Aus der Klinik für Innere Medizin
der Medizinischen Fakultät Charité
der Humboldt-Universität zu Berlin

Dissertation

GENOME-WIDE SCREENING OF LOSS OF HETEROZYGOSITY IN HUMAN MIDGUT CARCINOID TUMORS WITH FLUORESCENT TECHNIQUE

Zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät Charité
der Humboldt-Universität zu Berlin

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Datum der Promotion: 14. Juni 2004

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Widmung

Meinen lieben Eltern

Abbreviations

LOH	Loss of heterozygosity
MEN	Multiple endocrine neoplasia
APUD	Amine precursor uptake and decarboxylation
NSE	Neuronespecific enolase
5-HIAA	5-hydroxyindolaceticacid
EPT	Endocrine pancreatic tumors
CT	Computerized tomography
MRI	Magnetic resonance imaging
RFA	Radiofrequency ablation treatment
GH	Growth hormone
ACTH	Adrenocorticotrophic hormone
TSH	Thyroid stimulating hormone
VHL	Von Hippel Lindau
PP	Pancreatic polypeptide
PYY	Peptide YY
GEP	Gastroenteropancreatic
WDHA	Watery diarrhea hypocalcemia achlorhydria
GHRH	Growth hormone releasing hormone
ECL	Enterochromaffine-like
VIP	Vasoactive intestinal peptide
HTP	Hydroxy tryptophane
PET	Positron emission tomography
Cdk	Cyclin-dependant kinase
TNF	Tumor necrosis factor
IL-1	Interleukin 1
ICE	Interleukin-1 converting factor
FMTC	Familial medullary thyroid carcinoma
HCG	Human chorionic gonadotropin
CAG	Chronic atrophic gastritis
TSG	Tumor suppressor gene
APC	Adenomatosis polyposis coli
FCC2	Familial non polyposis type of colonic cancer
PG1	Prostaglandin 1
TGF	Tumor growth factor

EGF	Epithelial growth factor
NGF	Nerve growth factor
PDGF	Platelet derived growth factor
dNTP	2´-deoxynucleoside 5´-triphosphate
dATP	2´-deoxyadenosine 5´-triphosphate
dCTP	2´-deoxycytosine 5´-triphosphate
dGTP	2´-deoxyguanine 5´-triphosphate
dTTP	2´-deoxytyrosine 5´-triphosphate
RNAse	ribonucleotidacidase
FAL	Fractional allelic loss

Vorwort

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1 Zusammenfassung

1.1 HINTERGRUND

Karzinoid-Tumoren des embryonalen Mitteldarms sind seltene intestinale neuroendokrine Tumoren, bei denen zum Zeitpunkt der Diagnose häufig Metastasen vorliegen. Im Gegensatz zu Karzinoiden des Vorderdarms und Respirationstraktes sind sie nicht mit der Multiplen Endokrinen Neoplasie Typ 1 (MEN1) vergesellschaftet. Die Mechanismen ihrer Tumorigenesis sind weitgehend unbekannt.

1.2 METHODEN

Tumorgewebe acht sporadischer, maligner Dünndarm-Karzinoide war Objekt dieser Studie über Verlust der Heterozygotie („Loss Of Heterozygosity“ (LOH)) mit 131 fluoreszierenden Mikrosatelliten. DNA Sequenz-Analyse mit Oligonucleotid Primern, die Exon 8-11 des SMAD4/DPC4 Gens flankieren sowie immunhistochemische Färbung mit Smad4/DPC4 antikörpern wurde durchgeführt.

1.3 ERGEBNIS

Chromosom 18 wies Deletionen in 88% der Tumoren auf. Alle außer einem Tumor hatten sowohl 18p als auch 18q verloren, in einem der Tumoren war eine kleine Region telomer zu den *SMAD4/DPC4/DCC* Genen auf *18q21* verloren. Andere Chromosomen waren nur in drei Tumoren betroffen. LOH auf Chromosom 11q13, dem MEN1 Lokus, wurde nicht gefunden.Sequenzierung der DNA und immunhistochemische Färbung für das *SMAD4/DPC4* Gen zeigten keine Aberrationen.

1.4 DISKUSSION

Die Funde der Chromosom 18 Deletionen weisen eindeutig auf ein entscheidendes Ereignis in der Tumorigenese von Karzinoiden des Mitteldarms hin. An der Entstehung dieser Tumoren könnte ein mutmaßliches Tumor Suppressor Gen beteiligt sein, welches auf Chromosom 18 lokalisiert ist. Dahingegen ist SMAD4/DPC4 wahrscheinlich nicht in die Tumorneogenese von Carcinois Tumoren involviert.

Schlagwörter: Verlust der Heterozygotie, Chromosom 18, Mitteldarm, Karzinoid, SMAD4/DPC4

2 Abstract

2.1 BACKGROUND

Midgut carcinoid tumors are rare malignant tumors with origin in the neuroendocrine cells of the small intestine. Due to secretion of a variety of peptide hormones and biogenic amines they cause the carcinoid syndrome. Metastases are often present at first diagnosis. Despite this, patients have a realistic chance to survive for a prolonged period (30% (unresectable/metastatic disease) -79% (non-metastatic disease) 5-year survival rate) if treated by a combination of surgery and medication. Unlike their foregut counterparts, midgut carcinoid tumors are not or rarely associated with the multiple endocrine neoplasia type 1 (MEN1) syndrome. The genetic back-ground to tumorigenesis of these neoplasms is unknown. In contrast, the events involved in tumorigenesis of gastroenteropancreatic adenocarcinomas are better characterized with frequent mutations e.g. of the *Smad4/DPC4*, *Smad2/MADR2/JV18-1* and *DCC* genes on chromosome 18.

2.2 METHODS

Eight metastatic midgut carcinoids were analysed by a genome-wide screening for loss of heterozygosity using 131 PCR-amplified fluorescent-labelled microsatellite markers. DNA sequence analysis using oligonucleotide primers flanking exons 8-11 of the *Smad4/DPC4* gene and immunohistochemical staining with *Smad4/DPC4* antibodies was performed.

2.3 RESULTS

Chromosome 18 was deleted in seven out of eight tumors (88%). All but one of these tumors had lost both *18p* and *18q*, the remaining tumor had lost the long arm but retained the short arm. Several other chromosomal alleles were lost in a subset of the tumors. Loss of heterozygosity (LOH) on chromosome *11q13*, the *MEN 1* locus, was not found. *Smad4/DPC4* wild-type sequence and normal immunohistochemical staining for *Smad4/DPC4* protein was found for all analysed tumors.

2.4 CONCLUSIONS

Our finding of a high frequency of chromosome 18 deletions in 88% of the tumors strongly suggests that midgut carcinoid tumorigenesis might involve inactivation of a candidate tumor suppressor gene located in that region while *Smad4/DPC4* is unlikely to be involved in that process. A more detailed analysis of the genetic events in midgut carcinoid tumors is warranted to clarify their neogenetic origin.

Keywords: Loss of heterozygosity, chromosome 18, midgut, carcinoid tumor, SMAD4/DPC4

3 Introduction

Neuroendocrine gastrointestinal tumors are rare and usually slowly-growing neoplasms originating from the neuroendocrine cell system. These neoplasms may occur either sporadically or in association with familial syndromes, i.e. multiple endocrine neoplasia type 1 (MEN-1). The neuroendocrine cells can be subdivided into cells from the neuroectodermal cells or endodermal origin. Neuroectodermal cells are located in the suprarenal medulla from where pheo-chromocytomas and neuroblastomas arise and in the paraganglia where paragangliomas develop. Endodermal tumors originate from the pituitary and the parathyroid glands, the C-cells of the thyroid gland, the pancreatic islets or from the diffuse endocrine cell system of luminal organs (Wilander and Grimelius 1993). These tumors have been called APUDomas; the idea of the APUD (Amine Precursor Uptake and Decarboxylation) system was first brought up in 1974 by Pearse who detected that cells of neural crest origin move to other tissues such as the intestine, pan-creas and a number of endocrine glands (Pearse 1974). These cells are specialized to accumulate amine precursors (e.g. DOPA or 5-hydroxytryptophan) and to then decarboxylate them to biogenic amines (catecholamines or serotonin), they also produce peptides. Even though this concept was later abandoned by most researchers it still helps to understand the capacity of these cells to produce various hormones and amines. The assessment has been made that de-fective DNA-mismatch repair plays a role in the tumorigenesis of gastrointestinal cancers. For carcinoids, however, this could not be proved (Ghimenti, Lonobile et al. 1999). The prognosis of non-metastatic, resectable neoplasms is excellent (79% 5-year survival rate), whereas metastatic resectable tumors that have metastasized to the liver have a worse diagnosis (30% 5-year survival rate) (Tiensuu Janson and Oberg 1996). However, the patients have a realistic chance to survive for a longer period if treated by a combination of surgery and medication. Histopathological diagnosis is achieved by conventional HE-staining and immunohistochemistry of chromogranin A and synaptophysin. Silver staining (Grimelius and Wilander 1980) as well as the use of neuron-specific-enolase (NSE) as a histopathological marker has been abandoned. Chromogranin A is also analyzed in the patients´ plasma, 80-100 % of patients with diagnosed neuroendocrine tumors present with elevated levels of chromogranin A. Complementary to this and depending on the clinical manifestation of the tumor, other peptide hormones may be analyzed, as well as urine 5-HIAA in cases with midgut carcinoids. In recent years, somatostatin receptor scintigraphy and endoscopic ultrasonography have improved diagnostic results as adjuncts investigations to CT and MRI techniques. In almost 80% of the tumors, somatostatin receptor subtype 2 binding ^{111}Indium-labelled octreotide is found which can be used for both tumor staging and to give an indication of effect of treatment with somatostatin analogues. Rather aggressive surgery has emerged in the past years in order to improve the clinical conditions even if the patients are beyond cure. As for medical treatment, chemotherapy as well as somatostatin analogues and alpha-interferon (in particular for midgut carcinoids) are used. For the present, malignant tumors can be controlled but not cured by this treatment. More information about tumor proliferation, expression of adhesion molecules, growth factors and their receptors will help to focus on individual treatment in the future (Oberg 1996).

3.1 CARCINOID TUMORS

Cells of the neuroendocrine cell system are dispersed along the gastrointestinal tract mucosa as well as in many other organs. In 1907 Oberndorfer described small, slowly growing ileal tumors that showed a more benign course than more commonly recognized carcinomas in the same region introducing the term carcinoid. In 1949 their true malignant potential was emphasized by Pearson and Fitzgerald when they reported on several patients with metastasising carcinoid tumors (Pearson and Fitzgerald 1949). Carcinoid tumors contain well differentiated neuro-endocrine tumor cells, secreting various bioactive and hormonal products. The term carcinoid has been used for an enlarged spectrum of tumors. In 1963, Williams and Sandler classified carcinoid tumors, according to their embryological origin, into foregut carcinoids (occurring in the lungs, thymus, stomach, proximal duodenum and pancreas), midgut carcinoids (originating from the distal duodenum to the mid-transverse colon) and hindgut carcinoids (with origin in the distal colon and the rectum) (Williams and Sandler 1963). Carcinoids supposedly originate from heterogenous neuroendocrine cells and show common features such as specific staining reactions, e.g. argyrophilia (Grimelius and Wilander 1980) the presence of secretory granules and characteristic clinical features.

Midgut carcinoid tumors are usually argentaffin, the other two subgroups are not. They derive from enterochromaffin cells (Kulschinsky cells) in the small intestinal crypts of Lieberkuehn, and are usually depicted as the classical carcinoids. Histologically, midgut carcinoids tend to grow in nests disconnected from the normal tissue. Other carcinoid subtypes (foregut carcinoids) show a more trabecular pattern (Tiensuu Janson and Oberg 1996). Midgut carcinoids and occasionally foregut carcinoids characteristically produce high levels of serotonin (Lembeck 1953). The latter lesions may

also exhibit in rare cases the synthesis of adrenocorticotrophic hormone (ACTH), gastrin, calcitonin and histamine. Regarding hindgut carcinoids, no serotonin is produced but other hormones such as somatostatin and peptide YY (PYY) may occur within the tumor. In all carcinoids high levels of chromogranin A, pancreatic polypeptide (PP) and human chorionic gonadotropin (HCG)-alpha and-beta are often found (Wilander, Lundqvist et al. 1989).

In 1999, the World Health Organization (WHO) revised the clinicopathological classification of neuroendocrine tumors of the gastroenteropancreatic (GEP) tract (Rindi, Capella et al. 2000). Criteria for classification of these tumors are both tumor cell type and the clinical status of the patient, whether associated or not to a tumor-related hyperfunctional syndrome. As for the latter approach, tumors may be divided into functioning or non-functioning tumors. Carcinoids pre-senting with unique features corresponding to the secretion of biologically active substances are called functional tumors. Those tumors refraining from synthesis of biologically active peptide hormones are depicted as non-functioning tumors. The first approach refers to immuno-histochemical cell typing of GEP neuroendocrine tumors providing morphofunctional infor-mation. Correlation between these data and the level of circulating hormones as well as the patient´s clinical symptoms is required. Most tumors are composed of different cell types of which one may be related to a hyperfunctional syndrome. As for the tumor´s natural history and tumor behaviour the hyperfunctional syndrome is *per se* more predictive than identification of a specific hormone cell content in a tumor. Non-functioning tumors present either with a tumor mass or are an unexpected finding at operation. It is recommended to call such growths non-functional neuroendocrine tumors mainly composed of a specific cell type (e.g. „ non-functional tumors of the pancreas mainly composed of glucagon-producing A-cells“) while reserving the term glucagonoma of the pancreas for tumors causing a hyperfunctional syndrome.

