-93% for carcinoma in situ (Takayama et al 1998, Sakamoto&Hirohashi 1998). However, the remaining patients are still diagnosed at advanced stages (Bruix&Llovet 2002), and even for small HCC lesions, the 5-year survival rate after treatment is only 54-71% (Arii et al 2000). Tumours diagnosed at advanced stages are often not suitable for radical treatments, and survival of untreated HCC patients has been reported to be 10-72% after 1 year and 8-50% after 2 years (Llovet&Bruix 2003). Another study reported 1-, 2-, and 3-year survival of untreated HCC patients to be 54%, 40% and 28% respectively (Llovet et al 1999b). Additionally, in 70% of patients, tumour recurrence occurs at 5 years, which may represent true recurrence or de novo tumours (Bismuth&Majno 2000). In Asia and Africa, many patients are first diagnosed with end-stage HCC. Treatment of these patients confers no survival benefit, and with less than 6 months life expectancy, their outlook remains poor (Llovet et al 1999c, Llovet&Bruix 2003).

The presence of cancer-related symptoms and vascular invasion or extrahepatic spread dramatically reduces survival time (Llovet et al 2003). Prognostic factors predicting reduced survival include the existence of metastatic disease, the presence of a p53 mutation, older patient age, larger tumour size, partial or complete portal vein thrombosis, significant weight loss, high serum AFP levels, and evidence of poor hepatic function (Okuda et al 1993).

1.2 Gastric Cancer: Epidemiology, Pathology and Pathogenesis

The primary epithelial tumour of the stomach is the adenocarcinoma, and develops from the stomach mucosa, usually maintaining glandular differentiation. Other less common tumours of the stomach are the squamous cell carcinomas, and the adenosquamous carcinomas, combining characteristics of both the adenocarcinoma and the squamous cell carcinoma to approximately equal extent. Undifferentiated carcinoma lacks any differentiated features and does not fit into any of the above categories.

1.2.1 Epidemiology

Gastric cancer is one of the most common cancers worldwide, ranking fourth in overall frequency, and accounting for over 870,000 new cases and over 650,000 deaths annually (Stewart&Kleihues 2003). Mortality from gastric cancer is second only to lung cancer. Gastric cancer occurs more frequently in men than in women, with the estimated number of new cases worldwide being 558,000 for males and 317,000 for females, respectively, accounting for 5.5% and 3.1% of all malignancies, excluding skin cancer (Hamilton&Aaltonen 2000). The geographic distribution of gastric cancer varies from an annual incidence of more than 300,000 new cases in the more developed regions of Europe, Japan, Australia, New Zealand and North America, to nearly 550,000 new cases per year in the developing or less developed regions of Africa, Latin America and the Caribbean, Asia (excluding Japan), Micronesia, Polynesia and Melanesia. In high risk areas, the intestinal-type adenocarcinoma is more frequent, whereas the poorly differentiated diffuse-type carcinoma predominates in low risk areas.

The incidence and mortality rates of gastric carcinoma are steadily declining. However, due to the aging population, the absolute number of new cases per year is increasing (Hamilton&Aaltonen 2000, Munoz 1988). Below the age of 30, the incidence of gastric carcinoma is extremely rare, but thereafter rises quickly and continuously, with the oldest age groups having the highest rates. In males, the intestinal-type is more common than the diffuse-type and the incidence rises faster with age, whereas the diffuse-type mainly impacts younger individuals, frequently females. A decline in incidence of the intestinal-type carcinomas is largely responsible for the decline in overall incidence rates (Munoz&Connelly 1971, Amorosi et al 1988, Lauren 1965), and has been correlated with the corresponding decrease in prevalence of *Helicobacter pylori* infection (The Eurogast Study Group 1993, Konturek et al 2003). However, the incidence of the diffuse-type carcinoma may be increasing (Craanen et al 1992b), which is worrying given that these types of tumours have a worse prognosis (Blok et al 1997). An increase has also been observed for cancers localized

to the gastro-oesophageal junction, some probably originating from the distal oesophagus caused by gastro-oesophageal reflux (Yamada&Kato 1989).

1.2.2 Pathogenesis

The pathogenesis of gastric cancer involves multiple risk factors including dietary, infectious, occupational, genetic and preneoplastic risk factors (Correa 1992), most of which influence the gastric mucosal microenvironment, resulting in a state of chronic inflammation. Past research has concentrated on the identification of the complex aetiology of environmental and genetic risk factors, which may influence the initiation, promotion, and progression of gastric cancer (Correa 2002, Chan et al 2001, El-Rifai&Powell 2002, Kelley&Duggan 2003, Stadtländer&Waterbor 1999).

1.2.2.1 Helicobacter pylori infection

H. pylori is a Gram-negative, spiral-shaped bacterium that can survive and proliferate in the acidic environment of the stomach mucosa, living predominantly in the mucous layer overlying the normal gastric epithelium. *H. pylori* infection is usually acquired during childhood (Torres et al 2000), and more frequently in families of low socioeconomic status (Banatvala et al 1993, Webb&Forman 1995, Graham et al 1991, Goodman&Correa 2000, Blaser et al 1995). A range of epidemiological studies have provided evidence of the world-wide association between *H. pylori* infection and the development of gastric cancer (Asaka et al 1994, Nomura et al 1991, The Eurogast Study Group 1993, Parsonnet et al 1991, Huang et al 1996, Hansson et al 1995), and are summarized elsewhere (O'Connor et al 1996, Cover&Blaser 1995). The identification of the association between gastric carcinoma and *H. pylori* infection has been the most important development in gastric cancer epidemiology, and *H. pylori* has been classified as a Group I carcinogen by the World Health Organisation (WHO).

H. pylori infection results in chronic gastritis in nearly all infected persons (Kuipers et al 1995, Valle et al 1996), and is strongly associated with gastric atrophy and intestinal metaplasia (Kikuchi et al 1995, Parsonnet et al 1991, Wong et al 1999). In humans (Rugge et al 1996, Craanen et al 1992a) and in experimental animal models (Hirayama et al 1996, Sugiyama et al 1998, Watanabe et al 1998, Honda et al 1998), *H. pylori* induces the phenotypic changes of chronic gastritis, mucosal atrophy, intestinal metaplasia, and dysplasia, which are characteristic for progression to intestinal-type gastric cancer (Correa 1992), and in Mongolian gerbils, *H. pylori* infection causes gastric cancers (Watanabe et al 1998, Bergin et al 2003). In humans, *H. pylori* plays a role in approximately 60% of gastric cancer cases

(O'Connor et al 1996), and is associated with a 2.7 to 12-fold risk of developing gastric cancer (Cover&Blaser 1995). *H. pylori* infection is found in both intestinal- and diffuse-type gastric cancers (Parsonnet et al 1991, Nomura et al 1991, Talley et al 1991, Forman et al 1991, Eurogast Study Group, 1993), and the inflammatory response induced by the infection, as well as soluble products generated by *H. pylori*, influence gastric carcinogenesis. Chemokines, such as interleukin-8, pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα, and immunosuppressive peptides, such as IL-10, make up the complex network of inflammatory mediators involved in the host immune response to *H. pylori* infection. Polymorphisms in these genes shape the extent and magnitude of the host immune response (see Section 1.2.2.11).

Due to the genetic heterogeneity of the *H. pylori* genome, bacterial virulence factors also play a role in determining the outcome of a *H. pylori* infection. The Cag pathogenicity island is a large region of the *H. pylori* genome containing over 30 genes. Proteins encoded by this region form a Type IV secretion system, a cylinder-like structure that can directly transfer CagA protein into gastric epithelial cells, where phosphorylation of a CagA tyrosine residue triggers signal transduction pathways and induces morphological changes (Segal et al 1999, Stein et al 2000, Odenbreit et al 2000, Asashi et al 2000, Higashi et al 2002). A greater degree of inflammation, related to IL-8 production by epithelial cells, and a higher risk of developing gastric cancer is associated with CagA-positive, compared to CagA-negative *H. pylori* strains (Queiroz et al 1998, Blaser et al 1995). Another virulence factor, the vacuolating cytotoxin VacA, is found in almost all *H. pylori* strains, and the expression of VacA varies considerably between strains, which may be due to variations in the VacA gene structure. VacA is responsible for epithelial cell damage, and is also associated with gastric carcinogenesis (Soares et al 1998, Peek&Blaser 2000).

Chronic infection with *H. pylori* alters cell-cycle regulation and increases epithelial cell replication (Fiocca et al 1994), despite initially enhancing apoptosis (Yanai et al 2003). Gastric ascorbic acid concentrations are decreased, while the production of anti-oxidants and reactive nitrogen intermediates is increased (Correa 1992, Cahill et al 1994, Blaser&Parsonnet 1994, Forman 1998, Correa&Miller 1998). The combination of heightened proliferation with increased concentrations of DNA mutagens promotes the likelihood of critical DNA damage, and thereby the accumulation of mutations that drives the progression towards gastric cancer.

1.2.2.2 Epstein-Barr virus infection

The human herpesvirus 4, or Epstein-Barr virus (EBV), is an icosahedral herpesvirus containing double stranded DNA that has been connected with gastric cancer. The EBV has

been classified as a Group I carcinogen by the WHO and IARC, and is ubiquitous in all human populations. EBV is the cause of Burkitt's lymphoma, sino-nasal angiocentric T-cell lympoma, Hodgkin's disease and nasopharyngeal carcinoma (International Agency for Research on Cancer 1997). EBV-associated carcinomas are found in all geographic regions (Stadtländer&Waterbor 1999), and are approximately three-fold more frequently found in Japanese than in American populations (Watanabe et al 1997). EBV is associated with both intestinal- and diffuse-type gastric cancers (Shibata&Weiss 1992), but may be more prevalent in the male than in the female (Tokunaga et al 1993).

The mechanism of EBV-mediated gastric carcinogenesis is as yet unclear. Virus replication occurs in pharynx and salivary gland epithelial cells. The subsequent infection of lymphoid B-cells is mediated by the interaction of the gp350 viral envelope glycoprotein and CD21, the C3d complement component CR2 (International Agency for Research on Cancer 1997). The viral glycoproteins gp85, gp25 and gp42 are involved in host cell binding and viral envelope fusion, with the virus persisting in a latent state until triggering of the host cell results in shedding of infectious virus particles (International Agency for Research on Cancer 1997). Up-regulation of p53 is rarely observed in EBV-positive carcinomas, but found in over 30% of EBV-negative carcinomas (Ojima et al 1997, Chang et al 2005) and p27 loss, p16 loss, cyclin D1 expression and NF-κB nuclear positivity are found more frequently in EBV-positive gastric carcinomas (Chang et al 2005). Despite the association of EBV infection with the development of gastric carcinoma, there is no correlation with bcl-2 expression and p53 accumulation (Gulley et al 1996), leading to the conjecture that EBV induces gastric carcinomas via different mechanisms than EBV-negative carcinomas (Ojima et al 1997).

1.2.2.3 Diet

Dietary factors play an important role in gastric carcinogenesis, since the presence or the introduction of carcinogens in food, as well as possible synthesis through the interaction of ingredients during preparation, may contribute to the development of gastric cancer. A high intake of smoked, salted and nitrated foods, high intake of carbohydrates and low intake of fruit, vegetables and milk significantly increases the risk of developing stomach cancer (Howson et al 1986, Kramer&Johnson 1995). Smoked foods may contain polycyclic aromatic hydrocarbons, which have been shown to cause gastric cancer in animal experiments (Weisburger et al 1986), and high salt consumption has been consistently associated with gastric cancer risk (Joossens et al 1996, Lee et al 1995, Hansson et al 1993, Ramon et al 1993, Boeing et al 1991, Graham et al 1990, Buiatti et al 1989, Kono&Hirohata 1996). Salt causes stomach irritation, damaging the mucosa and leading to the development of atrophic gastritis,

as well as causing excessive cell replication and increasing the mutagenicity of nitrosated foods (Stadtländer&Waterbor 1999, Tatematsu et al 1975, Takahashi et al 1984, Hanawa et al 1980).

Foods high in nitrate may be harmful, since the conversion of nitrate to nitrite, and the subsequent reaction with other nitrogen-containing substances, results in the formation of N-nitroso compounds. N-nitroso compounds, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), have been demonstrated in animal experiments to be mutagenic and carcinogenic (Magee et al 1976, Druckrey 1975, Bulay et al 1979). In the human stomach, N-nitroso compounds can be formed from dietary nitrate or nitrite, which suggests that a diet with a high intake of nitrate or nitrite may predispose to gastric cancer (Bartsch et al 1987, Kelley&Duggan 2003). Patients with intestinal metaplasia, dysplasia and gastric cancer have exhibited increases in gastric nitrite (Ruddell et al 1978, Jones et al 1978, Stewart 1967). A positive correlation with gastric cancer has been observed for the consumption of pickled foods containing nitrosated products (Haenszel et al 1972, Sato et al 1959), and the utilization of fertilizers incorporating nitrate (Jones et al 1978, Schlag et al 1980, Frazer et al 1980). Additionally, inadequately stored foods may facilitate the growth of microorganisms that transform nitrate to nitrite, for subsequent endogenous nitrosation (Bartsch et al 1992)

A fruit and vegetable-rich diet is high in micronutrients, such as anti-oxidants and radical scavengers, which have a protective effect against gastric cancer. The consumption of fresh fruits and vegetables is consistently associated with a reduction in risk for developing gastric cancer (Kono&Hirohata 1996, Hamilton&Aaltonen 2000). A high intake of vitamin C (ascorbic acid) approximately halves the risk in case-control studies (Neugut et al 1996), but may require a longer duration of administration, since supplemental vitamin C had no effect of the gastric cancer risk in a 5-year intervention trial (Blot et al 1993). Vitamin C scavenges reactive radicals and may inhibit the formation of nitroso compounds (Stadtländer&Waterbor 1999), thereby inhibiting radical-mediated DNA mutation (Drake et al 1996). Vitamin C, selenium, β-carotene and calcium chloride have been shown to reduce the incidence of MNNG-induced gastric carcinomas (Kawasaki et al 1982, Kobayashi et al 1986, Santamaria et al 1987, Nishikawa et al 1992), and the effects of high salt intake may be opposed by β-carotene (Stadtländer&Waterbor 1999).

1.2.2.4 Lifestyle

The relationship between alcohol consumption and smoking, and the risk of gastric cancer has been intensively studied. However, the results are inconclusive. In contrast to HCC, the association between the development of gastric cancer and alcohol consumption is

particularly weak, and although a weak to moderate association has been found by most studies (Hansson et al 1994, Nomura et al 1990, Kabat et al 1993, Vaughan et al 1995), smokers may have a less than two-fold increased relative risk, with few studies finding a dose-response relationship (Hansson et al 1994, McLaughlin et al 1995, Ji et al 1996). Nevertheless, gastric adenocarcinomas have been induced experimentally in rats by catechol, a component of cigarette tar (Tanaka et al 1995), which has been shown to inhibit DNA synthesis (Li et al 1997) and interfere with the elimination of transformed cells by normal cells (Schäfer et al 1995). Exposure to workplace carcinogens may also play a role in the development of gastric carcinoma. Exposure to N-nitroso compounds, as well as the nitrate and nitrogen oxide precursors, ionising radiation, crystalline silica, organic and inorganic dusts, glycol ethers, hydraulic fluids and leaded gasoline have been suggested as occupational risk factors (Cocco et al 1996, Parent et al 1998). However, data are extremely limited, and no firm conclusions may be drawn.

1.2.2.5 Gastric surgery

A partial gastrectomy increases the risk of developing gastric cancer in the gastric remnant after 5-20 years (Werner et al 2001, Caygill et al 1986, Viste et al 1986, Lundegardh et al 1988). In particular, the Bilroth II operation, which increases bile reflux and leads to chronic inflammation, exhibits increased incidence of dysplasia (Grad 1984).

1.2.2.6 Chronic gastritis

Nearly all cases of chronic gastritis result from *H. pylori* infection (Kuipers et al 1995, Valle et al 1996), and chronic gastritis is a risk factor for the development of gastric cancer, being present in the great majority of gastric carcinoma cases (Sipponen et al 1994). The type of gastritis depends on the localization of the infection, and correlates with the clinical outcome (Konturek et al 2003, Macarthur et al 2004). Antrum-predominant gastritis has high-acid secretion and an increased risk of duodenal ulcer. The mixed antrum/corpus gastritis does not have a serious clinical outcome, as acid secretion is not affected. Corpus-predominant gastritis is associated with gastric atrophy, with the loss of acid-secreting parietal cells resulting in low acid levels, and has an increased risk of gastric cancer. Increased severity of gastritis increases the risk (Sipponen et al 1994), in extreme cases up to 10-fold (Sipponen et al 1985). Atrophic gastritis is often associated more with intestinal-type gastric cancers, whereas non-atrophic gastritis is more common in diffuse-type carcinomas (Sipponen et al 1994). Gastric acid secretion is altered by gastritis and atrophy, which results in elevated gastric pH and changed gastric flora, allowing anaerobic bacteria to colonize the stomach. In addition to the N-nitroso carcinogenic compounds produced by these bacteria (Yang et al

1984), gastritis is associated with increased production of oxidants and reactive nitrogen intermediates, which in combination with increased expression of nitric oxide synthase (Mannick et al 1996), increases the production of carcinogenic nitrosated compounds.

1.2.2.7 Intestinal metaplasia

Intestinal metaplasia (IM) often evolves as a response to chronic atrophic gastritis, and may be classified into different subtypes, according to the widely-used Jass and Filipe system (Jass and Filipe 1980). Complete IM (small intestinal type, type I) consists of absorptive cells, Paneth cells and goblet cells, and can be distinguished by decreased expression of the gastric mucin core proteins MUC1, MUC5AC, and MUC6, and expression of the intestinal mucin MUC2 (Reis et al 1999). Incomplete IM (types II and III) coexpresses the gastric mucins with MUC2, and is characterized by the presence of columnar and goblet cells, with type II secreting neutral and acidic sialomucins and type III sulphomucins.

Intestinal metaplasia is connected with an approximately 10-fold increased risk of developing stomach cancer (Filipe et al 1994), and is considered to be one of the most important risk factors for the development of intestinal-type gastric cancer (Leung & Sung 2002). Type III IM, but not type I or II, is strongly associated with early and advanced intestinal-type carcinomas, but not with diffuse-type cancers or benign gastric lesions (Filipe et al 1994, Rokkas et al 1991, Craanen et al 1992a, Jass & Filipe 1979, Heilmann & Höpker 1979, Segura & Montero 1983, Craanen et al 1992b). However, the association between the subtypes of intestinal metaplasia and the risk of developing gastric cancer has not been shown conclusively (Ectors&Dixon 1986, Ramesar et al 1987, Mentlein et al 2001, Genta & Rugge 2001, Petersson et al 2002), and it has been suggested that IM and gastric cancer may arise coincidentally (Hattori 1986).

1.2.2.8 Gastric ulcer

Gastric ulcer is usually characterized by accompanying gastritis, which may result from *H. pylori* infection. 6-20% of *H. pylori* infections result in peptic ulceration, of which no more than one percent lead to gastric cancer (Farthing 1998). Although not a frequent precursor of gastric carcinoma, the presence of gastric ulcer is moderately associated with the development of gastric cancers (Molloy&Sonnenberg 1997, Lee et al 1990, Hansson et al 1996, Hole et al 1987).

1.2.2.9 Autoimmune gastritis

One of the main causes of atrophic gastritis is autoimmune gastritis. The production of autoimmune antibodies directed against parietal cells leads to the distruction of parietal cells

and severe atrophy of the corpus mucosa. Antibodies directed against intrinsic factor, and the lack of parietal cells that secrete gastric intrinsic factor results in cobalbumin deficiency and pernicious anemia (Kapadia 2003). In patients with pernicious anaemia, the risk of gastric cancer increases threefold (Hsing et al 1993), and intestinal-type gastric cancer develops in approximately 10% (Siurala et al 1985).

1.2.2.10 Blood group A

An association between gastric carcinomas and the blood group A has been reported (Aird&Bentall 1953, Haenszel et al 1976), which may be related to the interaction between the Lewis^b blood group antigen and *H. pylori* (Carneiro et al 1996). The association of the blood group A with males, with diffuse-type gastric cancer is stronger than with females, or intestinal-type gastric cancer (Kramer&Johnson 1995, Nomura 1982).

1.2.2.11 Gene polymorphisms

In recent years, genetic polymorphisms have come to be recognized as crucial factors determining disease susceptibility. Host gene polymorphisms frequently influence the magnitude of the host response, and this interindividual variation contributes to the clinical outcome. The development of gastric cancer on the background of chronic inflammation induced by *H. pylori* infection is significantly influenced by host gene polymorphisms.

The proinflammatory cytokine interleukin-1 (IL-1) gene cluster containing *IL-1B* and *IL-1RN* encodes IL-1β and the IL-1β receptor antagonist, respectively, and the risk of gastric cancer and its precursor lesions is increased in the presence of *H. pylori* by polymorphisms in these genes. An increased risk of developing *H. pylori*-mediated hypochlorhydria and gastric atrophy is associated with the *IL-1B*-31*C or -511*T, and the *IL-1RN**2/*2 genotypes (El-Omar et al 2000), which are also associated with a two- to three-fold increase in the risk of developing gastric cancer (odds ratio 9.2; 95% CI 2.2-37), compared to individuals with less proinflammatory genotypes (El-Omar et al 2000, El-Omar et al 2003, Figueiredo et al 2002).

Although IL-1β is one of the most important proinflammatory cytokines mediating the effects of *H. pylori* infection (El-Omar et al 2000), polymorphisms in other proinflammatory cytokines, such as TNFα (308*A) and IL-10 (ATA/ATA), have also been associated with increased risk for gastric cancer (El-Omar et al 2003). The more proinflammatory genotypes an individual has, the higher the risk of developing gastric cancer, with the presence of three or more of these genotypes associated with an up to 27-fold increased risk of gastric cancer (Figueiredo et al 2002). Gastric cancer and *H. pylori* infection has been linked with the human leukocyte antigen (Magnusson et al 2001), with the *1601 allele significantly increasing the risk of gastric cancer (odds ratio 8.7; 95% CI 2.7-28). This association is seemingly

independent of *H. pylori* infection, being stronger in *H. pylori*-negative patients and in diffuse-type carcinomas (Correa 2002).

The MUC-1 mucin is a glycoprotein involved in the protection and lubrication of epithelial surfaces, detecting potential external insults and interacting with signal transduction and cell adhesion proteins (Gendler 2001). MUC-1 contains a highly variable number of tandem repeats, with higher numbers of repeats encoding larger proteins better able to respond to external stimuli. Gastric cancer patients exhibit higher proportions of smaller MUC-1 proteins, and smaller alleles have also been linked to gastric atrophy and intestinal metaplasia (Carvalho et al 1999, Correa 2002).

1.2.3 Pathology

Gastric carcinomas can be classified according their localization in the stomach. The antral-pyloric region of the stomach is the most common site of stomach cancer, and carcinomas of the body or corpus are located along the greater or lesser curvature. Cancers of the cardia are often unable to be distinguished from cancers of the gastroesophageal junction, and are believed to be a separate entity, probably originating from the distal oesophagus.

The diagnosis of gastric cancer is often delayed by the lack of early symptoms, with early gastric cancer causing non-specific gastrointestinal complaints, such as dyspepsia, in only 50% of patients. Up to 90% of Western gastric cancer patients first present with advanced carcinomas, which have more serious symptoms such as abdominal pain, bleeding, vomiting, or severe weight loss.

Endoscopic screening is considered to be the most sensitive and specific diagnostic test for gastric cancer. Dysplasia may present as a flat lesion or exhibit polypoid growth, with depressed, reddish or discoloured mucosa. Endoscopic detection of changes in colour, relief, and architecture of the mucosal surface enables the classification of gastric cancers according to their macroscopic growth pattern. Early gastric cancers may feature protruded (Type I), elevated (Type IIa), flat (Type IIb), depressed (Type IIc) or excavated (Type III) growth (Murakami 1971, Hamilton&Aaltonen 2000), whereas advanced gastric carcinomas are classified into polypoid (Type I), fungating (Type II), ulcerated (Type III) or infiltrative (Type IV) growth patterns (Hamilton&Aaltonen 2000, Borrmann 1926). Type II or III advanced gastric cancers are commonly ulcerating, and the risk of penetration of the submucosa is highest in early gastric cancers with a depressed growth pattern (Type IIc), and in infiltrative advanced gastric carcinomas (Type IV). The superficial spread of Type IV infiltrative (diffuse) tumours through the mucosa and submucosa result in flat, plaque-like lesions, which may exhibit shallow ulcerations. Serosal, lymphatic, and vascular invasion and lymph node

metastases are most frequent in the diffusely growing tumours. Tumour spread may involve direct extension to adjacent organs, such as the duodenum, or metastasis via the lymphatics or circulation, with intestinal-type carcinomas (Section 1.2.3.1) preferentially metastasising to the liver, and diffuse-type carcinomas to peritoneal surfaces (Mori et al 1995, Carneiro&Sobrinho 1996). The current TNM-classification and stage grouping of gastric cancers is given in Tables 1.3 and 1.4 (Hamilton&Aaltonen 2000).

Table 1.3: Tumour-Node-Metastasis (TNM) Classification of Gastric Tumours

Primary Tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial tumour without invasion of the lamina propria
T1	Tumour invades lamina propria
T2	Tumour invades muscularis propria or submucosa
T3	Tumour penetrates serosa (visceral peritoneum) without invasion of adjacent structures, such as the spleen, transverse colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestina, and retroperitoneum
T4	Tumour invades adjacent structures
Regional Lymph Nodes (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 6 regional lymph nodes
N2	Metastasis in 7 to 15 regional lymph nodes
N3	Metastasis in more than 15 regional lymph nodes
Distant Metastasis (M)	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 1.4: UICC Staging based on TNM Classification for Gastric Tumours

Stage	Tumour	Nodal	Metastasis
0	Tis	N0	M0
IA	T1	N0	M0
IB	T1	N1	M0
	T2	N0	M0
II	T1	N2	M0
	T2	N1	M0
	T3	N0	M0
IIIA	T2	N2	M0
	T3	N1	M0
	T4	N0	M0
IIIB	T3	N2	M0
IV	T1, T2, T3	N3	M0
	T4	N1, N2, N3	M0
	Any T	Any N	M1

1.2.3.1 Histological classification

Classification of gastric adenocarcinomas is based of the predominant histological pattern, which may either exhibit glandular histopathology, forming tubular, acinar or papillary structures, or be composed of a complex mixture of dissociated, isolated cells with variable morphologies. The differentiation state of the carcinoma may be classified as well-differentiated, moderately differentiated, or poorly differentiated, depending on the extent to which glandular structures are maintained. The most commonly used classification systems are those of the World Health Organisation and Laurén (Hamilton&Aaltonen 2000).

The WHO classification differentiates between four histological variations. Tubular adenocarcinomas consist of branching tubules of varying diameter exhibiting conspicuous dilated or slit-like growth patterns, with individual tumour cells being columnar, cuboidal, or flattened by intraluminal mucin. Papillary adenocarcinomas are well-differentiated exophytic carcinomas containing elongated cores of fibrovascular connective tissue supporting cylindrical or cuboidal cells, which usually maintain their polarity. Mucinous adenocarcinomas contain extensive extracellular mucinous pools, and may be glands of columnar mucous-secreting epithelium, or mucinous lakes containing free-floating irregular cell clusters or chains. Signet-ring cell carcinomas are mainly composed of isolated or small groups of intracytoplasmic mucin-containing malignant cells, which may exhibit a range of

morphologies, with the classical signet-ring cell being characterised by an expanded, globoid, optically clear cytoplasm pushing the nucleus against the cell membrane.

In the Laurén classification, intestinal-type carcinomas maintain the glandular phenotype, with well- to moderately-differentiated tumours forming identifiable glands, often with poorly differentiated tumour cells at the invasive front. Typically arising on a background of intestinal metaplasia, these tumours exhibit an intestinal, gastric and gastrointestinal mucinous phenotype. Diffuse-type carcinomas form no or very few glandular structures, instead usually infiltrating the gastric wall, appearing diffusely distributed as small, round single cells or poorly cohesive cell clusters. They may resemble signet-ring cells, and may contain small amounts of intestinal mucin. Additionally, mixed tumours exhibit both intestinal and diffuse characteristics, and undifferentiated tumors are classified as indeterminate. The natural history of gastric carcinoma, in particular the association with environmental factors, incidence trends, and precursor lesions, is often evaluated with respect to the Laurén classification.

1.2.4 Precursor Lesions

Although chronic atrophic gastritis and intestinal metaplasia may be considered to be *preneoplastic lesions*, this setting may only facilitate the development of what is generally regarded as a true precancerous or *precursor lesion*, dysplasia. Dysplasia encompasses a large range of cellular and structural atypias, which are defined under the term *intraepithelial neoplasia* (Hamilton&Aaltonen 2000), and lies between atrophic metaplasia and invasive cancer.

1.2.4.1 Intraepithelial neoplasia

Intraepithelial dysplasia may develop in the gastric or intestinal metaplastic gastric epithelium, and can be categorized into four categories: indefinite for intraepithelial neoplasia, low-grade and high-grade intraepithelial neoplasia, and suspicious for invasive cancer (Rugge et al 2000, Schlemper et al 2000). Regenerative or reactive changes unable to be definitely diagnosed are classified as *indefinite for intraepithelial neoplasia*. Intraepithelial neoplasia exhibits flat, elevated or polypoid, or slightly depressed growth patterns. Histological classification of intraepithelial neoplasia as low- or high-grade depends on the severity of architectural and cytological atypia. The mucosal structure is only slightly modified in *low-grade intraepithelial neoplasia*, maintaining tubular differentiation with the proliferative zone limited to the outward portion, whereas *high-grade intraepithelial neoplasia* exhibits increasing distortion of the mucosal architecture, resulting in crowded possibly irregular

glands with obvious cellular atypia, and proliferative activity distributed throughout the lesion. High-grade intraepithelial neoplasia is associated with a higher risk of developing gastric carcinoma (Rugge et al 1994), particularly in association with Type III intestinal metaplasia. The progression from *intraepithelial neoplasia* to *carcinoma* is diagnosed when the lamina propria or muscularis mucosae are invaded by the tumour.

1.2.4.2 Adenomas

Circumscribed tubular and/or villous structures exhibiting intraepithelial neoplasia constitute the benign lesions defined as adenomas. In contrast to Western countries, where adenoma only applies to macroscopic protruding lesions, Japan includes flat, elevated and depressed lesions in the adenoma classification. Gastric adenomas, in the Western sense, are uncommon (Tamura 1996), making up only 10% of all gastric polyps (Stolte et al 1994). Malignant transformation depends on the size and histological grade, occurring in 2% of lesions less than 2 cm and in 40-50% of lesions larger than 2 cm, and more frequently in flat adenomas.

1.2.4.3 Polyps

Hyperplastic gastric polyps typically arise in the antrum in the presence of *H. pylori* gastritis, but only proceed to carcinoma in a minority of cases. Fundic gland polyps are most common in Western populations, without a background of *H. pylori* infection. Sporadically occurring fundic gland polyps have no malignant potential, often affecting patients receiving long-term proton pump inhibitor treatment. Fundic gland polyps may also appear in the hundreds in familial adenomatous polyposis (FAP) patients (Watanabe 1977, Watanabe 1978), where dysplasia and subsequent carcinoma may develop in adenomatous polyps (Zwick et al 1997, McGarrity et al 2000), an elevated polypoid dysplastic lesion occurring seldom in the absence of FAP, with a cancer risk of up to 76% (Ming 1998).

1.2.5 Hereditary Syndromes

Although most gastric carcinomas arise sporadically, inherited familial genetic components are responsible for 8-10% of gastric cancer cases (Uemura et al 2001). An up to threefold increase in risk of developing gastric cancer has been reported for relatives of gastric carcinoma patients (Zanghieri et al 1990, La Vechhia et al 1992, Palli et al 1994, Lossowska et al 1999), which is associated with both hereditary and environmental factors (Lichtenstein et al 2000).

1.2.5.1 Hereditary diffuse gastric carcinoma

Familial diffuse gastric cancer is an autosomal dominant inheritable disease, usually developing at an early age (Huntsman et al 2001). Germline mutations in the E-cadherin (CDH1) gene generally result in truncated proteins, and lead to gastric cancer in 75% of patients (Caldas et al 1999), with an age of onset and diagnosis between 14 and 69 years. These tumors referred to as hereditary diffuse gastric carcinomas, and manifest as diffuse, poorly differentiated adenocarcinomas with an infiltrative growth pattern, perhaps containing signet-ring cells (Guildford et al 1999, Guildford et al 1998, Gayther et al 1998). Additionally, methylation of the CDH1 gene promoter has also been reported to lead to hereditary diffuse gastric carcinoma (Stone et al 1999).

1.2.5.2 Hereditary nonpolyposis colorectal cancer

Hereditary nonpolyposis colorectal cancer (HNPCC) results from an underlying defect in DNA mismatch repair, mainly involving the hMLH1, hMSH2, and the hMSH6 genes (Peltomaki 2001, Kinzler&Vogelstein 1996). Although chiefly causing colorectal cancer, this inherited cancer susceptibility syndrome also results in gastric cancers (Lee 1971), usually of the intestinal-type, with no accompanying *H. pylori* infection, and exhibiting microsatellite instability (Lynch et al 1993).

1.2.5.3 Polyposis syndromes

Rarely, gastric cancers also occur in gastrointestinal polyposis syndromes, such as familial adenomatous polyposis (FAP) and Peutz-Jeughers Syndrome (PJS). A higher risk of gastric cancer is associated with both FAP (Hofgartner et al 1999, Groden et al 1991) and PJS (Park et al 1998). The involvement of the germline mutations of the adenomatous polyposis coli (APC) or the serine/threonine kinase 11 (STK11) genes, respectively, and the accompanying polyposis in the development of gastric adenocarcinoma is as yet uncertain.

1.2.6 Prognosis

The prognosis of gastric cancer depends on various pathological factors, such as the macroscopic type, the depth of invasion, cancer-stromal relationship, histological growth pattern, lymph node involvement, lymphatic invasion, vascular invasion and tumour site (Yokota et al 2004), with the main prognostic factors being the TNM staging, along with the presence and extent of lymph node metastases. Diagnostic improvements and advances in treatment options have improved the long-term survival of early gastric cancer patients, and the 5-year survival rates of early gastric cancers without regional lymph node metastases infiltrating the mucosa or submucosa are greater than 90% (Antonioli 1994), although the

presence of regional lymph node metastases reduces the 5-year survival rate to 70% in tumours invading the submucosa (Inoue et al 1991). However, the prognosis of advanced gastric cancer remains poor, with survival rates rarely exceeding 15% (Stewart&Kleihues 2003), and the prognosis worsening as the degree of infiltration increases (Hermanek 1999). The 5-year survival rates of patients with tumours invading the mucosa or submucosa, the mucularis propria, and the subserosa or serosa are 95%, 60-80%, and less than 50%, respectively (Yoshikawa&Maruyama 1985). The depth of infiltration correlates with the presence of lymph node metastases, and the lymph node status and the ratio of metastasis-positive/metastasis-negative lymph nodes are the strongest markers of gastric cancer prognosis (Ichikura et al 1999, Msika et al 1989, Ichikawa et al 2003, Roder et al 1998, Yokota et al 2004). For patients with metastases in 1-6 lymph nodes, 7-15 lymph nodes, or more than 15 lymph nodes, the 5-year survival rates are 44%, 30% and 11%, respectively (Roder et al 1998). Unfortunately, most patients presenting with advanced gastric cancer already have lymph node metastases.

Other prognostic factors include lymphatic and vascular invasion, both being associated with lower survival rates (Hamilton&Aaltonen 2000, Gabbert et al 1991, Gabbert et al 1992, Yokota et al 2004), and the histological classification, whereby diffuse-type (Lauren classification) and mucous-rich (Goseki classification) tumours may predict a worse prognosis (Hamilton&Aaltonen 2000, Martin et al 1994, Songun et al 1999). In addition to the identification of reduced E-cadherin expression as an indicator of poor prognosis (28,29 in Yasui), the influence of further molecular genetic alterations on the prognosis of gastric cancer is currently the subject of numerous investigations (Becker et al 2000, Oue et al 2004, Yasui et al 2005).

1.3 Molecular Pathogenesis of Hepatic and Gastric Cancers

Both hepatic and gastric cancers have certain features in common. A long-lasting chronic inflammation, caused by viral or bacterial infection, initiates a cascade of reactive changes which ultimately lead to the development of cancer. It is generally believed that an ordered sequence of genomic changes, often referred to as the adenoma-carcinoma sequence, is reflected by the morphological and pathological transformation observed as normal gastric or hepatic tissue progresses through precancerous lesions to well-differentiated carcinoma, which then further evolves to a less-differentiated form. The adenoma-carcinoma sequence is exemplified in the pathogenesis of colon carcinoma (Vogelstein et al 1988), and can also be observed, in a less well-defined way, in intestinal-type stomach cancer (Tahara 2004), and to some extent in HCCs (Ozturk 1999). However, although the progression to diffuse-type gastric cancer and liver cancer is morphologically similar and encompasses some of the same molecular changes, evidence is lacking for an equivalent sequence of genetic events, despite the common development on a background of increased regenerative processes. The current status of the adenoma-carcinoma sequences of HCCs, and intestinal- and diffuse-type gastric cancers are shown in Figure 1.1 and Figure 1.2.