Current clinicopathological classification of neuroendocrine tumors of the GEP tract is, according to anatomy, as follows: Neuroendocrine tumors of the pancreas: Well-differentiated functioning (insulinoma) or non-functioning endocrine tumors with benign behaviour (1a), functioning (gastrinoma, insulinoma, vipoma, glucagonoma, somatostatinoma) or inappropriate syndrome tumor (inappropriate hormone syndromes: Cushing (ACTH), acromegaly or gigantism (GHRH), hypercalcemia, etc.) or non-functioning well-differentiated endocrine tumors with uncertain behaviour (1b), well-differentiated low grade malignant endocrine carcinoma, functioning (gastrinoma, glucagonoma, insulinoma, vipoma, somatostatinoma or inappropriate syndrome tumor) or non-functioning (2) and poorly differentiated, highly malignant endocrine carcinoma (3). Neuroendocrine tumors of the stomach: Well-differentiated tumors with benign behaviour (ECL cell tumor associated with chronic atrophic gastritis or MEN-1 syndrome or sporadic, serotonin-producing tumor, gastrin-producing tumor) (1a), well-differentiated tumors with uncertain behaviour (ECL cell tumor, gastrin-, serotonin- or somatostatin-producing tumors or sporadic) (1b), well-differentiated, low grade malignant functioning (gastrinoma, serotonin-producing tumor with carcinoid syndrome, ECL cell tumor with atypical carcinoid syndrome or ACTH-producing tumor with Cushing syndrome) or non-functioning endocrine carcinoma (ECL cell tumor, gastrin-, somatostatin- or serotonin-producing tumors) (2) and poorly differentiated, highly malignant endocrine carcinoma (3). Endocrine tumors of the duodenum and uppermost jejunum: Well-differentiated endocrine tumors with benign behaviour (gastrin- or serotonin-producing tumor, gangliocytic paraganglioma (1a), well-differentiated tumors with uncertain behaviour (somatostatin-producing tumors with or without Recklinghausen´s disease, gastrin- or serotonin-producing tumors) (1b), well-differentiated, low grade malignant endocrine carcinoma (gastrin-or serotonin-producing tumor, somatostatin-producing tumor with or without Recklinghausen´s disease) (2) and poorly differentiated, highly malignant endocrine carcinoma.

Midgut carcinoid tumors (Jejunum, ileum, right colon and appendix) or remaining colon and rectum (hindgut) tumors are classified, as shown above, into well-differentiated endocrine tumors with benign behaviour (serotonin- or enteroglucagon-producing tumors) (1a) and with un-certain behaviour (1b), well-differentiated low grade malignant endocrine carcinomas (sero-tonin-producing carcinoma with or without carcinoid syndrome) (2) and poorly differentiated highly malignant endocrine carcinoma.

The subdivision of carcinoids referring to the anatomical origin of the tumors is rather con-fusing. It has been suggested that the term midgut carcinoid or classical midgut carcinoid should be kept for traditional neuroendocrine midgut carcinoid neoplasms[1]. Other tumors should be referred to as neuroendocrine tumors followed by their primary site and, in addition the predominantly secreted hormone may be added, e.g. gastrin-producing neuroendocrine duo-denal tumor (Kloppel, Solcia et al. 1999).

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[1] In the present thesis I use the term carcinoid or carcinoid tumor for classical midgut carcinoid tumors.

3.1.1 Features regarding the different carcinoid subgroups

3.1.1.1 • Foregut carcinoids

In 1960, bronchial carcinoid tumors were declared to be related to carcinoid tumors arising in the gut (Williams and Azzopardi 1960). Bronchial carcinoids tend to become clinically manifest at earlier age than other carcinoids. Bronchial carcinoids can produce serotonin (these patients may develop the carcinoid syndrome) and other hormones such as ACTH, growth hormone releasing hormone (GHRH) and histamine. ACTH and GHRH production will lead to specific syndromes comprising Cushing´s syndrome and acromegaly, respectively. Histamin secretion may give rise to the histamine-flush, a bright red flush combined with face-swelling and lacrimation. Bronchial carcinoids can be classified according to their histological appearance: Typical carcinoids, atypical carcinoids and small cell lung carcinomas. Typical carcinoids are generally of a more benign nature than atypical carcinoids. However, both types are able to present with a high mitotic count and a high amount of cells staining positive for Ki-67, both prognostically unfavorable factors (Granberg, Wilander et al. 2000). Malignant bronchial carcinoids may metastasize to regional lymphnodes, liver, skin, central nervous system and bones. The 5-year survival rate for patients with typical carcinoids is 87-94% and 56% for atypical carcinoids (Granberg, Wilander et al. 2000).

Carcinoids with origin in the thymus occur more rarely. They show serotonin-, ACTH- and calcitonin-production and characteristically a tendency for local recurrences after surgery. Thymic carcinoid tumors may compress large vessels and the trachea and thus be symptomatic. Spreading of the disease goes hand-in-hand with bad prognosis and short survival.

Gastric carcinoids can be subdivised into well-differentiated tumors (1) (Argyrophil cell tumors, mainly composed by ECL cells or gastrin-producing cells (G cells)) or poorly differentiated tumors (2) (Rindi, Bordi et al. 1996). ECL-omas may be subgrouped into tumors with chronic atrophic gastritis (CAG), achlorhydria and pernicious anemia (type 1), tumors associated with hypertrophic gastropathy and hypergastrinaemia due to Zollinger-Ellison syndrome with MEN1 (type 2) and sporadic gastric carcinoids with sporadic Zollinger-Ellison syndrome and hypergas-trinaemia (type 3). ECL-omas originate from the histamine-producing and -storing entero-chromaffine-like cells. Type 1 ECL-omas are mostly multiple and occur in the gastric fundus and corpus. Gastrin-producing tumors (type 2) may present with G-cell hyperplasia in the gastric antrum. Gastric carcinoids are rarely malignant. ECL-omas may develop histopatho-logically from small ECL-cell nests via linear hyperplasia to solid polyps. Hypergastrinaemia and its trophic effect on ECL-cell seems to play the striking role pathogenetically. Also Zollinger-Ellison syndrome, sporadic or in association with MEN1, may be the cause of hyper-gastrinaemia and gastrin-dependent carcinoids (type 2). As for diagnosis, gastroscopy and histo-pathology are most efficient.

3.1.1.2 • Hindgut carcinoids

Hindgut carcinoids can be subgrouped into tumors of the transverse and descending colon or the rectum. Rectal carcinoids represent the majority and are malignant in 5-40% of the cases (Mani, Modlin et al. 1994). Colonic neoplasms are rare and diagnosed at later stages than rectal lesions. Hindgut carcinoids are generally non-functional but the tumor cells may contain hormones such as PP, PYY and somatostatin (Wilander, Lundqvist et al. 1989). At time of first diagnosis, patients more often suffer from intestinal obstruction, bleeding or having a large palpable abdominal mass abdomen rather than from symptoms due to excessive hormone production.

3.1.2 Midgut carcinoids

3.1.2.1 Incidence

Midgut carcinoid tumors represent a small percentage (0.5-1.5%) of clinically diagnosed intestinal neoplasms compared to e.g. colorectal adenocarcinomas which occur at least 60 times more frequently (Moertel, Sauer et al. 1961; Godwin 1975). Classical midgut carcinoids[2] occur with a clinical incidence of approximately 0.3/100000-0.7/100000 (Skogseid 2001). They are most often diagnosed

in patients of 50-60 years of age but do occur even in children patients. 40-70% of patients with midgut carcinoid syndrome have multicentric disease at time of first diagnosis (Skogseid 2001). However, carcinoid tumors may be detected in about 1% of routine autopsies, thus showing that those tumors often remain silent throughout lifetime (Moertel, Sauer et al. 1961; Godwin 1975). Still, small bowel carcinoids are the most common neuroendocrine tumors of the gastrointestinal tract. As midgut carcinoids are the most common cause of the carcinoid syndrome, they tend to prevail in clinical series with about the same frequency as adenocarcinomas of the small bowel (Thompson, van Heerden et al. 1985).

Midgut carcinoid tumors can be subdivided into two separate entities. Appendiceal carcinoids have by far the highest incidence and are mainly detected at appendectomy. They rarely become clinically manifest by the typical hypersecretive syndrome except for those large metastatic tumors associated with the carcinoid syndrome. Appendiceal carcinoids seem to arise from sub-epithelial cells in contrast to carcinoids with origin outside the appendix arising from the aforementioned enterochromaffine cells in the crypts of the bowel wall.

Those latter carcinoids with origin outside the appendix are strikingly more prevalent at autopsy and most tumors may not reach clinical significance during the patient`s lifetime.

3.1.2.2 Clinical presentation

Midgut carcinoid tumors are characteristically slowly-growing neoplasms and therefore most patients will experience prodromal symptoms for quite some time before the disease itself be-comes clinically manifest. Midgut carcinoids are typically located in the terminal ileum. The primary tumor characteristically is inconspicuous in size. It is located deep in the mucosal tissue and of fibrotic nature. Occasionally, intestinal bleeding might occur with large and ulcerating tumors or as a consequence of venous stasis in an intestinal segment. When the tumor is growing larger it may extend directly into mesenteric lymphatic glands. This almost invariably is the case with patients undergoing surgery for abdominal complaints (Davis, Moertel et al. 1973; Strodel, Talpos et al. 1983; Moertel 1987). The patients may initially exhibit the carcinoid syndrome or mainly show abdominal complaints and have to undergo surgery for intestinal obstruction, often without the actual diagnosis being overt (Moertel 1987; Feldman 1989). Most carcinoids with appendiceal origin are found at the tip of the appendix and thus seldom cause intestinal obstruction. Neoplasms evolving at the appendix base, however, might indicate surgery due to obstruction. Tumors of the appendix larger than 2 cm in diameter tend to metastasize. Also goblet cell carcinoids (those producing mucus) have malignant potential (Tiensuu Janson and Oberg 1996). These latter tumors are of endocrine origin apparently from specialized subepithelial neuroendocrine cells (Wilander, Lundqvist et al. 1989) and are thus mixed tumors of neuroendocrine- and adenocarcinoma-population. Prognosis for patients with these neoplasms is similar if not worse than for colorectal cancers.

Carcinoid metastases often are considerably larger than the primary tumor and characteristically can provoke pronounced desmosomic reactions. The mesenteric neoplasms and its fibrotic growth, rather than the primary lesion *per se* commonly tend to cause partial or complete small bowel obstruction by entrapping and kinking the small intestine.The tumor also tends to occlude or compress the neighbouring mesenteric vessels resulting in venous and, less commonly arterial ischemia. The intestinal vascular deterioration in advanced midgut carcinoid tumors may be intensified by a specific angiopathy exhibiting elastic tissue proliferation (elastic vascular scle-rosis) within the adventitia of the intestinal vessels (Anthony and Drury 1970; Eckhauser, Argenta et al. 1981). The mesenteric desmoplasia and the vascular elastosis have been suggested to result from local effects of growth factors and other substances released from carcinoid metastases (Funa, Papanicolaou et al. 1990). Carcinoid tumors commonly spread to the liver and might then become hormonally symptomatic with features of the carcinoid syndrome. Hormones released by gastroenteropancreatic primary tumors are generally metabolized by the hepatic drainage system, whereas those released from metastases of the liver or extraperitoneal sites might by-pass the liver. However, only at advanced disease stages do carcinoid tumors spread to extraabdominal sites such as peripheral lymph nodes, lungs, central nervous system, ovary, skin and skeleton, even though a neck lymph node may be a first clinical sign of the tumor (Sanders and Axtel 1964; Moesta and Schlag 1990; Makridis, Rastad et al. 1996).

3.2 THE CARCINOID SYNDROME

Diarrhea and cutaneous flushing are the prominent and often debilitating symptoms of the carcinoid syndrome. Further possible symptoms are bronchoconstriction, elevated urinary *5-hydroxy-indole aceticacid* (5-HIAA) levels and a fibrotic carcinoid heart disease with pulmonary stenosis and tricuspid regurgitation (Janson, Holmberg et al. 1997)**.**

Classical midgut carcinoids are the most common cause of the carcinoid syndrome and may generate a complex of symptoms comprising the carcinoid syndrome long before local growth or metastatic spread is otherwise apparent. Presence of the syndrome is synonymous with extensive disease and incurability in the majority of the cases. Presence of the carcinoid syndrome has been attributed to secretion of a number of bioactive agents by carcinoid hepatic metastases, e.g. serotonin, prostaglandin, kallikrein/bradykinin, dopamine, tachykinines etc. (Lucas and Feldman 1986)). About 5% of all patients with carcinoid tumors present with one ore more symptoms of the carcinoid syndrome, 30-60% of small intestinal carcinoids but only 3.5 % of bronchial, 1% of appendiceal and no rectal carcinoids are associated with the syndrome. Individual patients may present with symptoms to a different extent. For the development of the syndrome in patients with intestinal carcinoids the patient must have liver metastases (the secretion products by-pass hepatic metabolization). Bronchial and extraintestinal carcinoids whose hormones are not immediately detoxified by the liver may present with the syndrome without metastatic disease to the liver. Occasionally, manifestations of the syndrome may be expressed in patients with mere ovarian or large retroperitoneal metastases as a consequence of venous effluents directly draining into the systemic circulation (Makridis, Rastad et al. 1996).