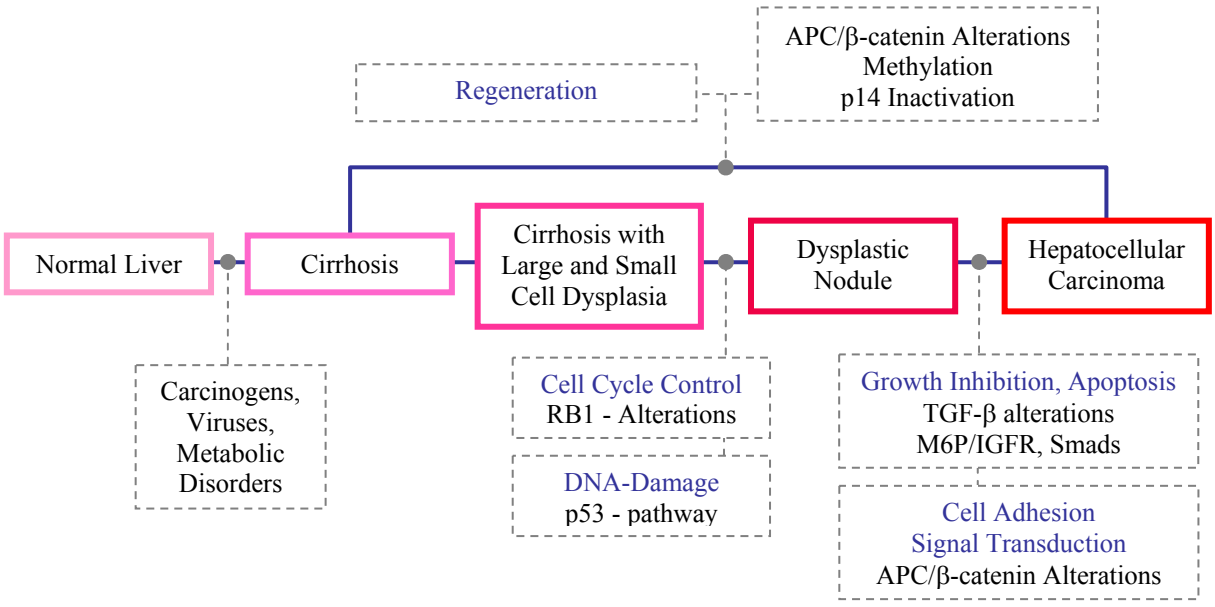

Figure 1.1: The adenoma-carcinoma sequence of hepatocellular carcinoma The currently recognized genetic alerations in HCC affect the main regulatory pathways involved in cell cycle regulation and apoptosis (modified from Tannapfel&Wittekind 2002)

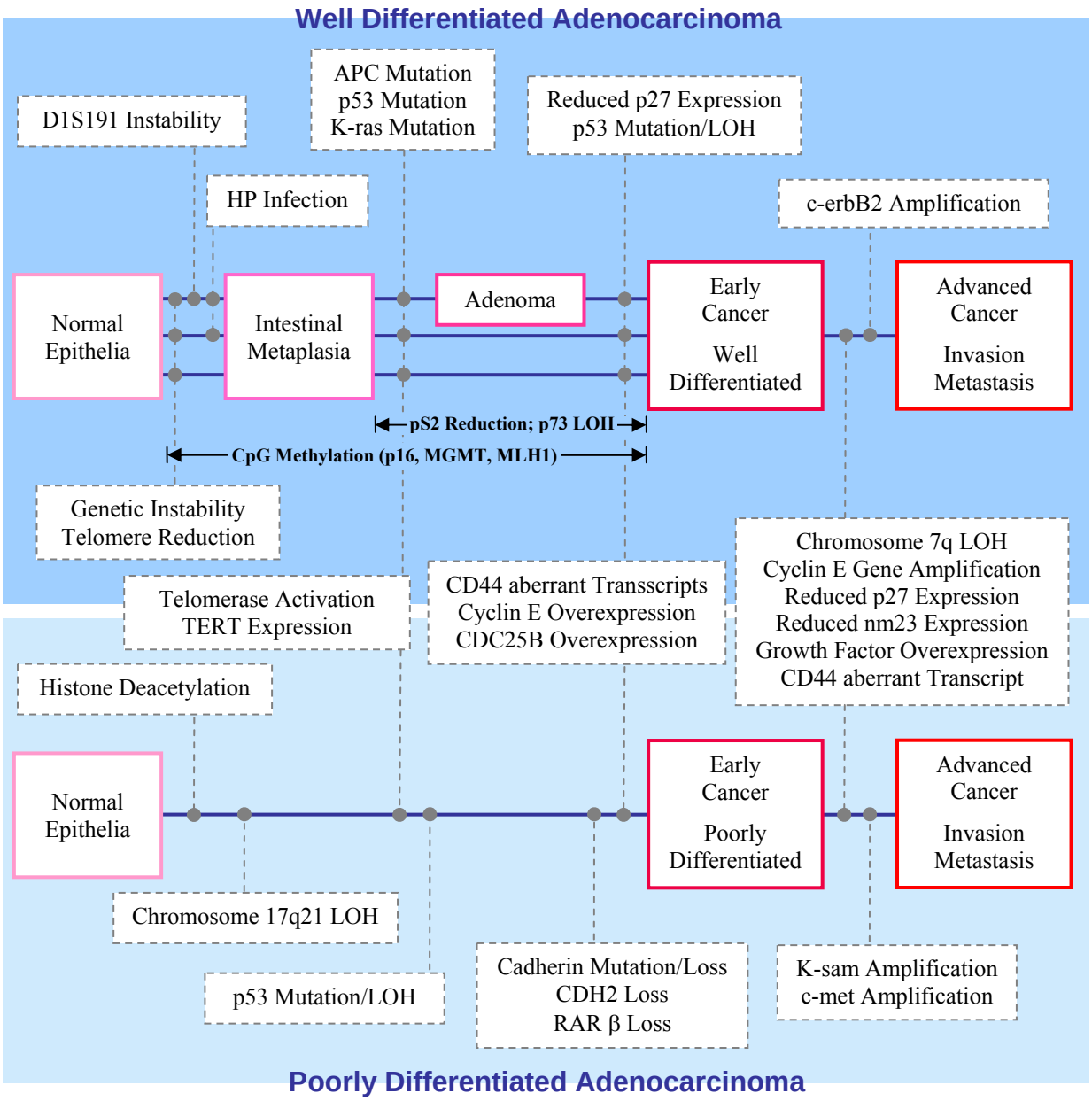

Figure 1.2: The adenoma-carcinoma sequence of gastric cancer The multistage process of gastric carcinogenesis is characterized by the accumulation of various genetic and epigenetic alterations, with intestinal-type adenocarcinomas exhibiting a different profile of alterations than diffuse-type gastric cancer (modified from Yasui et al 2001).

1.4 Tumour Biology

The accumulation of mutations during carcinogenesis results in six essential alterations in cell physiology that collectively dictate malignant growth: self sufficiency in cell growth, insensitivity to growth-inhibitory signals, evasion of cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan&Weinberg 2000). Most of these alterations affect cell signalling pathways, and the majority of oncogenes and tumour suppressor genes are integral components of cellular signalling circuits, which are up-regulated or constitutively activated in malignant cells. However, the true initiators of these circuits are the extracellularly-derived signalling molecules, which may be secreted by the host cells or the tumour cell itself.

Autocrine, paracrine and juxtacrine modulation of cell signalling by growth factors, cytokines, hormones and signalling peptides plays a key role in the promotion of proliferation, inhibition of apoptosis, and facilitation of invasion and migration through tissues, as well as the induction of angiogenesis. Synthesis and/or amplified secretion of growth factors and regulatory peptides is often a feature obtained during carcinogenesis, even of non-endocrine tumours, and a single tumour may express a number of different autocrine or paracrine loops to maintain malignant growth. Both autocrine and paracrine loops have been observed in HCCs and gastric cancers, and these cell signalling pathways are frequently composed of three main aspects. Extracellular signalling molecules bind to cell-surface receptors, which activate intracellular circuits to initiate the cellular effect (Figure 1.3). Alterations in any of these pathway components may give rise to tumour biology-relevant modifications in cell signalling and affect the sensitivity of the tumour cell to external stimuli: changes to the availability of extracellular signalling molecules by increased or decreased synthesis, to the transcellular transducers of those signals, such as the receptor molecules, or to the functioning of the intracellular circuits by structural changes to molecular components.

While the majority of molecular analyses in liver and gastric cancer searched for changes in the intracellular circuits, little is known about the biological function of cell surface molecules that modulate the immediate cellular environment, such as the availability of the extracellular signalling molecules. Many of these signalling pathways involve the extracellular regulation of ligand availability through proteolysis, which may be mediated by cell membrane-bound proteolytic enzymes (ectopeptidases) expressed on the surface of tumour or host cells.

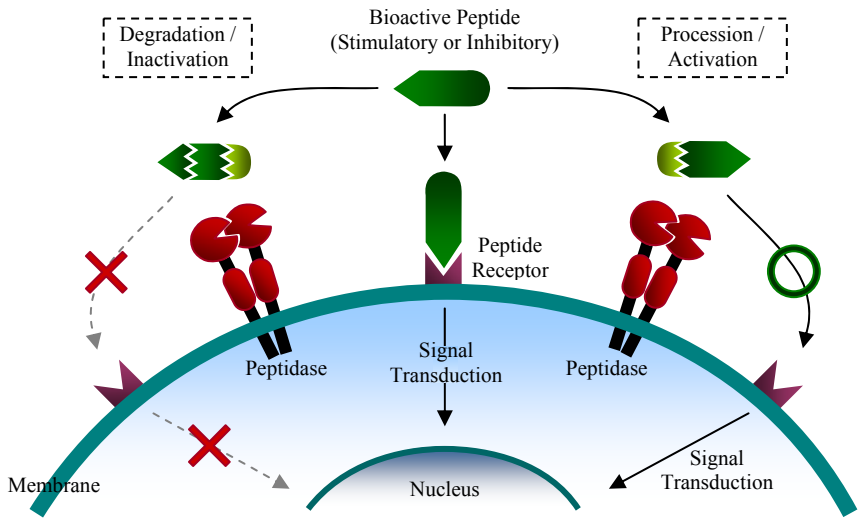

Figure 1.3: Ectopeptidase modulation of signal transduction pathways Inactivation or activation of extracellular signalling molecules by ectopeptidases may modulate intracellular signalling pathways by regulating the access of ligands to their receptors (modified from Ino et al 2004).

1.5 The Ectopeptidases in Tumour Biology

Cell membrane-bound proteolytic enzymes (ectopeptidases) are multifunctional membrane proteins, which are widely distributed among various cell systems. Ectopeptidases are integral membrane proteins, orientated asymmetrically with the catalytic site exposed to the extracellular surface, which enables a versatile range of physiological and pathological functions, ranging from proteolytic 'shedding' of signalling molecules, degradation of the extracellular matrix and tissue remodelling, to adhesion and cell migration, and the transduction of specific intracellular signals and involvement in inflammation.

Proteases may regulate the release of many growth factors and their receptors into the circulation, as well as activating or inactivating circulating signalling molecules. Additionally, many proteases have functions not limited to proteolysis, but are able in themselves to function as receptors, transducing intracellular signals. The individual ectopeptidases are each able to perform several, often overlapping functions, and, as a result, the expression of each ectopeptidase must be precisely regulated, in a tissue- and cell-specific manner. Even a minor disruption in the normal proteolytic equilibrium can influence the development of inflammatory and autoimmune diseases (Bank et al 2000). Functions, such as the modulation of cell-signalling, matrix degradation, cell adhesion and migration, which are particularly important for tumour cell growth and dissemination have been reported for a number of ectopeptidases.

1.5.1 Neutral Endopeptidase 24.11 (NEP)

Neutral endopeptidase 24.11 (NEP, neprilysin, enkephalinase, CD10, EC 3.4.24.11) is a 90-110 kDa zinc-dependent metallopeptidase belonging to the gluzincin family of metallopeptidases. The type II integral membrane protein is identical to the common acute lymphoblastic leukaemia antigen (CALLA). The 80kb NEP gene is located on chromosome 3q21-27 (Barker et al 1989), and the transcribed mRNAs range from 2.7 to 5.7 kb and exhibit tissue-specific and developmentally regulated expression (Li et al 1995). The encoded 749 amino acids are inserted asymmetrically into the membrane, with the large extracellular C-terminal catalytic domain anchored by the 23 amino acid transmembrane region and the short 27 amino acid cytoplasmic N-terminal tail (Crine et al 1997). The human NEP sequence contains 12 cysteine residues that may possibly form stabilizing disulfide bridges in the active enzyme, and 5 glycosylation sites, which are important for transport to the cell surface and full enzyme activity (Lafrance et al 1994). Usually found in homodimeric conformation, NEP cleaves peptide bonds on the amino side of hydrophobic residues, but also has peptidyl-dipeptidase activity with certain substrates (Roques et al 1993). The endogenous substrates of NEP include the enkephalins, atrial natriuretic peptides, substance P, and other tachykinins, as well as a wide range of other bioactive peptides (Matsas et al 1984, Kenny 1993), such as somatostatin, neurokinin, cholecystokinin-8, angiotensin I, angiotensin II, bradykinin, gastrin-releasing peptide, calcitonin, calcitonin gene-related peptide, interleukin-1, bombesin, and endothelin-1. NEP is expressed in various tissues (Kenny 1993), including immune cells, the brush border membranes of the kidney, intestine, and placenta, the brain, thyroid, lung and prostate, where it regulates proliferation and differentiation by degrading signalling peptides (Crine et al 1997). The association of NEP with acute lymphoblastic leukaemia, Alzheimer's disease, multiple sclerosis, asthma, inflammation, hypertension, and neoplastic transformation and progression is assumed to be due to the deregulation of peptide processing (Letarte et al 1997, Sumitomo et al 2005).

1.5.2 Aminopeptidase N (APN)

Aminopeptidase N (APN, CD13, EC 3.4.11.2), another member of the gluzincin family of metal-dependent proteases, is an approximately 150 kDa type II transmembrane protease encoded by the 35 kb APN gene, located on chromosome 15q25-26 (Noren et al 1997). The 967 amino acid sequence contains a single 24 amino acid transmembrane segment near the 8-10 amino acid cytoplasmic N-terminal. APN contains ten N-glycosylation sites and exists as a homodimer. A 40 amino acid stalk connects the transmembrane segment to the catalytic domain, which consists of two subunits. The C-terminal subunit is assumed to bind

substrates, while the N-terminal subunit contains the HELAH zinc-binding motif in the single catalytic site, preferentially cleaving N-terminal unsubstituted neutral amino acids from oligopeptides (Noren et al 1997). APN cleaves vasoactive peptides, such as angiotensin III and kallidin, neuropeptides, such as enkephalins and endorphins, and chemotactic peptides, such as MCP-1, as well as neurokinin A, somatostatin, and interleukin-8, but is unable to cleave bradykinin or substance P, which act as endogenous inhibitors. The broad substrate specificity reflects its wide expression pattern (Noren et al 1997). APN is found in the brush border membranes of intestine and kidney, on the synaptic membranes of the central nervous system, and on the surface of macrophages, granulocytes, and lymphocytes, as well as on endothelial cells, smooth muscle cells, and fibroblasts. In addition to its role in the regulation of cell growth and differentiation by modulating local peptide concentrations, APN is involved in antigen processing and presentation (Larsen 1996), serves as receptor for the human coronavirus 229E (Yeager 1992), transduces intracellular signals via MAP kinases (Lendeckel 1998), and mediates invasion and metastasis in a range of tumours and cell lines (Saiki et al 1993, Menrad et al 1993, Fujii et al 1995) via initiation of collagen IV degradation (Saiki et al 1993). APN is also associated with neoangiogenesis (Pasqualini et al 2000, Bhagwat et al 2001, Bhagwat et al 2003).

1.5.3 Dipeptidyl peptidase IV (DPIV)

Dipeptidyl peptidase IV (DPIV, CD26, EC 3.4.14.5) is a multifunctional type II cell surface glycoprotein with a molecular mass of approximately 110 kDa. The human DPIV gene is located on the long arm of chromosome 2q24.2 and covers 82 kb (Gorrel et al 2001). The predicted protein of 766 amino acids, with six amino acids in the cytoplasmic region and a 22 residue hydrophobic transmembrane domain, contains nine potential N-linked glycosylation sites, and has an α/β hydrolase domain and a seven-blade β-propeller domain, characteristic of members of the prolyl oligopeptidase gene family. DPIV may be cell bound or soluble, occuring in the serum (sDPIV). Cell-associated DPIV is widely expressed on T cells, B cells, natural killer cells, endothelial cells and epithelial cells. DPIV has three different functions: adenosine deaminase (ADA) binding, serine peptidase activity, and extracellular matrix (ECM) binding. These different biological activities of DPIV and its ubiquitous expression may reflect its diverse sometimes opposing functions in physiological and pathological settings. DPIV was found to be up-regulated in T-cell lymphoblastic lymphoma, thyroid cancer, adenocarcinoma of the lung and basal cell carcinomas of the skin and to be down-regulated in malignant melanomas (Dang&Morimoto 2002, Pro&Dang 2004). Serum levels of DPIV are increased in humans and animals suffering from HCCs or

hepatomas (Gorrel et al 2001, Hanski et al 1986), and the expression pattern of DPIV in HCCs and cirrhotic livers is different from that of non-cirrhotic livers (Stecca et al 1997, Matsumoto et al 1992). Serine peptidase activity of DPIV reverses malignant transformation of malignant melanomas (Wesley et al 1999) and prolongs survival and decreases invasive activity of ovarian carcinoma cell lines (Kajiyama et al 2003), while signal transduction via DPIV affects proliferation of T-cell lymphomas (Kähne et al 1999), and in hepatoma cell lines, activates a tyrosine kinase, thereby inducing apoptosis (Gaetaniello et al 1998).

1.5.4 Angiotensin-Converting Enzyme (ACE)

Angiotensin-converting enzyme (ACE, CD143, EC 3.4.15.1) is a 150-180 kDa type I integral membrane protein with 17 potential N-linked glycosylation sites (Soubrier et al 1988). The human ACE gene covers 21 kb on chromosome 17q23, and encodes 1306 amino acids, consisting of a 28 residue C-terminal cytoplasmic domain, a 22 amino acid transmembrane anchor, and the extracellularly-orientated carboxypeptidase domain. ACE is a unique metallopeptidase in that it has two functionally active catalytic sites (Soubrier et al 1988), probably due to gene duplication (Sidgel&Erdös 1987). Each site displays differences in affinity for substrates and inhibitors (Georgiadis et al 2003). A testis-specific soluble isoform of ACE, generated by alternative splicing, has only one catalytic site, and corresponds to the C-terminal region of full length ACE (Ehlers et al 1992). Both isoforms of ACE are transcribed from the gene by two alternative promoters (Howard et al 1990, Hubert et al 1991). Another soluble form of ACE is derived from the membrane-bound protein by proteolytic cleavage of the membrane-inserted C-terminal stalk (Ehlers&Riordan 1990), and there is evidence for the secretion of an alternative splicing variant lacking the transmembrane domain (Sugimura et al 1998). ACE is almost ubiquitously expressed. Apart from being expressed on the luminal surface of endothelial cells in vascular tissues (Rayan et al 1976, Igić&Kojović 1980), considerable amounts of ACE are expressed by epithelial cells in the gastrointestinal tract, predominantly in the intestinal mucosa, but also in the stomach (Kobayashi et al. 1991, Laliberte et al. 1991, Nonotte et al. 1993, Nonotte et al. 1995), where it may play a role in the metabolism of gastrointestinal hormones and regulatory peptides (Turner et al. 1987, Lendeckel et al. 2000). ACE is also found on epithelial cells in the brush border of the proximal tubule of the kidney, the small intestine and the placenta (Johnson et al 1984, Igić et al 1977), as well as in neuroepithelial and vascular smooth muscle cells, and fibroblasts and macrophages (Igić&Behnia 2003).

ACE plays a major role in the regulation of blood pressure, cleaving angiotensin I to generate the hypertensive angiotensin II, the major effector peptide of the renin-angiotensin

system, and inactivating the hypotensive bradykinin (Re 2003). Local angiotensin II-generating systems are believed to be responsible for the blood pressure-independent effects of renin-angiotensin system inhibitors. Ang II regulates cell growth and fibrosis in inflammatory processes (Suzuki et al. 2003), and induces the ADAMs-mediated shedding of epidermal growth factor receptor (EGFR) ligands (Schäfer et al 2004) via the G-protein coupled receptors (GPCR), type 1 (AT1) and type 2 (AT2). Upregulation of angiotensin II and its precursors has been observed in connection with stress-induced ulcers and gastric lesions (Mou et al. 1998, Seno et al 1997, Yang et al 1997) and the inhibition of ACE in animal models reduces the incidence and severity of stress-induced gastric ulcers (Bailey et al. 1987, Bhandare et al. 1992, Bhounsule et al. 1990, Cullen et al. 1994, Ender et al. 1993, Rao et al. 1995, Uluoglu et al. 1998).

Being a relatively non-specific enzyme, ACE also cleaves di- or tripeptides from a number of synthetic and naturally occurring substrates, including substance P, opioid peptides (Met-enkephalin-Arg^6-Phe^7, heptapeptide, β-neoendorphin, $dynorphin_{1-8}$, $dynorphin_{1-6}$), neurotensin, chemotactic peptide, luteinizing hormone releasing hormone, cholecystokinin-8, [Leu^{15}]$gastrin_{11-17}$, and B-chain of insulin (Skidgel 1990). This wide range of substrates may explain the involvement of ACE in a variety of physiological and pathological processes, such as neovascularization (Volpert et al 1996), fertilization (Krege et al 1995), atherosclerosis (Metzger et al 2000), kidney and lung fibrosis (Metzger et al 1999, Leehey et al 2000, Nguyen et al 1994), smooth muscle and myocardial hypertrophy (Naftilan et al 1989, Aceto&Baker 1990), and inflammation and wound healing (Sun&Weber 1996).

1.5.5 The ADAMs (A Disintegrin And Metalloproteinase)

Instead of degrading bioactive peptides and peptide hormones, another group of ectopeptidases are better known for their roles in the shedding of membrane-bound growth factors or receptors. The ADAMs (A Disintegrin And Metalloproteinase) are a family of membrane-anchored, cell-surface glycoproteins, containing pro-, metalloprotease, disintegrin (RGD-binding motif), cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains. Removal of the prodomain occurs during transport through the secretory pathways of the cell, with the mature, proteolytically active form being expressed on the cell surface. After removal of the prodomain and its cysteine switch, the metalloproteinase domain is proteolytically active, enabling the shedding or degradation of a wide range of substrates. Both the disintegrin and cysteine-rich domains bind adhesion molecules, such as integrins and syndecans. The EGF-like domain may play a role in the association between the

ADAM and the EGF-like ligands to be shed. The cytoplasmic tail is involved in intracellular signalling via Src-homology-3 (SH3) binding motifs.

1.5.5.1 ADAM9

ADAM9 (MDC-9, meltrin γ) is expressed in a wide range of tissues (Weskamp et al 1996). Shed substrates include heparin-binding epidermal growth factor (HB-EGF), β-amyloid precursor protein, fibronectin, β-casein, gelatin, TNFα, p75 TNF receptor and c-kit ligand-1 (Izumi et al 1998, Roghani et al 1999). The ECD integrin binding motif within the disintegrin domain mediates binding to integrin α6β1, resulting in enhanced cellular motility (Nath et al 2000). The disintegrin domain also mediates the binding of ADAM9 to another integrin, avb5 (Zhou et al 2001). The cytoplasmic tail contains potential SH3-binding motifs, to which the adaptor proteins endophilin I and SH3PX1 are assumed to bind (Howard et al 1999). The cytoplasmic tail can also be phosphorylated by protein kinase Cδ, which may activate ADAM9-mediated HB-EGF-shedding (Izumi et al 1998, Gechtman et al 1999). Increased expression of ADAM9 has been detected in breast cancer (Lendeckel et al 2005, O'Shea et al 2003, Borrel-Pages et al 2003), in pancreatic ductal adenocarcinoma (Grutzmann et al 2003), and liver cancer (Tannapfel et al 2003, Le Pabic et al 2003). Expression of ADAM9 was detected in prostate adenocarcinomas and tumour cell lines (Karan et al 2003).

1.5.5.2 ADAM12

ADAM12 (meltrin α) is broadly expressed in a variety of tissues. There are two alternate forms of ADAM12, created by alternative splicing (Gilpin et al 1998). The longer form (ADAM12-L) produces a transmembrane protein, whereas the shorter form lacks the transmembrane and cytoplasmic domains (ADAM12-S) and transits through the endomembrane system to be secreted (Cao et al 2002, Hougaard et al 2000, Kadota et al 2000). In addition to the shedding of HB-EGF (Asakura et al 2002), soluble ADAM12 degrades insulin-like growth factor (IGF) binding proteins 3 and 5, thereby increasing the available pool of IGF-1 and -2 (Loechel et al 2000). Although lacking a defined integrin-binding motif within the disintegrin domain, ADAM12 binds to α9β1 integrin (Eto et al 2000), as well as regulating β1 integrin function (Kawagucki et al 2003). ADAM12 also supports cell adhesion and migration through the interaction of its cysteine-rich domain with syndecans (Iba et al 2000, Thodeti et al 2003). The SH3-binding motifs in the cytoplasmic tail of ADAM12 are assumed to mediate binding to Src, Yes, and Grb2 (Kang et al 2000, Suzuki et al 2000) and p85α, a regulatory subunit of PI 3-kinase (Kang et al 2001), indicating its role in the activation of intracellular signalling pathways. In keeping with its important role in

myoblast fusion (Yagami-Hiromasa et al 1995), the cytoplasmic tail also binds to the muscle specific actin-binding proteins, α-actinin-1 and -2 (Galliano et al 2000). Increased expression of ADAM12 has been detected in giant cell bone tumours (Tian et al 2002), in breast cancers and cell lines (Lendeckel et al 2005), and in liver cancer (Tannapfel et al 2003, Le Pabic et al 2003).

1.5.5.3 ADAM15

ADAM15 (MDC-15, metargidin) also exhibits a wide expression pattern. ADAM15 is involved in the lysophosphatidic acid-induced EGFR transactivation (Schaefer et al 2004), and mediates the shedding of amphiregulin and TGFα (Schaefer et al 2004), as well as being involved in the degradation of type IV collagen and gelatin (Martin et al 2002). ADAM15 is the only member of the ADAMs family to contain the well-known RGD-integrin binding motif within the disintegrin domain, and can bind to αvβ3 and α5β1 integrins (Nath et al 1999). Binding to integrin α9β1 also occurs via the disintegrin domain, but in an RGD independent manner (Eto et al 2000, Eto et al 2002). With SH3 binding motifs and potential phosphorylation sites in the cytoplasmic tail, ADAM15 associates with the adaptor proteins endophilin I, SH3PX1 and Grb2, and the tyrosine kinases Src, Lck and Hck (Yasui et al 2004, Howard et al 1999, Poghosyan et al 2001). ADAM15 is up-regulated in lung carcinomas (Schutz et al 2005), high expression levels of ADAM15 have been reported in cell lines derived from haematological malignancies (Wu et al 1997), and over-expression of ADAM15 reduces ovarian cancer cell adhesion and motility (Beck et al 2005). ADAM15 may also play a role in pathological neovascularization (Horiuchi et al 2003). Additionally, elevated transcription of ADAM15 was recently detected in gastric cancer compared with non-neoplastic tissue (Yoshimura et al 2002).

1.5.6 The Ectopeptidases in Hepatogastrointestinal Cancers

It has long been recognized that the expression pattern of proteases may be changed in malignant tumours, indicating a putative involvement in tumour development and tumour growth. There is an increasing body of evidence that ectopeptidases also participate in the pathology of hepatogastrointestinal carcinomas. Their roles in cancer progression and invasion are evidenced by the ability to influence proliferation, angiogenesis, tumour cell migration, and metastatic behaviour. However, the putative role of these ectopeptidases during the development and progression of gastrointestinal cancers, and, in particular, the change in expression in carcinomas compared to non-neoplastic tissue, has rarely raised attention. A systematic analysis of the differential expression between normal tissue,

carcinomas, and metastases is the first step, lacking thus far, required for the elucidation of the involvement of the ectopeptidases in the tumour biology of HCC and gastric cancer.

1.6 Aims

In view of the high frequency and extremely poor prognosis of gastric and liver cancers, it is becoming imperative to identify new diagnostic markers and therapeutic options. The focus of this study was the identification of possible diagnostic markers or therapeutic targets for hepatocellular and gastric carcinomas. The analysis of gastrointestinal tumour biology was approached from two different directions:

1.6.1 Ectopeptidases in tumour biology

Alterations in the expression patterns of the ectopeptidases in hepatocellular and gastric carcinomas compared to non-neoplastic tissues were to be investigated, and the influence of these proteases on the *in vitro* behaviour (proliferation, invasion) of liver and gastric cancer cell lines evaluated.

1.6.2 A novel gene in tumour biology

Using differential display, a novel candidate gene that is up-regulated in hepatogastrointestinal cancer was to be identified. Investigation of the transcription and expression of the gene in liver and gastric cancers and non-neoplastic tissues should confirm whether the up-regulation of this gene and/or gene product plays a role in other hepatocellular and gastric carcinomas.

2 MATERIALS AND METHODS

2.1 Case Selection and Specimen Processing

Hepatocellular carcinoma, gastric cancer, and colon cancer patients operated on between 1995 and 2003 were retrieved from the archive of the Department of Pathology (Table 2.1). All cases were reviewed before study inclusion. Tissue samples were obtained from 25 HCC patients (14 men, 11 women), 69 gastric cancer patients (40 men, 29 women; 35 diffuse-type, 34 intestinal-type), and 14 colon cancer patients (5 men, 9 women). The age of patients ranged from 26 to 84 years (mean 63.96 ± 12.5 years): 28 to 79 years in HCC patients (mean 61.60 ± 12.0 years), 26 to 84 years in gastric cancer patients (mean 64.81 ± 12.7 years), and 45 to 87 years in colon cancer patients (mean 62.21 ± 11.0 years). Tissue samples (tumour, lymph node metastases, and corresponding non-neoplastic tissue) were obtained immediately after surgery, snap-frozen in liquid nitrogen, and stored at –80°C until required. For histological processing, tissue samples were fixed in 10% neutralized formalin and embedded in paraffin. Deparaffinized serial sections were stained using hematoxylin and eosin (H&E).

Table 2.1: Patient Characteristics

Differential Display (GSDML)	**N =**	**Age (mean ± SD); Range**	**M : F**
HCC	25	61.60 ± 12 0; 28-79	14 : 11
Gastric Cancer (Intestinal-Type)	12	69.42 ± 9.49; 51-83	10 : 2
Gastric Cancer (Diffuse-Type)	14	63.14 ± 10.76; 45-79	7 : 7
Colon Cancer	14	62.21 ± 11.0; 45-87	5 : 9
Ectopeptidases	**N =**	**Age (mean ± SD); Range**	**M : F**
HCC	25	61.60 ± 12 0; 28-79	14 : 11
Gastric Cancer (Intestinal-Type)	34	67.79 ± 11.18; 41-83	23 : 11
Gastric Cancer (Diffuse-Type)	35	61.91 ± 13.48; 26-84	17: 18
Gastric Inflammation	**N =**	**Age (mean ± SD); Range**	**M : F**
Control	17	62.6 ± 20.0	6 : 12
H. pylori Gastritis	14	62.1 ± 11.9	7 : 6
Gastric Ulcers	13	68.9 ± 11.2	5 : 8
Local Ang II System in Lymph Node Metastases	**N =**	**Age (mean ± SD); Range**	**M : F**
Gastric Cancer (Intestinal-Type)	19	68.85 ± 10.77; 40-82	11 : 8
Gastric Cancer (Diffuse-Type)	16	62.44 ± 10.8; 26-80	5 : 11

2.2 Differential Display

2.2.1 RNA extraction

Samples from a poorly differentiated HCC and corresponding non-tumorous hepatic tissue were obtained at the time of liver transplantation from a 66-year-old Caucasian woman, who had suffered from chronic viral Hepatitis B. Total RNA was extracted from frozen liver tissue using the High Pure Tissue Total RNA Extraction Kit (Roche Biochemicals, Mannheim, Germany), which included a DNase incubation step to remove contaminating DNA. cDNA was synthesized from 11 µl total RNA (6-10 µg) and 1 µl 3' T_{11}MN primer (25 µM), using the SUPERSCRIPT™ First Strand cDNA Synthesis Kit (GIBCO BRL, Karlsruhe, Germany).

2.2.2 DDRT-PCR

Differential Display PCR (Figure 2.1) was performed as previously described (Lohmann et al 1995), using 5' upstream arbitrary decamers designed for complete analysis of a gene expression profile (Bauer et al 1993). 2 µl cDNA were amplified in a 20 µl reaction, containing 0.5 µM 5' arbitrary decamer, 2.5 µM oligo-dT primer mix, 25 µM dNTP mix, 1x PCR Buffer, 2.5 mM $MgCl_2$, and 4.5 U Taq polymerase (GIBCO BRL, Karlsruhe, Germany) in sterile H_2O. Thermocycling was performed as follows: 40 x (94 °C, 30 sec; 42 °C, 1 min; 72 °C, 30 sec), terminating with 72 °C, 5 min. 14 µl Laemmli dye buffer were added to each 20 µl PCR sample, and the samples were denatured for 3 min at 95 °C, before placing on ice. The fragments were electrophoretically separated on a 6%, 7 M urea denaturing polyacrylamide gel, and silver stained.

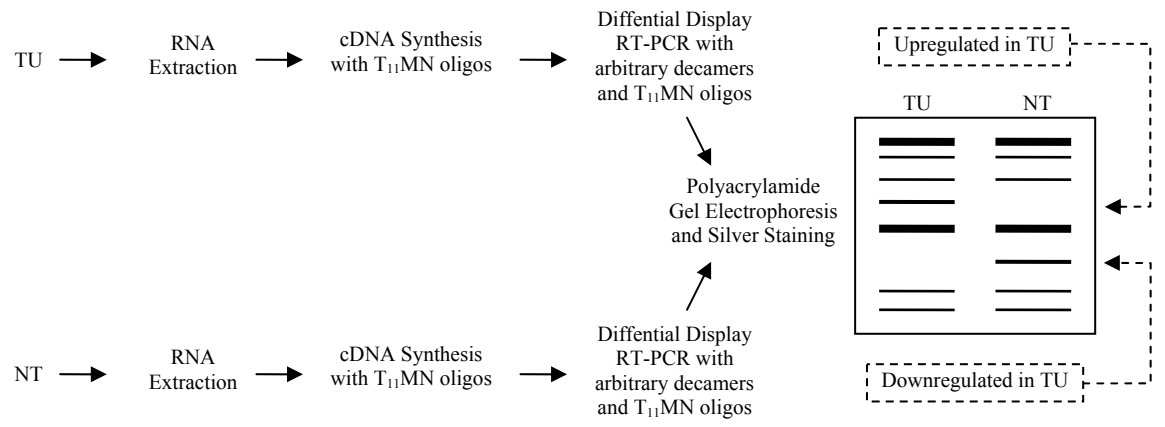

Figure 2.1: Differential Display RT-PCR Side-by-side display of cDNA fragments amplified using oligo-dT and arbitrary primers enables the identification gene fragments that are differentially expressed in tumour (TU) or non-lesional (NT) tissues (modified from Lohmann et al 1995).

2.2.3 Reamplification, Cloning and Sequencing

A cDNA fragment showing differential expression (designated CRSS) was finely cut out of the gel and eluted by boiling the gel piece in 100 µl H_2O for 15 min. The cDNA was recovered by ethanol precipitation in the presence of 0.3 M sodium acetate, and resuspended in 10 µl H_2O. A 2 µl aliquot of the eluted cDNA were reamplified in a 40 µl PCR, using the same PCR conditions and primers (Table 2.2) that generated the original fragments. The new PCR product was run next to the original product on a polyacrylamide gel to verify the purity and identity of the amplified DNA. The differentially expressed band was cloned into the pCR2.1-TOPO vector (Invitrogen, Groningen, Netherlands) and sequenced. A BLAST alignment (Altschul et al 1997) was carried out with the partial cDNA sequence obtained.

2.2.4 Verification of differential expression in HCCs by semi-quantitative RT-PCR

Using the GSDML reference sequence (NM_018530), primers were designed to amplify a 399bp portion of the GSDML cDNA (Table 2.2). The product was sequenced to verify its identity, and RNA obtained from ten additional patients with HCC was used in RT-PCR, with amplification of the β2-microglobulin (β2M) housekeeping gene as a control for normal expression. All reactions contained 1 µl of the cDNA template, 1X PCR Buffer (Promega, Mannheim, Germany), 1.5 mM $MgCl_2$, 0.6 µM of each primer, 0.8 mM dNTPs, and 0.2 U Taq polymerase in a final volume of 25 µl. Amplification was performed according to the following temperature profile: 95 °C, 5 min; 28 cycles of (94 °C, 1 min; 67 °C, 1 min; 72 °C, 2 min); 72 °C, 10 min. β2M RT-PCR reaction mixes were as for GSDML, with 0.4 µM each primer (Table 2.2) and 0.1 U Taq polymerase, with the following temperature profile: 94 °C, 1 min; 25 cycles of (94 °C, 1 min; 57 °C, 1 min 30 sec; 72 °C, 3 min); 72 °C, 10 min.

2.3 Analysis of mRNA expression in gastrointestinal cells and tissues

2.3.1 RNA extraction and cDNA synthesis

Total RNA was extracted from cell pellets or tissue samples, using the High Pure Tissue Total RNA Extraction Kit (Roche Biochemicals, Mannheim, Germany), or the Nucleospin RNA II Kit (Macherey&Nagel, Düren, Germany), following the recommended protocols. The resulting RNA was quantified spectrophotometrically and stored at –80°C until further required. One µg total RNA was transcribed into cDNA using either Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany) and Oligo-dT primers (Promega, Mannheim, Germany), or AMV reverse transcriptase (Promega, Mannheim, Germany) and random hexanucleotides (Boehringer Mannheim, Germany), both according to the recommended protocol.

2.3.2 RT-PCR

RT-PCR reactions were performed using the Taq PCR Core Kit (Qiagen, Hilden, Germany), as instructed. All reactions contained 2 µl of the cDNA template, 1X PCR Buffer containing 1.5 mM $MgCl_2$, 0.5 µM of each primer (Table 2.2), 0.2 mM dNTPs, and 0.5 U *Taq* polymerase in a final volume of 20 µl. Amplification was performed according to the following temperature profile: 95 °C, 5 min; 35 cycles of (94 °C, 1 min; 60 °C, 1 min; 72 °C, 2 min); 72 °C, 10 min. Amplification of housekeeping genes (β2-microgöobulin or β-actin) was used as a control for normal expression.

2.3.3 Agarose gel electrophoresis

The appropriate number of agarose tablets (0.5g/tablet; Eurogentec, Germany) for 1.5-3.5% agarose gels were dissolved in 50 ml 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), with 10 µl ethidium bromide (10 mg/ml). After the gels were poured and set, the PCR reaction mixture was diluted 1:1 with gel loading buffer (0.01% bromophenol blue, 50% glycerol) and electrophoresed in 0.5X TAE buffer for 40 min. Ethidium bromide-stained DNA strands were visualised under ultraviolet light.

2.3.4 Quantitative RT-PCR analyses

2.3.4.1 Establishment of external standards for fluorescence-mediated quantitative RT-PCR

For GSDML analysis, the 399bp GSDML fragment and the 269bp β2M fragment (Section 2.2.4) were cloned into pCR2.1-TOPO vectors (Invitrogen, Groningen, Netherlands), according to the manufacturer's instructions, and plasmid DNA was isolated using the Miniprep kit (Qiagen, Hilden, Germany), following the recommended protocol. For analysis of the ectopeptidases, the appropriate PCR product was purified using the Nucleospin Extract II kit (Macherey&Nagel, Düren, Germany), as instructed. The plasmid or PCR product DNA was quantified spectrophotometrically, and the copy number of the plasmids or PCR products was calculated. Serial dilutions of the purified DNA samples were used as external standards in every run to create a standard curve for calculation of mRNA levels.

2.3.4.2 Quantitative real-time RT-PCR for GSDML (LightCycler)

Quantitative RT-PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany). All reactions contained 2 µl of the cDNA template, 2 mM $MgCl_2$, 0.5 µM of each primer (Table 2.2), 2 µl 10xLightCycler – DNA Master SYBRGreen I (Roche Diagnostics, Mannheim, Germany) in a final volume of 20 µl, with the following temperature profile: 95 °C, 30 sec; 40 cycles of (95 °C, 1 second; 53 °C, 5 sec; 72 °C, 15 sec). Melting curve analysis of the products was performed between 65 °C and 95 °C, and verified the absence of substantial side products.

2.3.4.3 Quantitative real-time RT-PCR for the Ectopeptidases (iCycler)

Quantitative RT-PCR was performed using the iCycler (real-time PCR device, Bio-Rad, Munich, Germany). All samples were analysed in triplicate. A 30 µl reaction mixture consisted of 15 µl HotStarTaq™ Master Mix (Qiagen, Hilden, Germany), 0.25 µl of Fluorescein Calibration Dye (BioRad, Munich, Germany), 1.2 µl of the RT-reaction, 0.3 µl SYBR-Green I (1:10,000) (Molecular Probes, Eugene, USA), and 0.5 µmol/L of the specific primers for ADAM9, ADAM12, ADAM15, NEP, APN, DPIV, and ACE (listed in Table 2.2). Initial denaturation and activation of *Taq* polymerase at 95 °C for 15 min was followed by 40 cycles with denaturation at 94 °C for 30 sec, annealing at 60 °C (NEP, APN, DPIV, ACE) or 62 °C (ADAM9, ADAM12, ADAM15) for 30 sec, and elongation at 72 °C for 30 sec, followed by a final elongation at 72 °C for 5 min.

2.3.4.4 Quantitative RT-PCR calculations

The fluorescence intensity of the double-strand-specific SYBR-Green-I, reflecting the amount of actually formed PCR product, was measured at the end of each cycle during the 72 °C elongation each elongation step, after previously carrying out melting curve analyses to determine the melting points of the PCR products. The time point at which the linear increase of PCR product started (threshold cycle) was determined for each sample. Using the threshold cycle values, the mRNA copy number was calculated from the standard curve (serial dilutions of the corresponding plasmid or PCR product). The expression levels of various housekeeping genes (18S ribosomal RNA, α-tubulin, β-actin, β2-microglobulin) were calculated in the same manner and used to normalize cDNA contents for any variability in RNA amounts or integrity.

Table 2.2: Primer Details

	Forward (5'-3')	Reverse (5'-3')	Product (bp)
Differential Display			
DDA	-	TTTTTTTTTTT(A/C/G)A	-
P1	CTTGATTGCC	-	-
GSDML			
GSDML	GTTGGTCCAGGCGCAATGT	CTGCTGGGATATCCGGTTCTC	399
GSDML variants	GATCTCTCAGGGCCATCTCA	GCGAGGGAGTTTAGCACATC	234
Ectopeptidases			
ADAM9	GCTAGTTGGACTGGAGATTTGG	TTATTACCACAGGAGGGAGCAC	486
ADAM15	GCTGATGAAGTTGTCAGTGC	GAGACTGACTGCTGAATCAG	280
ADAM12	CAAATATAGGTGGCACTGAGGAG	TAGCAGCAGTTCTCCAAAGTGTG	285
NEP	GACCAACCTCGACTTGGCCT	CAACCAGCTGAATGGCTTCCC	316
APN	GATGCTACAGCTGACAGTCGC	TGGTGACCATGTGACCCACTG	239
DPIV	GGTGGTGCACCTCAAGGG	GGAAGCATGTTGGACAGGG	384
ACE	CTCAAGTACTTCCAGCCAGTC	GCAGAATCTTGCTGGTCTCTG	371
AT1	CTGATGCCATCCCAGAAAGT	ATCACCACCAAGCTGTTTCC	228
AT2	GAGTGTGTTTAGGCACTAAAGC	GTAAATCAGCCACAGCGAGG	369
Housekeeping Genes			
18S RNA	commercially available primer pair (Ambion Europe Ltd, Huntingdon, UK)		315
α-tubulin	CACCCGTCTTCAGGGCTTCTTGGTTT	CATTTCACCATCTGGTTGGCTGGCTC	528
β-actin	CATgTACgTTgCTATCCAggC	CTCCTTAATgTCACgCACgAT	250
β2-microglobulin	CCAGCAGAGAATGGAAAGTC	GATGCTGCTTACATGTCTCG	269
ACE Insertion/Deletion Polymorphism			
ACE I/D	CTGGAGACCACTCCCATCCTTTCT	GATGTGGCCATCAC-ATTCGTCAGAT	190 (D) 490 (I)

2.4 Protein Analysis

2.4.1 Coupled *in vitro* transcription/translation reaction

A plasmid construct from the Mammalian Genome Collection, containing a full-length cDNA sequence of GSDML (IRAKp961G0749Q; BC025682: 1556bp, 411aa, predicted 46.9kDa) was obtained from the German Resource Centre for Genome Research (RZPD; Berlin, Germany). The plasmid was sequenced to verify that the insert was GSDML. The protein encoded by the construct was generated using the TNT Quick Coupled Transcription/Translation System with biotinylated tRNA (Promega, Mannheim, Germany), following the manufacturer's protocol. One to 2µl aliquots were used in reducing SDS-PAGE and Western blotting as described below. Proteins were detected using alkaline-phosphatase-conjugated Streptavidin (dilution 1:5000; Rockland, distributed by Biotrend, Cologne, Germany), or the polyclonal rabbit anti-human GSDML antibody described below (dilution 1:250) with a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (dilution 1:1000; Dako, Hamburg, Germany).

2.4.2 Generation of peptide antibodies against putative GSDML protein sequence

PredictProtein (Rost 1996) and PSORT II (Nakai&Horton 1999) were used to analyze the protein sequence (NP_061000), predicted from the GSDML Reference Sequence (NM_018530). The following epitopical peptide sequences were selected from the putative GSDML protein sequence as being common to nearly all splicing variants (Figure 3.3): QGHLSYKHKGQREVT (184-198) and LASSPPDMDYDPEA (356-369). Anti-peptide antibodies directed against GSDML (anti-GSDML) were generated in two rabbits according to a standardized immunization protocol (Eurogentec, Seraing, Belgium). Briefly, the peptides were covalently bound to keyhole limpet hemocyanine and each rabbit was simultaneously immunized with both peptides. After the final bleeding, immunoglobulins were purified from antiserum using HiTrap Protein G columns (Amersham Biosciences, Freiburg, Germany) and concentrated to 5 mg/ml. ELISA and Western blotting confirmed that the polyclonal anti-peptide antibody (anti-GSDML) specifically bound to the synthesized peptide epitopes (data not shown).

2.4.3 Protein extraction

Cell pellets or tissues were immersed in denaturing lysis buffer (62.5 mM Tris, pH 6.8, 4 M urea, 0.5% SDS), or non-denaturing lysis buffer (50 mM sodium phosphate, pH 7.4, 200

mM NaCl, 1% Triton X-100), both containing 0.9 mg/ml protease inhibitor cocktail (Sigma, Deisenhofen, Germany), and homogenized using the Ultra-Turrax rotor-starter homogenizer (Merck, Darmstadt, Germany), and centrifuged at 11,000 rpm in a benchtop centrifuge for 10 min at 4 °C to remove cell debris. The protein concentration of the supernatants was measured using the DC Protein Assay Kit (Biorad Laboratories, Munich, Germany).

2.4.4 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed using the Mini-Protean 3 electrophoresis system (Biorad, Munich, Germany) with gels poured according to the manufacturers instructions (Table 2.3). Samples were diluted with 2X Laemmli Sample Buffer (Sigma, Deisenhofen, Germany), and boiled for 5 min. at 85 °C. For reducing SDS-PAGE, 1 µl β-2-mercaptoethanol was added to 50 µl 2X Laemmli buffer before diluting samples. After loading samples, the gel was immersed in running buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS) and electrophoresed until the dye front had exited the gel and all the molecular weight markers (Sigma, Deisenhofen, Germany) had progressed past the stacking gel (30 min, 50V; 60 min, 100V; 60-120 min; 150V).

Table 2.3: SDS-PAGE Gel Composition

Gel Components	Separating Gel (16.5%)	Spacer Gel (10%)	Stacking Gel (4%)
40% acrylamide (29:1)	2.5 ml	1.5 ml	0.6 ml
3.0 M Tris, pH 8.45 (0.3% SDS)	2 ml	2 ml	2 ml
Glycerol	1 ml	1 ml	1 ml
H_2O	0.5 ml	1.5 ml	3.4 ml
100 mg/ml ammonium persulphate (APS)	60 µl	60 µl	60 µl
N,N,N´,N´-tetramethyl-ethylenediamine (TEMED)	3 µl	3 µl	3 µl
Total volume	6 ml	6 ml	6 ml

2.4.5 Coomassie blue gel stain

To remove SDS, the unfixed and unstained gel was washed in H_2O (3 x 10 min.), before immersion in the staining solution (0.75% (w/v) Coomassie blue, 30% methanol, 10% acetic acid) overnight. The gel was repeatedly washed in destaining solution (30% methanol, 10% acetic acid) until bands could be clearly distinguished. Gel was dried and preserved using the DryEase Mini-Gel Drying System (Novex, San Diego, USA).

2.4.6 Western blotting

After briefly washing in H_2O, the unstained gel was equilibrated in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). The polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany) were prepared by immersing in 100%

methanol, then in blotting buffer. The nitrocellulose (NC) membranes (Amersham Biosciences, Freiburg, Germany) were first rinsed in H_2O, then in blotting buffer. Using the Mini-Protean 3 buffer tank and lid with the Mini-Trans-Blot cell (Biorad, Munich, Germany), according to the manufacturer's instructions, the proteins were electrotransferred from the gel to the appropriate membrane (100V, 90-120 minutes). To verify that the protein transfer had taken place, the unblocked membrane was rinsed in Tris-buffered saline (TBS; 0.25 M Tris, pH 7.4, 0.15 M NaCl), immersed 1-2 seconds in Ponceau S staining solution (Sigma, Deisenhofen, Germany), and washed in TBS containing 0.5% Tween 20 (TBST), before blocking overnight in 3% BSA (Serva Electrophoresis GmbH, Heidelberg, Germany) in TBST, in 3% skim milk powder (Roth, Karlsruhe, Germany) in TBST, or in Rotiblock (Roth, Karlsruhe, Germany).

2.4.7 Immunodetection

2.4.7.1 Chromogenic immunodetection

After washing with TBST (2 x 5 min, 2 x 10 min), the membranes were incubated for 2 hours at RT with the primary antibodies listed in Table 2.4 The membranes were washed with TBST (2 x 5 min, 2 x 10 min), then incubated for 1 hour with the alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:1000 dilution; Dako, Hamburg, Germany). After washing (TBST; 2 x 5 min, 2 x 10 min), the NBT/BCIP substrate (p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; Pierce Biotechnology, Bonn, Germany) was left on the membrane until distinct bands had developed. Immunoblots were analysed with the GeneGnome and GeneTools image scanning and analysis package (Syngene BioImaging Systems, Synoptics Ltd.), and the molecular weights of the proteins were calculated.

2.4.7.2 Chemiluminescent immunodetection

After washing with TBST (2 x 5 min, 2 x 10 min), the membranes were incubated for 2 hours at RT with primary antibodies directed against NEP, APN, DPIV and ACE, listed in Table 2.4. The membranes were washed with TBST (2 x 5 min, 2 x 10 min), then incubated for 1 hour with the horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:25,000 dilution; Dako). After washing (TBST; 2 x 5 min, 2 x 10 min), the membrane was covered in West Pico Supersignal substrate (Pierce Biotechnology, Bonn, Germany), and using the GeneGnome and GeneTools image scanning and analysis package (Syngene

BioImaging Systems, Synoptics Ltd.), the signal was observed until distinct bands had developed, the membrane images were evaluated and the protein expression quantified.

2.5 Immunohistochemical analysis

2.5.1 Histological processing of specimens/samples

Tissue samples from every tumour and the corresponding non-neoplastic tissue were fixed in 10% neutralized formalin and embedded in paraffin. Deparaffinized serial sections were stained using hematoxylin and eosin (H&E).

Table 2.4: Antibody Details

Antibody	Host	Clone	Distributor	IHC	HIER	WB
GSDML	Rb	polyclonal	-described above-	1:150	Citrate	1:250
Chromogranin A	Rb	polyclonal	Dako, Hamburg, Germany	1:200	EDTA	-
Gastrin	Rb	polyclonal	Quartett, Berlin, Germany	1:20	-	-
ADAM9	Rb	polyclonal	Cedar Lane; Biozol, Munich, Germany	1:75	-	n.d.
ADAM12	Rb	polyclonal	Cedar Lane; Biozol, Munich, Germany	1:200	-	n.d.
ADAM15	Rb	polyclonal	Cedar Lane; Biozol, Munich, Germany	1:75	-	n.d.
NEP	Ms	56C6	Novocastra; Medac, Wedel, Germany	1:10	EDTA	1:100
APN	Ms	38C12	Novocastra; Medac, Wedel, Germany	1:50	Citrate	1:100
DPIV	Ms	MA261	BioCarta, Hamburg, Germany	1:5	-	n.d.
ACE	Ms	CG2	Dianova, Hamburg, Germany	1:50	Citrate	1:200
AT1	Rb	polyclonal	Biotrend, Cologne, Germany	1:20	Citrate	-
AT2	Rb	polyclonal	Alpha diagnostics; Biotrend, Cologne, Germany	1:50	EDTA	-

HIER: heat induced epitope retrieval, IHC: immunohistochemistry, Citrate: 10 mM sodium citrate, pH 6.0 (3 x 10 min, 600 W microwave oven), EDTA: 1 mM EDTA, pH 8.0 (2 x 10 min, 450 W microwave oven), WB: immunoblotting, n.d.: not done

2.5.2 Immunostaining

For immunostaining of paraffin-embedded sections, the slides were deparaffinized and rehydrated in a graded alcohol series, and pre-treated either with 1 mM EDTA, pH 8.0 (2 x 10 min, 450 W microwave oven) or with 10 mM sodium citrate, pH 6.0 (3 x 10 min, 600 W microwave oven), if necessary (see Table 2.4). For immunostaining of frozen tissues, 12 µm sections were cut from tissue samples stored at -80 °C and fixed in acetone (10 min, –20 °C). Immunostaining was performed with antibodies directed against GSDML, chromogranin A, gastrin, somatostatin, ADAM9, ADAM12, ADAM15, NEP, APN, DPIV, ACE, AT1 and AT2 (Table 2.4). Slides were washed between steps with TBS. Incubation with the primary antibodies was performed in a moist chamber at 37 °C for 1 hour. Biotinylated polyvalent anti-mouse/anti-rabbit IgG (Immunotech, Marseilles, France) served as a secondary antibody (30 min, RT). Using an avidin-biotin complex, GSDML, ADAM9, ADAM12, ADAM15, APN, ACE, AT1, and AT2 immunoreactions were visualized via the Vectastain ABC alkaline

phosphatase (AP) kit (distributed by CAMON, Wiesbaden, Germany) with Fast Red/Napthol Mx (Immunotech, Marseille, France) as chromogen. Chromogranin A, gastrin and NEP immunoreactions were visualized via the UltraTech horseradish peroxidase (HRP) Streptavidin-Biotin Universal Detection System (Immunotech, Marseilles, France), with 3,3-diaminobenzidine-tetrahydrochloride as chromogen. DPIV immunoreactions were visualized via a double alkaline phosphatase-anti-alkaline phospatase (APAAP) complex, using the APAAP-Komplex (Immunotech, Marseilles, France) and Fast Red/Napthol Mx (Immunotech, Marseille, France) as chromogen.

The specimens were counterstained with hematoxylin. Incubation with pre-immune serum and omission of primary antibodies served as negative controls. A cross-check for reactive alkaline phosphatase in the brush border of intestinal metaplasia was carried out using horseradish peroxidase-based detection.

2.5.3 Immunoreactivity Score (IRS)

For the quantification of the immunohistochemical results, a numerical scoring system was applied. The observed expression of ADAM9, ADAM12, ADAM15, NEP, APN, DPIV, and ACE in epithelial cells was assessed using two categories. Category A documented the number of immunoreactive epithelial cells as 0 (no immunoreactive cells), 1 (<10%), 2 (11 to 50%), and 3 (>50%). A positive case was defined as having a Category A value of 1. Category B documented the intensity of the immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). Finally, the values for Category A and B were added to give the "*immunoreactivity score*" (IRS), which could range from 0 to 6. Note that the method of calculating the IRS does not allow the individual categories to add up to an IRS of 1.

2.6 Cell Culture Assays

2.6.1 Cell Culture

The human stomach (MKN28, MKN45, AGS, NCI-N87, KATOIII), liver (HepG2, C3A) and colon (HT29, T84, LoVo, DLD-1, SW480, SW620) cancer cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), the Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (Rockville, USA). C3A cells were grown in 90% ISCOVEs basal medium (Biochrom KG, Berlin, Germany), supplemented with 10% FCS (PAA Laboratories, Cölbe, Germany). All other cell lines were maintained in RPMI-1640 medium (PAA Laboratories, Cölbe, Germany), supplemented with 10-20% fetal calf serum (PAA Laboratories, Cölbe, Germany). The cells were grown in Nunclon-Surface cell culture flasks (NUNC, Wiesbaden, Germany) in a tissue culture hood at 37 °C with 5% CO_2. Cells were washed with phosphate-buffered saline (PBS) from Gibco-BRL Life Technologies (Eggenstein, Germany) and harvested with Trypsin-EDTA (PAA Laboratories, Cölbe, Germany). The cells were treated with BM Cyclin (Roche Molecular Biochemicals, Mannheim, Germany) and analysed with the mycoplasma-specific MycoSensor PCR Assay kit (Stratagene, Amsterdam, The Netherlands) to ensure the absence of mycoplasma contamination.

2.6.2 Determination of the ACE Insertion/Deletion Genotype

The ACE genotypes of the gastric cancer cell lines were determined by PCR as described previously (Yoshida et al. 1995). Genomic DNA was purified from the cells using the E.Z.N.A. Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH; Elangen, Germany). DNA was dissolved at 100 ng/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and 100 ng were used in a 50 µl reaction mixture, consisting of 25 µl HotStarTaq Master Mix (Qiagen, Hilden, Germany), 5 µM of each ACE I/D primer (Table 2.2), and 5% (v/v) DMSO. An initial 15 min denaturation at 95 °C was followed by 40 cycles of 1 min at 64 °C, 1 min at 72 °C, and 0.6 min at 94 °C. Amplified ACE gene fragments were separated on 1.6 % agarose gels and visualised by ethidium bromide staining. D or I alleles were identified by the presence of 190 bp or 490 bp fragments, respectively.

2.6.3 Cytokine Assays

Cells were seeded into 6-well plates at a density of 50,000 cells/well/ml cell culture medium. After allowing cells to adhere overnight, cells were incubated with serum-free

medium, before incubating a further 48 hours in culture medium containing 2% FCS, with and without (all samples 6-fold) the addition of 50 ng/ml IL-1β, IL-6, IL-8, TGFβ, and TNFα. (R&D Systems, Wiesbaden, Germany). The cells were then washed with PBS and frozen at –20°C for later use. Total RNA was extracted from the frozen cells using the Nucleospin II Kit (Macherey&Nagel, Düren, Germany), as described above. cDNA was synthesized from 1 µg RNA using Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany), and quantitative *real-time* RT-PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany). A 20 µl reaction mixture consisted of 10 µl Quantitect SYBR Green MasterMix (Qiagen, Hilden, Germany), 2 µl cDNA, and 1 µM of the specific primers for ACE or β-actin (listed in Table 2.2). Initial denaturation and activation of *Taq* polymerase at 95 °C for 15 min was followed by 40 cycles with denaturation at 94 °C for 15 sec, annealing at 62 °C for 20 sec, and elongation at 72 °C for 20 sec (ACE) or 15 sec (β-actin), followed by melting curve analysis between 65 °C and 95 °C to verify the absence of primer artefacts. Only samples without primer artefacts were included in the analyses. Specific initial template mRNA amounts were calculated as described above from a standard curve obtained by serial dilution of known copy numbers of the corresponding cloned PCR fragments. cDNA contents were normalized for any variability in RNA amounts or integrity by calculating the ACE/β-actin ratio.

2.6.4 Proliferation assays

Cells were seeded into 96-well plates at a density of 10,000 cells/well/100 µl cell culture medium with and without (all samples 6-fold) the addition of 1µg/100µl anti-ADAM9 (Chemicon, Hofheim, Germany), anti-ADAM12 (Biomol, Hamburg, Germany), and anti-ADAM15 (R&D Systems, Wiesbaden, Germany) ectodomain antibodies, or the addition of 1 µM each of thiorphan, phosphoramidon (NEP inhibitors), actinonin, phebestin (APN inhibitors), I49 (DPIV inhibitor), captopril, enalapril (ACE inhibitors), olmesartan (AT1 inhibitor), or PD123319 (AT2 inhibitor). Thiorphan, phosphoramidon, actinonin, phebestin, captopril and PD123319 were obtained from Sigma (Deisenhofen, Germany). I49 was synthesized by Drs. Jürgen Faust and Klaus Neubert from Halle, Germany. Enalapril and olmesartan were provided by MSD Sharp&Dohme GmbH (Haar, Germany) and Sankyo Pharma GmbH (Munich, Germany), respectively. Cells were incubated at 37 °C with 5% CO_2 for 48 hours.

Both thymidine incorporation and MTT conversion were used as measures of cell growth. Thymidine is incorporated into the genomic DNA during S-phase growth, with the

incorporation corresponding to DNA synthesis and implying progression through the cell cycle, whereas MTT conversion correlates with the amount and enzymatic activity of succinate dehydrogenase, which is a measure of the metabolic activity of the citric acid cycle and usually correlates with the number of viable cells.

2.6.4.1 Thymidine incorporation assay

For evaluation of DNA synthesis occurring during progression through the cell cycle, the cultures were pulsed for additional 6 hours with 0.2 µCi per well [^{3}H]-methylthymidine (Amersham Biosciences, Freiburg, Germany). Cells were harvested onto glass-fibre filters, and the incorporated radioactivity was measured by scintillation counting.

2.6.4.2 Succinate dehydrogenase inhibition assay

For evaluation of metabolic activity of the citric acid cycle, cells were incubated a further 2 hours at 37 °C with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltatrazolium bromide (MTT; Sigma, Deisenhofen, Germany). Formazan formed from MTT was extracted from the cells by adding dimethylsulfoxide and mixing for 15 min. The absorbance was measured at 562 nm, using a spectrophotometer (Anthos Labtec Instruments) with 620 nm as reference.

2.6.5 Invasion Assays

Cells were seeded into Transwell chambers (Corning, Bodenheim, Germany) with polycarbonate filters (0.8 µm pore size, 0.3cm^2/well) coated with 300 µg Matrigel reconstituted basement membrane (Becton Dickinson, Heidelberg, Germany) at a density of 30000 cells/well/300 µl cell culture medium with and without (all samples 3-fold) the addition of 1 µM each of enalapril (ACE inhibitor), olmesartan (AT1 inhibitor), or PD123319 (AT2 inhibitor). After 48 hours, cells that passed the synthetic basement membrane and polycarbonate filter were harvested by incubating the lower side of the filters with Trypsin/EDTA. The harvested cells were counted using a cytometer (Coulter Immunotech). The relative proliferation and percent of invasive cells were calculated using the cell numbers obtained from a simultaneously seeded 48-well plate (0.3cm^2/well), harvested and counted as above.

2.7 Statistical analysis

Statistical analysis was carried out with Microsoft Excel, or SPSS for Windows, Version 10.0. Dimensional values are expressed as mean ± standard deviation (SD), and population differences were evaluated using a χ^2-test, or a *two-sided* Student's *t*-test. For analysis of tissue samples obtained from the same patient, a *paired, two-sided* Student's *t*-test was applied. Paired categorical values, such as the IRS, and paired dimensional values with non-Gaussian distributions were analyzed using the non-parametric Wilcoxon Signed Ranks test. A P-value less than 0.05 were considered to be 'significant', and less than 0.01 to be 'highly significant'. Relationships between data sets were evaluated using Pearson's correlation coefficient. The results were verified using other parametric and non-parametric tests, including Fisher's Exact, Wilcoxon, and Mann-Whitney tests.

3 RESULTS AND DISCUSSION: GASDERMIN-LIKE PROTEIN

The focus of this chapter is the identification of a novel candidate gene that is up-regulated in hepatogastrointestinal cancer, which may be a possible diagnostic marker or therapeutic target for hepatic and gastric carcinomas. Using differential display, the gasdermin-like protein (GSDML) was identified to be up-regulated in HCC, and by investigating the transcription of the gene and expression of the protein in gastrointestinal cancer tissues, the putative role of GSDML in tumour biology was evaluated.

3.1 Results

3.1.1 Differential Display

Total RNA samples from a poorly-differentiated HCC and the corresponding non-neoplastic tissue were analyzed using differential display (Lohmann et al 1995). The DDRT-PCR products were run side-by-side on sequencing gels, where several bands showed differential expression between the two tissue types. A band of approximately 1 kb (designated CRSS) showed higher expression in tumour tissue than in non-tumour tissue (Figure 3.1) and was analyzed further. This band was purified from the gel and reamplified using the primer pair, which had been used to amplify the original product (DDA and P1 in Table 2.2). The differentially expressed fragment was cloned, and partially sequenced.

A BLAST alignment (Altschul et al 1997) with the partially recovered sequence information (268 bp) returned seven genomic DNA sequences corresponding to chromosome 17, as well as six cDNA sequences with very high identity (96-97%) with fragments of CRSS sized between 173 and 262 bp. All the cDNA sequences (listed in Table 3.1) exhibited significant sequence homology to each other, but bear little similarity to any other previously described nucleic acid sequence.

Table 3.1: A BLAST Alignment Identified Unigene Cluster Hs. 30667

Nucleotide Sequence (GenBank Accession)	Size (bp)	Homology to CRSS (bp CRSS/bp sequence)	Size of translated protein (aa/MW)	Protein Sequence (GenBank Accession)
AK025174	1963 bp	262/266 (99%)	-	-
AK000409	1518 bp	174/175 (99%)	403 aa/45.8 kDa	BAA91146
BC025682	1556 bp	174/175 (99%)	411 aa/46.7 kDa	AAH25682
NM_018530	1646 bp	173/175 (99%)	394 aa /45.0 kDa	NP_061000
AF119884	1646 bp	173/175 (99%)	394 aa /45.0	AAF69638
BX647700	5555 bp	174/175 (99%)	263 aa /	-
Additional sequences from UniGene cluster Hs. 30667				
AK057588	2207 bp	-	164 aa /18.1	-
AF258572	1794 bp	-	163 aa /18.1	AAG23775
BX538068	1282 bp	-	347 aa /	CAD97998

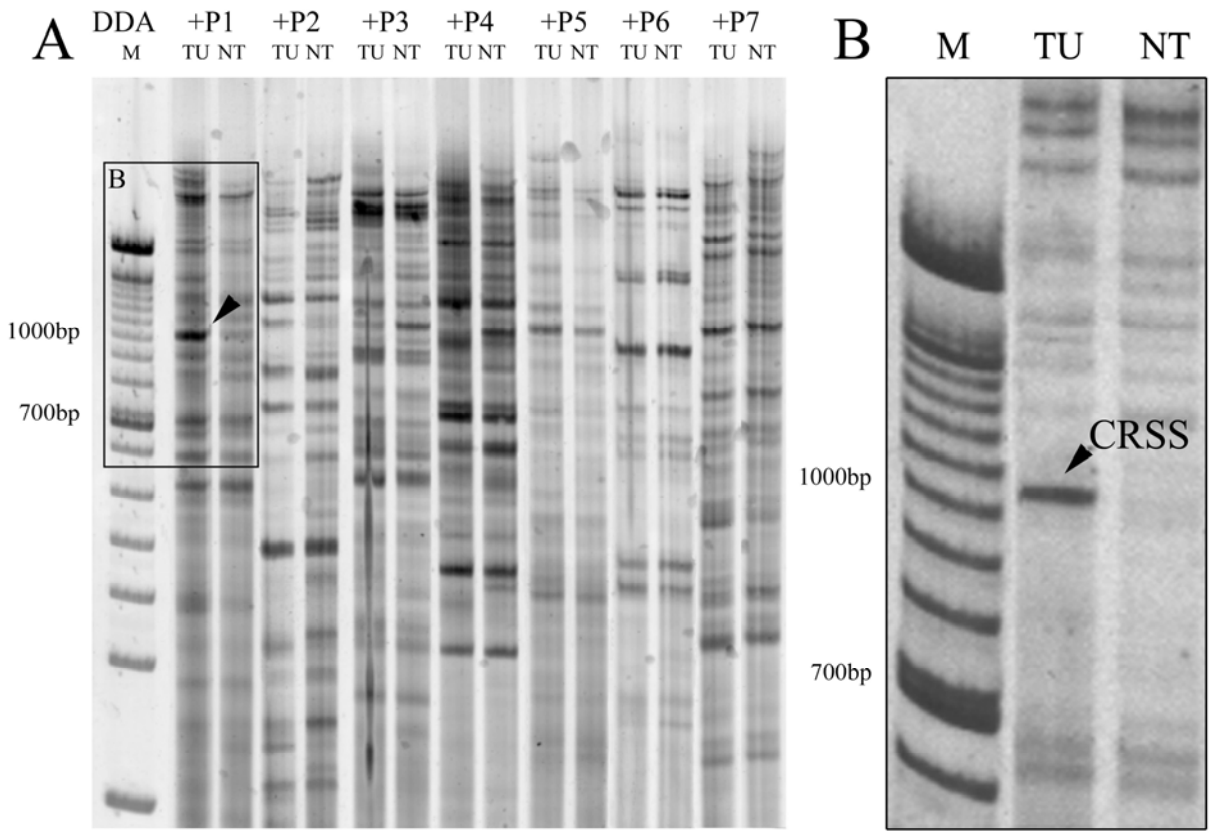

Figure 3.1: Differential display results Differential display RT-PCR analysis of total RNA samples from a poorly-differentiated HCC (TU) and the corresponding non-neoplastic tissue (NT) was carried out using poly-A tail directed T11MA primer, with seven 5' upstream arbitrary decamers (P1-P7), and polyacrylamide gel electrophoresis enabled a side-by-side comparison of the amplified fragments (A). An approximately 1 kb band (arrowhead) was expressed at a higher level in the HCC was chosen for further analysis and designated CRSS (B).

The 268 bp CRSS cDNA sequence showed almost 100% identity over the full 268 bp length of the partially sequenced CRSS fragment, when aligned with AK017254 (Figure 3.2). Since this sequence had been found in colon tissues, it was decided that colon cell lines and tissues should be included in further investigations.

The AK017254 sequence, along with the other highly homologous sequences, belongs to Unigene cluster Hs. 306777 Homo sapiens gasdermin-like) with reference sequence NM_018530. After a multiple alignment of all sequences, primers were designed to amplify a 399bp portion (210-608bp) of the GSDML cDNA Reference Sequence NM_018530 common to all splicing variants (Figure 3.3).

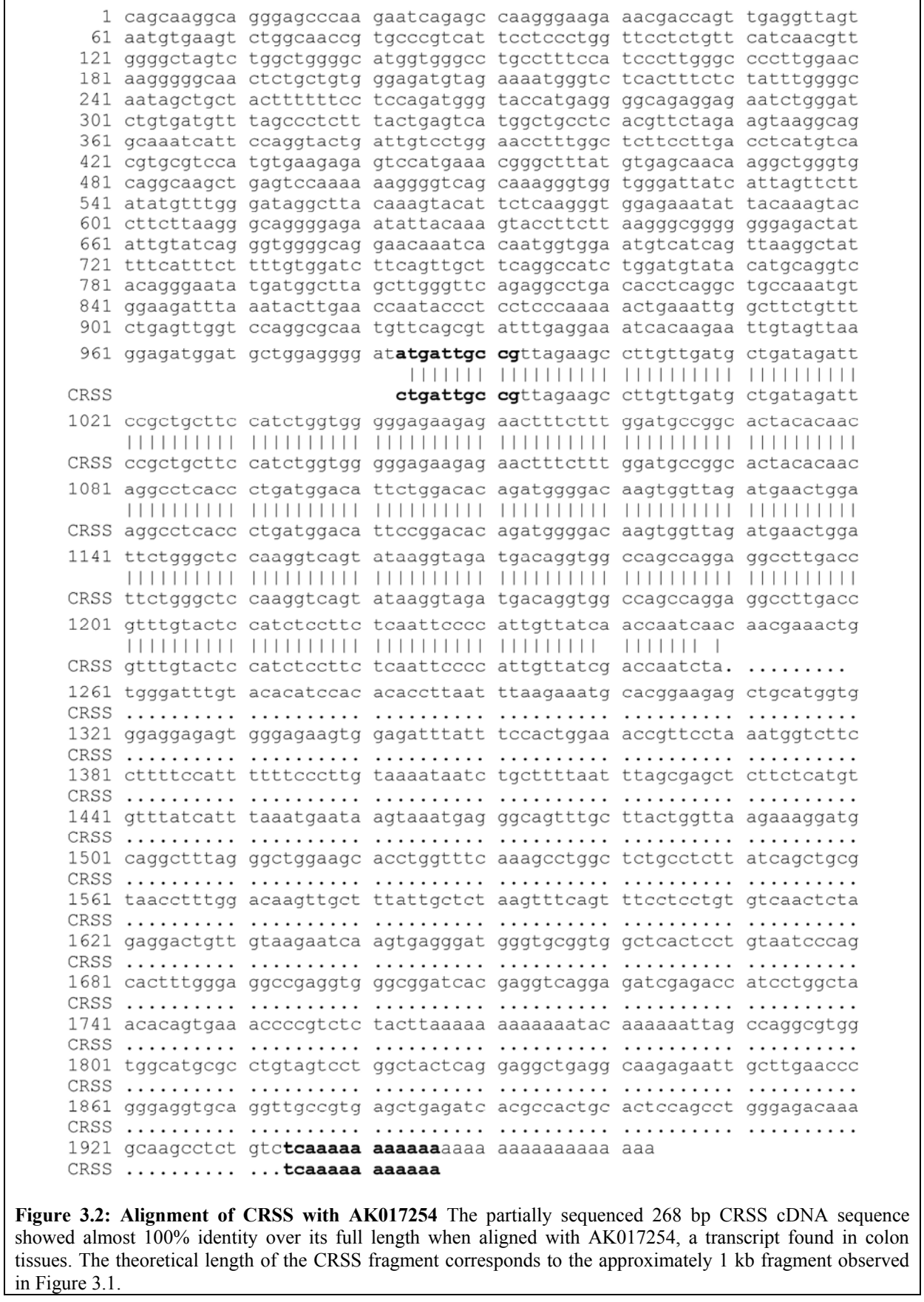

Figure 3.2: Alignment of CRSS with AK017254 The partially sequenced 268 bp CRSS cDNA sequence showed almost 100% identity over its full length when aligned with AK017254, a transcript found in colon tissues. The theoretical length of the CRSS fragment corresponds to the approximately 1 kb fragment observed in Figure 3.1.

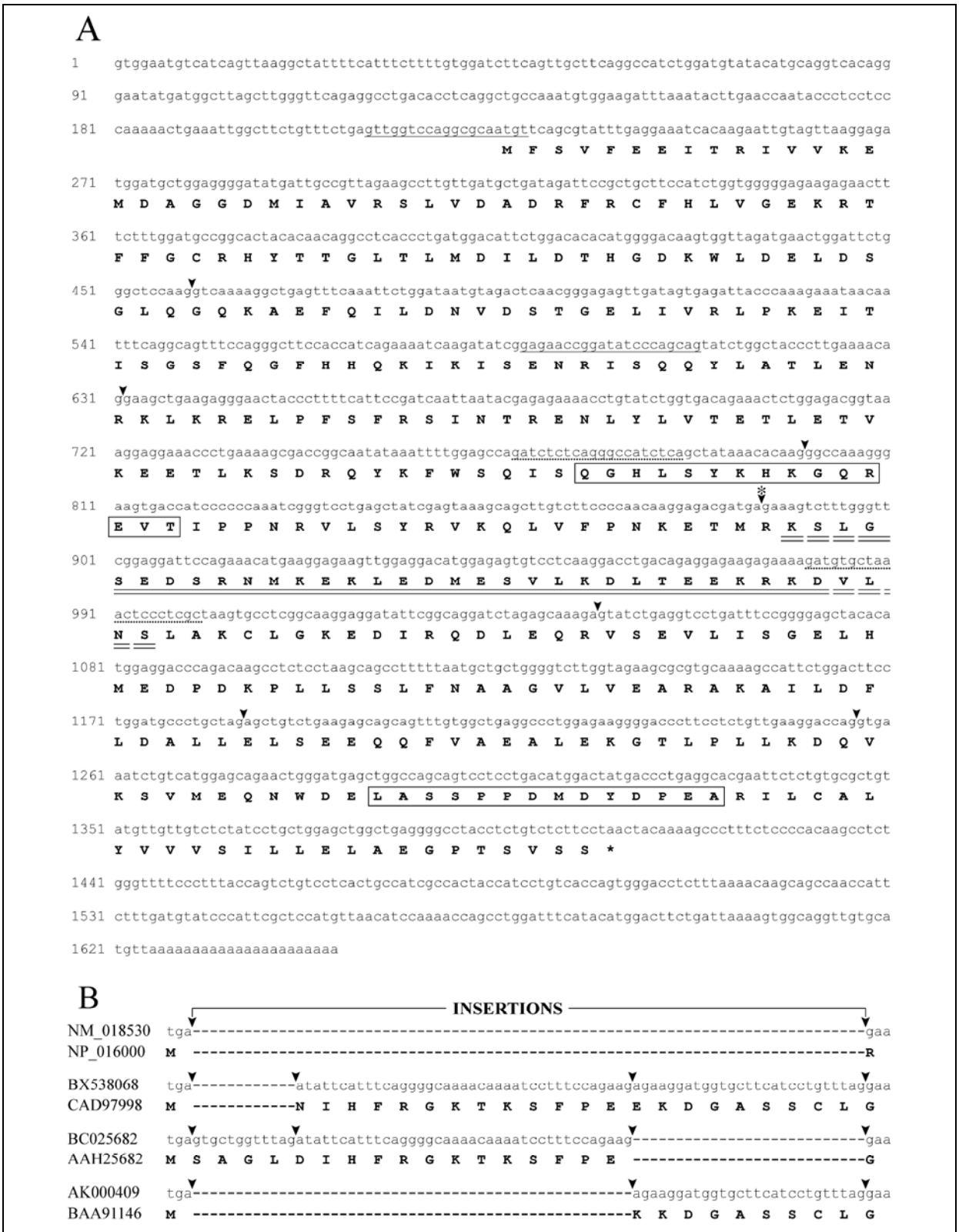

Figure 3.3: GSDML Reference Sequence cDNA and protein and splicing variants The human GSDML nucleotide sequence (RefSeq NM_018530) was used for the selection of primers for the semi-quantitative and real-time RT-PCR (underlined), and the corresponding amino acid sequence (NP_061000) for the selection of epitopical peptides for the production of anti-GSDML antibodies in rabbits (boxed) (**A**). The sequence contains eight exons (arrowheads), and sequence insertions have been documented for one of the exon junctions (asterisk). The protein contains a coiled coil motif directly following the point of insertion (double-underlined), and some of the insertion sequences will lead to longer proteins (**B**), whereas others result in premature truncation (not shown).

The amplified product was verified to be GSDML by sequencing, and the expression of GSDML mRNA was investigated in HCCs obtained from ten additional patients with HCC, using β2 microglobulin as a control for normal expression (Figure 3.4; not all shown). GSDML was detected in nine of the ten tumour (TU) samples (90%) and in only seven of the corresponding non-tumorous (NT) liver samples (70%). The same pattern of differential expression (TU>NT) as for the CRSS fragment was observed in the original HCC sample, as well as in five of ten other HCC cases (6/11; 54.5%).