Flush is the most striking feature of the syndrome, sometimes evoked by physical and psychic stress, meals and alcohol. Release of endothelium-derived vascular-relaxing factor upon stimu-lation with serotonin, substance P and VIP seems to contribute to an important paracrine mecha-nism (Regoli and Nantel 1991). Tachikinin secretion by carcinoids also contributes to the flush symptom, however, preventing the flush symptom pharmacologically has not always been asso-ciated with tachykinin level normalization (Makridis, Rastad et al. 1996).

Carcinoid heart disease includes morphological and functional changes of the tricuspid and pul-monary valves, enlargement of the right heart cavities and paradoxical septal contraction patterns. Cardiac sequelae may be diagnosed by echocardiography, especially by the trans-eosophageal route. Microscopically, the pathognomonic carcinoid cardiac lesions consist of fibrous tissue on mural and valvular endocardium, predominantly if not exclusively on the right side of the heart. The lesions may infiltrate into underlying endo- and myocardium. Knowledge about the etiology of carcinoid heart disease is scarce. However, there seems to be a relation bet-ween the extent of the disease and the amount of circulating substances secreted by the tumors, i.e. serotonin and tachykinins. Severe carcinoid heart disease resulting in right ventricular failure is an indication to reconstructive valvular surgery when the malignant disease is under control and when there are no possibilities to cure clinical signs of right heart failure medically (Lundin 1991).

Diarrhea is nearly as common as flush but both symptoms are not necessarily present simul-taneously (Davis, Moertel et al. 1973). Diarrhea in carcinoid patients can be caused by ileal resection resulting in reduction of bile salt absorption and dysfunction of motility and secretion in the distal ileum. A short bowel syndrome or intestinal bypass after surgery, partial intestinal obstruction, ischemia and venous stasis may also lead to severe watery diarrhea and malnutrition in some patients. Not only anatomical aberrations may induce diarrhea but also humoral factors produced by the carcinoid tumor, mainly excessive release of serotonin, motilin and substance P (Feldman and O'Dorisio 1986; Norheim, Theodorsson-Norheim et al. 1986). Serotonin, however, does not seem to mediate the diarrhea alone. Local intestinal paracrine mechanisms, such as increased intestinal secretion of prostaglandin (PG) E2 and tachykinins exerting substantial effects on intestinal motility and secretion may also be pathophysiologically involved in carcinoid diarrhea, especially substance P, neurokinin A, neuropeptide K and eledoisin (Brunsson, Fahrenkrug et al. 1990; Sjokvist, Brunsson et al. 1993). Bronchoconstriction and asthma occur in about 10-20% of patients suffering from the carcinoid syndrome.

3.3 DIAGNOSIS

Characterization and diagnosis of a carcinoid tumor can be achieved by considering the following aspects: hormone production, histopathological features and certain aspects of tumor biology, radiological and radionuclear examinations.

3.3.1 Hormones

Especially midgut carcinoid tumors and also foregut carcinoids but never hindgut carcinoids show characteristic serotonin- production (Lembeck 1953). The serotonin metabolite U-5-HIAA is the most commonly used biochemical marker to be measured in the urine as well as serotonin in plasma. Chromogranin A, chromogranin B/secretogranin I and chromogranin C/secretogranin II constitute a family of water-soluble acidic glycoproteins and are stored in large dense core vesicles in endocrine and neuroendocrine cells. Tumors originating from those cells are thus associated with elevated plasma levels of chromogranin A (99%), B (88%) and C (6%) which can serve as early markers for neuroendocrine tumors comprising foregut, midgut and hindgut carcinoid tumors. Although less reliable, urinary measurements usually also reveal elevated levels of chromogranins (Eriksson, Arnberg et al. 1990; Stridsberg, Oberg et al. 1995). Furthermore, elevated levels of substance P and neuropeptide K from the tachykinin family are found especially in patients with midgut carcinoids. Flush provocation with pentagastrin is followed by an increase in plasma levels of neuropeptide K so this test may indicate the carcinoid disease in patients with normal basal levels of the peptide at an early stage (Norheim, Theodorsson-Norheim et al. 1986). The serum concentration of the alpha-and beta-subunits of HCG may be raised in midgut carcinoids, the alpha-subunit may be raised in foregut and hindgut carcinoids. However, this increase usually is not impressive enough to serve as a means of monitoring.

3.3.2 Histopathology

Carcinoid tumors all react positively to the argyrophilic stain of Grimelius (Grimelius and Wilander 1980) and stain immunohistochemically with antibodies against chromogranin A (Stridsberg, Oberg et al. 1995). Additionally, midgut carcinoids show argentaffinity whereas foregut and hindgut carcinoids do not (Wilander, Lundqvist et al. 1989). Immunohistochemistry with antibodies against the proliferation marker Ki-67 antigen may serve as a method to observe the proliferation activity in carcinoid tumors. Findings of splice variants of CD44 in a primary tumor may indicate a more malignant nature of the tumor and a metastatic potential (La Rosa, Sessa et al. 1996).

3.3.3 Radiological and radionuclear examinations

Conventional radiology, computerized tomography (CT), magnetic resonance imaging (MRI) and ultrasonography are used to stage carcinoid tumors. Staging of the disease is important in order to aim at identification of those patients suitable for resection of the liver metastases, e. g. solitary or unilobular metastases and no further spread.

Often, the carcinoid causes only discrete stenosis which is difficult to detect by small bowel study. However, a plain abdominal film may reveal a distended small bowel loop or a thickened bowel wall if the patient is suffering from bowel ischemia or mechanical obstruction. Also, radiographic contrast examination by enteroclysm according to Sellink may detect a tumor in the small intestine in patients suffering from intestinal obstruction. CT scans, MRI and ultra-sonography are valuable tools in evaluating hepatic metastases whereas the sensitivity for de-tecting the primary tumor is low.

If the site of the primary tumor is unknown, further investigations will be necessary, e.g. selective arteriography or more frequently somatostatin receptor scintigraphy. Selective angio-graphy may show the affected branches in patients with abdominal angina. However, a normal angiogram does not exclude ischemia as it only shows larger vessels whereas ischemia expresses itself mostly in smaller vessels which are not visualized by angiography.

However, substances labelled with radioactive compounds have been more frequently serving as a base for tumor diagnosis and biological characterization of the tumors. Carcinoid tumors are well known for characteristic expression of somatostatin receptors, midgut carcinoids to a larger extent than other carcinoids and carcinoids with elevated urinary 5-HIAA to a larger extent than non-secreting carcinoids (Reubi, Kvols et al. 1990). So far, five subtypes of the somatostatin receptor have been cloned of which subtype 2 binds the somatostatin analogue used in the clinic with the highest affinity and subtypes 1 and 4 with the lowest affinity. A correlation is assumed between somatostatin receptor expression and the response to treatment with somatostatin analogues. Binding a somatostatin analogue to a radioisotope serves as a tool for visualizing the tumor. A technique in which indium-labelled (^{111}IN-DTPA-D-Phe) octreotide (=Octreoscan) is intravenously injected provides knowledge about the somatostatin receptor status of the patient´s tumor(s) and tumoric lesions outside the abdomen (Bakker, Albert et al. 1991). This somatostatin receptor status will then predict the success

regarding the decrease in hormone levels after medical treatment with somatostatin analogues. Somatostatin scintigraphy is not necessarily superior to CT or ultrasound in detecting primary tumors larger than 2 cm or hepatic metastases whereas it is superior in detecting extra abdominal metastases. However, 20% of the patients with tracer uptake in the lesions might not respond to treatment with somatostatin analogues. The reason for this might be the tracer binding to different receptor subtypes but not all of which inhibit hormone secretion. In contrast to midgut carcinoids, colorectal carcinomas are negative on Octreoscan. In contrast to octreotide, which is attached to cell surface receptors, Iodine^{131}-meta-iodobenzylguanidine (MIGB) is taken up in carcinoid cells or neuroendocrine cells in general and accumulates in the argentaffin granules. Combination of MIGB scintigraphy and octreotide scan may result in a rather high sensitivity.

VIP receptor scintigraphy as another imaging procedure resulted in positive scans in patients with carcinoids but even colorectal adenocarcinomas present with VIP receptors .

C-labelled 5-hydroxytryptophan (HTP), a precursor in the biosynthesis pathway of serotonin, is taken up by the carcinoid tumor in positron emission tomography (PET), the actual tumor detection limit is 5mm. PET gives information about tumor metabolism as well as effects of treatment. It is as sensitive as somatostatin receptor scintigraphy but less reliable than CT-scanning. Labelling of various tracer molecules helps to observe tumor biology in vivo (Tiensuu Janson and Oberg 1996).

3.4 TREATMENT

3.4.1 Medical treatment of metastatic carcinoid tumors

3.4.1.1 Somatostatin analogues

Treatment of somatostatin receptor positive tumors with somatostatin analogues labelled with radioactive substances, e.g. octreotide in patients with midgut carcinoid patients can improve or sometimes prevent flushing and diarrhea (Tiensuu Janson, Westlin et al. 1994). Long acting analogues require less frequent injections. Interference of somatostatin analogues with exo- and endocrine pancreas function may cause side effects such as diarrhea, steatorrhea, flatulence, nausea, vomiting and mild hyperglycaemia.

Also, Iodine^{131}-metaiodobenzylguanidine (I^{131}-MIBG) has treatment potential, both „cold“ and radiolabelled MIBG may alleviate symptoms of the carcinoid syndrome. Functioning tumors are resistant to radiotherapy.

3.4.1.2 Interferon-alpha

Interferon-alpha may be administered alone or in combination with somatostatin analogues (octreotide) depending on the individual tolerance of the medication. Symptomatic improvement of flush, diarrhea and bronchoconstriction after treatment with interferon-alpha is achieved in 60% of patients with metastasizing carcinoids. The anti-tumor effect in neuroendocrine tumors is due to biochemical and tumor response and includes induction of apoptosis and reduction of tumor size (Oberg, Eriksson et al. 1994; Imam, Eriksson et al. 1997). Stabilisation of the carcinoid disease is possible.

The patients may develop neutralizing antibodies against recombinant interferon-alpha resulting in abolishment of the anti-tumor effect and thus, a lower regress rate. Interferon-alpha-related adverse reactions include flu-like symptoms, fatigue and weight loss and are dose-dependent.

3.4.2 Chemotherapy

Interferone-alpha may also be combined with systemic chemotherapy with single-therapy or combinations of streptozotocin, 5-fluoruracil, cyclophosphamide, and/or doxorubicin (Plöckinger and Wiedenmann 2000) or a combination of dactinomycin (actinomycin D), dacarbazine (Van Hazel, Rubin et al. 1983).

Lower biochemical response rates (urinary 5-HIAA levels) and poor subjective improvement among

patients with carcinoid tumors originating in the small bowel was noted after chemo-therapy when compared to interferone-alpha treatment. This is suggestive of the fact that today, as for quality of life, interferone-alpha treatment is superior to chemotherapy (Oberg, Norheim et al. 1989). Side effects of chemotherapy are, nausea, vomiting, leukopenia, thrombocytopenia and nephrotoxity.

To conclude, patients with the carcinoid syndrome generally die from carcinomatosis rather than from the pharmacological effects of the tumor.

3.4.3 Surgery

Radical surgery, i.e. curative resection of the primary tumor, is indicated when the tumor is re-sectable. In most cases, patients present with multiple primary tumors. The metastatic mass in the mesentery and liver are usually larger than the primary tumor. A potentially curative, radical re-section of liver metastases is recommended if the metastases constitute a substantial part of the tumor burden. In most cases, resection of liver metastases can only lead to palliation. However, liver metastases are often irresectable due to multiplicity. In that case, palliative resection of the primary tumor may help to prevent from obstruction and ischemia and thus, maintain bowel function. Intestinal resection of mesenteric lymphnode metastases must be performed. Tumor re-moval may result in clinical and biochemical complete remission and the patient may live symptom-free for a long time after tumor removal. Life expectancy and quality of life may im-prove after curative and even palliative resection. The perioperative treatment with octreotide gives surgery a chance even in advanced stages of the carcinoid disease and helps to avoid a car-cinoid crisis with circulatory chock, excessive flush and bronchoconstriction.

3.4.4 Hepatic embolization

For patients with liver metastases not exceeding 50% of the liver volume, embolization of the hypervascularized liver is a possibility to achieve a biochemical and tumor response. The blood supply of the liver is mainly arterial. The hepatic tissue is supplied by both hepatic artery (20-25%) and portal vein (75-80%). Hepatic embolization (dearterialisation of the liver metastases) will result in ischaemia and necrosis of the metastases but will affect normal tissue to a lesser degree and be followed by regeneration of the parenchyma. However, this procedure might pro-voke severe complications such as liver abscesses and intestinal ischemia. Hepatic embolisation may require laparotomy but may also be performed by radiological intervention. In case of laparotomy, cholecystectomy is advocated prior to embolisation in order to prevent gall-bladder necrosis. Prophylactic octreotide should be given when patients undergo hepatic emboli-zation and surgery in order to prevent a carcinoid crisis with the aforenamed complex of symptoms (Makridis, Rastad et al. 1996; Eriksson, Larsson et al. 1998).

Side effects of dearterialisation of liver metastases is the post embolization syndrome character-ized by pain in the liver region lasting for several days as well as febrile temperatures in the patient.

3.4.5 Radio frequency ablation (RFA)

More recently, liver metastases that are unresponsive to hepatic artery embolization have been treated with thermal ablation using radio frequency ablation as a salvage treatment. RFA therefore is a useful adjunct to decrease symptoms, to lower octreotide treatment and to slow the progression of the disease (Wessels and Schell 2001).

3.4.6 Cryosurgery

The role of cryosurgery in palliative care has yet to be assessed. To treat hepatic metastases by cryotherapy an ice ball is formed around the metastatic structure. However, this makes it difficult to reposition the metastasis later on for optimal targeting. Side effects of cryotherapy might be cracking of the liver and thus causing massive haemorrhage after thawing.

3.4.7 Heart valve surgery

Reconstructive tricuspidal valve replacement is indicated for patients with carcinoid heart disease and right heart failure.

3.5 SURVIVAL AND PROGNOSIS

Although carcinoid tumors were first believed to be of benign nature, it is nowadays known that for tumors in the midgut region this is only true for small neoplasms arising in the appendix. Other midgut carcinoids may very well be malignant. However, there is a realistic chance for the patient to be cured if the primary tumor and regional lymph nodes are surgically removed. Over-all median survival is 12 years from the onset of any features of the carcinoid syndrome.

Survival of 14 years is found in patients who have undergone radical removal of the primary tumor and mesenteric carcinoid metastases. Irresectable mesenteric lymph node dissemination, however, decreases survival to 11 years, presence of liver metastases to 5-7 years, depending on the degree of liver envolvement (Tiensuu Janson, Westlin et al. 1994). Expected survival for individuals with extended regional metastases or peritoneal carcinoidosis with massive weight loss (>9 kg) as well as for patients with diagnosed valvular heart disease or clinically manifest heart failure is 2.5-5 years. Even shorter survival is found in patients with extraabdominal metastases. Other unfavourable prognostic parameters are raised plasma chromogranin A and neuropeptide K levels as well as high urinary 5-HIAA levels (>500µmol/24h) (Tiensuu Janson and Oberg 1996; Makridis, Ekbom et al. 1997).

A 3-4-year period before recurrence of the tumor has been reported for carcinoid patients but might as well be as long as 16 years (Moertel 1987). Massive liver involvement thus requires more radical treatment but also patients with less metastases to the liver should be given the best medical treatment as they have a long life expectancy (Tiensuu Janson and Oberg 1996). Carcinoid-related heart disease and cachexia account for the principal cause of death in gastro-intestinal carcinoid patients. Increasing age, advanced disease stage, tumor location in the large bowel and presence of other malignancies are related to increased risk of death in these tumors (Greenberg, Baumgarten et al. 1987). Another investigation, however, did report that the male gender and the amount of metastases were predictive factors for the lethal outcome of the disease but that age was not. Also, the site of the primary tumor was of prognostic significance, with poor survival for carcinoids of the small intestine compared to appendiceal carcinoids, as well as the mode of discovery: Accidentally diagnosed carcinoids had a better prognosis (McDermott, Guduric et al. 1994). However, the patients may benefit markedly from a combination of surgical and medical treatment (Makridis, Ekbom et al. 1997).

3.6 MULTIPLE ENDOCRINE NEOPLASIA (MEN)

3.6.1 Clinical features

Multiple Endocrine Neoplasia belongs to the group of pluriglandular syndromes with endocrine tumors developing in more than one organ but also non-endocrine expressions of the disease. There are five major MEN syndromes: Multiple Endocrine Neoplasia type 1 (Wermer-Syndrome (Wermer 1963), which is characterized by a combined emergence of endocrine tumors in the anterior pituary and parathyroid gland (parathyroid adenoma), pancreas and duodenum (submucosal duodenal carcinoids) as well as less frequent tumors such as adenoma and carcinoma of the thyroid gland, adrenal cortical hyperplasia, hepatic focal nodular hyperplasia and renal angiomyolipoma, foregut carcinoid tumors (gastric enterochromaffin-like-cell carcinoids, thymus and bronchial carcinoids) (Lamberts and Gregor 1999) and non-endocrine tumors such as angiofibroma, lipoma, leiomyoma, as well as facial angiosarcomas and collagenomas, recently found but common skin manifestations (Marx, Agarwal et al. 1999). Multiple Endocrine Neoplasia type 2a (Sipple-Syndrome) (Wermer 1963) presents with c-cell carcinoma of the thyroid glands, hyperparathyroidism and pheochromocytoma. Type 2b includes mucosal neurinomas, intestinal ganglioneuromatosis and occasionally a marfanoid habitus. Von Hippel-Lindau Syndrome presents with pancreatic neuroendocrine tumors, pheochromocytoma and different neoplastic non-endocrine tumors in the CNS, retina, kidney, pancreas, endolymphe and epididymis. The Carney complex comprises endocrine neoplasms in Sertoli and Leydig cells, the pituitary, thyroid and adreno-cortical gland and non-endocrine neoplasms such as myxomas and lentigines. Mc Cune-Albright Syndrome presents with the symptoms of precocity, pituitary, thyroid and adrenocortical gland neoplasms as well as café-au-lait spots and non-neoplastic affection of heart and liver (Marx, Agarwal et al. 1999).

MEN-1 is an autosomal-dominant hereditary disease and shows high penetration but irregular expressivity (Metz 1995). The population prevalence of the disease is advanced with 2-10 per 100000 (Marx, Agarwal et al. 1998). In MEN 1 tumor multiplicity is a characteristic feature referring both to tumors in multiple organs and to multicentric tumors in one organ, often bilateral neoplasms (Pipeleers-Marichal, Donow et al. 1993; Debas and Mulvihill 1994). 95% of the MEN-1 patients

present with hyperparathyroidism, often as the primary manifestation, 80% show pancreatic tumors, 50-65 % pituitary tumors are detected in autopsies. Diffuse hyperplasia or multiple adenomas and postoperative recurrence due to the multicentric origin of the disease, are characteristic for primary hyperparathyroidism. MEN-1 patients often show symptoms of sporadic hyperparathyroidism (hypercalcemia, nephrourolithiasis and ostitis fibrosis cystica). Important diagnostic features are raised parathyroid hormone (PTH), raised serum-calcium, lowered serum-phosphate, lowered urinary calcium and raised urinary phosphate levels. Surgery is considered as firstline therapy, either subtotal parathyroidectomy or total parathyroidectomy with simultaneous autogenous parathyroid transplantation e.g. to the forearm (Mallette 1994). Also the pancreatic lesions (nesidioblastosis, microadenoma or carcinoma) are multicentric and of endocrine differentiation, of which up to 40% are situated in the duodenal wall or the triangle between duodenal C, ventricle antrum and pancreatic head. The majority of MEN-1 patients with pancreatic tumors are asymptomatic (Skogseid, Eriksson et al. 1991). 50% of the cases present with clinical or pathological malignancy criteria (lymphnode or visceral metastases). The pancreatic lesion within MEN-1 is the most striking factor concerning the prognosis of the disease, that in up to 50% is malignant. The pituitary tumors are almost only benign, singular adenomas or multicentric tumors, of which about 15% are prolactinomas or growth hormone (GH)-producing tumors as well as raised ACTH (adenocorticotropic hormone) production (McCutcheon 1994), TSH-secreting and non-secreting tumors. Most common are chromophobe adenomas with the clinical manifestation of expanding growth or pituitary functional loss. Characteristic for prolactinomas are secondary amenorrhea, galactorrhea, loss of libido or infertility in females, impotence and loss of libido in males (Farid, Buehler et al. 1980) and, for GH-producing tumors, acromegalia. Stimulation tests with pituitary releasing and inhibiting factors as diagnostic means have proved to be more sensitive than basal hormone measurement. Ophtalmologic and perimetric examination of the patients is important. Therapy depends on the tumor entity: Prolactinomas and GH-producing tumors respond to medical treatment with dopamin analogues and/or transsphenoidal tumor resection as the surgical alternative, with or without radiotherapy. GH-producing tumors may also be treated with subcutaneous injections of somatostatin analogues (Lamberts and Gregor 1999).

3.7 ENDOCRINE PANCREAS TUMORS (EPT)

3.7.1 Clinical features

Endocrine pancreas (EPT) tumors are a rare tumor type with an incidence of approximitely 4 per year and million population (Eriksson, Larsson et al. 1989). They behave more indolently than their highly malignant exocrine counterparts. They may arise within the tumor syndromes MEN 1 or von Hippel-Lindau (VHL) as well as sporadic neoplasms. When no metastases or local invasiveness are present, there are no indisputable clinical or histopathological methods to declare EPT as malignant. On the other hand, absence of cellular atypia, perineural infiltration, intracapsular growth and lymph or blood vessel invasion does not allow to exclude malignant nature (Pelosi, Bresaola et al. 1996). The majority of these tumors are clinically functioning tumors, i.e. they are associated to a syndrome related to hypersecretion of a specific hormone, 50% of them are gastrinomas, 25% insulinomas, 10% non-functioning tumors, including tumors secreting pan-creatic polypeptide (PP), chromogranin A, peptide YY (PYY) and neurotensin, and 2% glucagonomas, VIPomas (vasoactive intestinal peptide), and somatostatinomas (Lamberts and Gregor 1999). Gastrinoma or Zollinger-Ellisons syndrome can also be caused by duodenal carcinoids and more than 70% of these tumors have malignant potential (Zollinger, Ellison et al. 1980; Oberg 1996). Clinically they present gastric and duodenal ulcerations, diarrhea and malabsorption. Insulinomas are rarely malignant and small in size but nevertheless cause severe hypoglycemia syndrome. Signs of neuroglucopenia and increased catecholamine release are typical symptoms related to insulin/ proinsulin overproduction (Oberg 1996). Glucagonoma, somatostatinoma, Verner-Morrison or Watery Diarrhea Hypocalcemia Achlorhydria (WDHA) syndrome caused by VIP are more seldom syndromes but are more frequently of malignant potential. Glucagonomas are clinically characterized by a necrolytic migratory erythema, diabetic glucose tolerance, anaemia, weight loss and tromboembolism (Stacpoole 1981), while increased somatostatin production sometimes leads to gall bladder dysfunction, gall stones and diabetic glucose tolerance, malabsorption and diarrhea (Krejs, Orci et al. 1979).

4 TUMOR BIOLOGY

A malignant cancer cell is characterized by autonomous and invasive growth as well as the capability to metastasize to distant sites of the body and to infiltrate blood and lymph vessels. The pathway to tumor formation is a multi-step journey, it takes genetic alterations in at least four pathways to convert a normal cell to a cancerous cell and to evoke derangement of numerous gene products (Weitzman and Yaniv 1999). The phenotype might change with every new genetic event succeeding, e.g. normal epithelia progresses from dysplasia to adenoma to carcinoma in situ to invasive carcinoma while it aquires additional genetic aberrations (Vogelstein and Kinzler 1993). Cells are quite resistant to neoplastic formation by a number of intrinsic mechanisms controlling the cell cycle and they might compensate loss of function in one pathway with gain of function in another. Loss of normal growth control is due to mutation in three categories of genes: Proto-oncogenes, tumor suppressor genes and DNA repair enzymes. All cells are able to replicate themselves but will eventually be bound to reach a non-dividing state, the so-called senescence. Genetic alterations, however, may make it possible for the cell to escape this state. After living through a crisis and usually massive cell death the cell may become immortalized and duplicate eternally, without growth factors and anchorage to a solid ground.

4.1 THE CELL CYCLE

The mechanism of cell division is necessary for the understanding of transformation in neoplasia. The cell cycle is subdivided into G (gap) 1 phase, in which the cell decides whether or not to continue to S phase, S (synthesis) phase, during which the genome is replicated, G2 phase, where replication errors are detected and corrected and M (mitosis) phase, which gives room to separation of the replicated chromosomes and packaging them into two new nuclei as well as division of the cytoplasm (cytokinesis). Replication errors occurring during S phase are corrected by the cell. Various mechanisms are available and mismatch repair is essential for preventing mistakes to be passed on to the daughter generation. Numerous cancer types display a defect mismatch repair system and are strikingly involved in neogenesis (Eshleman and Markowitz 1996). Telomere biology also is another key word in neoplastic events. Telomeres are the structures at the end of our chromosomes. With each cell cycle the telomeric DNA is left somewhat shorter and this telomeric erosion is thought to be one of the restricting factor to the cell´s lifespan. Normal cells naturally have low telomerase activity, the RNA-dependent DNA polymerase that replicates telomeric DNA and holds the responsibility for keeping telomere length, while cancer cells express telomerase at higher levels and thus escape normal lifespan control (Weitzman and Yaniv 1999). Transition between G1 and S and G2 and M is subjected to strict control by checkpoints, and checkpoint regulation mechanisms imply two cyclin-dependent kinase (cdk)/cyclin complexes: cyclinD/cdk4 or -6 and cyclinE/cdk2. Activation of cdk/cyclin complexes initiates transcription of factors enhancing growth and differentiation, whereas inhibition of cdk/cyclin complexes by cdk inhibitors (cdki) leads to cell cycle arrest or apoptosis, e.g. p21, which upregulates the *p53* gene product upon detection of DNA damage (Gartel, Serfas et al. 1996). Apoptosis is a form of programmed cell death, functioning as the regulatory mechanism in cell homeostasis opposite to mitosis. Signals from the extra- or intracellular space, e.g. tumor necrosis factor (TNF) binding to its receptor or *p53* (upgraded by vast DNA damage and subsequent pathways) initiate activity of the interleukin 1 (IL1)-converting enzyme (ICE) family of proteases and result in DNA degradation on the nuclear level and successively, cell death (Carson and Lois 1995; Martin and Green 1995; Ledgerwood, Pober et al. 1999).