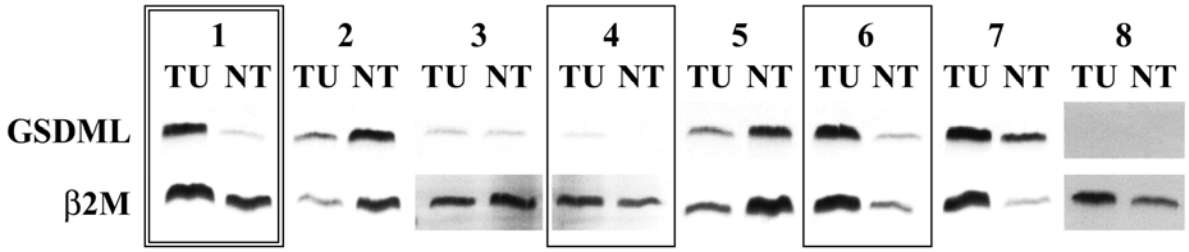

Figure 3.4: Verification of GSDML differential expression The expression of GSDML mRNA was investigated in HCCs obtained from additional patients with HCC, using β2 microglobulin (β2M) as a control for normal expression. The pattern of differential expression observed in the DDRT-PCR was confirmed (1; double line), and was also found in other cases (4, 6; single line). Note that not all investigated cases are shown here.

3.1.2 Transcription of GSDML in gastric, hepatic and colon carcinoma cell lines and tissues

Since the sequence with highest homology to CRSS had been found in colon, the expression of the GSDML mRNA in gastrointestinal cell lines and tissues was then examined, not only in liver and stomach, but also in colon cell lines and tissues. The GSDML gene transcript was expressed in each of the gastric, liver, and colon carcinoma cell lines investigated (Figure 3.5 A). We also analyzed the transcription of GSDML by quantitative RT-PCR in tumorous (TU) and non-tumorous (NT) samples obtained from twenty-one patients with gastric cancer (ten diffuse-type, eleven intestinal-type), fifteen patients with HCC, and nine patients with moderately differentiated adenocarcinomas of the colon (Figure 3.5 B). The GSDML transcript was expressed in diffuse-type gastric cancer tissues (TU: 3.1 ± 6.2, NT: 2.0 ± 4.1, P = 0.65), in intestinal-type (TU: 6.1 ± 5.5, NT: 36.0 ± 58.0, P = 0.11), in liver (TU: 1.3 ± 2.7, NT: 0.60 ± 0.55, P = 0.34), and in colon samples (TU: 0.14 ± 0.13, NT: 0.036 ± 0.028, P = 0.06). The expression pattern in intestinal-type was significantly different from diffuse-type gastric carcinomas. Samples from patients diagnosed with intestinal-type tumours exhibited higher expression levels than diffuse-type, with the expression in several non-neoplastic samples, as shown by the high mean and SD values, being many times higher than in tumour samples. However, the majority of the non-neoplastic samples demonstrated

expression levels close to the tumour samples, illustrated by the median bars in Figure 3.5 B, and there was no statistically significant difference between the expression in liver, gastric and colon carcinomas and the corresponding non-neoplastic tissues.

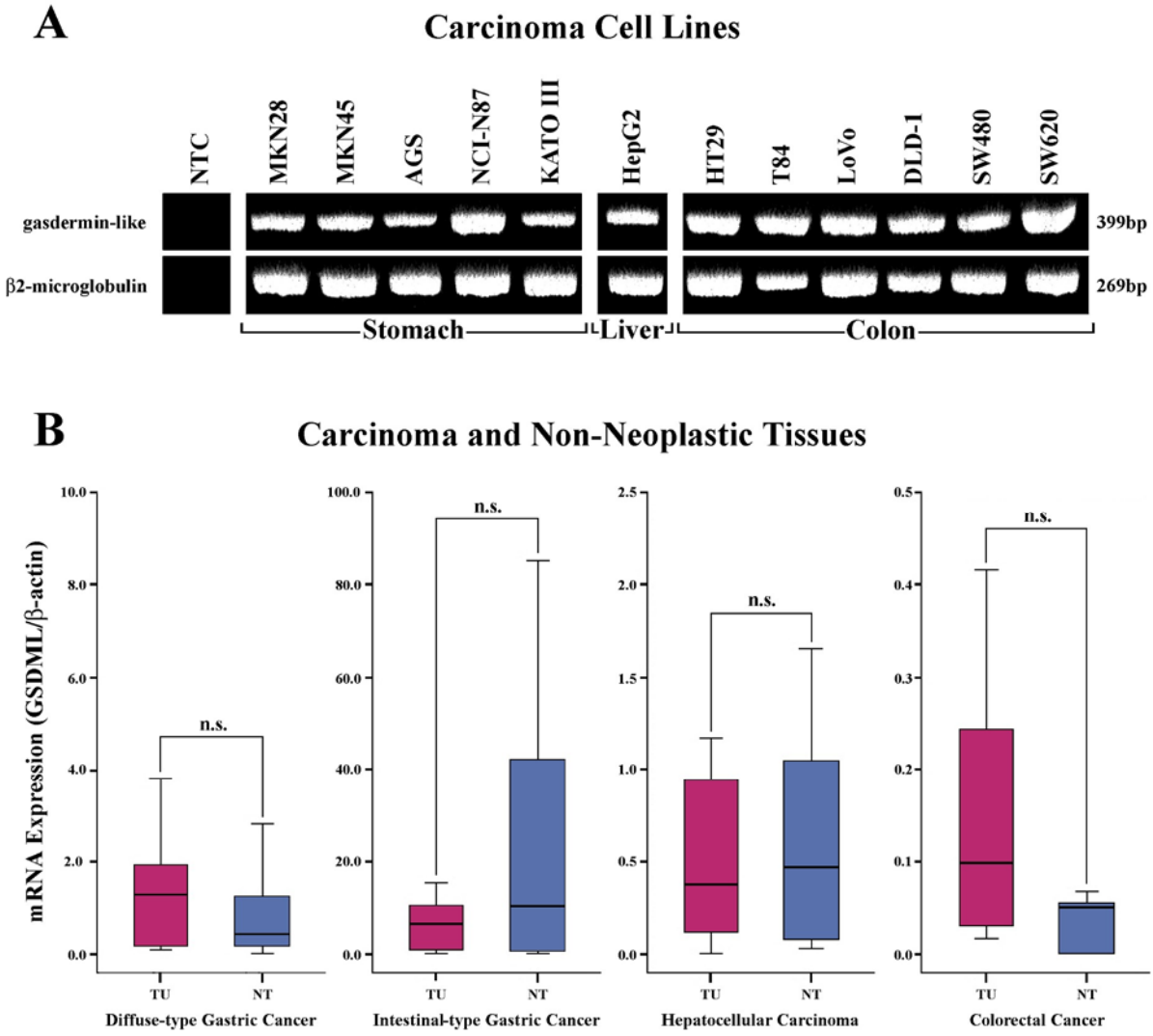

Figure 3.5: GSDML transcription in gastric, liver and colon cancer cell lines and tissues GSDML-mRNA was shown to be expressed in gastric, liver and colon cancer cell lines using semi-quantitative RT-PCR (**A**). Fluorescence-mediated quantitative *real-time* RT-PCR assays demonstrated that the expression level of GSDML-mRNA was not significantly different between the tumorous (TU) and corresponding non-tumorous (NT) samples obtained from 21 patients with gastric cancer (10 diffuse-type, 11 intestinal-type), fifteen patients with HCC, and nine patients with moderately-differentiated adenocarcinomas of the colon (**B**). Note that the scales differ for each graph, and the outliers and extreme values are not shown. Box boundaries: 25th and 75th percentiles; whiskers: 10th and 90th percentiles; solid line: median.

3.1.3 *In vitro* transcription and translation of GSDML

The full-length cDNA sequence of GSDML, cloned into a plasmid, was added to a single-tube, cell-free system, based on rabbit reticulocyte lysate. GSDML mRNA was then transcribed *in vitro* from the GSDML sequence, and subsequently translated, whereby biotinylated lysine was incorporated into the encoded recombinant protein. The *in vitro*

transcription/translation (IVTT) reaction was followed by SDS-PAGE and immunoblotting with streptavidin, or the anti-GSDML antibody described in section 2.4.2. The molecular weights (MW) of the encoded proteins were determined to be approximately 45 kDa and 47 kDa (Figure 3.6), which correspond to the predicted MW of the GSDML protein encoded by the plasmid (46.9 kDa). Immunoblotting with both streptavidin and the anti-GSDML antibody resulted in the detection of equivalent bands in samples with biotinylated protein, as well as in samples without biotinylation, indicating that the antibody specifically detects GSDML.

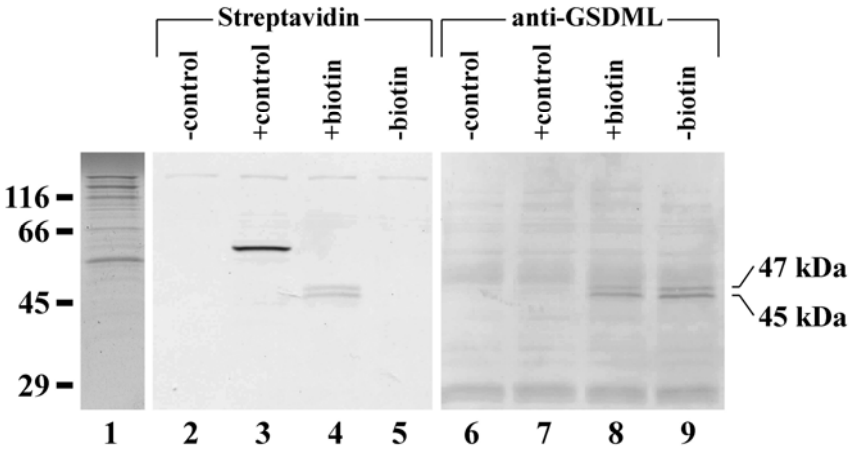

Figure 3.6: *In vitro* transcription/translation - GSDML protein size and antibody specificity A plasmid construct containing a full-length GSDML cDNA (BC025682) was transcribed and translated *in vitro*, and used in SDS-PAGE (Lane 1; Coomassie Blue staining), followed by immunoblotting. The protein was encoded with (Lanes 4,8) and without (Lanes 5,9) biotinylated lysine residues. A negative control containing no plasmid DNA (Lanes 2,6), and a positive control, encoding a 62 kDa product (lanes 3,7) were also included. Immunoblotting with streptavidin (left) specifically detected the Luciferase positive control and the biotinylated GSDML protein (Lane 4), but not the unbiotinylated GSDML protein (Lane 5). The anti-GSDML antibody (right) detected both biotinylated and unbiotinylated GSDML proteins (Lanes 8,9), without detecting positive control (Lane 7) or any of the proteins in the stained SDS-PAGE gel (Lane 1). A double band was detected by both streptavidin and the anti-GSDML antibody, and the molecular weights of the encoded GSDML proteins were determined to be approximately 47 kDa and 45 kDa, which corresponds to the predicted MW of 46.7 kDa.

3.1.4 Immunoblotting: Cells and Tissues

Stomach, liver and colon tumour cell lines, and tumour and non-neoplastic tissue samples were examined for GSDML protein expression (Figure 3.7). The anti-GSDML antibody consistently detected a number of bands, ranging between 35 kDa and 50 kDa in molecular weight, which were compatible with the experimental MW of the GSDML protein (47 kDa; see Section 3.1.3), as well as the predicted MWs of the GSDML splicing variants (Table 3.1), retrieved from the public databases.

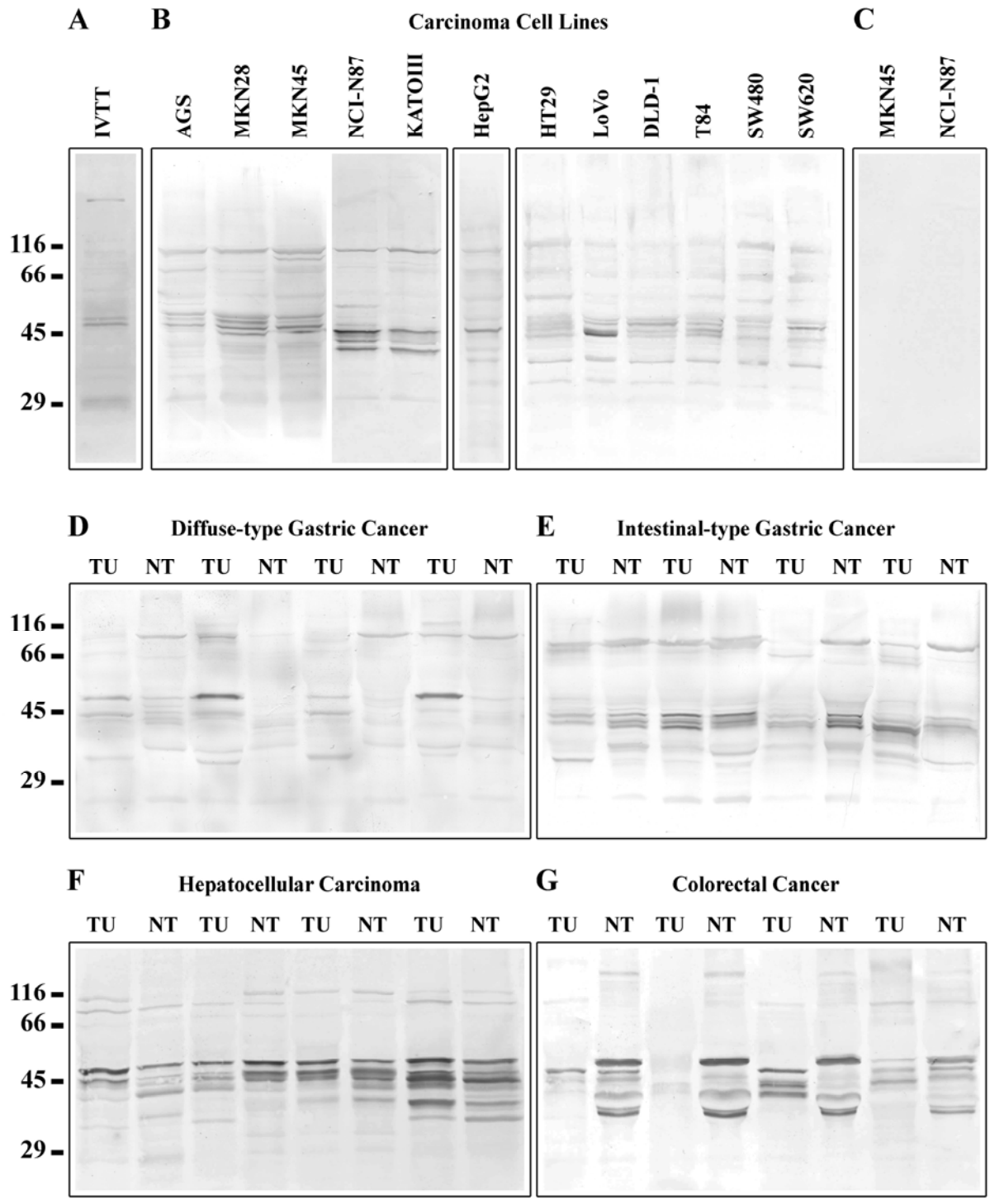

Figure 3.7: GSDML protein expression in gastric, liver and colon cancer cell lines and tissues Carcinoma cell lines and tissue samples from stomach, liver and colon cancer patients were examined for GSDML expression. The anti-GSDML antibody consistently detected a number of bands, ranging between 35 kDa and 50 kDa in molecular weight (MW), which were compatible with the experimental MW from the in-vitro transcription/translation (IVTT; **A**). Stomach, liver and colon tumour cell lines (**B**) reflected the general expression profile of the corresponding tumours, with the gastric cancer cell lines exhibited banding patterns related to their tumour of origin. Omission of the primary antibody served as a negative control (**C**). The expression profile of the tumour (TU) and non-neoplastic (NT) tissue samples (**D-G**) were compared: hepatocellular (**F**) and intestinal-type gastric (**E**) carcinomas demonstrated no obvious differences, whereas diffuse-type gastric (**D**) and colon tumours (**G**) exhibited distinctly different expression profiles.

3.1.5 Detection of GSDML splicing variants

Using RT-PCR, the expression of the GSDML mRNA splicing variants was examined in gastric cancer cell lines (Figure 3.8) with primers that were designed to bind either side of the insertion point (Figure 3.3). Various bands, ranging in size from 158 bp to 304 bp, were detected and isolated. Several bands were sequenced, and were clearly identified as GSDML. Unfortunately, the sequences of the exon insertions were unable to de defined.

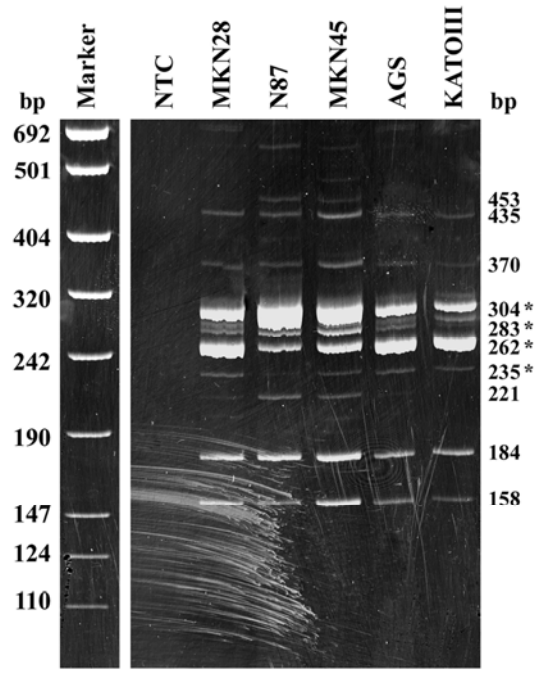

Figure 3.8: Detection of GSDML splicing variant mRNA Using RT-PCR, the expression of the GSDML mRNA splicing variants was examined in gastric cancer cell lines with primers that were designed to detect a majority of the splicing variants. After electrophoretic separation, the image was analysed and the size of the fragments was calculated. The fragments marked with asterisks correspond in size to the theoretical fragments expected from the amplification of the sequences with the insertions shown in Figure 3.3 B.

3.1.6 Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens were obtained from ten gastric cancers (5 intestinal-type, 5 diffuse-type), ten HCCs, and ten colorectal cancer specimens. The putative GSDML protein was localized using immunohistochemistry to the cytoplasm of epithelial cells in each tumour and non-tumorous tissue (Figure 3.8). The GSDML cytoplasmic immunoreactivity was usually characterized by a distinctive vesicular staining pattern in stomach, liver and colon tissues.

In the stomach, GSDML was not expressed in the foveolar epithelium (Figure 3.9 A,B). However, the chief cells of the gastric glands demonstrated the GSDML vesicular staining (Figure 3.9 C), and in intestinal metaplasia (Figure 3.9 D), GSDML was expressed towards the apical membrane. Diffuse-type gastric carcinomas showed very little immunoreactivity, with only isolated cells expressing GSDML (Figure 3.9 E). Intestinal-type tumours also expressed GSDML towards the apical surface (Figure 3.9 F). In the liver, normal hepatocytes (Figure 3.9 G,H) expressed GSDML in vesicles, and HCCs (Figure 3.9 I) exhibited higher immunoreactivity in a similar staining pattern to normal hepatocytes. The normal colonic surface mucosa and crypt epithelial cells expressed GSDML towards the apical and luminal surface (Figure 3.9 J), and, as for intestinal-type gastric cancers, colon cancers displayed an apically-orientated expression of GSDML (Figure 3.9 L).

In contrast to the subapical distribution of vesicles in chief cells and intestinal metaplasia of the stomach, and the surface mucous and crypt epithelial cells of the colon, certain cells of some of the gastric and colon glands (Figure 3.9 D and 3.9 K, respectively) exhibited exclusively basal expression for GSDML. Through double staining for chromogranin A and GSDML, this cell-specific expression was localized to a subset of neuroendocrine cells (Figure 3.10). The possibility that these cells were G-cells was excluded by subsequent staining of serial sections for gastrin. Staining for somatostatin (data not shown) also eliminated D-cells.

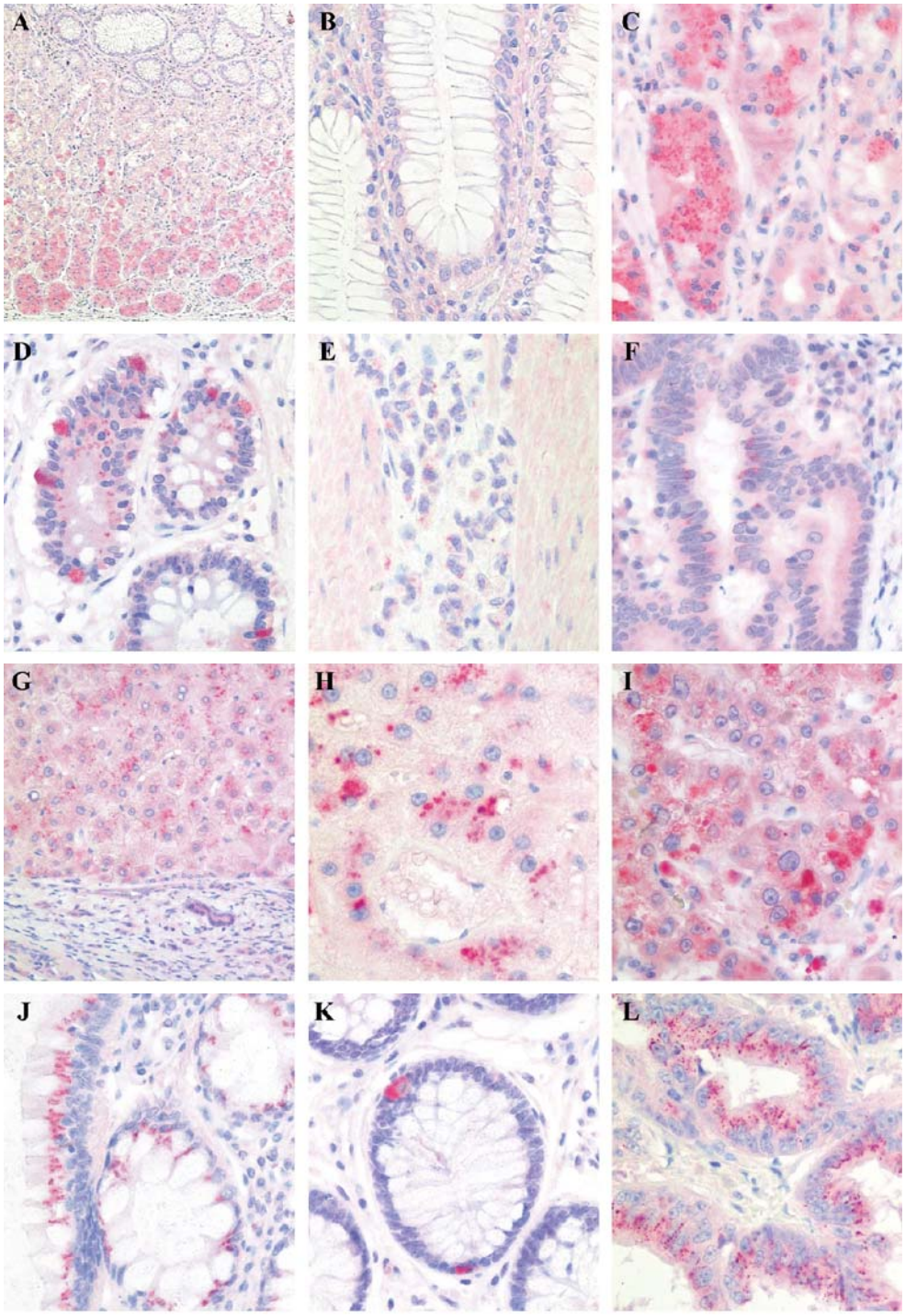

Figure 3.9: Immunohistochemical localization of GSDML protein in gastrointestinal tissues Using immunohistochemistry on paraffin-embedded tissue specimens, GSDML was found in the cytoplasm of each tumour and non-tumorous epithelium. In the stomach, the foveolar epithelium was negative (A,B), but chief cells of the gastric glands demonstrated the vesicular staining pattern that is distinctive for GSDML (A,C). However, certain sub-populations of the glandular cells exhibited a strong cytoplasmic immunoreactivity towards the basal surface (D). Diffuse-type gastric carcinomas showed very little immunoreactivity, with only isolated cells expressing GSDML (E). In contrast, intestinal-type tumours also expressed GSDML towards the apical surface (F). In the liver, fibrous septum was always negative (G) and normal hepatocytes surrounding the central hepatic vein expressed GSDML in vesicles (G,H). Hepatocellular carcinomas exhibited a similar staining pattern to normal hepatocytes, with the extent of immunoreactivity being higher (I). The normal colonic surface mucosa and crypt epithelial cells expressed GSDML towards the apical and luminal surface (J), and again, specific crypt cells exhibited a basal staining pattern (K). As for intestinal-type gastric cancers, colon cancers displayed an apically-orientated expression of GSDML (L). Original magnifications: 100X (A), 200X (G), 400X (B-F,H-L)

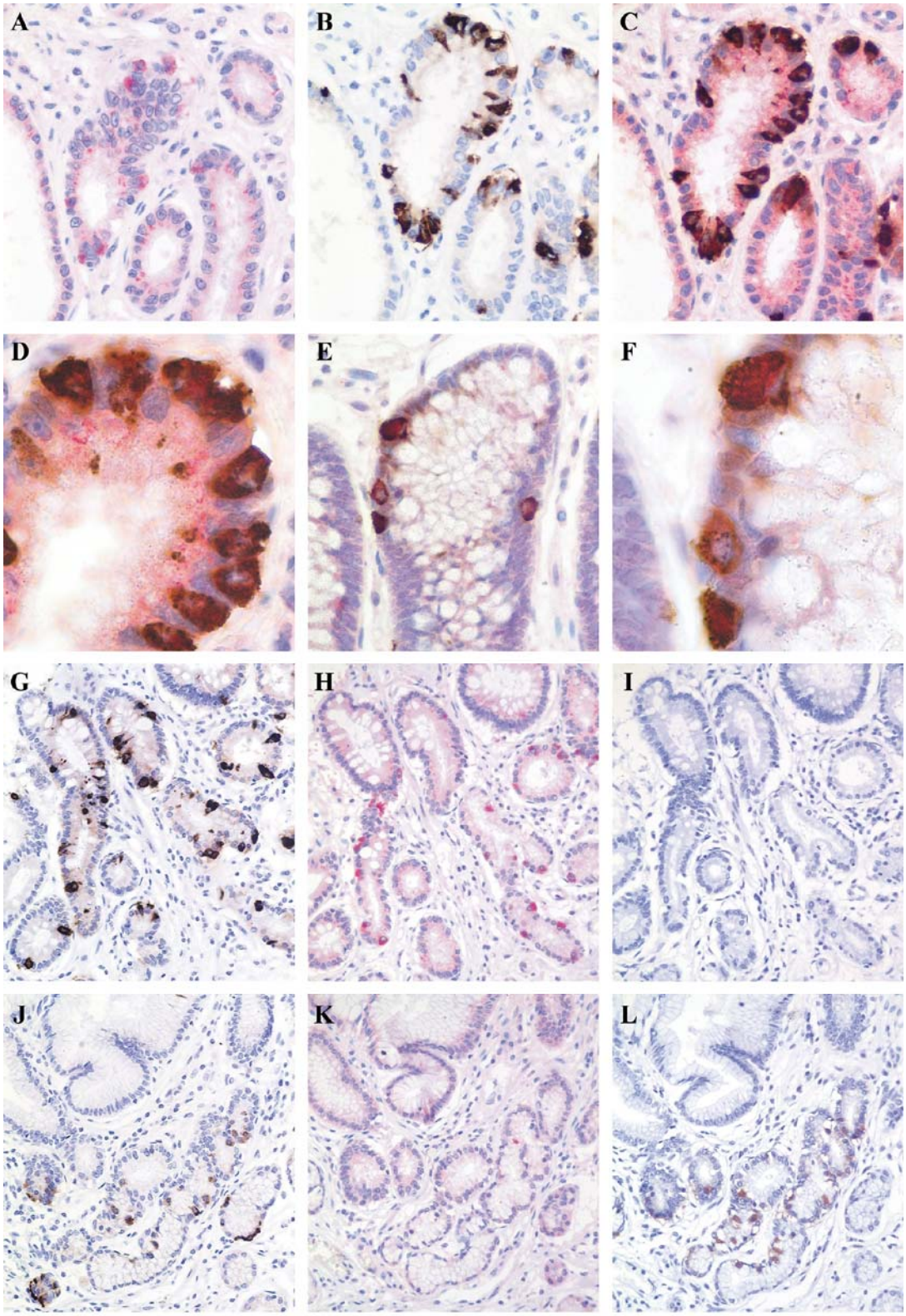

Figure 3.10: GSDML is localized to a subset of neuroendocrine cells Using immunohistochemistry on paraffin-embedded tissue specimens, GSDML (**A**) and chromogranin A (**B**) were shown to be co-expressed (**C, D**) in a subset of neuroendocrine cells in the stomach. This co-localization was also observed in the glands of the colon (**E, F**). By staining serial sections for chromogranin A (**G, J**), GSDML (**H, K**) and gastrin (**I, L**), it was demonstrated that the GSDML-expressing cells are a subset of neuroendocrine cells distinct from the gastrin-expressing cells (G-cells) of the stomach. Original magnifications: 200X (**G-L**), 400X (**A-C,E**), 1000X (**D,F**)

3.2 Discussion

Using differential display (DD) on HCC and non-tumorous liver tissue samples, we identified a candidate oncogene, which showed higher expression in tumorous than in the corresponding non-tumorous tissue (Figure 3.1). The approximately 1 kb fragment, designated CRSS, was only able to be partially sequenced. However, a BLAST alignment with the recovered 268 bp CRSS sequence returned, apart from genomic DNA sequences corresponding to chromosome 17, one transcribed mRNA sequence (AK025174) with 99% homology for the full 268 bp sequence (264 bp identical) alignment shown in Figure 3.2. Another seven mRNA sequences also exhibited very high homology for a 175 bp segment (94-99%; 166-174 bp identical). All eight returned sequences belonged to the UniGene cluster Hs. 306777 for Homo sapiens GSDML, and the transcribed protein belongs to the gasdermin domain-containing (GSDMDC) protein family (Bateman et al 2002). The cluster reference sequence (NM_018530) was used to select new primers for verification of the differential expression pattern (Figure 3.3). The 399bp product was sequenced and a BLAST alignment returned the same result as for CRSS, confirming that the newly selected primers were specific for GSDML. RT-PCRs carried out for the new GSDML product showed the same pattern of differential expression in the original HCC sample, and also in five of ten other HCC cases (Figure 3.4, not all shown).

Using differential display, the novel gasdermin-like (GSDML) gene was here first identified as being up-regulated in HCC. The protein encoded by this gene belongs to the cancer-associated gasdermin-domain containing (GSDMDC) protein family. Other members of the GSDMDC protein family, GSDM, DFNA5, and MLZE, have been brought into association with cancer development and progression, and it is probable that the other members are also involved in cancer. Although none of the members of this family have been functionally characterized, this protein family seems to contribute to a diverse range of cellular processes, known to be related to the development and progression of cancer, such as differentiation (Thompson&Weigel 1998, Runkel et al 2004, Lunny et al 2005), cell-cycle control (Gregan et al 2003), apoptosis (Gregan et al 2003, Van Laer et al 2004, Lage et al 2001), extracellular matrix production (Busch-Nentwich et al 2004), and invasion (Watabe et al 2001). Additionally, the spatial expression of the members of the GSDMDC family is highly restricted. GSDM is expressed exclusively in skin and stomach in humans (Saeki et al 2000) and the GSDM homologues in mouse exhibit a similar expression pattern (Runkel et al 2004, Lunny et al 2005). MLZE is expressed primarily in the trachea and spleen (Watabe et al 2001). Despite the detection of DFNA5 ESTs in libraries from a wide range of tissues

(Unigene), northern blot analyses do not reflect the UniGene data, but demonstrate that DFNA5 is predominantly expressed in placental, fetal, and cochlear tissues (Van Laer et al 1998, Thompson&Weigel 1998, Maeda et al 2001). In addition to these family members, it is probable that the other members are also involved in cancer.

Here, the expression and putative involvement of GSDML, a novel member of the GSDMDC family, in gastrointestinal cancer biology was studied. The GSDML gene does not exist within the mouse or rat genome (Katoh&Katoh 2004a), and may have evolved independently in humans through a GSDM-gene duplication (Runkel et al 2004, Katoh&Katoh 2004a, Katoh&Katoh 2004b). The GSDML gene is situated close to the GSDM gene on chromosome 17q21.1 (Katoh&Katoh 2003). This chromosomal region, for which loss of heterozygosity, amplifications or allelic gains have been reported in cancers of the stomach (Kokkola et al 1997, Sakakura et al 1999), liver (Nishida et al 2003, Wang et al 2001), and colon (Garcia-Patino et al 1998, Knosel et al 2002, Knuutila et al 2000), is an evolutionary recombination hotspot closely linked to the oncogenic ErbB2 locus (Katoh&Katoh 2004a). Katoh and Katoh suggest that, since oncogenes and tumour suppressor genes are usually clustered around recombination hotspots or fragile sites, and other family members are known to be down-regulated in cancer, GSDML is a candidate tumour suppressor gene (Katoh&Katoh 2004a, Katoh&Katoh 2004b). Contrasting with the restricted expression observed for GSDM, GSDML has previously been detected in libraries from a wide range of tissues, including those of the gastrointestinal tract (UniGene). This investigation is the first ever to compare the expression of GSDML in hepatocellular, gastric and colorectal carcinomas with non-lesional tissues. In addition to examining the differential transcription of GSDML in tumorous and non-tumorous epithelial tissue, an antibody directed against GSDML was developed. This has enabled the tissue-associated localization of GSDML in liver, stomach and colon tissues, as well as the determination of its molecular weight, and the expression profile in tumorous and non-tumorous tissue. Since the functions of this protein family are as yet unknown, it is hoped that information about GSDML will provide clues towards the role of GSDMDC proteins, not only in cancer, but also in cellular biology and metabolism.

In contrast to GSDM, which could not be detected in any of the cancer cell lines investigated (Saeki et al 2000), we found GSDML gene transcription in all of the gastric, liver and colon cancer cell lines investigated, and also in human gastric, hepatic and colorectal tumours. GSDML expression was also found in non-tumorous tissues, with no significant difference in transcription levels observed between tumour and non-tumour samples.

Although DFNA5 and MLZE are not at all expressed in the gastrointestinal tract and GSDM is spatially restricted to the stomach and esophagus, GSDML ESTs (UniGene) have been detected in blood, bone, lymph node, pancreas, stomach, colon, kidney, liver, lung, mammary gland, muscle, brain, peripheral nervous system, placenta, prostate, testis, thymus, and uterus, indicating that GSDML may not be subject to the extremely defined tissue- and organ-specific regulation of the GSDMDC family. However, as for DFNA5, the EST expression data may be unreliable, and might not reflect the true expression pattern.

Recombinant GFP- or BFP-constructs of DFNA5 and GSDM, respectively, have been localized to the cytoplasm of cultured cells (Van Laer et al 2004, Saeki et al 2000), and the expression of DFNA5, a mouse GSDM homologue, and MLZE has been examined in zebrafish embryos, mouse skin, and human melanomas and skin tissues, respectively, by in situ hybridization (Busch-Nentwich et al 2004, Runkel et al 2004, Watabe et al 2001). However, of the GSDMDC protein family members, only GSDM protein expression has been investigated. Lunny and colleagues developed a polyclonal antibody that detects both human GSDM and the mouse GSDM homologues, and examined the expression of the gasdermins in mouse and human skin and mouse stomach (Lunny et al 2005). In both mouse and human skin, GSDM expression was restricted to the well-differentiated cells of the upper layer of the epidermis, the inner root sheath, the hair shaft, and the sebaceous glands. In the gastrointestinal tract, gasdermins are expressed in the suprabasal layers of the esophagus, the layers of the fore-stomach epithelium, and in chief cells of the glandular stomach.

In this study, the protein expression of GSDML, a novel GSDMDC protein family member, was examined for the first time, using antibodies developed against peptide epitopes from the putative GSDML protein sequence (Figure 3.3). The characterization of accessible epitopes was based on the probable tertiary structure of the putative protein (Rost 1996). The specificity of the antibodies for GSDML was verified with an IVTT reaction, and the molecular weights of the transcribed GSDML recombinant proteins were determined to be 45 kDa and 47 kDa, in keeping with the predicted MW of 46.9 kDa. Analysis of the putative GSDML protein sequence (NP_061000) using PSORTII (Nakai&Horton 1999) predicted that the GSDML protein would be located in the cytoplasm or in the vesicles of the secretory system. The cellular localization and tissue-related expression pattern of the GSDML protein was examined using the anti-GSDML antibody. The GSDML protein was localized with immunohistochemistry to the cytoplasm of cells in both tumour and non-lesional tissues, and exhibited a distinctive vesicular staining pattern. Since GSDM is the closest relative to GSDML, the expression pattern of GSDM may shed some light on the possible functions of

GSDML. While GSDM expression is restricted to the upper gastrointestinal tract, with high expression in esophagus, and fore-stomach, and lower expression levels in glandular stomach, we have shown that GSDML is expressed in the stomach, liver and colon tissues of the lower gastrointestinal tract. Both GSDM and GSDML are expressed in the chief cells of the glandular stomach. GSDML was localized to subapical vesicles of gastric chief cells, and the colonic surface mucosa and crypt epithelial cells, as well as in the basal vesicles of a subset of neuroendocrine cells. All these cell types are involved in the secretion of cytoprotective growth factors, proteinases, peptide hormones and other signaling molecules. In addition to the vesicular staining pattern, this leads to the conjecture that GSDML may be a secretory or metabolic product involved in a secretory pathway. Interestingly, GSDM expression is restricted to well-differentiated cells in both stomach and skin, with the GSDM-expressing cells of the epidermis in very late stages of differentiation, immediately prior to the initiation of programmed cell death (Lunny et al 2005). Since DFNA5 has also been brought into association with apoptosis (Van Laer et al 2004, Gregan et al 2003, Lage et al 2001), this may indicate that GSDMDC family members may play a role here as well.

The vesicular expression of GSDML in the majority of cells in intestinal-type gastric, hepatocellular and colon carcinomas suggested that the function of GSDML was maintained in these tumours, compared to the diffuse-type carcinomas, where only isolated cells expressed GSDML. This reflects the expression pattern reported for GSDM in basal and squamous cell carcinomas, and the possible role in differentiation suggested for GSDM [16] may well also apply to GSDML.