4.2 CANCER GENES

Tumor formation is thought to be induced by cooperation of mutations causing telomerase upregulation and mutations in proto-oncogenes or tumor suppressor genes.

Proto-oncogenes are normal genes controlling cell growth and are contained in normal cells. They might, however, be transformed and activated to oncogenes, by point mutation, amplifi-cation or chromosomal rearrangements. About 100 such oncogenes have been identified so far and are activated in numerous human cancers, e.g. *ret,* the gene for a receptor tyrosine kinase which displays mutations in MEN2A and FMTC (familial medullary thyroid carcinoma) (Calender 1998).

Table 1. Examples of oncogenes

oncogene	classification
INT2	Growth factor
RET	Tyrosine kinase
MAS	Receptor lacking proteine kinase activity
KRAS	Membrane-associated G protein
RAF/MIL	Cytoplasmic protein serine kinases
CRK	Cytoplasmic regulator
INK4A	Cell cycle regulator
MYC	Transcription factors
ELL	Transcription elongation factors
BCL2	Intracellular membrane factor
NUP98	Nucleoporin
SHC	Adapter protein
EWS	RNA binding protein

Tumor suppressor genes (TSGs) are mutated genes found in the majority of human neoplasms (Weinberg 1991). Analysis of retinoblastoma cases lead to the emergence of Knudson´s two-hit hypothesis about carcinoneogenesis (Knudson, Di Ferrante et al. 1971). A *TSG* is typified by the very critical function of suppressing uncontrolled cell growth and to enhance cell differentiation. A *TSG* being the cause of familiar cancer types presents with the following features: One allele generally experiences loss of gene function by a germline mutation of the respective allele and a somatic mutation leads to loss of the second wildtype allele (Marshall 1991). The retinoblastoma gene *(Rb)* and the gene causing familiar adenomatosis (*APC*) are examples for *TSGs.* Analysis of retinoblastoma cases lead to the emergence of Knudson´s two-hit hypothesis about carcinoneogenesis (Knudson, Di Ferrante et al. 1971).

Table 2. Examples of tumor suppressor genes

gene	Function	associated tumors
p53	cell cycle regulator, promotes growth arrest and apoptosis	most sarcomas, breast carcinoma, leukaemia
APC	binds alpha-and beta-catenin: may mediate adhesion, cell cycle progression	Colon carcinoma
CDH1/ e-cadherin	Ca^{2+}pendent intercellular adhesion, signalling	many: Breast, ovarian
VHL	modulates RNA polymerase-II via elongin	Renal cell carcinoma, pheochromocytoma
MSH2, MLH1	DNA mismatch repair	Hereditary non-polyposis colon cancer
Smad4/DPC4	cell growth inhibitor	Pancreas, colon

4.3 KNUDSON´S TWO-HIT HYPOTHESIS ABOUT NEOPLASIA

Two sequential mutations in a neoplasm precursor cell can result in the development of neoplasia. Each cell can be hit postzygotically by a first mutation of a TSG in the germline cell (all cells are mutated identically, hereditary neoplasia) or, in the somatic cell as a more rare event (non-hereditary neoplasia). This usually is a small mutation such as a point mutation and does not result in detectable biological effects on the cell but in a heterogenous carrier predisposed to neoplastic process. A second somatic mutation (=hit) of the remaining wildtype allele of a tumor suppressor gene results in loss of function of the gene and evokes neogenesis. This second hit is more likely to occur early when the first hit is a germline mutation compared to somatic mutations (Knudson 1978). Both mutational events finally lead to uncontrolled cell growth resulting in a tumor clone (Marx, Agarwal et al. 1999).

LOH analysis of retinoblastoma cases leads to findings of somatic hits in retinoblastoma as chromosomal deletion or loss of a whole chromosome (Cavenee, Dryja et al. 1983; Cavenee, Hansen et al. 1985). The second hit can occur as e.g. point mutation, somatic recombination or chromosomal deletion (Knudson 1978). *TSG*s may be silenced by still another process: hypermethylation of regions promoting gene regulation (CpG islands) thus resulting in inhibition of transcription of that gene. Examples for genes hit by hypermethylation are i.g. *p16* and *Rb* (Schmutte and Jones 1998).

4.4 DNA REPAIR

6 billion base pairs of DNA are copied in each cell division. The DNA can suffer various kinds of damage and the proof-reading activity of DNA polymerase has a certain error rate, it is therefore crucial to have DNA repair mechanisms to grant propagation of the correct DNA sequence to the next generation. There are several DNA repair mechanisms acting upon different types of DNA damage, e.g. DNA mismatch repair and nucleotide excision repair (Kolodner 1996).

These repair systems can be damaged by both acquired and inherited mutations, thus initiating accumulation of genome-wide molecular alterations under cell division. Deficient repair systems causing alterations in *TSGs* or oncogenes may lead to canceroneogenesis.

The ***DNA mismatch repair*** systems hold the function of detecting errors in recently synthesized DNA, attaching to the defective base pairs and excising them. Re-synthesis of the gap and re-ligation by an

enzyme complex terminate the process. MutS and MutL are mismatch repair proteins, first found in procaryotes, with five known human MutS homologues (hMSH2-6) and three MutL homologues (hMLH1, hPMS1 and hPMS2) (Fishel and Kolodner 1995; Fishel 1998). Germline mutations in hMSH2 and hMLH1, e. g. are detected in 90% of hereditary non-polyposis colorectal cancers (HNPCC) (Liu, Parsons et al. 1994) and LOH on the hMLH1 and hMSH3 loci has been found in non-small lung cell cancer (Benachenhou, Guiral et al. 1998). Mutations in mismatch repair genes are associated with a generally increasing mutation rate and cancerogenesis as well as microsatellite length instability. Microsatellites (or simple repeated sequences, SRSs) are up to 6 bp long DNA sequences repeated 10-50 times. They are characterized by relatively low inherent mutation rate, and individual variability.

The ***nucleotide excision repair*** system repairs DNA damaged by ultraviolet radiation (UV). Global genomic repair and transcription-coupled repair cut the DNA on both sides of the lesion, re-synthesize the correct sequence and ligate it back into the gap. *p53* plays an important role in activation of the global genomic repair pathway and activation is mediated through p48 transcription (Ford and Hanawalt 1997).

Several human cancers have manifested defects of global genomic repair and transcription-coupled repair, among those e.g. various types of Xeroderma pigmentosum, a form of skin cancer caused by exposure to UV (Lambert, Kuo et al. 1995; Chu and Mayne 1996).

4.5 DEFINITION OF LOH - EXPRESSION OF A TUMOR SUPPRESSOR GENE

The second, carcinogenic insult on a neoplasm precursor cell usually results in removal of the normal copy of the mutated gene, often as large deletions or the whole remaining chromosomal copy. Occasionally, a replacement of the lost DNA might happen by so-called *gene conversion*, a procedure by which lost DNA is replaced with the respective sequence from the other copy. Heterozygosity (i.e. two distinct alleles in germline DNA) is the essential requisite for evaluation of LOH. If a heterozygous individual loses one allele of a polymorphic site this can be detected as allelic loss or *loss of heterozygosity (LOH).* The germline DNA remains heterozygous at that site. Thus, screening of LOH has been taken advantage of to narrow and identify regions with putative tumor suppressor genes.

5 GENETIC FEATURES OF ENDOCRINE TUMORS

5.1 MEN

In 1988, the MEN1 gene was first mapped to chromosome *11q13* (Larsson, Skogseid et al. 1988) and closely linked to the *PYGM* locus. Screening of chromosome *11q13* using highly polymorphic markers frequently showed somatic loss of heterozygosity (LOH) in MEN1 tumors (Larsson, Skogseid et al. 1988; Friedman, Sakaguchi et al. 1989; Thakker, Bouloux et al. 1989; Bystrom, Larsson et al. 1990; Skogseid, Rastad et al. 1995). These findings are strongly suggestive of a tumor suppressor function of the MEN1 gene. Following Knudson`s two-mutational hit theory of hereditary neoplasm etiology, MEN-1 tumorigenesis results from a germline mutation followed by a second somatic chromosomal hit (Knudson, Di Ferrante et al. 1971). After progressive restriction of the *MEN1* candidate region the gene causing MEN1 was finally cloned in 1997 and the gene was located approximately 70 kb telomeric of *PYGM* (Chandrasekharappa, Guru et al. 1997; Lemmens, Van de Ven et al. 1997). The gene of 9 kb contains 10 exons. The first exon and part of exon 10 are untranslated and a 2.8 kb transcript has been found in all human tissues. The gene encodes for a 610 aminoacid protein, menin with no homology to previously known proteins (1. 1997; Chandrasekharappa, Guru et al. 1997). Menin is a nuclear protein which translocates from the nucleus to the cytoplasma during the cell cycle (Huang, Zhuang et al. 1999) and is known to bind JunD, a member of the transcription factor family AP1, and to repress transcriptional activity in transfection assays (Agarwal, Guru et al. 1999; Gobl, Berg et al. 1999).

Close to 250 different mutations of the *MEN1* gene have been described since 1997 (1. 1997; Agarwal, Kester et al. 1997; Aoki, Tsukada et al. 1997; Chandrasekharappa, Guru et al. 1997; Mayr, Apenberg et al. 1997; Basset, Forbes et al. 1998; Olufemi, Green et al. 1998). They are spread over the whole gene and not accumulated on known functional domains.

LOH on chromosome *11q13* has even been found in sporadic endocrine tumors with a frequency

ranging from 30-70% in tumors of the parathyroids and the endocrine pancreas whereas pituitary tumors rarely exhibited losses. Only in a subset of these tumors (30-58%), however, the re-maining MEN1 gene was mutated (Hessman, Lindberg et al. 1998; Tanaka, Kimura et al. 1998; Wang, Ebrahimi et al. 1998; Heppner, Reincke et al. 1999). LOH on chromosome *11q13* without any MEN1 gene mutation has also been detected in a number of neoplasms, e.g. adrenocortical and follicular thyroid tumors. This leads to the assumption that there might exist another tumor suppressor gene at this locus involved in endocrine tumorigenesis (Heppner, Reincke et al. 1999; Kjellman, Roshani et al. 1999; Nord, Larsson et al. 1999).

5.2 EPT

Homozygous somatic mutations of the MEN1 gene have been found in about one third of non-familial EPT (Zhuang, Vortmeyer et al. 1997; Hessman, Lindberg et al. 1998; Wang, Ebrahimi et al. 1998). Nonfamilial tumors show *3p* deletions as well as allelic loss on chromosomal arms *3q*, *11p*, *11q*, *16q* and *22q*. No mutations have been found for the VHL gene on *3p26* (Chung, Brown et al. 1998). In contrast, a striking association between LOH at *11q13* and *3p* and malignant phenotype was found for nonfamilial tumors in another tumor deletion study (Hessman, Lindberg et al. 1999).

Chromosome *18q21* is frequently deleted in a variety of human cancers including exocrine pancreas tumors. Chromosome *18q21* harbours the putative tumor suppressor genes *DCC*, *Smad4/DPC4* and *Smad2/MADR2/JV18-1* genes(Fearon, Cho et al. 1990; Eppert, Scherer et al. 1996; Schutte, Hruban et al. 1996; Kong, Choi et al. 1997; Toliat, Berger et al. 1997).