Immunoblotting of the stomach, liver and colon cell lines and tissues detected an array of bands of the approximate expected MW, the range of which varied not only with the tissue of origin (stomach, liver, colon), but also between tumour and non-tumour, particularly in diffuse-type gastric and colon cancer patients. Although not always all bands were detected and the relative amounts of each band varied, the tissue-handling procedures and the loading amount of protein were the same for each sample analysed. Additionally, the appearance of several bands is highly unlikely to be due to proteolytic activity, since the tissues were resected and shock-frozen within half an hour of the operation, then prepared on ice in buffers containing protease-inhibitors. Since tumour samples were compared only with non-tumorous tissue samples from the same patient, the observed differences could be attributed to the presence of the tumour. As can be seen in Figure 3.7, there is no noticeable difference in the banding pattern between tumour and non-lesional tissue samples in patients with hepatocellular or intestinal-type gastric carcinomas. However, in diffuse-type gastric and

colon cancer patients, the GSDML expression profile is distinctly different in tumour compared to non-tumorous tissues in each patient investigated. Interestingly, the gastric cancer cell lines exhibited banding patterns related to the tumour of origin, and the liver and colon cancer cell lines reflect the general expression profile of the corresponding tumours.

A database search (Entrez) retrieves several different full-length GSDML transcript sequences, predicting a range of amino acid sequences with various lengths (Table 3.1, Figure 3.3). From sequence analysis, it can be seen that the range of transcripts results from sequence insertions at one of the exon junctions, indicating that alternative splicing at this intron-exon junction may be a method of regulation of GSDML expression. To ascertain whether the range of protein bands could be the result of variations in exon splicing, primers were designed to amplify a product containing the insertion site. According to the sequence databases, the sizes of the splicing variants PCR products should theoretically range between 135bp and 951bp. The gastric cancer cell lines were examined using RT-PCR (Figure 3.8), and bands ranging in size from 158bp to 453bp were detected. Interestingly, the four bands identified with asterisks in Figure 3.8 correspond in size to the expected fragments of NM_018530 (235bp), AK000409 (262bp), BC025682 (283bp), and BX538068 (304bp), containing the insertion sequences shown in Figure 3.3. A number of bands were isolated and sequenced. The initial segments of the each of the amplified fragments was able to be clearly identified as GSDML. However, the exon insertions were unable to de defined, believed to be due to difficulties in precisely isolating the individual bands.

This range of possible splicing variants may explain why the overall level of gene transcription does not significantly differ between tumour and non-tumour (Figure 3.5), despite the differential display identification of GSDML as being differentially expressed in HCC. The quantitative RT-PCR analysis was not designed to differentiate between the splicing variants, and any alteration in the relative expression levels of the different transcripts would not have been detected. A more detailed analysis of GSDML mRNA expression, with particular reference to the differential expression of the diverse splicing variants may provide further evidence for the role of GSDML in gastrointestinal cancers.

The antibody seems to detect all GSDML alternative splicing variants and specific GSDML proteins seem to be up- or down-regulated in tumour samples (Figure 3.7). The altered regulation of GSDML protein variants between tumours and non-lesional tissues can be seen clearly in diffuse-type gastric cancers and colon carcinomas and may either be directly involved in cancer biology, or simply a symptom of a cancer-related dysfunction in transcriptional or translational regulatory pathways. The mechanism that accounts for the

distinctive expression pattern is not known, and thus far, attempts to purify the protein(s) for sequencing and further analysis have been unsuccessful.

3.3 Conclusion

The expression of the GSDMDC proteins is highly regulated, in an organ- and tissue-specific manner, with the expression of DFNA5, MLZE and GSDM being spatially restricted. GSDML is not at all co-expressed with DFNA5 and MLZE, and only in the stomach do GSDM and GSDML coincide, which is to be expected given their close evolutionary relationship. The expression consistently detected in gastric, liver and colorectal cancer cell lines is intriguing and indicates that GSDML might be directly or indirectly involved in tumour biology, particularly at the normal organ- or tissue-expression site. The vesicular expression pattern of GSDML does not change during the progression to tumour, as is also reflected by the transcript amounts detected by RT-PCR, suggesting that the possible secretory or metabolic function of GSDML is still carried out at the tumour site. However, the immunoblotting demonstrated that the expression profile of the GSDML splicing variants can differ between tumour and non-tumour samples. The observed alteration in transcriptional or translational regulation may affect the tumour-related metabolism, thereby influencing the tumour growth and development. A larger tissue- and/or tumour-specific analysis of the expression of the GSDML gene and transcripts, as well as more information about the actual protein sequence and structure, will be necessary before a function can be assigned to this protein and its possible role in cancer biology clarified.

4 RESULTS AND DISCUSSION: ECTOPEPTIDASES IN GASTROINTESTINAL CARCINOMA

This chapter focuses on the elucidation of the possible role played by a number of ectopeptidases in the tumour biology of gastrointestinal cancers, with a view to identifying possible diagnostic markers or therapeutic targets. Alterations in the expression patterns of the ectopeptidases (NEP, APN, DPIV, ACE, ADAM9, ADAM12, ADAM15) in hepatocellular and gastric carcinomas compared to non-neoplastic tissues were investigated, and the influence of these proteases on the *in vitro* proliferation of liver and gastric cancer cell lines evaluated.

4.1 Results

4.1.1 Immunohistochemistry

The expression of NEP, APN and ACE was examined in paraffin-embedded sections from 24 HCC patients (17 men, 7 women; mean age 63.4 ± SD years), and 40 gastric cancer patients (24 men, 16 women; mean age 65.4 ± 11.9 years; 19 diffuse, 21 intestinal). DPIV immunostaining was carried out on frozen sections of tumour and non-tumour tissue from 3 HCC patients, and 10 gastric cancer patients (4 men, 6 women; mean age 66.9 ± 10.8 years; 5 diffuse, 5 intestinal). ADAM9, ADAM12, and ADAM15 expression was examined in paraffin-embedded sections from 24 gastric cancer patients (11 men, 13 women; mean age 65.8 ± 12.8 years; 12 diffuse, 12 intestinal). The expression of the ectopeptidases was examined in HCCs and normal hepatocytes in the liver, and in the stomach, the non-tumorous luminal and foveolar epithelium, any occurrences of intestinal metaplasia, and the carcinomas were investigated. The distribution patterns of the ectopeptidases in tumour and non-lesional tissues were compared. In stomach tissues, the differential expression between tumour cells and the mucosal regenerative zone evaluated, whereby the expression in the mucous neck, chief, and parietal cells was disregarded. Ten gastric cancer lymph node metastases were also investigated for the expression of NEP, APN, and ACE. Expression of ACE was also evaluated in 31 gastric biopsy specimens (14 with and 17 without histological evidence of *H. pylori* infection), and 13 gastric ulcer resection specimens (9 with adjacent mucosa).

4.1.1.1 NEP is down-regulated in HCCs, but up-regulated in gastric cancer

In liver tissues, NEP was expressed in tumour cells in 13 of 20 (55.0%) cases, and in non-tumorous tissues in 19 of 20 (95.0%) cases. As shown in Figure 4.1, NEP was found in the cytoplasm and at the cell surface of both neoplastic and non-neoplastic hepatocytes, with a prominent membranous staining pattern being observed in seven (35%) HCCs and a canalicular pattern in 11 (55%) HCCs. NEP was also present at the luminal surface of bile duct epithelium. NEP was expressed significantly more commonly in non-neoplastic liver tissue than in HCCs (P=0.02).

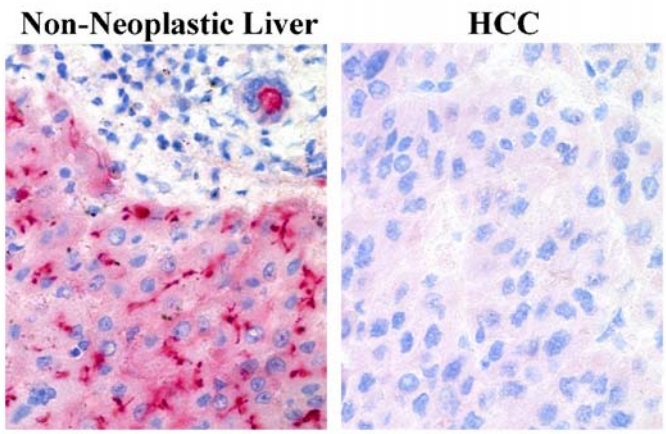

Figure 4.1: NEP expression in HCCs The distribution pattern of NEP was investigated by immunohistochemistry in non-lesional tissues and in HCCs. NEP was expressed at a significantly lower level in neoplastic than in non-neoplastic hepatocytes. Anti-NEP antibody and hematoxylin counterstain; Original magnification: x400

In the stomach (Figure 4.2), the non-tumorous mucosal epithelium of the regenerative zone (surface mucosa and foveolar epithelium) was negative, with the mucous neck cells, chief cells and parietal cells of the deep gastric glands expressing NEP. NEP was expressed in tumour epithelial cells of 31 of 40 (77.5%) cases. NEP exhibited intense immunoreactivity in the intestinal metaplasia in 13 of 15 (86.6%) cases. NEP expression was observed in 5 of 10 (50%) lymph node metastases, and in 8 of 10 (80%) corresponding tumours. Highly significant up-regulation of NEP was observed in tumour tissues (χ^2-test; P<0.01)

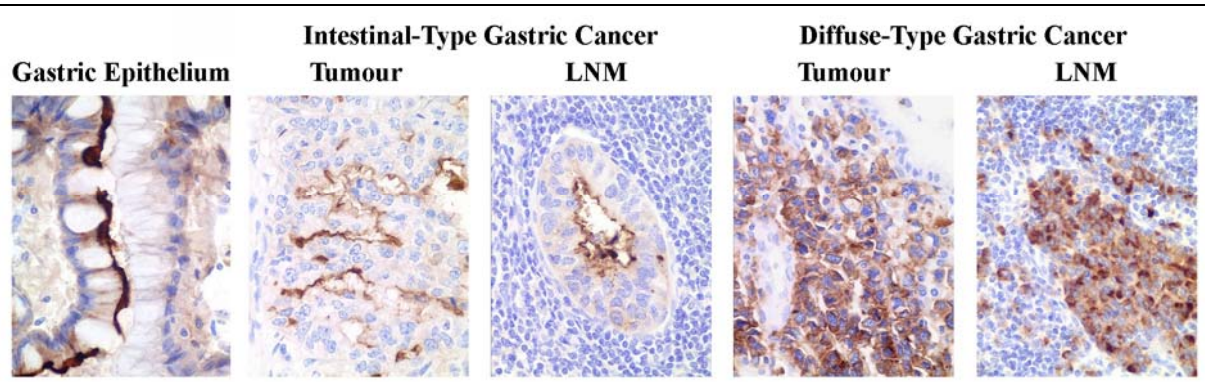

Figure 4.2: NEP expression in gastric cancer The distribution pattern of NEP was investigated by immunohistochemistry in non-lesional and gastric cancer tissues. NEP was not expressed in the foveolar epithelium, but was expressed in the deep gastric glands, strongly in intestinal metaplasia, in both intestinal-type and diffuse-type gastric cancers, and in metastatic tumour cells. Anti-NEP antibody and hematoxylin counterstain; Original magnification: x400.

4.1.1.2 APN is expressed in bile canaliculi of the liver, and is upregulated in gastric cancer

The prevalence of APN in non-neoplastic liver tissue and HCCs was identical, with both tumour (20/20; 100%) and non-lesional tissues (20/20; 100%) exhibiting APN immunoreactivity. However, overall immunostaining was more intense in HCCs than in non-neoplastic liver tissue (Figure 4.3). APN showed the same distribution pattern as NEP (Figure 4.1) with membranous staining in 14 of 20 (70%) cases and a canalicular staining of neoplastic and non-neoplastic hepatocytes in 20 of 20 (100%) cases.

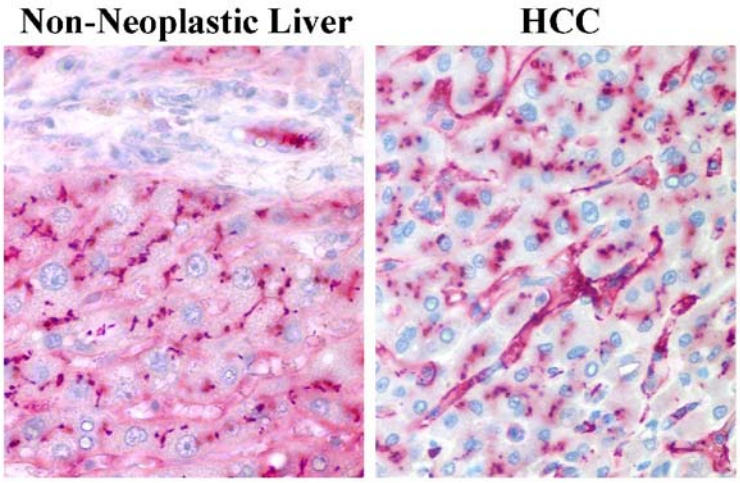

Figure 4.3: APN expression in HCCs The distribution pattern of APN was investigated by immunohistochemistry in non-tumor hepatocytes and HCCs. APN was expressed by non-neoplastic and neoplastic hepatocytes, and bile duct epithelium. Anti-APN antibody and hematoxylin counterstain; Original magnifications: x40.

The gastric surface and foveolar epithelium, and the cells of the deep gastric glands were all negative for APN, whereas tumour epithelial cells expressed APN in 21 of 40 (52.5%) cases. The intestinal metaplasia was consistently positive, with strong staining observed in all cases where it could be found (13/13; 100%). Expression of APN was found in the lymph node metastases of 8 of 10 (80%) cases, and in the corresponding tumours of 7 of 10 (70%) cases. Since the non-lesional tissues were completely negative, the expression of APN was found to be highly significantly different between tumour and non-tumorous tissues (χ^2-test; $P<0.01$).

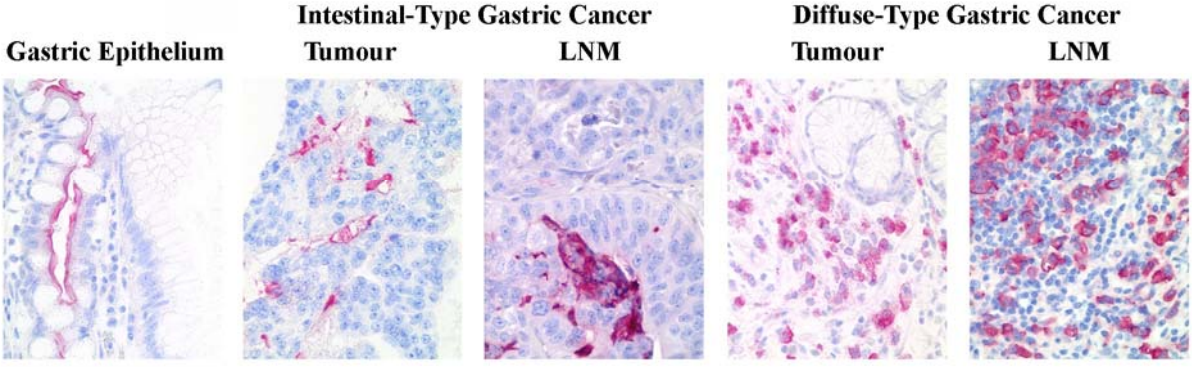

Figure 4.3: APN expression in gastric cancer The immunohistochemical distribution pattern of APN was investigated in non-lesional and gastric cancer tissues. APN was strongly expressed by the intestinal metaplasia and in the lymphatic metastases, and by both intestinal-type and diffuse-type gastric cancers, but not by the foveolar or glandular epithelium. Anti-APN antibody, hematoxylin counterstain; Original magnifications: x400.

4.1.1.3 DPIV is expressed in both tumour and non-lesional tissues of the liver and stomach

In the liver, DPIV was found at the cell surface in tumour cells, hepatocytes and bile duct epithelium, and showing a canalicular distribution pattern in HCCs and non-neoplastic liver parenchyma (Figure 4.5). However, since frozen tissue was available from only three patients, a statistical comparison of the expression in tumour with non-tumour tissue was not possible.

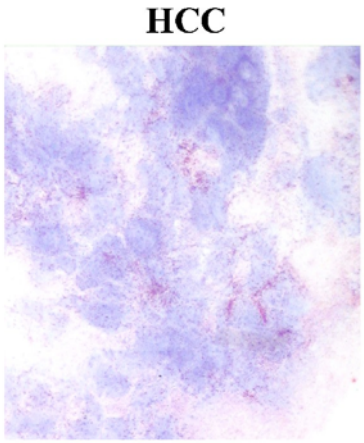

Figure 4.5: DPIV expression in HCCs Using immuno-histochemistry on frozen sections of unfixed tissue, CD26 was found at the cell surface in tumor cells, hepatocytes and bile duct epithelium showing a canalicular distribution pattern. Anti-DPIV antibody and hematoxylin counterstain; Original magnification: x400.

In gastric tissues, DPIV was expressed in 7 of 10 samples (70%) of tumorous epithelium and also in 4 of 10 samples (40%) of non-tumour tissue. The luminal surface and foveolae were occasionally positive for DPIV, whereas the gastric glands were always negative. Incidences of intestinal metaplasia were all positive (3/3; 100%). DPIV expression could not be investigated in the lymph node metastases, as unfixed tissue of lymph node metastases was not available. No significant differences were found for the expression pattern of DPIV (χ^2-test; P=0.18).

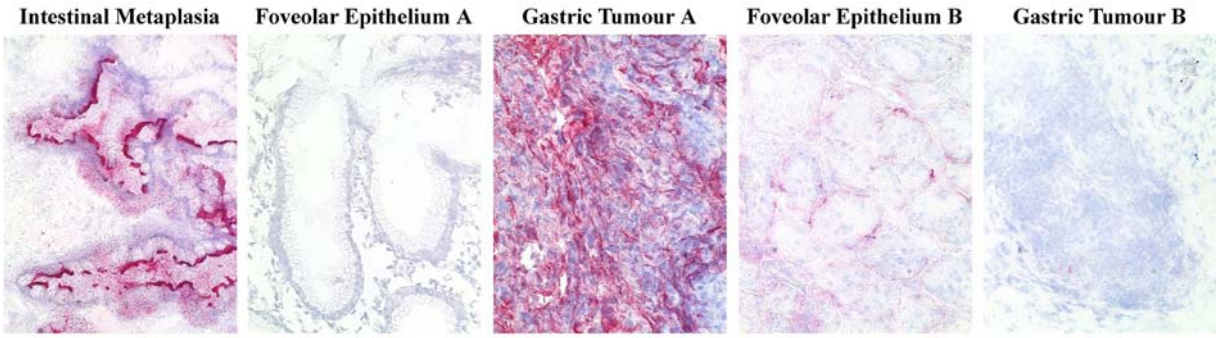

Figure 4.6: DPIV expression in gastric cancer Using immunohistochemistry on frozen sections of unfixed tissue, the distribution and expression pattern of DPIV was investigated in non-tumorous epithelium, and gastric carcinomas. DPIV was strongly expressed in intestinal metaplasia. Interestingly, in cases exhibiting no DPIV expression in the non-lesional foveolar epithelium, the expression of DPIV in the corresponding tumour was invariably higher (**A**), whereas when the non-lesional tissues expressed DPIV, the tumour lacked DPIV expression (**B**). Anti-DPIV antibody and hematoxylin counterstain; Original magnification: x400.

4.1.1.4 ACE is expressed by endothelial cells, and upregulated in gastric cancer

In non-neoplastic liver, ACE was expressed by endothelial cells of portal vessels and sinusoids, by the epithelium of ductular reactions in cirrhotic livers and occasionally as a dot-like staining in the cytoplasm of hepatocytes (Figure 4.7). HCCs expressed ACE in tumour vessels and in the cytoplasm of tumour cells, and in a single HCC, a canalicular staining was found with anti-ACE in some areas (Figure 4.7). The prevalence of ACE in non-neoplastic liver tissue (20/20; 100.0%) and HCCs (18/20; 90.0%) specimens (χ^2-test; P=0.147) showed no significant difference (χ^2-test; P=0.147). However, immunostaining of endothelial cells was more intense in HCCs than in non-neoplastic liver tissue (Figure 4.7).

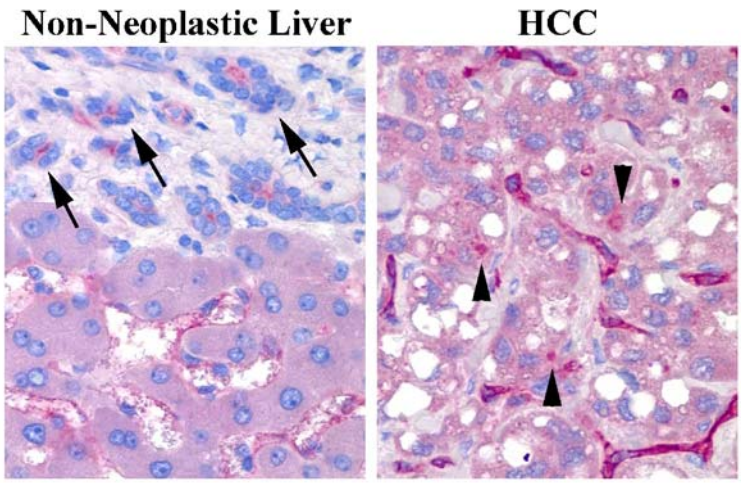

Figure 4.7: ACE expression in HCCs The distribution pattern of ACE was investigated by immunohistochemistry in non-tumor tissues and in HCCs. ACE was mainly found in endothelial cells, ductular reactions (arrow) and as cytoplasmic dots (arrowheads) in the cyoplasm of tumor cells. Anti-ACE antibody and hematoxylin counterstain; Original magnification: x400.

In the stomach, ACE was expressed in tumour epithelial cells in 23 of 40 (57.5%) cases. ACE exhibited both membranous and cytoplasmic staining in the lymph node metastases of 7 of 10 (70%) cases, and in the corresponding tumours of 9 of 10 (90%) cases. ACE was highly significantly up-regulated in tumour epithelial cells (χ^2-test; P<0.001), and was significantly more often expressed in intestinal-type than in diffuse-type carcinomas (χ^2-test; P<0.001). The intestinal metaplasia strongly expressed ACE in 10 of 11 (90.9%) cases.

The non-lesional luminal and foveolar epithelium did not express any ACE, while chief cells located in the deep gastric glands of the fundic region regularly showed weak to moderate cytoplasmic expression. The mucin-secreting cells of the antral and pyloric region showed membranous expression of ACE at the apical cell surface, which seemed in some cases to be related to the presence of local inflammation. Additional immunostaining of 31 gastric biopsy specimens (14 with and 17 without histological evidence of *H. pylori* infection), and 13 gastric ulcer resection specimens (9 with adjacent mucosa) was carried out to further investigate this (Table 4.1).

Table 4.1: Immunohistochemical Expression of ACE in Gastric Inflammation.

	Gastric biopsy specimens		Gastric ulcer specimens
	Control	H. pylori	
Patient Number [n]	17*	14*	13
Age in years (mean ± SD)	62.6 ± 20.0	62.1 ± 11.9	68.9 ± 11.2
Sex ratio (m:f)	6:12	7:6	5:8
H. pylori gastritis [n (%)]	0	14	6
Intestinal metaplasia [n (%)]	0	4 (29)	4 (31)
Grading of chronic inflammation (mean±SD)	0.6±0.5	1.8±0.4	n.a.
Grading of activity (mean±SD)	0	1.2±0.4	n.a.
ACE^+ intestinal metaplasia [n (%)]	0	4 (100)	4 (100)
ACE^+ fundic glands [n (%)]	15 (88)	13 (93)	3 (100)**
ACE^+ antral glands [n (%)]	6 (35)	9 (64)	6 (100)**
ACE^+ endothelial cells	17 (100)	14 (100)	13 (100)

*Biopsies from gastric fundus and antrum were studied in each case; ** Intact mucosa was not enclosed in four gastric ulcer resection specimens; n.a. not applicable

Interestingly, the expression of ACE in the gastric body and fundus appeared not to be influenced by inflammation. However, antral glands in inflamed mucosa expressed more commonly ACE than antral glands in non-inflamed mucosa: 9 of 14 (64%) *H. pylori*-positive gastric biopsy specimens infection compared to only 6 of 17 (35%) of the *H. pylori*-negative controls (Table 4.1). In the mucosa adjacent to the gastric ulcers, ACE was expressed by chief cells and mucin-secreting cells of the antral and pyloric glands in each specimen. ACE was also found in all samples in endothelial cells of the ulcer granulation tissue (Figure 4.8 F, G).

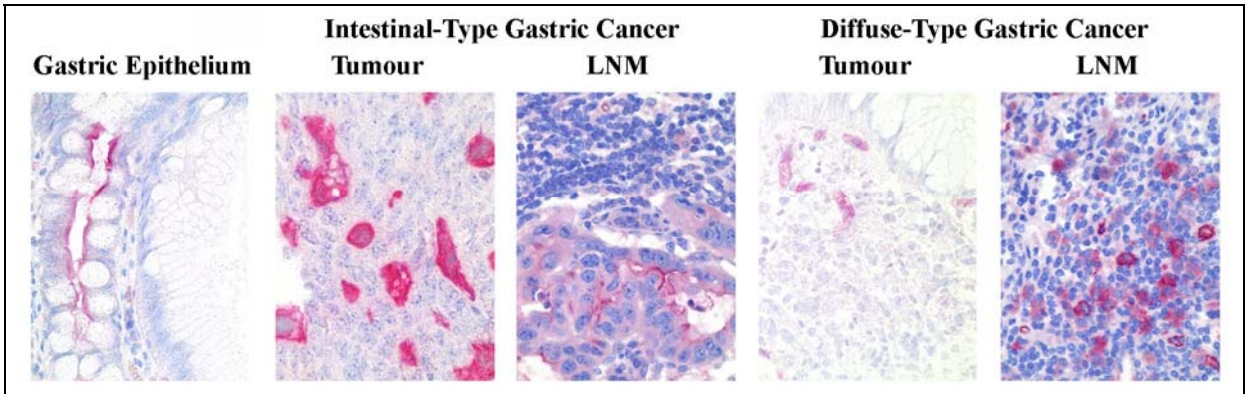

Figure 4.8 A: ACE expression in gastric cancer The distribution and expression pattern of ACE was investigated by immunohistochemistry in non-tumorous epithelium, gastric carcinomas and lymph node metastases ACE was found at the brush border of the intestinal metaplasia (arrowhead), gastric cancer (intestinal type and diffuse type) and in lymph node metastases. Non-neoplastic gastric foveolar epithelium (arrow) did not express ACE. Note the minimal immunostaining in the primary tumor (diffuse-type) and the moderate expression of ACE in the corresponding lymph node metastasis. Anti-ACE antibody and hematoxylin counterstain; Original magnification: x400.

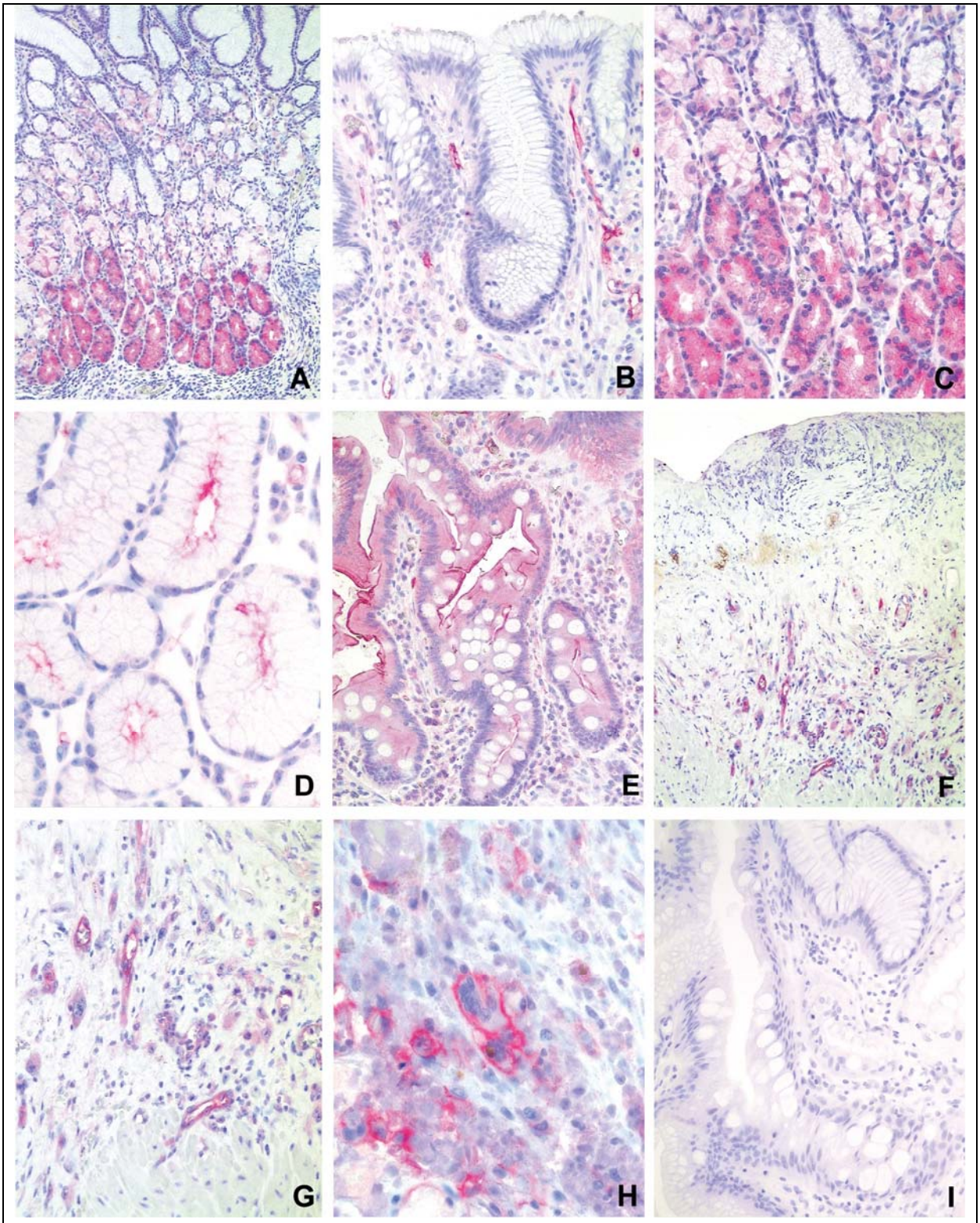

Figure 4.8 B: ACE expression in gastric inflammation und gastric ulcer Expression of ACE was studied immunohistochemically, and was found in chief cells of the gastric body and fundus (**A, C**), while normal gastric foveolar epithelium did not express any ACE (**A, B**). Mucin-secreting cells of the antral and pyloric glands showed membranous staining at the apical surface (**D**) more commonly in gastritis than in non-inflamed gastric mucosa. The brush border of intestinal metaplasia always strongly expressed ACE (**E**). In gastric ulcers, ACE was found in endothelial cells of blood vessels of granulation tissue (**F, G**) and occasionally at the cell surface of macrophages and multinucleated foreign body-type giant cells (**H**). Occasionally, stromal cells of scarring tissue in gastric ulcer specimens expressed ACE (**I**). No immunostaining was found after the omission of the primary antibody (**J**). Anti-ACE antibody and hematoxylin counterstain. Original magnifications 100x (**A, F**), 200x (**B, C, E, G, J**), and 400x (**D, H, I**).

4.1.1.5 The ADAMs are almost ubiquitously expressed in stomach tissues

The ADAMs were found at both the cell surface and in the cytoplasm in both diffuse-type and intestinal-type gastric carcinomas. ADAM9, 12 and 15 were expressed in the regenerative zones of non-tumorous gastric epithelium in 19 of 24 (79.2%), 19 of 24 (79.2%) and 20 of 24 (83.3%) cases, and in tumour epithelial cells of 24 of 24 (100%), 22 of 24 (91.7%) and 22 of 24 (91.7%) cases, respectively. The intestinal metaplasia demonstrated strong immunoreactivity at the brush border for ADAM9, ADAM12 and ADAM15 in 7 of 7 (100%), in 8 of 8 (100%), and in 6 of 7 (85.7%) cases, respectively. Using the χ^2-test for proportions, no significant differences were found between tumour and non-tumour tissues for ADAM12 (P=0.22), or ADAM15 (P=0.38) expression, but ADAM9 (P=0.02) was significantly upregulated in tumour tissues.

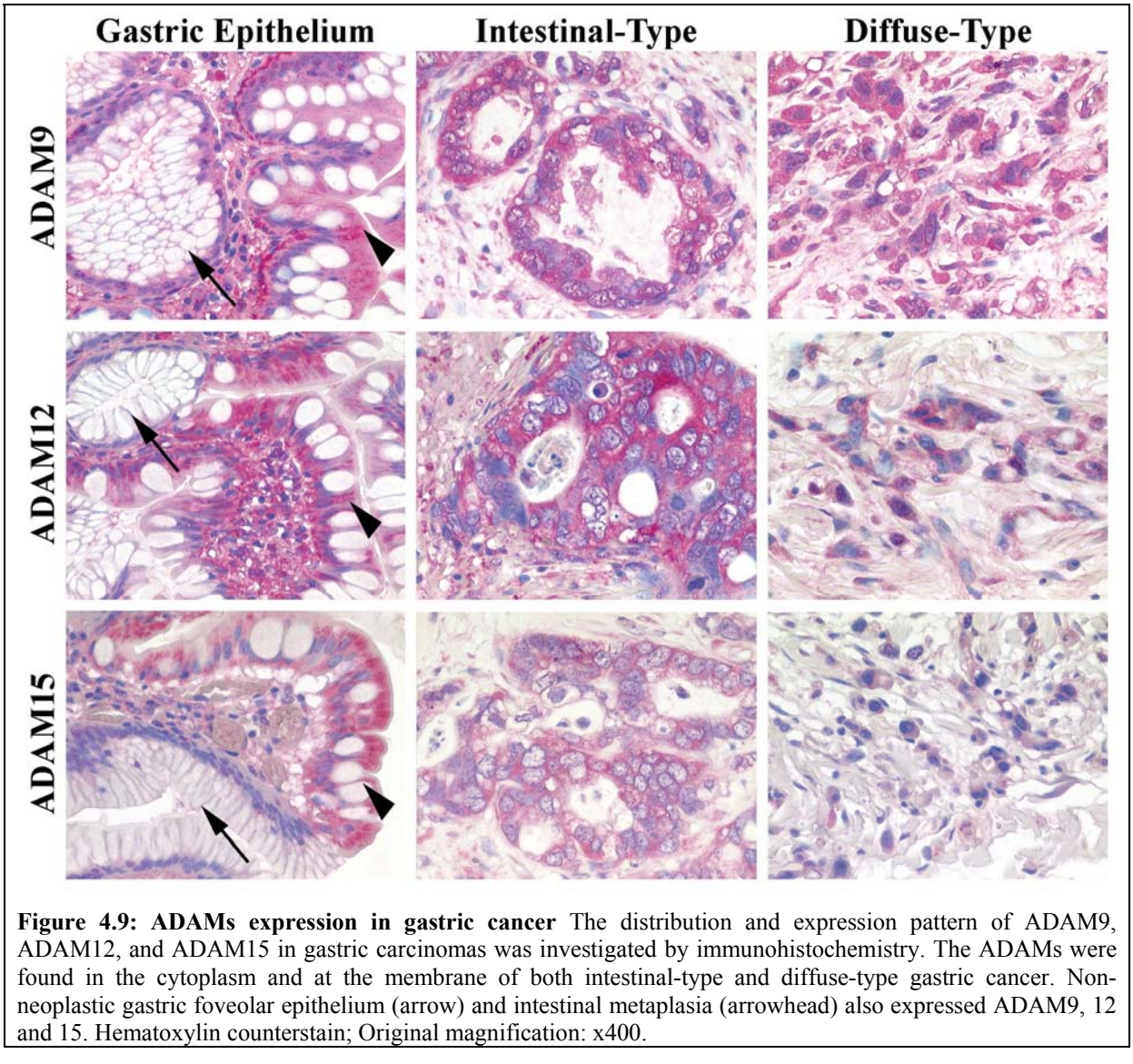

Figure 4.9: ADAMs expression in gastric cancer The distribution and expression pattern of ADAM9, ADAM12, and ADAM15 in gastric carcinomas was investigated by immunohistochemistry. The ADAMs were found in the cytoplasm and at the membrane of both intestinal-type and diffuse-type gastric cancer. Non-neoplastic gastric foveolar epithelium (arrow) and intestinal metaplasia (arrowhead) also expressed ADAM9, 12 and 15. Hematoxylin counterstain; Original magnification: x400.

4.1.2 Immunoreactivity Scores (IRS)

To elucidate any differences in the amount and intensity of the immunostaining between gastric tumour epithelial cells and non-tumorous foveolar epithelium, the expression of NEP, APN, ACE, ADAM9, ADAM12, and ADAM15, were further quantified by determining the IRS values. For comparison of expression, the mean IRS values (±standard deviation), the median IRS values and the interquartile ranges (IQR: 25–75%) were calculated.

The non-tumorous foveolar epithelium expressed low levels of DPIV (1.70±2.21; 0; IQR: 0-4), but was completely negative for NEP, APN, and ACE, compared to IRS values of 2.98±1.94 (3; IQR: 2-4) for NEP, 2.23±2.27 (2; IQR: 0-4) for APN, 2.80±2.15 (3; IQR: 0.75-4) for DPIV and 2.05±1.96 (2; IQR: 0-4) for ACE for tumour cells. The available lymph node metastases demonstrated IRS values of 2.70±2.91 (2; IQR: 0-5.75) for NEP, 3.30±2.31 (3.5; IQR: 2-5.5) for APN, and 2.40±1.96 (3; IQR: 0.5-3.75) for ACE. These IRS values (Table 4.2) reflect the moderate levels (Category B = 1-2) of mainly localized expression (Category A = 1-2) observed in the immunohistochemistry.

In non-tumorous foveolar epithelium, IRS values of 2.04±1.40 (2; IQR: 2–2) for ADAM9, 2.08±1.38 (2; IQR: 2–3) for ADAM12, and 2.54±1.50 (2.5; IQR: 2–4) for ADAM15 were observed, compared to IRS values of 3.75±1.07 (4; IQR: 3–5) for ADAM9, 3.50±1.56 (4; IQR: 2–5) for ADAM12, and 3.75±1.59 (4; IQR: 3–5) for ADAM15 for tumour cells.

4.1.3 Statistical Analyses

Since no IRS was calculated for the liver tissue samples, population differences were evaluated using a χ^2-test for proportions (Table 4.2). NEP was significantly down-regulated in HCCs, whereas no significance was found for APN or ACE expression. Since only 3 samples were available for analysis of DPIV expresssion, statistical analysis was not carried out.

Table 4.2: Immunohistochemical Expression in HCCs and non-lesional liver.

Peptidase	Normal Hepatocytes	Hepatocellular Carcinoma	χ^2-test (P-value)
NEP [n/N (%)]	19/20 (0%)	13/20 (0%)	0.02
APN [n/N (%)]	20/20 (0%)	20/20 (0%)	n.s.
DPIV [n/N (%)]	3/3 (100%)	3/3 (100%)	-
ACE [n/N (%)]	20/20 (0%)	18/20 (0%)	0.147

IHC = immunohistochemistry; n/N = number of positive cases/total number of cases; n.s. = not significant

The statistical analyses of gastric cancer, and the corresponding non-lesional tissues and lymph node metastases were carried out using a *paired, two-sided* Student's *t*-test and the non-parametric Wilcoxon Signed Ranks test, since the tissue samples were each obtained from the same patient (Table 4.3). The P-values were found to be highly significant for ADAM9, ADAM12 and ADAM15, and since NEP, APN, and ACE were not expressed at all in the foveolar epithelium, the P-values were also highly significant. We also compared intestinal-type with diffuse-type carcinomas using Analysis of Variance with repeated measurement. ADAM12 and ACE, but not ADAM9, ADAM15, NEP, DPIV or APN, exhibited significantly higher IRS values in intestinal-type carcinomas.

No significant differences were found between expression in the selected primary gastric cancers and their lymph node metastases. Relationships between data sets were evaluated using Pearson's correlation coefficient, with the level of expression of NEP, APN, and ACE in the primary tumour correlating substantially (r>0.5) with the level of expression in the lymph node metastases. However, due to the small sample size (n=10), evaluation of the IRS values according to the lymph node status (with or without lymph node metastases) demonstrated that the expression of the ectopeptidases was not significant for the presence of metastasis, which may change if a larger sample were to be investigated.

Table 4.3: Immunohistochemical Expression in Gastric and Tumour Tissue

Peptidase		Foveolar Epithelium	Gastric Carcinoma	P-value
NEP	IHC [n/N (%)]	0/40	31/40 (77.5%)	<0.0001 [a]
	IRS [median (IQR)]	0 (0-0)	3 (2-4)	<0.0001 [b]
	IRS [mean (± SD)]	0±0.00	2.98±1.94	<0.0001 [c]
APN	IHC [n/N (%)]	0/40 (0%)	21/40 (52.5%)	<0.0001 [a]
	IRS [median (IQR)]	0 (0-0)	2 (0-4)	<0.0001 [b]
	IRS [mean (± SD)]	0±0.00	2.23±2.27	<0.0001[c]
DPIV	IHC [n/N (%)]	4/10 (40%)	7/10 (70%)	0.18 [a]
	IRS [median (IQR)]	0 (0-4)	3 (0.75-4)	>0.5 [b]
	IRS [mean (± SD)]	1.70±2.21	2.80±2.15	0.33 [c]
ACE	IHC [n/N (%)]	0/40 (0%)	23/40 (57.5%)	<0.0001 [a]
	IRS [median (IQR)]	0 (0-0)	2 (0-4)	<0.0001 [b]
	IRS [mean (± SD)]	0±0.00	2.05±1.96	<0.0001 [c]
ADAM9	IHC [n/N (%)]	19/24 (79.2%)	24/24 (100%)	0.02 [a]
	IRS [median (IQR)]	2 (2-2)	4 (3-5)	<0.01 [b]
	IRS [mean (± SD)]	2.04±1.40	3.75±1.07	0.00020 [c]
ADAM12	IHC [n/N (%)]	19/24 (79.2%)	22/24 (91.7%)	0.22 [a]
	IRS [median (IQR)]	2 (2-3)	4 (2-5)	<0.01 [b]
	IRS [mean (± SD)]	2.08±1.38	3.50±1.56	0.00032 [c]
ADAM15	IHC [n/N (%)]	20/24 (83.3%)	22/24 (91.7%)	0.38 [a]
	IRS [median (IQR)]	2.5 (2-4)	4 (3-5)	<0.01 [b]
	IRS [mean (± SD)]	2.54±1.50	3.75±1.59	0.0087 [c]

IHC = immunohistochemistry; n/N = number of positive cases/total number of cases; IRS = Immunoreactivity Score; IQR = interquartile range (25-75%); differences between values analyzed with [a] Chi-square test, [b] Wilcoxon's Signed Ranks Test, or [c] Student's t-test.

4.1.4 Quantitative real-time RT-PCR

The relative amounts of the ectopeptidase transcripts were determined with quantitative *real-time* RT-PCR in liver and gastric cancer cell lines, and tumour and corresponding non-tumorous tissue. NEP, APN, DPIV and ACE mRNA was quantified in 14 HCC patients (5 men, 7 women; mean age 63.1 ± 15.8), and 10 gastric cancer patients (4 men, 6 women; mean age 59.46 ± 18.32; 4 diffuse, 6 intestinal). The ADAMs transcript levels were determined in 12 gastric cancer patients (5 men, 7 women; mean age 63.1 ± 15.8; 4 diffuse, 8 intestinal). Gastric cancer patients without intestinal metaplasia were selected in order to exclude the possibility of false messages caused by the expression of the ectopeptidases in the intestinal metaplasia.

4.1.4.1 mRNA expression levels in hepatoma and gastric cancer cell lines

The expression of the ectopeptidase mRNA transcripts was investigated in hepatoma (HepG2, C3A), and gastric cancer (AGS, MKN28, N87, KATO III) cell lines. Both HepG2 and C3A cells were found to express mRNAs for NEP, APN and DPIV. Interestingly, ACE mRNA was not detected in either hepatoma cell line. Of the gastric cancer cell lines, DPIV was detected only in N87 cells, whereas NEP, APN, ACE, ADAM9, ADAM12 and ADAM15 were expressed in all five cell lines. However, comparison with β2-microglobulin expression demonstrated that NEP, APN and ACE expression varied noticeably between the different cell lines. APN was mostly weakly expressed, with strong expression in the MKN28 cell line, whereas NEP and ACE exhibited very strong expression in MKN28 and N87 cell lines.

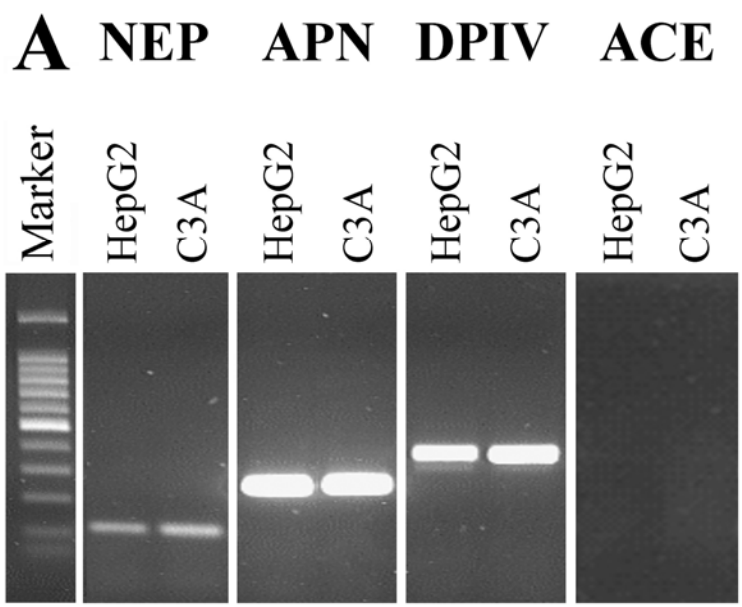

Figure 4.10A: Ectopeptidase mRNA expression in liver cell lines The level of expression of the ectopeptidase transcripts were investigated in the HepG2 and C3A hepatoma cell lines (**A**).

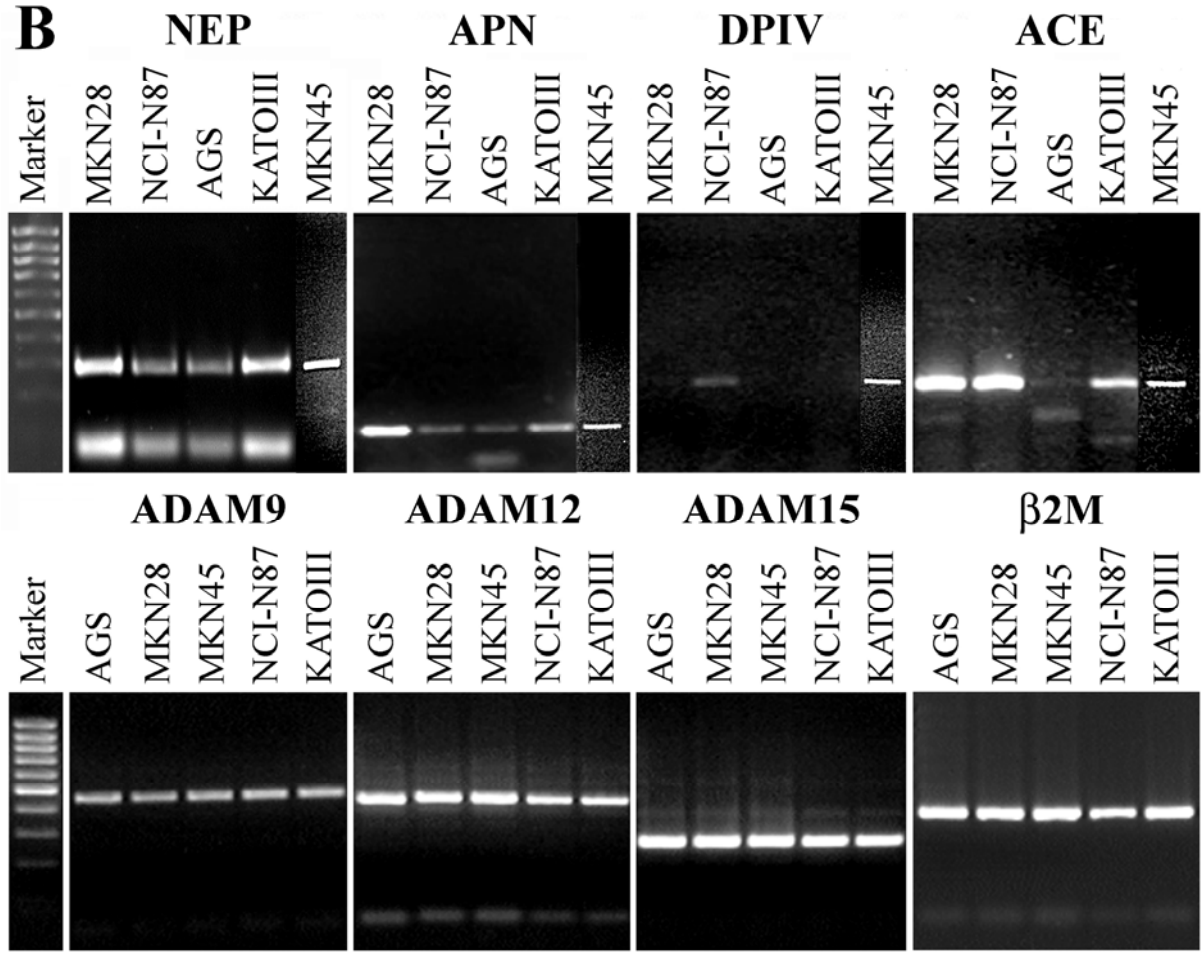

Figure 4.10B: Ectopeptidase mRNA expression in gastric cancer cell lines The level of expression of the ectopeptidase transcripts were investigated in in the AGS, MKN28, MKN45, NCI-N87 and KATOIII gastric cancer cell lines (**B**). Equality of RNA amounts was verified by β2-microglobulin (β2M) expression.

4.1.4.2 mRNA expression in tumour and non-lesional tissues

Using quantitative *real-time* RT-PCR, the relative amounts of the ectopeptidase transcripts were determined in liver and gastric carcinomas and the corresponding non-tumorous tissue. However, although the general trend was similar, the expression pattern observed did not reflect the unequivocal results seen for NEP, APN, and ACE in the immunohistochemistry. This discrepancy can be traced back to the heterogeneous, localized expression of these ectopeptidases in the tissue samples. Immunohistochemically, these ectopeptidases demonstrated moderate levels of mainly localized expression, reflected in the IRS values of between 3 and 5 (Table 4.3). This expression pattern means that one small sample of tissue may contain very little antigen, hindering detection and subsequent quantification. Although the possibility of false messages from intestinal metaplasia was excluded, the heterogeneous distribution of other cell types, such as inflammatory cells, within the tissue samples may also contribute to erroneous results using the homogenized samples.

NEP, APN, DPIV and ACE were found in HCCs and non-neoplastic liver of each case investigated. Transcription levels in the HCCs were significantly down-regulated for NEP (P=0.03), and up-regulated for DPIV (P=0.01) and ACE (P=0.04), compared to non-tumour tissue (Table 4.4, Figure 4.11). No difference was observed for APN.

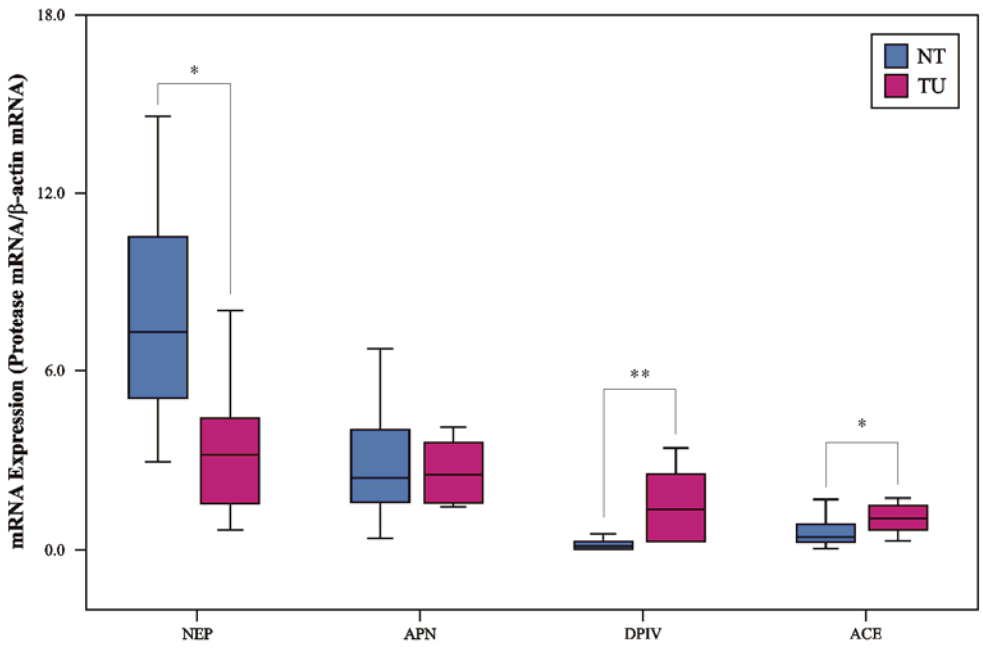

Figure 4.11: Ectopeptidase mRNA expression in liver tissues The transcription levels of NEP, APN, DPIVand ACE were determined with quantitative *real-time* RT-PCR in tumor and corresponding non-tumorous tissue from 12 patients. *: P<0.05; ** P<0.01

In the stomach tissues, NEP was detected only sporadically in the tumours, and was below detection level in non-tumorous tissues. APN was expressed mainly in gastric carcinomas, with a few cases also expressing APN in non-tumour tissues. DPIV was found in both tissue types, with higher expression exhibited in the tumour in some cases, and in the non-tumorous tissue in others. ACE was also detected sporadically in tumour, and in only one non-tumour sample. Both ADAM9 and ADAM15 were detected in virtually every tumour and non-tumour sample, whereas ADAM12 was found in almost all tumour samples, but in only half of the non-tumour samples. The general trend for higher expression in tumour than in non-tumour tissue parallels the immunohistochemistry results for all the ectopeptidases. However, statistical analysis using a t-test (Table 4.4) showed that the difference in expression in the tumour compared to the non-tumorous tissue was significant for NEP (P=0.048), APN (P=0.02), and ADAM12 (P=0.02). The t-test analysis of ACE (P=0.30), DPIV (P=0.08), ADAM9 (P=0.26), and ADAM15 (P=0.14) mRNA levels did not reach significance. However, the data values for the expression levels exhibited a non-Gaussian distribution, with the group variances being enlarged by outliers. Using the more suitable Wilcoxon Signed Ranks analysis, a significant up-regulation in gastric cancer was observed for DPIV (P<0.01), ACE (P<0.01), ADAM9 (P=0.01), and ADAM15 (P<0.01).

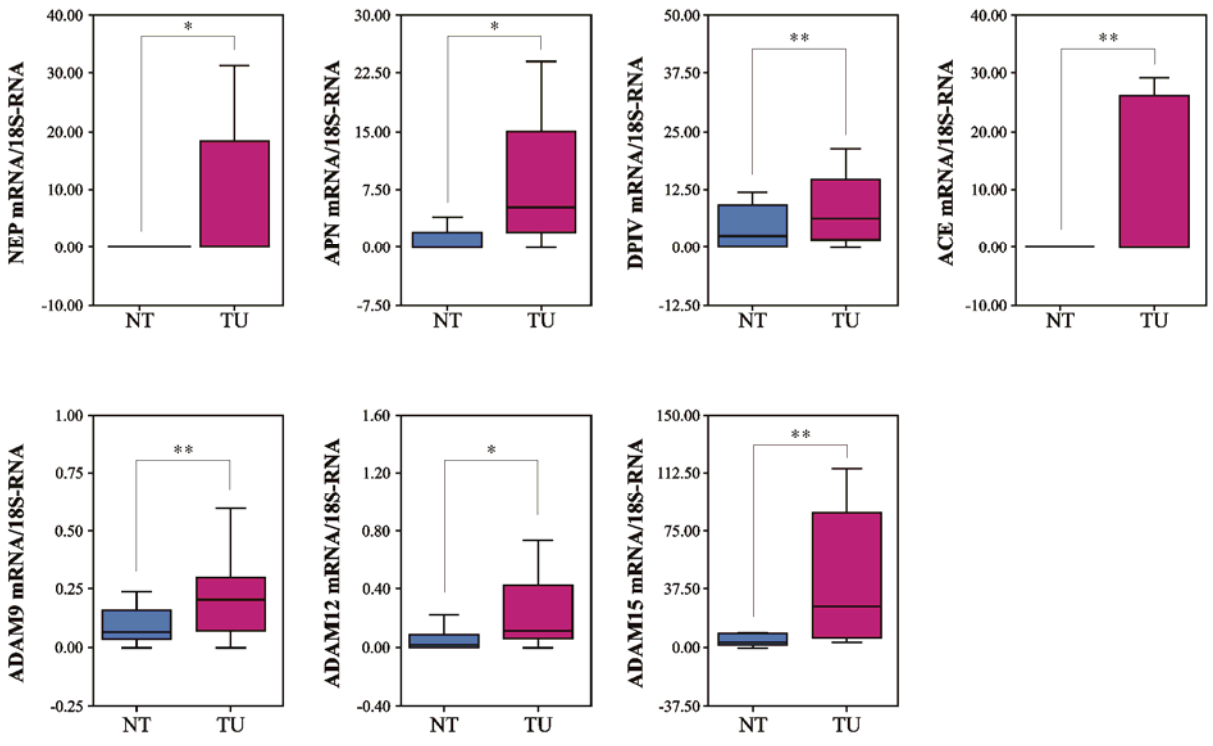

Figure 4.12: Ectopeptidase mRNA expression in stomach tissues The transcription levels of NEP, APN, DPIV, ACE, ADAM9, ADAM12, and ADAM15 were determined with quantitative *real-time* RT-PCR in tumor and corresponding non-tumorous tissue from 12 patients. *: P<0.05; ** P<0.01

Table 4.4: Ectopeptidase mRNA Expression in Liver and Stomach Tissues

Peptidase mRNA	Normal Hepatocytes (mean ± SD)	Hepatocellular Carcinoma (mean ± SD)	P-value (*t*-test)
NEP	15.55 ± 17.66	3.59 ± 2.79	0.03
APN	3.24 ± 2.67	4.07 ± 2.71	0.94
DPIV	0.33 ± 0.68	1.43 ± 1.24	0.01
ACE	0.59 ± 0.14	1.34 ± 1.05	0.04
Peptidase mRNA	**Mucosal Epithelium (mean ± SD)**	**Gastric Carcinoma (mean ± SD)**	**P-value (*t*-test)**
NEP	Below detection level	8.04 ± 12.80	0.04
APN	1.57 ± 2.97	8.12 ± 7.96	0.02
DPIV	4.09 ± 4.72	10.57 ± 13.26	0.08
ACE	2.45 ± 8.49	225.44 ± 717.63	0.30
ADAM9	0.12 ± 0.12	0.76 ± 1.90	0.26
ADAM12	0.05 ± 0.07	0.32 ± 0.40	0.02
ADAM15	8.98 ± 13.22	124.27 ± 265.86	0.15

4.1.5 Immunoblotting with liver tissues

Using Western blotting on HCCs and non-neoplastic liver tissues, protein bands were found at approximately 110-120 kDa (NEP), 130-160 kDa (APN), 180-190 kDa (DPIV), and 190-210 kDa (ACE) in keeping with the expected molecular weights. Quantification of the protein amount by chemiluminescence showed that the amount of NEP protein was decreased in HCCs as compared with non-neoplastic liver tissue ($P<0.05$), while DPIV ($P<0.05$) was significantly upregulated in HCCs (Figure 4.13). Although APN and ACE proteins showed no significant differences in expression levels between HCCs and non-neoplastic liver samples, Figure 4.13 shows that both APN and ACE tended to be expressed at higher levels in HCCs. Again, due to the non-Gaussian distribution, the group variances were enlarged by outliers, and statistical analysis was carried out with the Wilcoxon Signed Ranks test. Due to the heterogeneous expression of the ectopeptidases in gastric tissues and the accompanying difficulties for immunoblotting (described in Section 4.1.3.2), the protein expression in gastric tissues was not further evaluated.

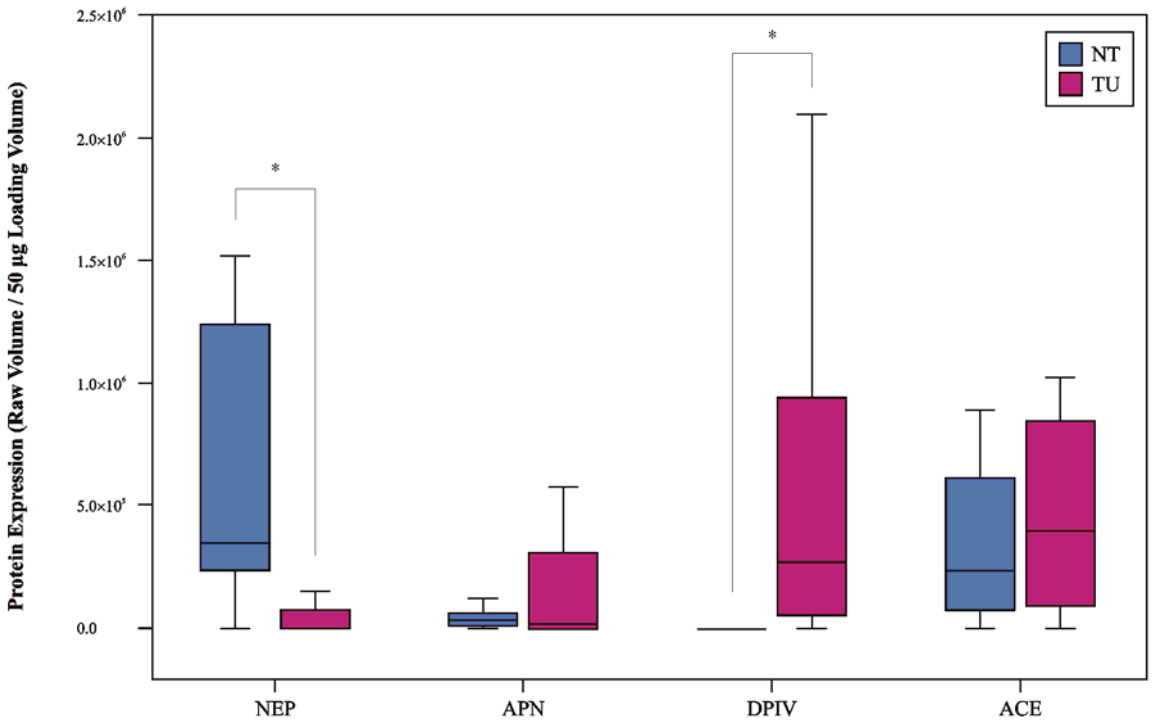

Figure 4.13: Ectopeptidase protein expression in liver tissues The expression levels of NEP, APN, DPIV, and ACE proteins were determined with chemiluminescent immunoblotting in tumor and corresponding non-tumorous tissue from 12 patients. *: P<0.05

4.1.6 Proliferation assays

To study the effect of the different ectopeptidases on gastrointestinal cell proliferation, *in vitro* experiments were performed with hepatoma (HepG2, C3A) and gastric adenocarcinoma (AGS, MKN28, N87, MKN45) cell lines. [^{3}H]-methylthymidine incorporation was assessed with and without the addition of specific protease inhibitors (thiorphan/phosphoramidon, actinonin/phebestin, I49 and captopril), or specific antibodies (no specific inhibitors of ADAMs available). The anti-ADAM antibodies detect both precursor and mature forms of the ADAMs (previously verified by Western blotting). Differences between control and treated cells were evaluated with a two-sided Student's t-test.

4.1.6.1 Hepatoma cell lines

In the hepatoma cell lines (Figure 4.14), blocking NEP and APN ectopeptidase activity retarded cell growth significantly in C3A cells (NEP: P=0.002; APN: P=0.05), but not in HepG2 cells. Inhibition of DPIV ectopeptidase activity with I49 retarded cell growth significantly in both cell lines (C3A: P=0.002; HepG2: P=0.05). In keeping with the lack of ACE expression observed in C3A cells (Figure 4.10), no effect was observed with captopril. However, captopril treatment resulted in a significant increase in proliferation in HepG2 cells, despite the absence of ACE.

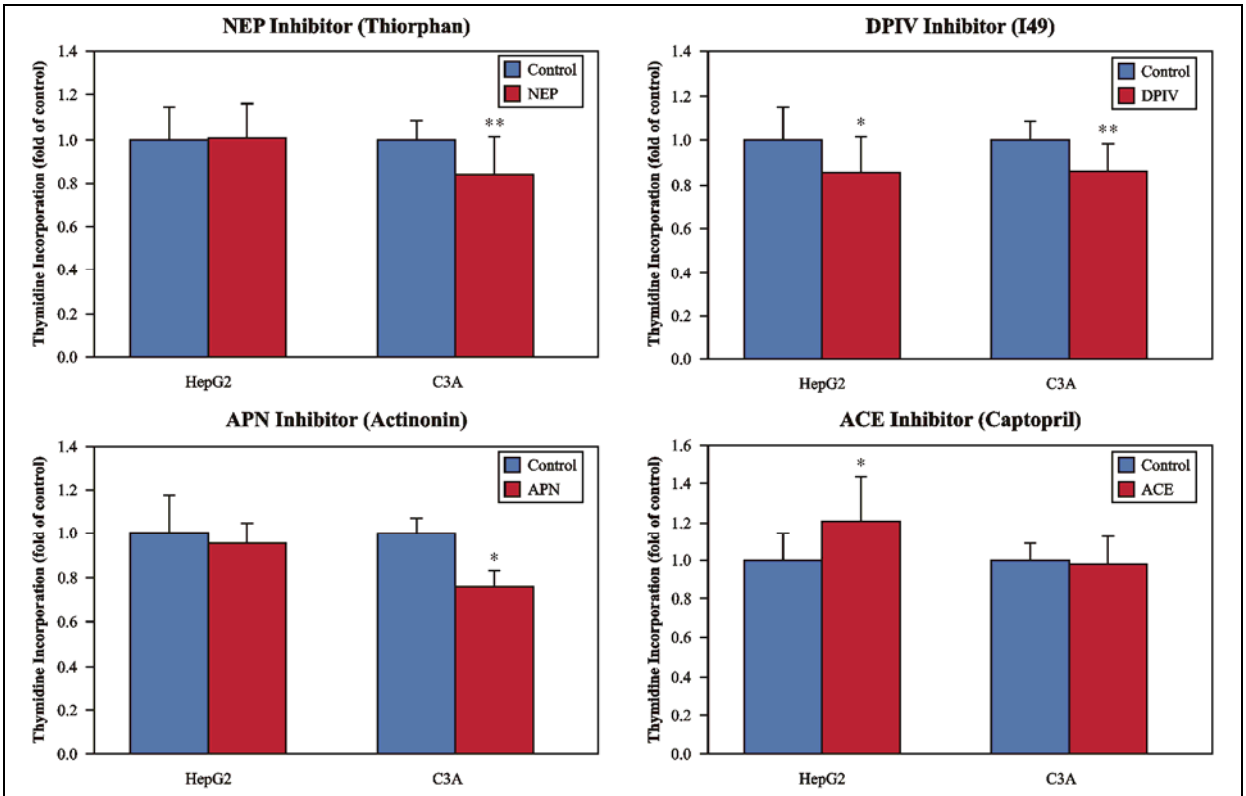

Figure 4.14: Ectopeptidase inhibition influences the proliferation of hepatoma cells Cell proliferation assays were performed with HepG2 and C3A hepatoma cell lines in the absence (Control) or presence (Inhibitor) of inhibitors of NEP (thiorphan), APN (actinonin), DPIV (I49), and ACE (captopril). Proliferation was assessed by ^{3}H-thymidine incorporation. *: P<0.05; **: P<0.01

4.1.6.2 Gastric cancer cell lines

In the gastric cancer cell lines (Figure 4.15), inhibition of NEP ectopeptidase activity resulted in significant reductions in cell growth in all four cell lines (AGS, MKN28, N87, MKN45), in comparison to the controls. After inhibition of APN, the MKN28 cells exhibited a non-significant growth retarding effect, whereas the proliferation of the AGS and N87 cells was significantly increased. The only significant change in the rate of growth when DPIV was inhibited was in N87, the only cell line expressing DPIV, where a highly significant increase in thymidine incorporation was observed. After the peptidase activity of ACE was blocked, AGS and MKN28 cell proliferation were non-significantly decreased, the growth of MKN45 cells was highly significantly decreased, and the proliferation of N87 cells highly significantly increased. Anti-ADAM9 antibodies significantly decreased the proliferation of MKN45 cells, and increased N87 cell growth without quite reaching significance, but had no effect on the other cell lines. Application of anti-ADAM12 antibodies significantly increased the proliferation rate of MKN28 and N87 cells. AGS and MKN45 cells also showed a higher growth rate in anti-ADAM12-treated cells than in the controls, although not significant. Antibodies against ADAM15 induced significant reductions in the proliferation rates of MKN45 cells.

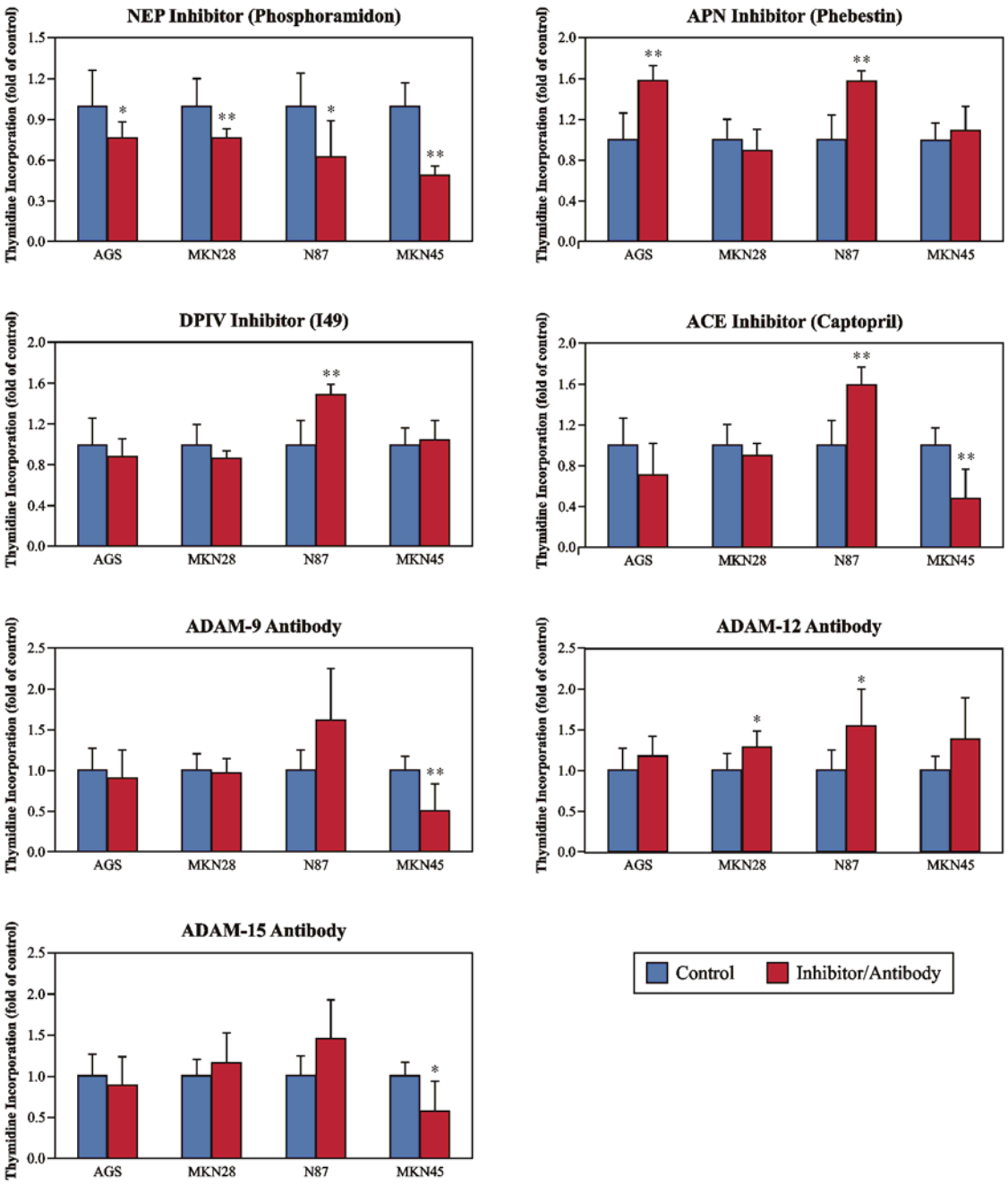

Figure 4.15: Ectopeptidase inhibition influences gastric cancer cell proliferation Cell proliferation assays were performed with AGS, MKN28, NCI-N87, and MKN45 gastric cancer cell lines in the absence (Control) or presence (Inhibitor/Antibody) of inhibitors of NEP (phosphoramidon), APN (phebestin), DPIV (I49), and ACE (captopril), or anti-ADAM9, anti-ADAM12, and anti-ADAM15 antibodies. Proliferation was assessed by ^{3}H-thymidine incorporation. *: $P<0.05$; ** $P<0.01$

4.2 Discussion: The Ectopeptidases in Gastrointestinal Cancer

Neprilysin (NEP, CD10), aminopeptidase N (APN, CD13), dipeptidyl peptidase IV (DPIV, CD26) and angiotensin-I converting enzyme (ACE, CD143), and the disintegrin-metalloproteinases, ADAM9, ADAM12 and ADAM15, belong to a large group of multifunctional, extracellularly orientated, membrane-bound proteolytic enzymes classified as ectopeptidases, which all have been shown to participate in the post-secretory processing of neuropeptides, peptide hormones and growth factors. Each ectopeptidase has the potential to possess proteolytic, adhesion, and signalling abilities, enabling a versatile range of physiological and pathological functions. Tumour cell growth, invasion, and metastasis depend on timely balanced changes of proteolytic activity and cell-cell and cell-matrix interactions, which could be influenced by the activity of these ectopeptidases. They are all widely distributed and have been found in various different cell types of different organs and tissues, including benign and malignant tumours (Antczak et al 2001, Nanus et al 1997). Accumulating evidence associates an increased expression of individual ectopeptidases with various types of cancer, and their extracellular orientation makes them particularly accessible, and therefore interesting, with regard to therapeutical applications. Differential expression of the ectopeptidases may contribute to the development and/or progression of gastrointestinal cancers in different ways, and this seems to also be true for cancers of the liver and stomach.

4.2.1 NEP is down-regulated in HCCs and up-regulated in gastric cancers, but facilitates in vitro cell proliferation in both liver and gastric cancer cell lines

In the liver, the overall expression pattern of NEP appeared to correspond to proliferation and/or differentiation of hepatocytes. NEP is generally expressed by bile canaliculi in normal liver and many HCCs (Borscheri et al 2001, Chu et al 2002, Chu&Arber 2000). However, NEP is only occasionally expressed by foetal hepatocytes (Röcken et al 2004). The association between proliferation and NEP expression in the liver has been evaluated by comparing the proliferation indices (number of Ki-67-positive nuclei) with expression of NEP (Röcken et al 2004). In foetal livers, almost all hepatocyte nuclei were immunoreactive for Ki-67, which correlated with the lowest expression level of NEP in the foetal hepatocytes. In contrast, NEP immunostaining was most intense in non-tumorous liver, which showed the lowest amount of Ki-67-positive nuclei, while HCCs exhibited moderate expression of NEP and an intermediate number of Ki-67-positive nuclei. Thus, overall

expression of NEP in non-neoplastic and neoplastic hepatocytes appears to correlate inversely with their state of proliferation or differentiation.

Contrasting strongly with the down-regulation of NEP in HCCs, NEP expression was significantly up-regulated in gastric cancers. NEP is expressed in more than 50% of investigated gastric carcinomas (Sato et al 1996), with membranous and cytoplasmic staining being found in well- or moderately-differentiated adenocarcinomas, whereas the expression level was decreased or absent in poorly differentiated type of adenocarcinoma (Sato et al 1996). NEP expression has also been observed in normal epithelial cells of the human stomach (Sato et al 1996). However, no distinction was made between the expression in normal luminal and foveolar epithelium and the expression in the gastric glands, and from the published figures, it can be seen that NEP is not expressed in normal foveolar epithelial cells, but detected only deeper in the gastric gland (Sato et al 1996). In combination with other markers, NEP has been used for phenotyping intestinal-type carcinomas and background mucosa, according to the types of intestinal metaplasia (Endoh et al 1999, Kabashima et al 2000, Tajima et al 2001), where only membranous staining was considered as a positive reaction for the monoclonal antibody 56C6. The specificity of this antibody was verified using Western blotting, and positive cytoplasmic staining was included in our evaluations as evidence of protein expression. NEP was found in both intestinal- and diffuse-type tumours, and in both complete- and incomplete-type intestinal metaplasia, as also previously reported (Endoh et al 1999, Kabashima et al 2000, Tajima et al 2001). Although membranous staining was also observed, predominantly cytoplasmic staining was seen in tumour cells of diffuse-type carcinomas. NEP also exhibits a preferentially cytoplasmic location in prostate cancer cells (Renneberg et al 2001), attributed to alterations in intracellular targeting (Gomes et al 2005). This may indicate that the up-regulation of NEP in these cancers is not only related to its extracellularly-orientated peptidase function. Indeed, up-regulation of NEP has been reported in melanomas (Carrel et al 1993), and it has been suggested that this may reflect a regulatory response to excessive growth, resulting from exposure to trophic peptides (Letarte et al 1997).