5.3 MIDGUT CARCINOID TUMORS

So far, only a limited number of carcinoid tumors have been investigated (Jakobovitz, Nass et al. 1996; Toliat, Berger et al. 1997; Debelenko, Emmert-Buck et al. 1997a; Ghimenti, Lonobile et al. 1999; Gortz, Roth et al. 1999; Zhao, de Krijger et al. 2000; D´Adda, Pizzi et al. 2002; Kytola, Nord et al. 2002). Sporadic foregut carcinoids frequently display allelic losses at *11q13* and somatic *MEN1* mutations have been revealed in about a third of the investigated tumors (Debelenko, Emmert-Buck et al. 1997a; Hessman, Lindberg et al. 1998; Zhao, de Krijger et al. 2000). In contrast to foregut carcinoids, midgut carcinoids are not overrepresented in the MEN1 syndrome and only infrequently display LOH at *11q13.* When pooling the results from all previous studies, LOH on chromosome 11 has been detected in 16 of 83 analyzed midgut carcinoids (Jakobovitz, Nass et al. 1996; Debelenko, Emmert-Buck et al. 1997a; Ghimenti, Lonobile et al. 1999; Gortz, Roth et al. 1999; Zhao, de Krijger et al. 2000) (D´Adda, Pizzi et al. 2002; Kytola, Nord et al. 2002). One somatic missense *MEN1* mutation in one of sixteen midgut carcinoid tumors has been described (Toliat, Berger et al. 1997; Gortz, Roth et al. 1999). Two constitutional putative missense mutations, H50R and G12S on the *SDHD* (*TSG*) (succinate-ubiquinone oxidoreductase subunit D) gene locus were found in two midgut carcinoids, both mutations were associated with LOH of the other allele (Kytola, Nord et al. 2002). Microsatellite instability was detected in one of six analyzed midgut carcinoid tumors (Ghimenti, Lonobile et al. 1999). Only one study, using comparative genomic hybridization could find LOH on chromosome 18 so far: LOH on chromosome *18p* in eight (38%) and on *18q* in seven (33%) out of 21 gastro-intestinal tumors whereas in none of the bronchial carcinoids (Zhao, de Krijger et al. 2000). In a recently published X-chromosome inactivation study, the same X-chromosome had been inactivated in multiple ileal tumors from the same patient. These results suggest that the multiple lesions result from intraintestinal spread (Guo, Li et al. 2000). Both TGF-α and EGF receptors are expressed in midgut carcinoids in vitro and in vivo (Nilsson, Wangberg et al. 1995). Mostly TGF-ß2 was found in 2/3 of midgut carcinoid cells and their stroma and was lacking expression in normal small intestine tissue. TGF-ß has also been found in stromal tissue only, leading to the suggestion that TGF-ß might stimulate matrix growth and angiogenesis in the stroma surrounding the neoplastic tissue whereas the tumor cells remain unaffected (Chaudhry, Oberg et al. 1994). PDGF seems to play a role in the growth of carcinoid tumor and stroma cells and is likely to add to the fibrosis often found in carcinoid tumors (Funa, Papanicolaou et al. 1990; Chaudhry, Papanicolaou et al. 1992). Carcinoid tumor growth may furthermore be stimulated by IGF-I and IGF-I receptors (Nilsson, Wangberg et al. 1993). *p53* mutations appear to play a role in the pathogenesis of a small subset classical and goblet cell carcinoids of the appendix and classical midgut carcinoids whereas *K-ras* mutations are absent in midgut carcinoids.

6 AIMS OF THE INVESTIGATION

A genome-wide search for molecular alterations using fluorescent technique has been performed on eight sporadic midgut carcinoid tumors in order to find a commonly deleted chromosomal region. Deletions of genomic regions containing candidate tumor suppressor genes may play an important role in the initiation and progression of some neoplasms. However, there is a lack of knowledge about the sequence of events leading to tumorigenesis in midgut carcinoid tumors and whether the MEN-1 gene is involved in the tumor development. The molecular basis of the neoplastic transformation of neuroendocrine cells is still not well understood (DeLellis 1995), it is not known if a carcinoid tumor evolves from a precancerous lesion or if various molecular alterations found in a tumor occur in a specific order or at random. Growth factors such as TGF alpha, EGF and NGF might induce malignant progression of midgut carcinoids as this has been shown to hold true for endocrine cell lines (Bold, Ishizuka et al. 1995; Nilsson, Wangberg et al. 1995). Furthermore, chromosomal instability and apoptotic mediators might influence the neogenetic process. In order to gain insight into this process it is necessary to accumulate data on genetic alterations in midgut carcinoids.

In sharp contrast to the scarce findings of *p53* mutations and the absence of *K-ras* mutations in midgut carcinoids are frequent findings of such alterations in half or more of examined adenocarcinomas of the gastrointestinal tract, both *K-ras* and *p53* mutations as well as mismatch repair deficiency have been described (Bos, Fearon et al. 1987; Weckstrom, Hedrum et al. 1996; Arber, Neugut et al. 1997; Younes, Fulton et al. 1997; Ramnani, Wistuba et al. 1999). Thus, small bowel carcinomas, which originate from the same organ and occur with approximately the same frequency, seem to have a different genetic background.

The availability of a series of tumor samples in which deletions are molecularly detected on a genome-wide basis could help make progress on these questions.

7 PATIENTS AND TUMORS

Frozen tumor tissue and corresponding blod samples were available from eight patients dia-gnosed with and operated for midgut carcinoid tumors during a 13-year period (August 1983 till February 1996) at the University hospital Uppsala, Sweden. Patients were identified through the medical archives and pathological reports. Access to the pathological and clinical data was approved by the institutional board and the regional research ethics committé. Six of the primary lesions originated in the ileum, one in the ileo-cecal valve and one in the ascending colon. Tissue from two primary tumors, four mesenteric metastases and two liver metastases were investi-gated. All eight tumors had metastasized to the liver and/or the small intestine mesenterium and/ or other sites at the time of surgery. In all patients the carcinoid tumors occurred sporadically as diagnosed by the lack of both personal and family history of the same tumor. The patients were four females and four males with an age range of 43 to 73 years. Clinical and pathological characteristics are displayed in Table 3.

Table 3. Clinical characteristics of cases with malignant midgut carcinoids subjected to genome-wide LOH screening (AWD=alive with disease)

Tumor number	Localization of primary tumor	Sample origin	Age at surgery (years)	Localization of metastases	Follow-up (years)
2283	Ileum	Lymph node metastasis	50	Mesenteric lymph nodes, Liver	AWD (10)
2762	Ileum	Primary tumor	73	Mesenteric lymph nodes, Liver	AWD (9)
4017	Ileo-cecal valve	Liver metastasis	71	Mesenteric lymph nodes, liver	AWD (7)
5184	Ileum	Primary tumor	46	Mesenteric lymph nodes, liver	AWD (5)
5216	Ileum	Liver metastasis	72	Mesenteric lymph nodes, liver	AWD (5)
5692	Right Colon	Lymph node metastasis	70	Mesenteric lymph nodes, liver	AWD (4.5)
5718	Ileum	Lymph node metastasis	55	Mesenteric lymph nodes, liver, breast	AWD (4)
5807	Ileum	Lymph node metastasis	43	Mesenteric lymph nodes, liver, bone	AWD (4)

8 METHODS

8.1 THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) provides a powerful technique for directly amplifying short segments of the genome, i. e. specific segments of the DNA strands. Effecting a PCR re-quires knowledge of the sequence on either side of the target region and allows amplification of a region between two defined sites.

The PCR protocol starts with denaturation of the DNA preparation at 94°/95° (generally an extract of the whole genome). The double-stranded DNA is separated by heat into single-stranded DNA serving as a template for amplification. Amplification of the DNA is achieved by DNA polymerase producing a complementary DNA-strand from the 5´OH end to the 3´OH end. As a starting point DNA polymerase needs a double-stranded sequence of DNA. To produce this double-stranded DNA the single-stranded DNA is annealed with two short primer sequences (20 bases each), one forward an one reverse primer. Each primer is complementary to a site on the opposite strand determining the target region (up to 2 kb). The reaction cocktail contains the two primers, the template DNA, thermostable DNA-polymerase (*taq* DNA-polymerase), all four 2`-deoxynucleoside 5`- triphosphates (dNTPs) dATP,

dCTP, dGTP, dTTP, a buffer and magne-sium chloride ions. After denaturation the temperature is lowered in a second step (annealing) to 55°-57° so both primers can ideally anneal to their complementary regions on the template DNA. In a third step (extension) the temperature is raised to 72°, the temperature optimum for the *taq* DNA-polymerase, and new DNA-strands are synthesized complementary to the template DNA. The entire cycle is repeated 25-32 times resulting in copies of non-determined length (with only one primer at one end) but also copies with a length defined by the two primers. Throughout amplification the number of copies of non-determined length grows linearly whereas the number of copies of determined length grows exponentially. Therefore only products of determined length exist after 25-32 cycles. The number of copies of the target sequence practically doubles with each cycle until reaching a plateau at which more primer-template accumulates than the enzyme manages to amplify during the cycle. A given target sequence may be amplified 4×10^6 times in 25 cycles. At this point the number of target product no longer increases exponentially. The PCR is accomplished in programmable incubation blocks which guaranty quick and precise temperature changes. The availability of thermostable *taq*-DNA polymerase from a thermophilic bacterium, able to withstand even multiple denaturation steps at 95° without losing all of its activity made automatization of the PCR possible. Before that, DNA polymerase had to be added after each denaturation step.

This method provides a powerful possibilitiy to investigate individual alleles and potential candidate genes involved in a disease. PCR is as sensitive as to genotype a single cell, offering analysis of a circumscribed cell population, f. ex. spermatozoa, but also amplification of rather small tissue material.

8.2 THE RESTRICTION TO PCR SENSITIVITY: GENOTYPING ERRORS CAUSED BY *TAQ* DNA POLYMERASE

• A potential source of genotyping errors is contributed to unspecific annealing, i.e. non-templated addition of a single nucleotide, predominantly adenosine, to the 3´ OH end of the DNA strand by *taq* DNA polymerase. *Taq* DNA polymerase is marker-specifically catalyzing the amplification of microsatellite loci, however, experimental variation of the frequency of adenosine addition is often difficult to avoid. The likelihood with which a marker undergoes "+A" (+ adenosine) modification also is marker specific but the factors promoting this phenomenon have not been defined. Allelic misidentification is commonly generated by incorrect labelling of spurious noise peaks or peaks one nucleotide greater in size than the true allele. Consequently, genotyping errors occur and the same allele may be idetified as the true allele in some family members and as the product one nucleotide greater than the true allele in other members. But even an allele in a single individual can be identified inconsistently in repeated amplifications or electrophoreses. One possibility to compensate for unspecificity (incorrect products) is by determining the exact size of the products, e.g. by computer-based analysis (e.g. GENESCAN).

• Using PCR technique, one must compromise between a low temperature which gives a high amount of products but also a lot of unspecific annealing, and a high temperature which gives high specificity but little products. The extent of range within one can modulate temperature depends on primer design and content of GC basepairs in the target sequences. Thus, another approach to decrease the error rate in PCR is the modification of thermocycling protocols to dinucleotide repeat markers to avoid problematic partial modification. A first protocol cycles between denaturation and anealing leaving out both the extension step to each cycle and the final extension step, thus diminuishing the degree of non-templated nucleotide addition by *taq* DNA polymerase and generating more product, a second protocol lengthens the final extension period to 90 minutes so that the enzyme catalyzes non-templated nucleotide addition to it´s maximum. Alternatively, a thermostable *taq* DNA polymerase version lacking all "+A" activity might be used but which is currently not available (Smith, Carpten et al. 1995).

Preferential PCR amplifying implies that when getting close to the plateau phase of the amplifying process or when having a for short elongation phase shorter products are amplified relatively more effectively and maybe more rare porducts as well, due to diminished dimerisation of products.

• A dinucleotide repeat and its "stutter bands" (one to two peaks characteristically smaller in size and in peak height) can be detected in the form of a ladder of peaks seperated by one nucleotide as a result of partial "+A" and partial true allelic detection. It is inferential that a dinucleotide repeat microsatellite marker modified to a 50% degree (probably due to "polymerase slipping") would imply the greatest potential for error. One way of lowering the error rate in genotyping would be the

substitution of tri- and tetranucleotide repeats for dinucleotide repeats. Tri- and tetra nucleotide repeats present with more faint or absent stutter bands. On the other hand, fewer markers may be multiplexed (scored) per gel due to greater allele size range of tri- and tetranucleotide repeat markers. In addition, dinucleotide repeats provide the advantage of their prominent stutter pattern, on the contrary minimal for tetranucleotide repeats, quite helpful in distinguishing noise and background peaks from true alleles. Furthermore, a higher number of dinucleotide repeats have been identified throughout the genome. These characteristics demand development of further methods for their optimized use.

8.3 DNA EXTRACTION AND PCR AMPLIFICATION

The tumors were snap frozen at the time of surgery, then cryosections were made from each tumor sample and tumorous tissue from each patient was identified on a Hematoxylin-Eosin stained slide. This slide served as a road map to process the tumor tissues into one Eppendorf tube each. Lesions with a low proportion of contaminating fibroblasts were selected for analysis. For tumors 5962 and 5807 the cryosections were microdissected in order to avoid gross con-tamintion by non-tumorous cells. From these frozen tissues DNA was extracted by standard proteinase K/SDS digestion and phenol extraction. Paired germline DNA was extracted from leucocytes with Wizard Genomic DNA Purification Kit (Promega) or normal intestine tissue.

DNA extraction from tumor tissues

On day one, according to the size of the tumor sample, 10 to 30 microdissected 20 µm slices were processed into one Eppendorf-tube filled with 450 µl SE-buffer (15 ml 5M NaCl, 50 ml 0,5M EDTA, 30 ml 1M Tris pH 8,0 and double-destilled water to a total of 1000 ml, then autoclaved). 10 µl proteinase K (20 µg/ml), 25 µl 10% SDS and 1 µl RNAse A (10 µg/ml) were added and all was incubated over night at 37°. On day two, 1 volume phenol/chisam (1:1) was added, carefully shaken and vortexed. Then, the extraction mixture was centrifuged for 3 minutes at 14000 rpm in room temperature. The upper phase was processed into a new Eppendorf-tube, the phenol/chisam extraction was repeated and the upper phase transferred to yet another Eppendorf-tube. 3M NaAc, ph 5,2 were added to a final concentration of 0,3 M NaAc, after which one volume Isopropanol was added. All was mixed carefully untill white strands of DNA precipitated. The DNA was centrifugated for 15 minutes at 14000 rpm at room temperature. The water phase was removed with a Pasteur pipet and the DNA pellet was rinsed with 70% ethanol and air-dried for 10 minutes under warm light. The DNA was then dissolved in 100 µl TE-buffert (1 ml 1M Tris pH 7,9, 200 µl 0,5M EDTA and sterile water to a total of 100 ml). The DNA was left standing over night and on day three a test gel was run in order to check the degree of DNA degradation and to get a preliminary DNA concentration measurement. DNA concentration was then attained by density (OD) measurement.

DNA extraction from leucocytes:

(Please, refer to Wizard´s protocol "DNA-extraction from leucocytes"). 900 µl of cell lysis solution were added to a sterile 1,5 ml Eppendorf-tube. The tube of patient blood was gently rocked until thoroughly mixed and 300 ml of blood were transferred into the tube containing the cell lysis solution. the tube was inverted 5-6 times to mix and incubated for 10 minutes at room temperature to lyse the red blood cells. Then, the tube was centrifuged for 20 seconds at 12000 rcf at room temperature. As much supernatant as possible was removed and discarded without disturbing the visible white pellet. The tube was vigorously vortexed until the white bloodcells were resuspended. 300 µl of nuclei lysis solution were added to the tube and the content pipetted several times to lyse the cells. 100 µl of protein precipitation solution were added to the nuclear lysate and vortexed vigorously for 10-20 seconds. The sample was then centrifugated for 3 minutes at 12000 rcf at room temperature until a dark brown pellet was visible. The supernatant was transferred to a clean 1,5 ml Eppendorf-tube containing 300 µl of isopropanol. The solution was mixed by inversion until the white thread-like strands of DNA formed a visible mass. The tube was centrifugated for 5 minutes at 12000 rcf at room temperature until the DNA was visible as a white pellet. The supernatant was decanted and 300 µl of room temperature 70% ethanol were added to wash the DNA, then the tube was centrifugated for one minute at 12000 rcf at room temperature. The ethanol was aspirated with a Pasteur pipet, the tube inverted on clean ab-sorbent paper and the DNA pellet air-dried for 10-15 minutes. Finally, 100 µl of

DNA hydration solution were added to the tube and the DNA rehydrated by incubating at 65° C for one hour. DNA was stored at 4° C.

PCR amplification

Paired tumor and non-tumor DNA from the same patient served as a template for PCR ampli-fications. Two sets of oligonucleotide primers were used, microsatellite markers (simple repeated sequences of DNA, mono-, di-, tri- and tetranucleotide repeats) of the first set were obtained from the department of clinical genetics at Uppsala University hospital. These primers are ampli-fying the polymorphic loci in the human genome as defined in the screenig set 6 released by the Cooperative Human Linkage Center (CHCL) in the U.S.. The second set was purchased from Research Genetics, Inc., U.S. These primers are amplifying markers within the human linkage map as defined in the screening set 9A from CHCL. For both sets the forward markers are labelled with a fluorescent dye, either 6-FAM (blue), HEX (yellow) or TET (green) to be ana-lyzed on the ABI PRISM Genetic Analyzer. PCR reactions were performed in an ABI PRISM 877 thermal cycler (Perkin Elmer Applied Biosystems). For primers from the first set the PCR reactions contained 10-20 ng of template DNA, 2-6 pmol of each forward and reverse primer, 0,2 mM each dNTP (2´-deoxynucleotide 5´- triphosphate) (Life Technologies, Inc.), 1x PCR buffer, 1,5 mM Magnesium Chloride (Life Technologies, Inc.), 0,5 units *Taq* DNA Polymerase (Life Technologies, Inc.) and autoclaved distilled water to a final volume of 5-10 µl. Cycling was achieved as follows: denaturation at 95° for 3,5 minutes, followed by 30 cycles of de-naturation at 95° for 30 seconds, annealing at 55° for 30 seconds and extension at 72° for 30 seconds. A ten-minute final extension at 72° was carried out to finish the amplification. PCR re-actions for markers from the second set contained 10 ng of template DNA, 1,2 pmol of each for-ward and reverse primer, 0,2 mM each dNTP (2´-deoxynucleotide 5´- triphosphate) (Life Technologies, Inc.), 1x PCR buffer, 1,5 mM Magnesium Chloride (Life Technologies, Inc.), 0,5 units *Taq* DNA Polymerase (Life Technologies, Inc.) and autoclaved distilled water to a final volume of 5 µl. Cycling was performed by a denaturation step ar 95° for 2 minutes, 30 cycles of denaturation at 94° for 45 seconds, annealing at 56° for 45 seconds and extension at 72° for 60 seconds. A six-minute final extension at 72° was carried out to finish the amplification. Different temperatures and times for the different steps of amplification were chosen according to re-commendation by the authors of the screening set 9 edition by CHLC.

8.4 LOH SCREENING

After cycling, 1µl of GENESCAN TAMRA lane standard (Perkin Elmer Applied Biosystems) and 17µl of Formamid were added to a screening pool consisting of 1-4µl of each of 5-8 micro-satellite PCR products of a given panel (markers grouped together according to their size) to be pooled together and coelectrophoresed unambiguously. Then, the screening volumes (27-37µl PCR reaction) were denaturated at 95° for 5 minutes and transferred to an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Corporation) consisting of a laser-induced fluorescence capillary electrophoresis instrument and a Macintosh computer including "Genescan Perkin-Elmer Corporation" software for data collection and analysis of fluorescent-labelled DNA frag-ments for size and quantification. Each sample was loaded on Performance Optimized Polymer 4 (POP4) and the products separated by electrophoresis through the capillary at 15 kV electro-phoresis voltage, 9 µA electrophoresis current, laser power of 9,9 mV and 60° for 24 minutes. The light intensities of each product were stored as electric signals and displayed in the form of coloured peaks (one peak representing one allele) and the peak amplitudes were analyzed.

Heterozygosity, i.e. the presence of two distinct alleles in normal tissue has been the essential requisite for evaluation of LOH. Decreased peak amplitude of either tumor allele in hetero-zygous individuals was calculated in relation to peak amplitudes of paired normal DNA.

A reduction of the relative amplitude of 40% or more (a retention of 60% or less, respectively) was considered LOH. Given the peakheights of two of different-sized alleles in non-tumorous DNA in heterozygous individuals (N_1 and N_2) and of loss of one allele of the corresponding tumorous DNA (T_1 and T_2) the retention level was calculated as follows:

$$\text{Retention level} = (T_1/T_2)/(N_1/N_2).$$

72 fluorescent microsatellite markers from the first set and 64 markers from the second set as well as two custom-made *11q13* primers, in total, 131 different mikrosatellite markers were used to genotype DNA from the eight midgut carcinoids. The markers were distributed over the entire genome exept for chromosomes X and Y, with at least two markers per chromosomal arm. The microsatellite markers used in the analysis are listed beneath.

Fluorescent microsatellites used in our genome-wide screening for LOH in midgut carcinoid tumors

custom-made primers (Perkin Elmer Corp.)
PYGM
INT2
Weber screening set 6 (Nordic Consortium Primer Resource Center at the department of Clinical Genetics, Uppsala, Sweden
D1S1622, D1S551, D1S1589, D1S549,
D2S1356, D2S1649, D2S434,
D3S2387, D3S1768, D3S2427, D3S2398,
D4S2639, D4S2397, D4S2408, D4S2368, D4S2431,
D5S2505, GATA7C06, D5S2501, D5S816,
D6S1281, D6S1009, D6S1003, D6S1277,
D7S513, D7S1802, D7S821, D7S1804,
D8S1099, D8S592, D8S1179, D8S373,
D9S925, D9S1118, D9S302,
GAAT5F06, D10S1239,
D12S374, D12S391, D12S373, GATA32F05,
D13S173,
D14S749, D14S611, D14S118,
D15S652, D15S642,
D16S748, D16S769, D16S2624,
D17S1308, D17S1298, D17S1299, D17S809, D17S1290,
D18S843, D18S64, D18S541,
D19S247, GGAT2H06, D19S601,
D20S95, D20S604, D20S481, D20S1085,
D21S1435, D21S1270, D21S156, D21S1446,
D22S685, D22S683, D22S445

Weber screening set 9 (Genetic Research Inc.)

D1S1612, D1S552,

GATA165C07, D2S1356, D2S1394, D2S139,

D3S2387, GATA164B08,

D4S2639, D4S2431, D4S1652,

D5S2488, D5S807, GATA134B03, D5S2500, D5S1505, D5S820, D5S1456,

GATA163B10, FA3A1, D6S1053,

GATA137H02

GATA62F03, D9S925, D9S910, D9S934, D9S1838,

D10S1435, D10S1430, D10S1426, D10S677,

D11S1999, D11S1392, D11S1984, D11S2000,

D12S391, D12S1042, PAH, D12S395,

D13S317, D13S285,

D14S617, GATA136B01,

D15S643, D15S657,

ATA41E04, D16S764, D16S753, D16S3253, D16S2624, D16S539,

D17S1293,

D18S481, D18S877, D18S858, D18S844,

D19S433,

D20S482, D20S470, D20S481, D20S480, D20S171,

GATA188F04

Smad4/DPC4 analysis

In order to investigate a possible role of the TSG *SMad4/DPC4*, located on *18q21,* in the neo-genesis of our tumors, sequence analysis of exon 8-11 was performed. Only exon 8-11 were ana-lysed since these exons are were most often mutated in previously investigated tumors (Bartsch et al. 1999).

All tumor samples underwent PCR amplification using oligonucleotide primers flanking exon 8-11. The amplified samples were subjected to semiautomated sequencing on ABI 310 using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli Taq DNA Polymerase FS (Perkin Elmer Corp.).

Immunohistochemical staining with monoclonal antibodies to *Smad4/DPC4* (clone B8, Santa Cruz) was additionally performed on all tumors exept for 5216. The latter tumor was excluded due to lack of tissue material.

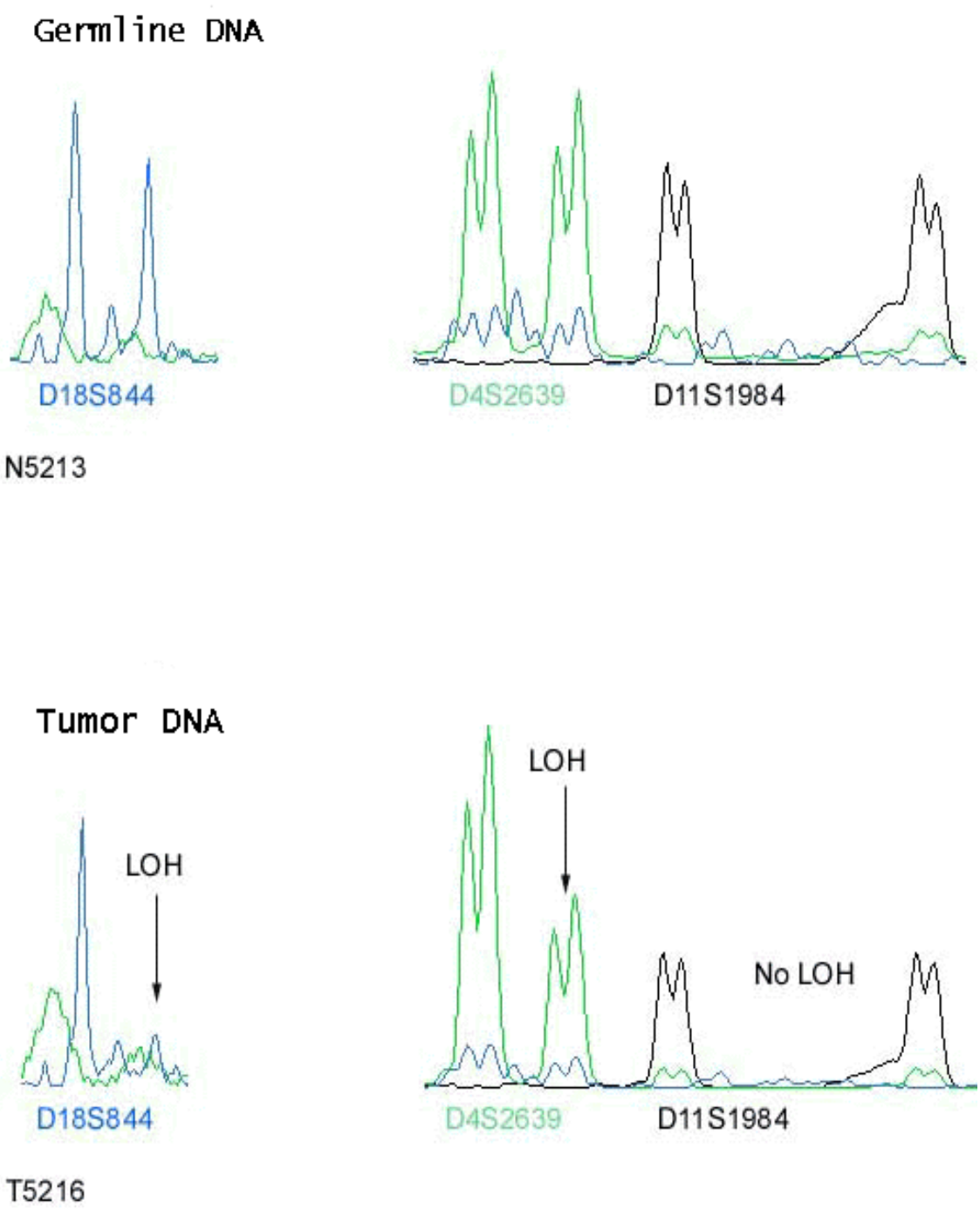

Figure 1. Examples of LOH in tumor T5216. Extensive LOH at chromosome 18q (marker D18S844). Partial LOH at chromosome 4p (marker D4S2639). No LOH at chromosome 11q (marker D11S1984). LOH revealed by allele reduction of one allele in the tumor tissue.