Of the ectopeptidases, predominantly NEP has been associated with peptide-mediated proliferation. There is ample evidence for the growth retarding effects of NEP, with the effect of NEP being mediated by regulatory peptides and peptide hormones (Shipp et al 1991a). In the lung, NEP regulates growth and maturation by inactivating the mitogenic activity of bombesin-like peptides (King et al 1993), and down-regulation of NEP occurs frequently in lung cancer (Shipp et al 1991b). Furthermore, the expression of NEP correlates inversely

with tumour cell proliferation of small cell (Shipp et al 1991b) and non-small cell lung cancers (Ganju et al 1994). Similarly, in prostate cancer NEP inhibits cancer progression by inactivating growth promoting androgens (Papandreou et al 1998), promoting apoptosis (Sumitomo et al 2000a) and inhibiting migration (Sumitomo et al 2000b). However, the *in vitro* experiments showed that inhibition of NEP ectopeptidase activity retarded cell growth in both liver and gastric cancer cells, contradicting the growth inhibiting effect of NEP expected from the literature, and the observations made in liver tissue specimens.

Both HCCs and gastric cancers respond to regulatory peptides and peptide hormones, and the *in vitro* experiments provide evidence that NEP ectopeptidase activity is capable of modulating cell proliferation in both hepatocytes and gastric cell lines. However, it is not yet known which of the many biological substrates of NEP influence gastrointestinal tumour biology, and the *in vivo* range of relevant substrates is defined by the specific tissue and organ systems.

4.2.2 APN is up-regulated in both HCCs and gastric cancers and has a proliferative effect in vitro

The expression and putative pathophysiological role of APN has been studied in many different malignant tumours (Mechtersheimer&Moller 1990, Tokuhara et al 2001). APN is up-regulated in melanomas (Menrad et al 1993), colon cancer (Hashida et al 2002) and various tumour cell lines, including those obtained from renal cell carcinomas (Stange et al 2000, Gohring et al 1998), prostate cancer (Ishii et al 2001), choriocarcinoma (Ino et al 1994), melanoma (Menrad et al 1993), fibrosarcoma (Fujii et al 1996), osteosarcoma (Kido et al 1999), and leukaemia (Mishima et al 2002).

APN was up-regulated in HCCs as compared with non-tumorous liver, which was in keeping with observations made in many other tumour types. However, detection of APN in the bile canaliculi of the liver may point to another role, unique to the liver, in the formation of bile canaliculi, and production and secretion of bile acids. APN-positive bile canaliculi are found in every foetal liver, every focal nodular hyperplasia, every non-tumorous liver and every HCC (Röcken et al 2004). This reflects its ubiquitous expression and its close association with the formation and function of bile canaliculi (Lian et al 1999). APN positive bile canaliculi have been detected as early as 16 to 18 weeks of gestation (Liu et al 2001), underscoring its role for morphogenesis. Expression of APN is maintained at a constant level during liver regeneration (Bartles et al 1991), and APN is found in focal nodular hyperplasia (Röcken et al 2005b), indicating that the expression is also maintained during a hyperplastic

response. Thus, compared with NEP, expression of APN in the liver is less sensitive to proliferation and differentiation, and this has proved useful in diagnostic pathology, where a canalicular staining pattern for APN, biliary glycoprotein 1 and NEP can be used to differentiate HCCs from non-HCCs metastatic to the liver (Röcken et al 2005b, Borscheri et al 2001, Chu et al 2002, Chu&Arber 2000). The common expression and close association of APN with bile canaliculi in HCCs leads to the conjecture that APN expression in bile canaliculi is associated with tumour biology. APN is an amphiphilic protein secreted into the bile and forms lamellar particles with bile acids (Rigotti et al 1993). Bile acids, in turn, induce polarization of hepatocytes *in vitro* (Ng et al 2000), and can effectively and reversibly inhibit APN ectopeptidase activity in the bile (Sasaki et al 1990), which suggests that the ability of HCCs to synthesize and secrete bile modulates the expression pattern, activity and biological function of APN. Thus, while cell polarity is lost during dedifferentiation of HCCs, APN may gain new additional functions.

The observed expression pattern of APN in intestinal metaplasia and gastric cancer has been previously reported (Matsukura et al 1980, Osborn et al 1988). Since APN is not at all expressed by either the luminal mucosa or the deeper gastric glands of the normal stomach epithelium, the new expression of APN observed in intestinal metaplasia and both intestinal-type and diffuse-type gastric carcinomas results in the deregulation of the local balance of peptide and growth factor activation/inactivation. Indeed, the expression of APN has been linked to tumour cell proliferation, degradation of extracellular matrix and metastatic behaviour (Tokuhara et al 2001, Menrad et al 1993, Hashida et al 2002, Ishii et al 2001, Kido et al 1999, Saiki et al 1993). Almost all of these biological effects were attributed to the ectopeptidase activity, and, like in other tumours, APN may mediate its pathophysiological effect on gastrointestinal carcinogenesis by cleaving regulatory peptides, such as bradykinin, enkephalins, or somatostatin, so promoting tumour cell proliferation. Interestingly, previous reports have demonstrated that APN also mediates IL-8-induced apoptosis of leukaemia cell lines (Mishima et al 2002). Here, the proliferation assays on the cell lines also demonstrated that APN can have opposing effects on cell proliferation of both hepatoma and gastric cancer cells, which may reflect the involvement of APN in different pathways. Inhibition of APN enzymatic activity in the hepatoma cells reduced the proliferation of C3A cells, but had no effect on HepG2 cells, and in the gastric cancer cells, APN inhibition suppresses the growth of MKN28 cells, but enhances the growth of AGS and N87 cells.

In addition to proliferation and apoptosis, APN has been shown to be involved in invasion. The invasive potential of rodent and human tumour cells could be significantly

inhibited by anti-APN antibodies or peptide inhibitors of APN enzymatic activity (Saiki et al 1993, Menrad et al 1993, Fujii et al 1995, Kido et al 1999). These results were paralleled by anti-sense strategies (Kido et al 2003), and are believed to be due to proteolytic degradation of and adhesion to the extracellular matrix. In this study, APN was found in a majority of the lymph node metastases, often demonstrating high intensity staining in over 50% of the cells. Although no correlation was found with lymph node status of the tumours in this study, the role of APN in metastasis is supported by the results of various investigations. In colon and pancreatic carcinoma patients, survival is significantly lower in patients with APN-positive tumours (Hashida et al 2002, Ikeda et al 2003), especially in patients already exhibiting lymph node metastasis (Hashida et al 2002). These studies were confirmed by clinical studies using Bestatin, an aminopeptidase inhibitor, which has been shown in a range of cell lines to inhibit proliferation and induce apoptosis (Sekine et al 2001). In the treatment of stomach cancer (Niimoto&Hattori 1991), the cancer patients receiving Bestatin showed higher survival rates than the control groups, particularly in patients exhibiting deeper tumour invasion. Additionally, treatment with Bestatin reduced the incidence of returning peritoneal dissemination. One of the factors essential for successful distant metastasis is angiogenesis, and APN has been identified as a specific marker of neoangiogenic vasculature endothelial cells (Pasqualini et al 2000). Additionally, blocking APN activity inhibited capillary network formation and reduced tumour growth in animal models (Bhagwat et al 2001, Bhagwat et al 2003).

4.2.3 DPIV is upregulated on mRNA level in HCCs and gastric cancer and has opposing effects on in vitro cell proliferation

It has long been recognized that the expression pattern of DPIV is changed in malignant tumours indicating a putative involvement in tumour development and tumour growth: DPIV is up-regulated in T-cell lymphoblastic lymphoma, thyroid cancer, adenocarcinoma of the lung and basal cell carcinomas of the skin and is down-regulated in malignant melanomas (Dang&Morimoto 2002, Pro&Dang 2004). Here, DPIV is also significantly up-regulated on the mRNA and protein level in HCCs and is expressed by two hepatoma cell lines. DPIV was found at the cell surface of both tumour cells and hepatoma cell lines, suggesting that DPIV is involved in tumour biology of HCCs. These observations are in accordance with previous reports. Serum levels of sDPIV are increased in humans and animals suffering from HCCs or hepatomas (Gorrel et al 2001, Hanski et al 1986), and the expression pattern of DPIV in HCCs and cirrhotic livers is different from that of non-cirrhotic livers (Stecca et al 1997,

Matsumoto et al 1992). While hepatocytes of non-neoplastic liver parenchyma expressed DPIV only at the canalicular plasma membrane, tumour cells expressed DPIV both at the basolateral and apical membrane (Stecca et al 1997). Increased protein expression of DPIV is associated with significantly increased amounts of mRNA, and it has been shown that DPIV activity is also significantly increased in HCCs (Stecca et al 1997). Together, this and previous studies suggest that DPIV is involved in the tumour biology of HCCs.

DPIV is expressed by normal stomach tissue in the foveolar epithelium, occasionally in deep gastric glands, and also in carcinomas. While DPIV has been previously detected in well- and moderately-differentiated gastric cancers, but weakly or not at all in poorly differentiated gastric cancers (Sakamoto et al 1993), no significant difference was found between DPIV expression in diffuse-type and intestinal-type carcinomas in this study. Interestingly, when DPIV was expressed in the non-tumorous epithelium, the IRS scores of the corresponding carcinoma tissue were invariably lower, contrasting with the relatively high IRS obtained in the tumour tissue where the non-tumorous foveolar epithelium was negative. This may indicate that the observed expression pattern in gastric cancer may be related to more than just the ectopeptidase activity. The enzymatic activity may contribute, but is not essential for DPIV-mediated signal transduction (Morimoto&Schlossman 1998, von Bonin et al 1998), and DPIV signal transduction influences hepatocarcinogenesis (Gaetaniello et al 1998), by activating tyrosine kinases and thereby inducing apoptosis in hepatoma cell lines (Gaetaniello et al 1998). In keeping with this observation, DPIV inhibition significantly increased N87 gastric cancer cell proliferation. However, inhibition of DPIV ectopeptidase activity with I49 unexpectedly retarded cell growth significantly in both hepatoma cell lines, which suggests that DPIV may be involved in the pathology of gastrointestinal carcinomas in different ways in different tumours.

4.2.4 ACE is up-regulated in gastrointestinal cancers, and regulates *in vitro* proliferation

Observations made in retrospective cohort studies suggested that ACE-inhibitors decrease the risk of cancer (Lever et al 1998) including those of the liver (Friis et al 2001). In addition to epidemiological studies supporting the involvement of the angiotensin II system in cancer progression (Lever et al 1998), there is also strong experimental evidence that this system plays an important role in tumour biology, influencing tumour cell proliferation (Reddy et al 1995, Yasumaru et al 2003), the remodelling of the interstitial matrix (Suzuki et al 2003), the local peritumorous inflammatory reactions (Smith&Missailidis 2004),

neoangiogenesis (Yoshiji et al 2001, Yoshiji et al 2002, Fujita et al 2002), and metastatic behaviour (Röcken et al 2005a).

4.2.4.1 ACE mRNA is up-regulated in HCCs, but is not expressed in hepatoma cell lines

The expression of ACE mRNA is significantly higher in HCCs and ACE has also been found in foetal livers (Röcken et al 2004), which implies that it is also involved in organogenesis. However, the amount of ACE in HCCs, as demonstrated by chemiluminescent immunoblotting, was only moderately increased compared with non-tumorous liver, and there is a lack of ACE expression in focal nodular hyperplasia indicates that ACE is not necessary for the development of a hyperplastic response (Gräntzdörffer et al 2004). In fact, the renin-angiotensin-system was shown to be strongly involved in matrix remodelling. Both inhibition of ACE activity by ACE-inhibitors and blockade of the angiotensin-II type 1 receptor significantly attenuated the development of liver fibrosis (Jonsson et al 2001). HCCs, in turn, often occur in cirrhotic livers and progression requires degradation and remodeling of the surrounding matrix (Theret 2001). The binding of angiotensin II to the angiotensin-II type 1 receptor has a trophic and mitogenic effect on cell growth. Angiotensin-II induces dose-dependent vascular endothelial growth factor (VEGF), which in turn correlates with tumour progression of HCCs (Torimura et al 1998). Interestingly, and despite the lack of ACE expression in HepG2 cells, captopril inhibition in HepG2 resulted in a significant increase in proliferation, which may be due to non-specific effects of captopril. Because of its reactive thiol group, captopril is known to have secondary functions not related to ACE inhibition, such as matrix metalloproteinase inhibition (Molteni et al 2003, Williams et al 2005). Thus, either ACE expression in HCC cells is not mandatory for tumour development and progression, or other proteases cover for ACE activity, such as mast cell chymase (Matsuo et al 2003).

4.2.4.2 Up-regulation of ACE in gastric cancer may be related to inflammation

In the stomach, expression of ACE was demonstrated in the deep gastric glands, but not in the normal foveolar epithelium. This result is contrary to previous reports that ACE is expressed in the rabbit fundic mucosa on the apical plasma membrane and in granules of surface mucous cells (Laliberte et al. 1991). However, the expression previously observed in lower gastric glands is confirmed (Kobayashi et al. 1991, Laliberte et al. 1991, Nonotte et al. 1993), and ACE was frequently detected in the secretory granules of chief cells, as observed here and previously reported (Laliberte et al. 1991). Soluble ACE may be derived from the membrane-bound form by proteolytic cleavage of its C-terminal domain and shedding from

the cell surface (Naim 1993), or by alternative splicing of the ACE-mRNA and subsequent secretion via an intracellular processing pathway (Sugimura et al. 1998). Peptide degradation in the stomach may therefore not be carried out by membrane-bound ACE on the luminal surface, as proposed by Laliberte et al. (1991), but may instead involve secreted ACE in the fundic lumen, or membrane-bound or soluble ACE in the antral lumen. The observed immunohistochemical expression pattern indicates that the expression of ACE in the stomach is highly regulated, with two pathways of expression offering a high degree of control, not only over the expression of membrane-bound ACE on the cell surface, but also of the secretory pathways that result in the secretion of ACE into the lumen, where it may degrade gastrointestinal peptides and growth factors.

The expression of ACE in mucin-secreting cells of the antral and pyloric glands was sensitive to inflammation, as observed in *H. pylori* gastritis and the mucosa adjacent to gastric ulcers. ACE cleaves several gastrointestinal regulatory peptides and peptide hormones, and the secretion of these signalling peptides is a necessary part of the response to gastric mucosal damage. Many of these peptides have been shown to alleviate or inhibit the extent of stress-related gastric lesions (Brzozowski et al. 1999, Hernandez et al. 1983, Konjevoda et al. 2001, Mercer et al. 1997), and cleavage of these peptides by ACE may result in attenuation of their bioactivity. Additionally, an increase in angiotensin II expression occurs concurrently with an increase in ulcer index and gastric mucosal damage (Mou et al 1998), which could then be decreased through administration of enalapril (Mou et al 1998). An angiotensin II-mediated mechanism was responsible for the cigarette-smoke-induced increase in gastric ulcer size in rats (Seno et al 1997), with enalapril inhibition of ACE decreasing the ulcer size (Seno et al 1997). Treatment with other ACE inhibitors also significantly reduces the incidence and severity of gastric ulcers (Bailey et al. 1987, Bhandare et al. 1992, Bhounsule et al. 1990, Cullen et al. 1994, Ender et al. 1993, Rao et al. 1995, Uluoglu et al. 1998). Interestingly, antagonism of the angiotensin II Type 1 receptor (AT1) also decreased the size and severity of gastric ulcer, as well as reducing the gastric mucosal damage (Bregonzio et al. 2003, Ender et al. 1993, Heinemann et al. 1999).

This indicates that, in addition to the degradation of cytoprotective factors, the ACE-mediated cleavage of angiotensin I to form angiotensin II may add to the damaging effect on gastric mucosa. Angiotensin II is involved in fundamental processes of the inflammatory cascade (Suzuki et al 2003), and the levels of angiotensin II in plasma and stomach tissues increase considerably during stress (Yang et al 1993). In the animal models of stress- or toxin-induced gastric ulcers described above, an increase in angiotensin II expression occur

concurrently with increases in ulcer index and gastric mucosal damage (Mou et al 1998, Seno et al 1997). In our study, ACE was expressed in the microvasculature within the granulation tissue of gastric ulcers, as well as in the endothelial cells within the muscularis propria and mucosae, as previously reported (Matsuo et al 2003). A decrease in gastric mucosal blood flow is frequently observed as a consequence of stress-induced ulcers (Bregonzio et al. 2003, Ender et al. 1993, Mou et al. 1998, Seno et al 1997, Yang et al 1997), and is thought to be mediated by the increase in angiotensin II levels (Heinemann et al. 1999, Mou et al. 1998, Seno et al 1997, Yang et al 1997). The protective or healing effect of ACE inhibitors and AT1 blockers may be mediated by the regulation of local blood pressure and vascular permeability via the attenuation of angiotensin II expression. Inhibition of ACE or AT1 results in increased blood flow to the gastric mucosa (Bregonzio et al. 2003, Ender et al. 1993, Mou et al. 1998, Yang et al 1997). The gastric mucosal blood flow plays an important role in the protection of normal and damaged gastric mucosa, by supplying the inflamed area with cytoprotective factors that promote regeneration (Sorbye&Svanes 1994). The stimulation of angiogenesis improves the reconstruction of capillary vessels, thereby accelerating ulcer healing (Tarnawski et al 2001). Inhibiting the production or function of angiotensin II may also improve ulcer healing, not only by increasing the supply of protective components, but also by working against the angiotensin II-mediated pro-inflammatory effects, and thereby improving tissue repair and regeneration. ACE inhibitors have been implemented to improve gut perfusion in critically injured patients (Kincaid et al 1998) and, in animal models, treatment with ACE inhibitors (Mou et al 1998, Seno et al 1997, Bailey et al 1987, Bhandare et al 1992, Bhounsule et al 1990, Cullen et al 1994, Ender et al 1993, Rao et al 1995) or AT1 antagonists (Ender et al 1993, Bregonzio et al 2003, Heinemann et al 1999, Bregonzio et al 2004) significantly reduces the incidence and severity of gastric ulcers, as well as reducing the gastric mucosal damage. This has potentially important therapeutic implications. ACE inhibitors and AT1 blockers are well-characterized, and well-established for clinical applications, and animal models have already successfully shown that treatment with ACE inhibitors and AT1 blockers may assist the healing process for gastritis and ulceration. In humans, ACE inhibitors prescribed for hypertension also normalize the proliferative processes in the gastric mucosa (Alekseenko et al 2004), which seems to be unrelated to the anti-hypertensive effect of ACE inhibitors. Here, it has been verified that ACE is locally expressed in healthy and in diseased human gastric tissue, and therefore, represents a useful therapeutic target for the treatment of inflammatory conditions, such as gastritis and gastric ulcers.

Since chronic inflammation, intestinal metaplasia and gastric ulcers are considered to be risk factors for the development of gastric cancer, treatment with ACE inhibitors or AT1 antagonists may assist the healing process for gastritis and ulceration, thereby reducing the risk of developing gastric cancer. Indeed, ACE has been shown to play a role in the development of early gastric cancers (Ebert et al 2005), with the ACE DD polymorphism, associated with higher ACE activity, being significantly more frequently found in patients with early gastric cancer, than in a control group of patients without gastric cancer. However, although ACE expression has been demonstrated in preneoplastic lesions, ACE expression in the precursor lesions of intraepithelial neoplasia and adenomas must also be evaluated to clarify the role that ACE and the local angiotensin II system may play during the development of gastric cancer.

4.2.4.3 ACE enzymatic activity regulates in vitro cell proliferation

Angiotensin II is involved in the regulation of cell proliferation via its receptors, AT1 and AT2 (De Gasparo et al. 2000, Suzuki et al. 2003), and can induce the metalloproteinase-mediated secretion of EGF-like growth factors (Schäfer et al. 2004), and the triple-membrane passing signal for EGFR transactivation (Mifune et al 2005, Olivares-Reyes et al 2005). Here, the inhibition of ACE peptidase activity resulted in both increased and decreased cell proliferation, which may reflect the cell-type specific expression profile of metalloproteinases and EGFR ligands.

Further putative roles of the local angiotensin II system, which have not yet been studied in gastric cancer, may include the regulation of differentiation and angiogenesis. Here, ACE was detected in tumour cells at a significantly higher level in intestinal-type, compared to diffuse-type gastric cancers, which is consistent with the elevation of ACE expression observed in well-differentiated intestinal epithelial cells (Naim 1996). ACE may also be involved in tumour progression through the conversion of angiotensin I to angiotensin II, which induces neovascularization (Tamarat et al 2002). Inhibition of ACE activity by captopril inhibits angiogenesis and slows tumour growth in rats (Volpert et al 1996), and the risk of cancer is reduced in hypertensive patients receiving ACE inhibitors (Lever et al 1998, Friis et al 2001). Thus, all these observations indicate that ACE and the local angiotensin II system may be involved in the pathogenesis of human gastrointestinal cancers (see Chapter 5).

4.2.5 ADAM9, ADAM12 and ADAM15 are up-regulated in gastric cancer, but have opposing effects of cell proliferation

Using immunohistochemistry, membranous and cytoplasmic expression of ADAM9, 12 and 15 were observed, which is in keeping with their processing pathways through other intracellular compartments on their way to the cell surface (Roghani et al 1999, Cao et al 2002, Lum et al 1998). Since the investigated ADAMs were so widely expressed in both gastric tissue and carcinomas, the IRS was applied to quantify any differences that may occur between non-neoplastic and tumorous tissue. As can be seen in Table 4.3, the median IRS values for non-neoplastic foveolar epithelium differ considerably from the median values for the tumours, with non-tumorous tissue exhibiting very low expression levels (IRS=2), and all three ADAMs were found to be significantly up-regulated in the tumours. Interestingly, it has been suggested that differential processing or post-translational modification of the ADAM9 proteins may occur in breast carcinomas compared to non-neoplastic tissue (O'Shea et al 2003). In this study, the applied antibodies detect both precursor and mature forms of the ADAMs, and we were therefore unable to evaluate any differences in immunohistochemical expression of the various protein forms.

The levels of ADAM9, 12 and 15 transcripts were also examined and demonstrated that mRNA amounts were significantly increased in human gastric cancers in comparison to adjacent non-tumour tissue (Figure 4.12). Very low levels of mRNA were observed in non-neoplastic tissue for all three ADAMs, whereas in the tumour tissue samples, ADAM9 and 12 were generally expressed at moderate levels, with the expression levels of ADAM15 being much higher. The five human gastric cancer cell lines investigated also expressed ADAM9, 12 and 15, with the level of expression reflecting the levels observed in the tumour tissue samples (Figure 4.10). A difference in the level of ADAM15 mRNA according to the localization in the stomach has been previously reported (Yoshimura et al 2002). No significant differences were observed in expression of mRNA or protein between samples obtained from the antrum, cardia or corpus of the stomach, which could be partly due the relatively small number of cases obtained from each anatomical site in this series. However, the level of expression of ADAM12 was significantly lower in diffuse-type, compared with intestinal-type carcinomas. Down-regulation of adhesion molecules has been frequently observed in diffuse-type carcinomas (Tahara 2004), including syndecan-1 (Watari et al 2004) and β1-integrin (Solcia et al 1996), adhesion molecules involved in ADAM12-mediated formation of focal adhesions and cell spreading (Thodeti et al 2003, Iba et al 1999). The lower

expression of ADAM12 in diffuse-type may reflect the different pattern of tumour-host interactions that distinguishes diffuse- from intestinal-type gastric cancers.

Although ADAMs are differentially expressed in malignant tumours and may therefore participate in the pathology of carcinomas, as yet, the role of individual members of the ADAM family in tumour development is largely unknown. Since specific inhibitors of metalloprotease activity of the individual ADAMs are not available, the gastric cancer cell lines were treated with anti-ADAM antibodies, which significantly affected the proliferation of the gastric cancer cell lines. However, the implication of ADAM9, 12 and 15 in the regulation of cellular proliferation and the malignant growth of gastric cancer cells requires further clarification. Due to the multifunctional aspects of these ADAMs, antibody binding could affect various tumour biology relevant processes, such as adhesion, intracellular signalling, and growth factor shedding.

ADAMs are able to modulate cellular adhesion to adjacent cells and extracellular matrix by binding to adhesion molecules - a prerequisite for expansion, invasion and spreading of tumour cells. The presence of an RGD motif in a β-loop structure of the disintegrin domain of ADAM9 and 15 facilitates binding to the integrins α6β1, α9β1, αvβ3, or αIIβ3 (Nath et al 2000, Eto et al 2002, Nath et al 1999, Tomczuk et al 2003, Tselepis et al 1997, Zhang et al 1998), and ADAM-integrin interactions have been implicated in tissue and matrix remodelling (Le Pabic et al 2003, Arndt et al 1998). With the disintegrin domain of ADAM9 and 15 blocked by antibodies, the normal integrin-dependent cell-cell/cell-matrix interaction of the tumour cells could be hindered, and may also result in a reduction in proliferation.

Interestingly, the binding of the anti-ADAM12 antibodies resulted in a proliferative effect on the cell growth in four of the five cell lines. ADAM12 lacks an RGD motif, and binding to syndecans via the cysteine-rich domain indirectly triggers β1-integrin-dependent cell spreading, perhaps via intracellular signalling (Iba et al 2000, Thodeti et al 2003). ADAM12 contains Src-homology-3 (SH3) domain binding motifs, and is known to bind to several Src family protein-tyrosine-kinases and SH3 domain-containing proteins, which are involved in the transduction of intracellular signals (Kang et al 2000, Suzuki et al 2000, Abram et al 2003). Antibody-binding may result in the activation of these signal transduction pathways, and a corresponding increase in cell proliferation and spreading.

In particular, the ADAMs metalloprotease activity influences cell proliferation. In the stomach, proliferation and migration of gastric epithelial cells, and the cellular response to infection and mucosal injury are regulated, in part, by the EGFR transduction pathway

(Miyazaki et al 2001, Miyazaki et al 1996, Zushi et al 1997, Wallasch et al 2002, Keates et al 2001, Noble et al 2003, Chen et al 2002), which plays a key role in tumour proliferation, angiogenesis, invasion and metastasis (Jonjic et al 1997, Fischer et al 2003, Eccles 2000). Transactivation of the EGFR by hormones and regulatory peptides that are found in the gastrointestinal tract, such as gastrin, angiotensin II, bradykinin, bombesin, or substance P, is apparently mediated by disintegrin-metalloproteinase shedding of EGFR-ligands (Schäfer et al 2004, Fischer et al 2003, Asakura&Kitakaze 2002, Miyazaki et al 1999, Tsutsui et al 1997, Dong et al 1999). Shedding can be induced by various activating and pathological stimuli, and the specific ligand released is defined by cellular context and stimulus. There is compelling evidence that ADAM9, 12 and 15 can release some of the transmembrane protein-derived EGFR ligands, such as HB-EGF, amphiregulin, and TGFα (Izumi et al 1998, Asakura et al 2002, Schäfer et al 2004). Up-regulation of these ADAMs could increase ligand-shedding, and thereby the availability of EGF-like ligands for the EFGR, and, like overexpression of the EGFR ligands (Ruck&Paulie 1997, Ruck&Paulie 1998), promote transformation and proliferation by autocrine mechanisms. In the cell proliferation assays, blocking the metalloprotease domain and the associated protease activity by antibody binding could result in a decrease in ligand-shedding and a corresponding reduction of the growth rate, as was observed for ADAM9 and ADAM15.

4.2.6 The ectopeptidases and intestinal metaplasia

In connection with tumour development, it was interesting to note that all of the investigated ectopeptidases are consistently expressed on the apical surface in intestinal metaplasia, with the controls verifying that the immunoreactivity observed in the intestinal metaplasia was due to specific antibody-antigen reactions, and not to brush border alkaline phosphatase activity. The ectopeptidases exhibited high intensity immunostaining in virtually every occurrence of intestinal metaplasia, with the intestinal metaplasia being associated with both intestinal-type and diffuse-type tumours.

Intestinal metaplasia is considered to be one of the most important risk factors for gastric carcinoma. It is connected with an approximately 10-fold increased risk of developing stomach cancer, and particularly intestinal-type gastric cancer is believed to develop from or surrounding this lesion (Leung&Sung 2002). Secretion of neuropeptides forms part of the response to gastric mucosal damage, and many of these peptides have been shown to alleviate or inhibit the extent of stress-related gastric lesions (Brzozowski et al 1999, Hernandez et al 1983, Konjevoda et al 2001, Mercer et al 1997). Intestinal metaplasia often evolves as a

response to chronic atrophic gastritis (Petersson et al 2002), and the disruption of the proteolytic balance in the local stomach region caused by the high level of ectopeptidases in the intestinal metaplasia, may increase the degradation of gastric peptides, thereby further reducing their mucosal cytoprotective effect. This is supported by reports that treatment with ACE inhibitors reduces significantly the incidence of gastric ulcers (Rao et al 1995, Uluoglu et al 1998). However, it has been suggested that intestinal metaplasia and gastric cancer arise coincidentally (Meining et al 2001), and the up-regulation of the ectopeptidases in gastric cancers is not necessarily related to their expression in intestinal metaplasia. Ectopeptidase-positive gastric cancers were also found in patients without evidence of intestinal metaplasia, and the expression of the ectopeptidases in the intestinal metaplasia may merely reflect the intestinal phenotype of these metaplastic cells.

4.2.7 Ectopeptidases and lymph node metastases

Ectopeptidases have also been linked to metastatic behaviour, and the primary gastric cancers in this study showed a heterogeneous expression pattern with some areas demonstrating strong expression and others of the same tumour being almost completely devoid, which raised the question whether expression of a particular ectopeptidase indicates a specific metastatic potential. The expression level of NEP, APN, and ACE in our lymph node metastases, as evaluated by the IRS, correlated strongly (r>0.5) with the level of expression in the primary tumour. Predominantly APN has been brought into association with metastasis (Saiki et al 1993, Menrad et al 1993), and a high expression level in our primary tumours was usually associated with high expression in the corresponding lymph node metastasis. However, evaluation of the IRS values according to the lymph node status demonstrated that the expression of the ectopeptidases was not significantly different in tumours that metastasised to the lymph node, compared to those without metastases. Interestingly, the same heterogeneous pattern of expression was observed in both the lymph node metastases and the primary tumours, indicating that, although the ectopeptidases may be important for the tumour cell biology, the expression of a particular ectopeptidase may not be associated with a particular metastatic phenotype.

4.3 Conclusion

In summary, strong evidence is provided that ectopeptidases are differentially expressed in both liver and gastric cancer on both the transcriptional and protein level. There are various, often conflicting explanations for the observed cancer-related up- or down-regulation of these ectopeptidases. Here, it has been demonstrated that the inhibition of the ectopeptidase activity affects tumour cell proliferation in different ways, being able to both activate and inactivate *in vitro* proliferative processes. Ectopeptidases have broad, partially overlapping substrate specificities, and as shown here, their expression pattern is not generally uniform, with different ectopeptidases cleaving the same substrate at different anatomical sites. The deregulation of the subtle balance between interactions with adhesion molecules and the extracellular matrix, and the shedding of surface proteins including growth factors and their receptors, affects inter- and intracellular signalling pathways. Indeed, the maintenance of malignant growth is dependent on an extremely complex system of proteolytic regulation. In particular, the triple membrane passing signal concept of EGFR transactivation, which involves the GPCR stimulation of ADAMs-mediated shedding of EGFR ligands, is becoming more and more established (Wallasch et al 2002). As shown in Figure 4.16, the ectopeptidases investigated here are involved in the regulation of the local GPCR ligand concentration (NEP, APN, DPIV, ACE), as well as regulating the release of the EGFR ligands from the cell surface (ADAM9, ADAM12, ADAM15).

The ability to degrade and inactivate peptide hormones and growth factors, with the resultant modulation of the tumour-host interface, may play an important role in the pathogenesis, development or progression of gastrointestinal cancers. Ectopeptidase inhibitors have been suggested as treatment for cancer (Antczak et al 2001, Nanus et al 1997), and the aminopeptidase inhibitor, Bestatin, is currently being studied in clinical trials (Ichinose et al 2003, Ota et al 1992). ACE inhibitors and AT1 blockers are already used to treat hypertension. However, although the deregulation of the ectopeptidases leaves no doubt that they are important for the tumour cell biology, the use of ectopeptidase inhibitors, particularly when orally administered, may be contraindicated in some cancer patients. In those gastrointestinal cancers where the up-regulation of the ectopeptidases is an internal response to uncontrolled growth, treatment with ectopeptidase inhibitors may increase the proliferative effect mediated by systemic neuropeptides and growth factors.

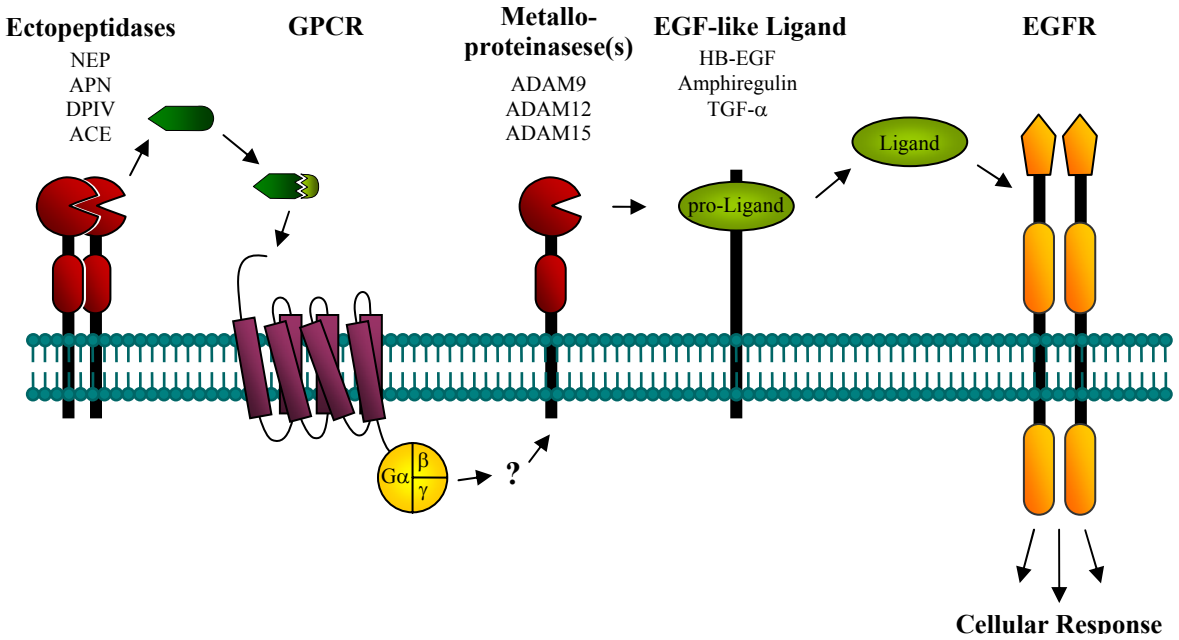

Figure 4.16: The role of the ectopeptidases in epidermal growth factor receptor transactivation The triple membrane passing signal concept of epidermal growth factor receptor (EGFR) transactivation involves the stimulation of G-protein coupled receptors (GPCR) by GPCR ligands, which induces the ADAMs-mediated shedding of EGFR ligands. Ectopeptidases are involved in the regulation of the local GPCR ligand concentration (NEP, APN, DPIV, ACE), as well as regulating the release of the EGFR ligands from the cell surface (ADAM9, ADAM12, ADAM15) (modified from Fischer et al 2003)

5 ACE AND THE LOCAL ANGIOTENSIN II SYSTEM IN GASTRIC CANCER

In this chapter, one of the ectopeptidases investigated in Chapter 4 and one of its substrates were selected for further investigation. The biological significance of ACE for gastric cancer has been recently demonstrated, with the gene polymorphism associated with higher ACE expression correlating significantly with the development of early gastric cancer (Ebert et al 2005), as well as with nodal status and tumour staging (Röcken et al 2005a).

ACE was here shown to be up-regulated in gastric cancer, and expressed in the preneoplastic conditions of gastritis, gastric ulcer, and intestinal metaplasia. These are all environments of inflammation, and in keeping with the major role played by angiotensin II-mediated chronic inflammation in gastrointestinal carcinogenesis, ACE influences the development of early gastric cancers (Ebert et al 2005). The role of angiotensin II and its receptors in inflammation is relatively well-characterized, and the members of the local angiotensin II system, ACE, AT1 and AT2, are up-regulated in gastric cancer (Carl-McGrath et al 2004, Röcken et al 2006). Additionally, ACE and AT1 are strongly associated with tumour staging and lymph node metastasis, functions not usually associated with the local angiotensin II system (Röcken et al 2005a, Röcken et al 2006). In order to clarify the involvement of this system in lymph node metastasis and invasion, the expression patterns of ACE, AT1 and AT2 in lymph node metastases, and the effect of ACE inhibition and AT1 and AT2 antagonism on cell proliferation and invasion were investigated.

5.1 Results

5.1.1 Immunohistochemistry of gastric cancers and corresponding lymph node metastases

Lymph node metastases from 45 patients were examined for the expression of ACE, AT1, and AT2 in tumour cells metastasised to the lymph node. ACE, AT1 and AT2 were found in 26 (57.8%), 30 (66.7%), and 40 (88.9%) lymph node metastases of 45 patients, respectively. Interestingly, a very distinctive expression pattern was occasionally observed for ACE (6/45; 13.3%), but not for AT1 or AT2 (Figure 5.1). Tumour cells were identified using an antibody directed against cytokeratins (AE1/AE3), with those directly surrounding the lymph follicle strongly expressing ACE, while tumour cells further away showed no expression of ACE (Figure 5.1).

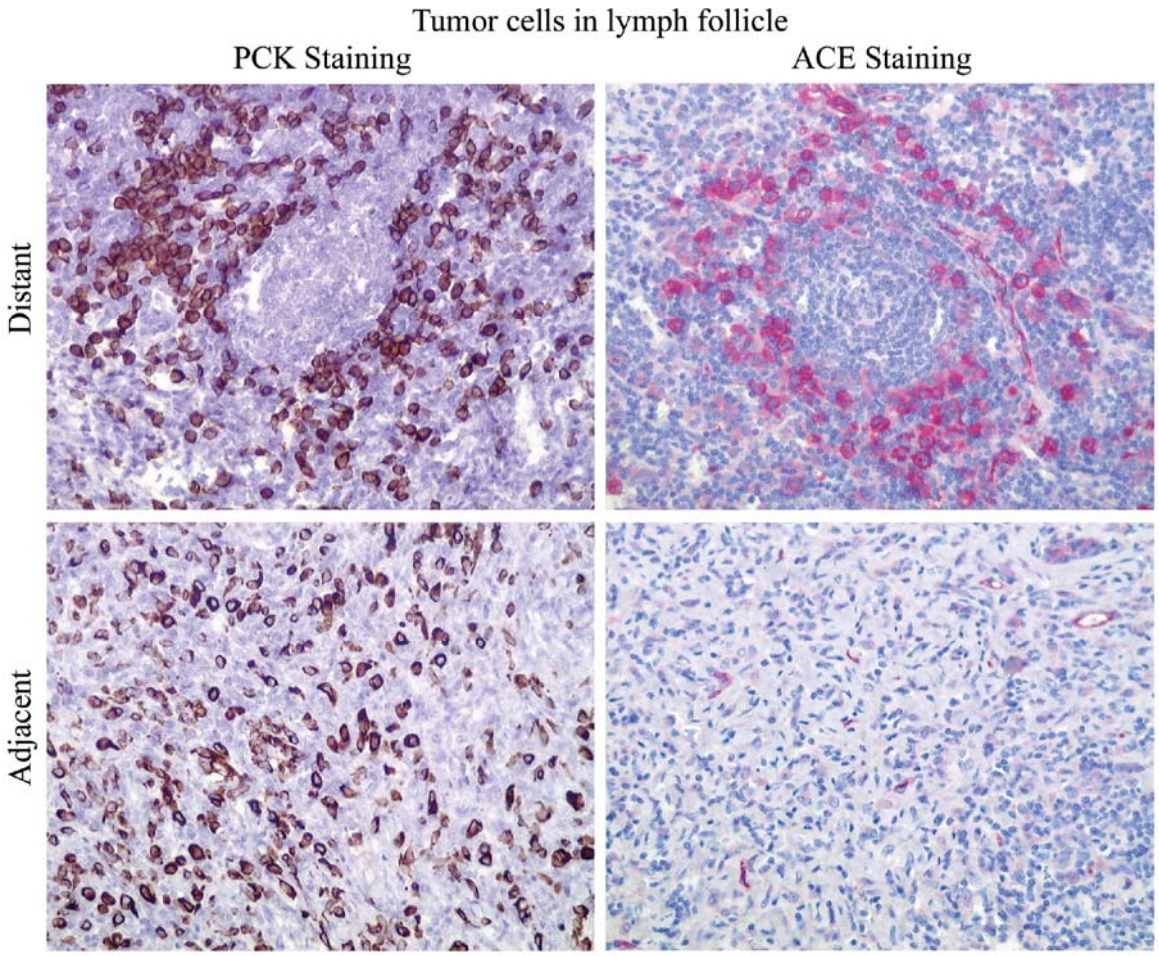

Figure 5.1: Expression of ACE in lymph node metastases Paraffin-embedded sections of lymph node metastases were examined for the expression of ACE in tumour cells metastasised to the lymph node, where a very distinctive expression pattern was occasionally observed. Tumour cells were identified using an antibody directed against cytokeratins AE1/AE3 (PCK), with those directly surrounding the lymph follicle strongly expressing ACE, while tumour cells further away showed no expression of ACE

5.1.2 ACE, AT1 and AT2 mRNA expression in gastric cancer cell lines

Using RT-PCR, it was shown that all the gastric cancer cell lines expressed ACE-, AT1-, and AT2-mRNA (Figure 5.2). MKN28, N87, MKN45 and KATOIII strongly expressed ACE, whereas AGS showed only very low expression levels. AT1 and AT2 were expressed at high levels in AGS, MKN28 and N87 cells, and at lower levels in MKN45 and KATOIII cells (Figure 5.2).

5.1.3 ACE-insertion/deletion gene polymorphism in gastric cancer cell lines

The ACE insertion/deletion gene polymorphism was determined for each gastric cancer cell line. MKN28 and MKN45 have an II-genotype, AGS a DD-genotype, and N87 and KATOIII an ID-genotype (Figure 2). KATOIII was not analyzed further, as the following assays were unsuitable for suspension cultures.

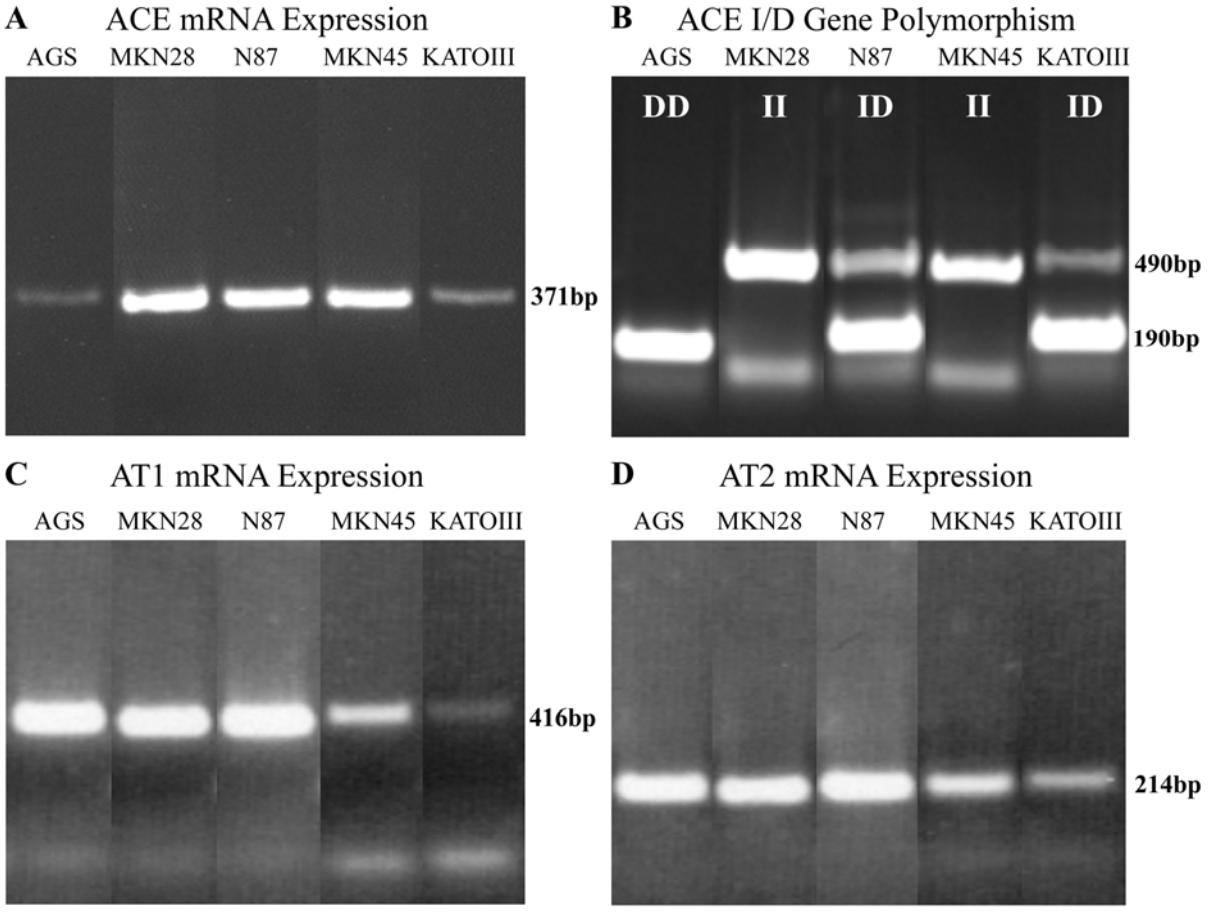

Figure 5.2: ACE, AT1 and AT2 mRNA expression and the ACE-insertion/deletion gene polymorphism in gastric cancer cell lines Using RT-PCR, it was shown that all the gastric cancer cell lines expressed ACE-, AT1-, and AT2-mRNA. The ACE insertion/deletion gene polymorphism was determined for each gastric cancer cell line. Interestingly, AGS has the DD-genotype associated with higher ACE expression, but exhibits comparatively low expression of ACE mRNA.

5.1.4 The effect of cytokine treatment on ACE mRNA expression in gastric cancer cell lines

After treatment of the gastric cancer cell lines MKN28, MKN45, N87 with the cytokines (IL-1β, IL-6, IL-8, TGFβ, TNFα), the induced changes in ACE expression were measured by quantitative real-time RT-PCR, and normalized against β-actin (Figure 5.3). Since ACE levels in AGS cells were too low to be reproducibly detected, these cells were not evaluated. After incubation with IL-1β, ACE expression was significantly increased in MKN28 ($P<0.01$), and significantly decreased in N87 cells ($P<0.01$). IL-6 incubation resulted in increases in ACE mRNA levels, with only N87 reaching significance ($P<0.05$). Expression of ACE was significantly increased in MKN28 cells after IL-8 treatment ($P<0.05$), but no effect was observed for N87 or MKN45. TGFβ induced significant increases in ACE expression in both MKN28 ($P<0.05$) and N87 cells ($P<0.01$). Treatment with TNFα also induced significant increases in ACE expression levels in MKN28 ($P<0.05$) and N87 ($P<0.05$), but had no effect on MKN45 cells.

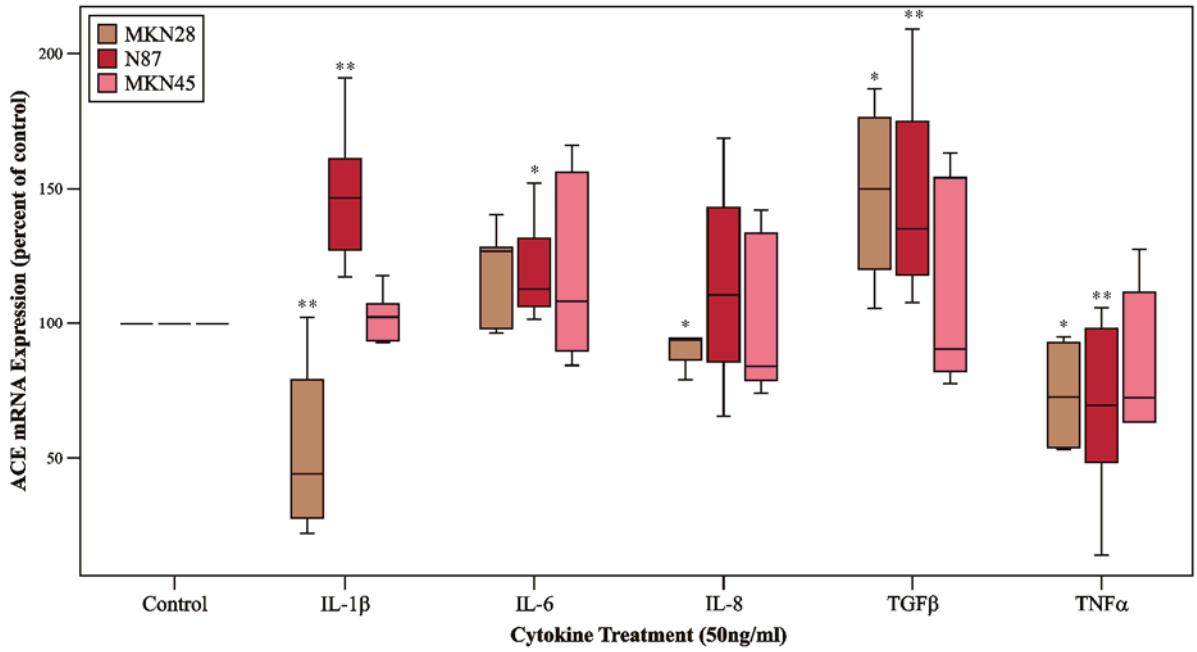

Figure 5.3: Effect of cytokine treatment on ACE mRNA expression After treatment of the gastric cancer cell lines MKN28, MKN45, N87 with the cytokines (IL-1β, IL-6, IL-8, TGFβ, TNFα), the induced changes in ACE expression were measured by quantitative real-time RT-PCR, and normalized against β-actin.

5.1.5 ACE, AT1, and AT2 inhibition in gastric cancer cell proliferation and invasion

The cancer cell lines AGS, MKN28, MKN45, N87 were treated with inhibitors of ACE (enalapril), AT1 (olmesartan), and AT2 (PD123319) for 48 hours, and metabolic activity (Figure 5.4 A) and DNA synthesis (Figure 5.4 B)were evaluated, and in a Matrigel invasion assay, the relative proliferation and invasiveness of the cells was determined (Figure 5.4 C,D).

ACE inhibition did not change MTT conversion, but thymidine incorporation was significantly increased in all four cell lines. For N87 and MKN45, this was reflected in the increase in cell number seen in the invasion assays, which was accompanied by significant decreases in invasive ability. AGS and MKN28 showed no change in cell number or invasion.

The pattern observed after treatment with AT1 inhibitor was similar to that of ACE inhibition. No change in MTT conversion was observed, and thymidine incorporation increased in N87 and MKN45 cell lines. The increased cell number observed for these two cell lines during the invasion assay corresponded to significant decreases in invasive ability. Again, AGS and MKN28 showed no effect.

Inhibition of AT2 resulted in significantly increased MTT conversion for MKN28, N87 and MKN45 cells. AGS showed no change in MTT conversion or thymidine incorporation. Both N87 and MKN45 also exhibited significantly increased thymidine incorporation. These increases were not reflected in the invasion assays, where no change in cell number could be observed for these cells. However, the invasive abilities of all four cells were significantly reduced after AT2 inhibition.

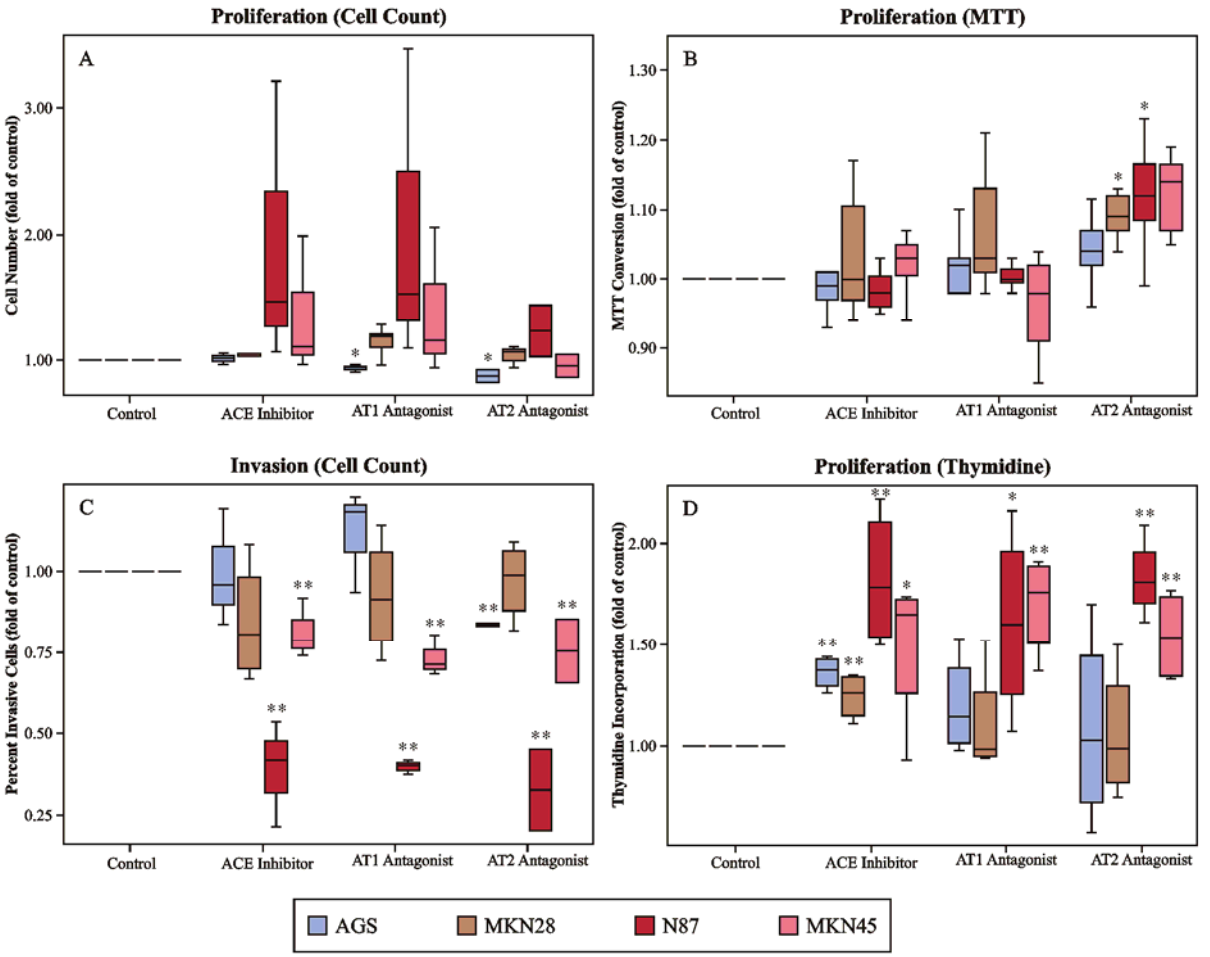

Figure 5.4: ACE, AT1, and AT2 inhibition in gastric cancer cell proliferation and invasion The cancer cell lines AGS, MKN28, MKN45, N87 were treated with inhibitors of ACE (enalapril), AT1 (olmesartan), and AT2 (PD123319) for 48 hours, and metabolic activity and DNA synthesis were evaluated, and in a Matrigel invasion assay, the relative proliferation and invasiveness of the cells was determined.

5.2 Discussion: The Local Angiotensin II System in Lymph Node Metastasis

Invasion and metastasis are fundamental processes involved in cancer progression, with the metastasis to regional lymph nodes representing an early step in the spread of solid tumours (Bogenrieder &Herlyn 2003). The poor prognosis of gastric cancer is often related to the presentation in an advanced tumour stage and is most strongly influenced by lymph node involvement (Yokota et al 2004). Identifying factors contributing to nodal spread may help to improve gastric cancer prognosis. The function of the local angiotensin II system in metastasis has generally been attributed to the promotion of angiogenesis (Yoshiji et al 2001, Yoshiji et al 2002, Fujita et al 2002). However, the ACE insertion/deletion gene polymorphism is also associated with nodal status (Röcken et al 2005a), with the DD genotype being significantly correlated with a greater number of lymph node metastases and advanced UICC tumor stage than the ID or II genotype. AT1 and AT2 are also expressed in gastric cancer and contribute to nodal spread (Röcken et al 2006), and the combination of AT1-expression and the ACE gene polymorphism allows the step wise risk assessment of nodal spread in gastric cancer (Röcken et al 2006). This and other investigations have shown that components of the angiotensin II system, such as angiotensin converting enzyme (ACE), angiotensin II receptor, type 1 (AT1) and type 2 (AT2), are up-regulated in gastric cancer (Carl-McGrath et al 2004, Röcken et al 2005a, Röcken et al 2006). All these data provide abundant evidence for the biological significance of the angiotensin II/angiotensin II receptor system in gastric cancer biology. Here, the previous investigations were extended to investigate the mechanisms by which the local angiotensin II system might mediate its pathological function in gastric cancer. The expression of ACE, AT1 and AT2 in gastric cancers and lymph node metastases, and the possible influence of the angiotensin II system on *in vitro* proliferation and invasive ability of gastric cancer cell lines were examined.

5.2.1 AT1, but not ACE or AT2, is more frequently expressed in metastatic gastric cancers

The protein expression of ACE, AT1 and AT2 was demonstrated immunohistochemically in a series of metastatic gastric cancers and the corresponding lymph node metastases. Interestingly, AT1 was more prevalent in tumour cells of metastatic gastric cancers (67%) than was previously observed in an unselected series of gastric cancers (26%) (Röcken et al 2006). This provides further evidence that AT1 expression correlates significantly with nodal spread (Röcken et al 2006). In contrast, the prevalence of ACE and AT2 in gastric cancer metastases does not significantly differ from their prevalence in primary

tumours and confirms previous investigations, where immunohistochemical detection of ACE and AT2 did not correlate with the presence and extent of lymph node metastases.

5.2.2 ACE expression is occasionally up-regulated near the lymph follicle, which may be due to the local presence of cytokines

In a few cases, a peculiar expression pattern of ACE was observed in lymph node metastases. Tumour cells surrounding lymph follicles showed high levels of ACE-expression, while tumour cells distant to lymph follicles were immunonegative. This distinctive immunohistochemical staining pattern, along with the up-regulation of ACE observed in gastritis and gastric ulcers (Sections 4.2.4.2), led to the conjecture that the expression of ACE may be influenced by differences in the local cytokine concentrations. To test this, a number of cell culture experiments were conducted. Using recombinant cytokines and four different gastric cancer cell lines, it could be shown that the transcription of the ACE-gene can be modulated by various pro- and anti-inflammatory cytokines (IL-1β, IL-6, IL-8, TGFβ and TNFα), with both up- and down-regulation of ACE mRNA levels observed. Interestingly, each cell line showed an almost unique transcriptional response to the cytokines and these *in vitro* observations reflect those made *in vivo*. The lymph node metastases of only a small number of patients showed a focal expression of ACE in lymph node metastases, while in others no difference in the local expression of ACE could be found, indicating that the ACE expression may be regulated by the regional cytokine profile, with the final observable ACE expression level resulting from a balance of up- and down-regulation. Thus, each gastric cancer and cancer cell line may have a unique and as yet unpredictable susceptibility of ACE expression to cytokines.

5.2.3 The ACE gene polymorphism does not necessarily reflect the ACE expression level

The level of mRNA expression, and hence the activity of ACE, is influenced by the insertion/deletion gene polymorphism, with an increased amount of ACE mRNA originating from the D allele (Suehiro et al 2004). All the gastric cancer cell lines expressed ACE, AT1 and AT2. Although the highest mRNA expression level is associated with the DD genotype, ACE expression in AGS cells with the DD-genotype is particularly low compared to the other cell lines, which all express ample amounts of ACE, despite having genotypes associated with lower ACE expression levels (ID/II). Thus, ACE mRNA levels in gastric cancer cells appear to depend on additional factors, such as cytokines, and not solely on the genotype.

5.2.4 The local angiotensin II system mediates tumour cell proliferation and hypertrophy

Angiotensin II stimulates cellular proliferation or hypertrophy via AT1, as well as suppressing cell growth by inducing apoptosis or differentiation via AT2 (Wolf&Wenzel 2004). Angiotensin II binding to AT1 induces EGFR transactivation, via the activation of metalloproteinase-mediated shedding of EGFR ligands, and can thereby influence cell proliferation (Schäfer et al 2004, Mifune et al 2005, Olivares-Reyes et al 2005). However, GPCR-mediated hypertrophy is also mediated by transactivation of the EGFR (Asakura et al 2002, Fujiyama et al 2001, Moriguchi et al 1999, Shah&Catt 2003), and is characterised by elevated protein content and synthesis, without concurrent changes to DNA levels, in addition to phenotypic changes (Hannan et al 2003). The decrease in cell proliferation expected after AT1 inhibition did not occur. Instead, the cell lines increased thymidine incorporation and absolute cell number, but showed no change in MTT conversion after inhibition of ACE or AT1, suggesting the cells were then able to progress into the S phase of the cell cycle, as well as maintaining the levels of protein synthesis. This is in keeping with the role angiotensin II and AT1 plays in hypertrophy.

The involvement of AT2 in hypertrophy is less clear, and may be due to constitutive activation of the AT2 receptor (D'Amore et al 2005, Miura&Karnik 1999), which is unable to be inhibited by PD123319, the AT2 inhibitor used here (D'Amore et al 2005). PD123319 does selectively inhibit the binding of angiotensin II to AT2, but may be unable to inhibit all the effects of the AT2 receptor (D'Amore et al 2005, Miura&Karnik 2000). However, AT2 opposes AT1 signalling and mediates the anti-proliferative and apoptotic functions of angiotensin II, so the observed increase in DNA synthesis and MTT conversion may result from the attenuation of the growth inhibitory actions of the AT2 receptor after inhibition of AT2. Interestingly, despite the increase in DNA synthesis and MTT conversion, the actual cell number did not change after AT2 inhibition in comparison to the control, indicating that perhaps apoptosis was still being carried out. This is in keeping with reports that the presence of AT2 induces apoptosis in the absence of angiotensin II, an effect unable to be modulated by PD123319 (Mioura&Karnik 2000).

5.2.5 Inhibition of the local angiotensin II system reduces invasion of gastric cancer cells

Tumour cell proliferation and invasion are essential for successful nodal spread of tumour cells, and the influence of the angiotensin II/angiotensin II receptor-system on tumour cell proliferation and invasion was investigated. However, it was not possible to determine the specific receptor subtype mediating the effect of inhibition, since receptor inhibition not only

limits the functions carried out by the inhibited receptor, but also shifts the availability of the signalling peptide towards the second receptor.

Inhibition of angiotensin II binding to the AT1 and AT2 receptors, as well as ACE inhibition, had a significant effect on the invasive potential of the cells. Angiotensin II is involved in the AT1 receptor-mediated invasion and migration of carcinoma cells, including cervical carcinoma (Kikkawa et al 2004), ovarian carcinoma (Suganuma et al 2005) and choriocarcinoma cells (Ishimatsu et al 2005). Here, inhibition of invasion was achieved in the N87 and MKN45 cell lines by blocking ACE activity and the AT1 receptor. EGFR expression is implicated in tumour progression and survival in gastric cancers (Lin et al 2000, Kopp et al 2003), and transactivation of the EGFR is associated with migration and invasion in head and neck squamous cell carcinomas (Gschwind et al 2002, Gschwind et al 2003). Inhibition of the AT1-mediated transactivation of the EGFR may reduce the invasive potential of the gastric cancer cells. Additionally, up-regulation of the vascular endothelial growth factor (VEGF) via the AT1 receptor is also directly associated with the angiotensin II-mediated induction of invasion (Kikkawa et al 2004). In addition to AT1, AT2 also up-regulates VEGF expression (Rizkalla et al 2003, Zhang et al 2004), and the invasive ability of all four cell lines could be significantly reduced by AT2 inhibition. AT2 inhibition may also influence the regulation of cytoskeletal elements and adhesion molecules, since activation of AT2 receptors has been shown to induce neurite outgrowth, via the regulation of neurofilaments (Laflamme et al 1996, Meffert et al 1996). Blockade of AT2 may also result in the augmentation of AT1-mediated functions, such as the up-regulation of adhesion molecules including E-selectin, P-selectin and the vascular cell adhesion molecule-1 (Alvarez et al 2004), which may enhance cellular adhesion, thereby inhibiting cellular migration.

The angiotensin II-mediated modulation of gastric cancer cell growth and invasion is highly dependent on the cell type, expression of receptor subtypes, and growth factor and cytokine signalling. In the gastric cancer cell lines, although inhibition of ACE and AT1 did inhibit invasion, it was accompanied by an increase in tumour cell proliferation, which in the clinical situation could be a distinct disadvantage, when attempting to reduce tumour progression and metastasis. In this respect, it was particularly interesting that AT2 blockade also significantly reduced the invasive potential of the tumour cells, without a concurrent effect on the absolute cell number.

5.3 Conclusions

The poor prognosis of gastric cancer is often related to the presentation in an advanced tumor stage and is most strongly influenced by lymph node involvement (Yokota et al 2004). Early identification of a high risk gastric cancer group might allow the identification of patients, who should undergo extensive lymph adenectomy (D2 versus D1) in gastric cancer surgery, as well as enabling the therapeutic approach to be tailored towards a more aggressive regimen in these patients. The combination of AT1-expression and ACE I/D gene polymorphism allows the step wise risk assessment of nodal spread in gastric cancer, possibly already before metastases have developed.

Here, the previously reported involvement of the ACE genotype and AT1 expression in lymph node metastasis of gastric cancer was investigated. It was demonstrated that Ang II mediates invasion in gastric cancer cell lines and that this invasion can be attenuated by ACE inhibitors, and AT1 and AT2 antagonists. Inhibitors of local Ang II system components have already been considered for “novel” anti-neoplastic treatment and cancer prevention strategies, and AT1 or AT2 antagonists might prove to be useful for the treatment of gastric cancer, particularly by preventing or reducing nodal spread in high risk patient groups.

6 SUMMARY

Hepatocellular and gastric carcinomas are among the most frequent gastrointestinal tract cancers, and have an exceedingly poor prognosis. Decreasing the high incidence and mortality of hepatogastrointestinal cancers will require earlier diagnosis of these cancers, as well as a wider range of therapeutic alternatives. In this study, the identification of new markers or therapeutic targets that may be able to improve the diagnosis and treatment of hepatocellular and gastric carcinomas was approached from two different directions.

For the identification of novel genes relevant for gastrointestinal tumour biology, differential display was used to compare non-tumour tissues and HCC. A novel candidate gene that was up-regulated in HCC was identified as GSDML, a novel member of the cancer-associated GSDMDC protein family. GSDML is expressed in both tumour and non-lesional tissues, but the expression profile of GSDML splicing variants may be altered, and a larger tissue- and/or tumour-specific analysis of the expression of the GSDML gene and transcripts, as well as more information about the actual protein sequence and structure is required before its role in tumour biology can be clarified.

Using a different approach, the putative role of multifunctional membrane proteases during the development and progression of gastrointestinal cancers was evaluated. NEP, APN, DPIV, ACE, ADAM9, ADAM12, and ADAM15 are ectopeptidases known to be important for tumour growth and dissemination, which was here also observed for liver and gastric carcinomas. Further investigations, based on the immunohistochemical results described here for HCC, have shown that the expression patterns of NEP and APN can be applied as diagnostic markers for HCC, and in combination, have proved useful in diagnostic pathology to differentiate HCCs from non-HCCs metastatic to the liver (Röcken et al 2005). Additionally, the low expression of ACE in focal nodular hyperplasia can assist diagnosis of these lesions (Gräntzdörffer et al 2004). All the investigated ectopeptidases were up-regulated in gastric cancer, and expressed in intestinal metaplasia and lymph node metastases. Gastric cancer cell proliferation was retarded by inhibition of NEP, APN, and ACE. Inhibitors of these ectopeptidases are already established for treatment of, for example, hypertension, and may well provide future therapeutic options for the treatment of gastric cancer. The ADAMs also influence gastric cancer cell proliferation. However, although the ADAMs are up-regulated in gastric cancers, their expression pattern is not tumour-specific enough to be practical for diagnosis, or as therapeutic targets. Since the investigated ADAMs are almost ubiquitously expressed, the GPCR stimulation of ADAMs-mediated growth factor shedding

and subsequent EGFR transactivation (Wallasch et al 2004) makes the regulation of GPCR ligands by the ectopeptidases increasingly interesting.

Ang II is generated by ACE-mediated cleavage of Ang I, and its GPCRs, AT1 and AT2 are involved in EGFR transactivation. ACE and AT1 have previously been shown to be biologically significant for the development and progression of gastric cancer. The association of ACE with the development of early gastric cancer (Ebert et al 2005) may reflect the upregulation of ACE in inflammatory conditions of gastritis and ulcers observed here, and the subsequent increase of proinflammatory Ang II. Indeed, ACE expression is regulated by cytokines, possibly also in the lymph nodes. ACE and AT1 are strongly associated with tumour staging and lymph node metastasis, functions not usually associated with the local angiotensin II system (Röcken et al 2005). Here, it has been shown that the involvement of the local angiotensin II system in metastasis and invasion is not limited to the generally accepted promotion of angiogenesis, but the expression of ACE, AT1, and AT2 by the tumour cells directly facilitates invasion, and thereby lymphatic metastasis. This invasion can be attenuated by ACE inhibitors, and AT1 and AT2 antagonists, and inhibitors of local Ang II system components might prove to be useful for the treatment of gastric cancer, particularly by preventing or reducing nodal spread in high risk patient groups.

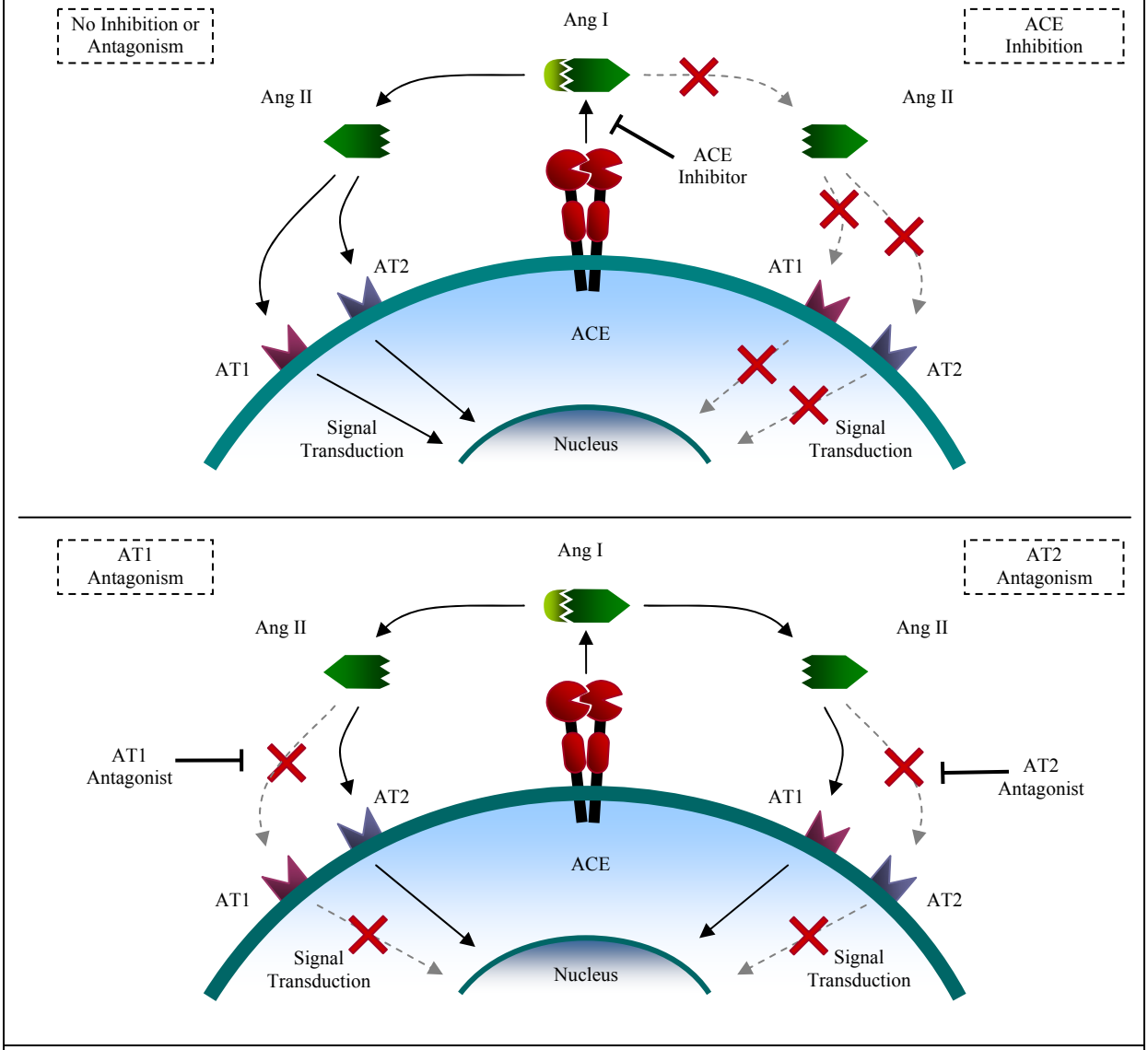

Figure 6.1: The Angiotensin II System provides valid targets for gastric cancer therapy Angiotensin II (Ang II) is generated from angiotensin I (Ang I) via cleavage by the angiotensin converting enzyme (ACE). The angiotensin II G-protein coupled receptors, AT1 and AT2, initiate a range of signal transduction pathways involved in tumour biology, such as the transactivation of the EGFR, that regulate proliferation, hypertrophy, neoangiogenesis and invasion. ACE, AT1, and AT2 are extracellularly orientated transmembrane proteins, for which a large number of well-characterized inhibitors and antagonists are available, making them attractive targets for chemotherapy.

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8 APPENDICES

8.1 List of Abbreviations

aa	amino acid
AAT	α1-antitrypsin
ABC	avidin-biotin complex
ACE	angiotensin-converting enzyme
ADA	adenosine deaminase
ADAM	A Disintegrin And Metalloproteinase
AE1/AE3	cytokeratins
AFB	aflatoxin B_1
Ang I	angiotensin I
Ang II	angiotensin II
AP	alkaline phosphatase
APAAP	alkaline phosphatase-anti-alkaline phospatase
APC	adenomatous polyposis coli
APN	aminopeptidase N
AT1	angiotensin II Type 1 receptor
AT2	angiotensin II Type 2 receptor
β2M	β2-microglobulin
BALB/3T3	mouse embryonic fibroblast cell line
BFP	blue fluorescent protein
BSA	bovine serum albumin
CagA	*H. pylori* pathogenicity factor
Cag	*H. pylori* pathogenicity island
CALLA	common acute lymphoblastic leukaemia antigen
CD	cluster of differentiation
cDNA	complementary DNA
CRSS	268bp fragment from differential display
CTL	cytotoxic T cell
DEAD	RNA binding motif of RNA helicase
DDRT-PCR	differential display polymerase chain reaction
DN	dysplastic nodule
DNA	desoxyribonucleic acid
dNTP	d-nucleotide triphosphate
DPIV	dipeptidyl peptidase IV
EBV	Epstein-Barr virus
ECD	integrin binding motif
ECM	extracellular matrix
EDTA	ethylene-diaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Elk1	ets related protooncogene 1
FAP	familial adenomatous polyposis
Fas	member of TNF receptor family
FADD	Fas-associated death domain
FCS	foetal calf serum
GFP	green fluorescent protein
Gp	glycoprotein
GPCR	G-protein coupled receptor

GSDM	gasdermin
GSDMDC	gasdermin-domain-containing protein family
GSDML	gasdermin-like protein
H. pylori	*Helicobacter pylori*
HB-EGF	heparin-binding epidermal growth factor
HBsAg	Hepatitis B serum antigen
HBV	Hepatitis B virus
HBx	transcript of theHBV X gene
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HELAH	zinc-binding protein motif
HNPCC	hereditary nonpolyposis colorectal cancer
HRP	horseradish peroxidase
IARC	international agency for research on cancer
IHC	immunohistochemistry
IgG	immunoglobulin G
IGF	insulin-like growth factor
IL-1	interleukin 1
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IM	intestinal metaplasia
IQR	interquartile range
IRS	immunoreactivity score
IVTT	*in vitro* transcription/translation
kb	kilobase
kDa	kilodalton
LCD	large cell dysplasia
MCP-1	monocyte chemoattractant protein 1
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltatrazolium bromide
MUC	mucin core protein
MW	molecular weight
NBT/BCIP	p-nitroblue tetrazolium chloride/5-bromo-4- chloro-3-indolyl phosphate
NC	nitrocellulose
NEP	neutral endopeptidase
NF-κB	nuclear factor kappa B
NT	non-neoplastic tissue
NS3	Heptitis C virus NS3 helicase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCK	pan-cytokeratin
PI 3-kinase	phospho-inositol-3 kinase
PJS	Peutz-Jeughers Syndrome
PKR	double stranded RNA (dsRNA)-activated serine/threonine kinase
PVDF	polyvinylidene difluoride
Rb1	retinoblastoma 1 gene
RGD	integrin binding protein motif
RPMI	Roswell Park Memorial Institute media formulation
RT	room temperature

RT-PCR	reverse transcription polymerase chain reaction
RNA	ribonucleic acid
SCD	small cell dysplasia
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophorese
SH3	Src-homology-3
SNARE	soluble NSF attachment protein receptors
STK11	serine/threonine kinase 11
TAE	Tris-acetic acid-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.5% Tween 20
TGF-β	transforming growth factor β
TNF	tumor necrosis factor
TNM	tumor-node-metastasis staging
TU	tumor
UICC	International Union Against Cancer
VacA	vacuolating cytotoxine A
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

8.2 List of Figures

8.3 List of Tables

8.4 Curriculum Vitae

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