9 RESULTS

For the genome-wide LOH analysis two primary tumors and six metastases with relatively low amount of contaminating fibroblasts were chosen from the collection of metastatic midgut carcinoid tumors at the Department of Surgical Sciences, Endocrine Unit, University Hospital, Uppsala, Sweden (**Table 3.**). The microsatellite markers used in the analysis are listed in **Figure 1.** and the LOH results of our midgut carcinoids are shown in **Table 4.**.

A tumor was scored positive for LOH on one arm when at least one of the markers that define that region showed allelic loss. To investigate if a limited region of loss could be revealed by using denser genetic mapping we studied additional nearby microsatellites for all chromosomes with more frequent deletions and for chromosomes with a single LOH event to reconfirm our findings.

In total, 298 out of 312 examined chromosomal arms (96%) presented with at least one infor-mative marker. 45 different markers were lost and a various percentage of tumors was affected by LOH on the respective chromosomal arms, from 12,5 to 83%. Deletions were found on 11 chromosomes. LOH was found for a contiguous set of markers on chromosome 18 in all tumors except 5807. Two tumors (5216 and 5807) had lost parts of eight and seven chromosomes (chromosomes 4, 5, 7, 9, 12, 14, 18, 20 and 3, 4, 5, 7, 14, 19, 20 respectively) and tumor 2762 had deletions on chromosomes 9, 16, and 18. With exeption of tumor 5216, similar levels of allele retention were detected on both chromosomal arms. Fractional allelic loss (FAL) per tumor is defined as the number of markers displaying LOH per total number of informative microsatellites. The FAL was ranging from 1,4 to 35,% for all tumors. The highest number of deletions was found in tumors 5216 and 5807, with a FAL of 28,6% and 35,4% respectively. Tumor 5216 showed allelic retention levels differing from 11-25% (chromosomes 5, 9 and 18) to 46-60% (chromosomes 4, 7, 12, 14 and 20) whereas all other tumors revealed similar retention levels in all their chromosomes (**Table 4**). Tumors without LOH had retention levels of 88-100%.

No correlation between malignant features and number of allelic deletions was revealed, all tumors had metastasized to the liver and the levels of LOH were greatly differing.

LOH on chromosome 18.

Chromosome 18 showed the most significant rate of LOH with allelic loss in seven of eight tumors (88%). In six of these lesions the deletions spanned all informative markers on both the short and long arm of chromomsome 18. Tumour 2762 displayed LOH for microsatellite marker *D18S541* and *D18S844* at *18q21* while retaining *D18S858* mapped more centromerically to *18q21* and all informative markers on *18p*. This tumor, with a limited deletion on chromosome 18, also displayed LOH on chromosomal arms *9p* and *16q* but not *9q* and *16p*. Tumor 5807 did not display any LOH on either arm of chromosome 18, paradoxically this tumor showed most LOH on all other chromosomal arms.

No Smad4/DPC4 mutations could be revealed by the sequencing analysis of this TSG. Only exons 8-11 were examined since the majority of previously described mutations are found within this region (117).

The immunohistochemical staining with antibodies to the *Smad4/DPC4* protein revealed ex-pression of *Smad4/DPC4* in the seven analyzed tumors (data not shown). Tumor 5216 was not analyzed due to shortage of tumor material. Thus, no evidence of aberrant *Smad4/DPC4* was found.

Table 4. Genome-wide LOH screening of midgut carcinoid tumors

Chromosomal arms	Tumor numbe							
	2283	2762	4017	5184	5216	5692	5718	5807
1p	○	○	○	○	○	○	○	○
1q	○	○	○	○	○	○	○	○
2p	○	○	○	○	○	○	○	○
2q	○	○	○	○	○	○	○	○
3p	○	○	○	○	○	○	○	●
3q	○	○	○	○	○	○	○	●
4p	○	○	○	○	●	○	○	●
4q	○	○	○	○	●	○	○	●
5p	○	○	†	○	●	○	○	●
5q	○	○	○	○	●	○	○	●
6p	○	†	○	○	○	○	○	○
6q	○	○	○	○	○	○	○	○
7p	†	○	○	○	●	○	○	●
7q	○	○	○	○	●	○	○	●
8p	†	○	○	○	○	○	○	†
8q	○	○	○	○	○	○	○	○
9p	○	●	○	○	●	○	○	○
9q	○	○	○	○	●	○	○	○
10p	○	○	○	○	○	○	○	○
10q	○	○	○	○	†	†	○	○
11p	○	○	○	○	○	○	○	○
11q	○	○	○	○	○	○	○	○
12p	○	○	○	○	●	○	○	○
12q	○	○	○	○	●	○	○	○
13q	○	○	○	○	○	○	○	○
14q	○	○	○	○	●	○	○	●
15q	○	○	○	○	○	○	○	○
16p	○	○	○	○	○	○	○	○
16q	○	●	○	○	○	○	○	○
17p	○	○	○	†	○	○	○	○
17q	○	○	○	†	○	○	○	○
18p	●	○	●	●	●	●	●	○
18q	●	●	●	●	●	●	●	○
19p	○	○	○	○	○	○	○	†
19q	†	○	○	○	○	○	○	●
20p	○	○	○	○	†	○	○	●
20q	○	○	○	†	●	○	○	●
21q	○	○	○	○	○	○	○	○
22q	○	○	○	†	○	○	○	○

●=Loss of heterozygosity, ○=Retention of heterozygosity,†=Non-informative

10 DISCUSSION

Search for LOH has provided a strong tool for gaining insight into the process of cancer neo-genesis and the involvement of a subset of deletions and mutations in the initiation and pro-gression of tumor development. Midgut carcinoids are rare malignant tumors of the small intes-tine. The primary tumor is often inconspicious in size but nevertheless associated with generally larger mesenteric lymph node or liver metastases. The rather indolent malignant behaviour as well as their low incidence has evoked an interest in the genetic events in tumorigenesis of these neoplasms.

Only few midgut carcinoids have been investigated so far (Toliat, Berger et al. 1997; Ghimenti, Lonobile et al. 1999; Jakobovitz, Nass et al. 1996; Debelenko, Emmert-Buck et al. 1997a; Gortz, Roth et al. 1999; Zhao, de Krijger et al. 2000; D´Adda, Pizzi et al. 2002; Kytola, Nord et al. 2002). In contrast, the molecular mechanisms involved in the tumorigenesis of more common highly malignant gastrointestinal carcinomas are better characterized including frequent mutations of the *Smad4/DPC4* and *DCC* genes on chromosome *18q21*, *APC* on *5q21* and *p16* on *9p2* (Fearon, Ekstrand et al. 1994; Eppert, Scherer et al. 1996; Schutte, Hruban et al. 1996; Kinzler, Nilbert et al. 1991; Sun, Hildesheim et al. 1995).

This genome-wide screening for LOH of eight midgut carcinoid tumors revealed multiple allelic deletions with losses found in all tumors and in most cases encompassing both chromosomal arms. The rate of fractional allellic loss (FAL, in %) varied greatly between the different lesions. Even though the tumor samples still may have contained a certain amount of contaminating fibroblasts after microdissection, the LOH results were striking, especially in tumors 5216 and 5807, presenting with most deleted chromosomes.

Most conspicious were the findings of LOH on chromosome 18 with allelic losses in seven of eight lesions (88%). Only one study, using comparative genomic hybridization, could reveal LOH on chromosome 18 so far, LOH on chromosome *18p* in 7/15 and on *18q* in 8/15 midgut carcinoids (Zhao, de Krijger et al. 2000). In all but one tumor (2762) the deletions were large and included all informative markers on chromosome 18. LOH on chromosome 18 is a common event in a high proportion of gastroenteropancreatic carcinomas (Fearon, Cho et al. 1990; Schutte, Hruban et al. 1996; Uchida, Nagatake et al. 1996) as well as other tumor types (Papadimitrakopoulou, Oh et al. 1998; Hessman, Lindberg et al. 1999). Moreover, colorectal cancers with LOH on chromosome 18 behave clinically more aggressive than those without LOH (Fearon, Ekstrand et al. 1994). Three candidate tumor suppressor genes have been identified in this region: The *DCC* (deleted in colorectal cancer), *Smad4/DPC4* and *Smad2/MADR2/JV18-1* genes located on *18q21* (Fearon, Ekstrand et al. 1994; Eppert, Scherer et al. 1996; Schutte, Hruban et al. 1996). The *Smad4/DPC4* gene has been found homozygously mutated in both exocrine and endocrine pancreatic tumors as well as colorectal cancers (Schutte, Hruban et al. 1996; Takagi, Kohmura et al. 1996; Howe, Roth et al. 1998; Bartsch, Hahn et al. 1999; Friedl, Kruse et al. 1999). *DCC* has also been described to be homozygously deleted in a subset of pancreatic and other cancers (Fearon, Ekstrand et al. 1994; Hilgers, Song et al. 2000) while *Smad2/MADR2/JV18-1* alterations have been detected in a limited fraction of colorectal and lung cancers (Eppert, Scherer et al. 1996; Uchida, Nagatake et al. 1996). Although the deletion pattern suggested a tumor suppressor gene telomeric of the gene cluster at *18q21*, we chose to analyze the *Smad4/DCP4* gene in more detail since exon-specific PCR-primers were available and the described anti-serum for immunohistochemical labeling of *Smad4/DPC4* has proven specificity and sensitivity for homozygous gene inactivation of 94% and 91%, respectively (Wilentz, Su et al. 2000). For *Smad2/MADR2/JV18-1* and *DCC*, the correlation of immunohistochemical labeling and gene inactivation is unknown.

In our series of midgut carcinoids we did not identify any mutations in exon 8-11 of the *Smad4/DPC4* gene, including the intron-exon boundaries. Although homozygous deletions are difficult to exclude, all exons of all tumors were PCR-amplified with the same efficiency. Immunohistochemical staining of seven tumors, revealed normal expression of the *Smad4/DPC4* protein in all investigated lesions. The sequencing results and the distinct staining of *Smad4/DPC4* protein strongly suggest the idea that the *Smad4/DPC4* gene is unlikely to be in-volved in the development of midgut carcinoids. Tumor 2762, however, displayed a more limited deletion telomeric to *D18S858* and the *Smad4/DPC4* and *DCC* loci on *18q*, allelic loss of *18q* markers *D18S541* and *D18S844.* This same tumor had remained *D18S858* on *18q* as well as all *18p* markers. These findings suggest that this region of LOH might harbour yet another *TSG* distal to *Smad4/DPC4* and *DCC* loci on *18q* unknown to date and that its inactivation is more likely to be involved in the initiation of midgut carcinoid neogenesis.

Previous studies on chromosome 11 have described one somatic missense *MEN1* mutation (V531 in

exon 2) in one of 16 midgut carcinoid tumors (Toliat, Berger et al. 1997; Gortz, Roth et al. 1999). Two constitutional putative missense mutations, H50R and G12S on the *SDHD* (*TSG*) (succinate-ubiquinone oxidoreductase subunit D) gene locus were found in two midgut carcinoids, both mutations were associated with LOH of the other allele (Kytola, Nord et al. 2002). Microsatellite instability was detected in one of six analyzed midgut carcinoid tumors (Ghimenti, Lonobile et al. 1999). LOH on chromosome 11 was analyzed in 16 of 83 midgut carcinoids (Jakobovitz, Nass et al. 1996; Toliat, Berger et al. 1997; Debelenko, Emmert-Buck et al. 1997a; Ghimenti, Lonobile et al. 1999; Gortz, Roth et al. 1999; Zhao, de Krijger et al. 2000; D´Adda, Pizzi et al. 2002; Kytola, Nord et al. 2002. In contrast to these findings, we could not detect LOH on *11q13* in our midgut carcinoids even though both *PYGM* and *INT2* flanking the *MEN1* gene were informative in six of eight tumors and for the other two tumors markers *D11S2000* at *11q22* was informative. This discrepancy might be due to different patient series and different microsatellites used in the studies. However, the lack of LOH on chromosome 11, especially at *11q13,* holds with the fact that sporadic midgut carcinoids are not associated with the MEN1 syndrome and MEN1 deletions only occur in a subset of these tumors. Clearly more tumors have to be analyzed in order to gain a more true insight into involvement of *11q13* in midgut carcinoid neogenesis.

In addition to the frequent LOH on chromosome 18, several other chromosomes were deleted in a subset of tumors. Only one tumor (5807) showed LOH for all informative markers on chromo-some 3, two tumors (5216, 5807) presented with LOH for all informative microsatellites on chromosome 4, 5, 7, 14 and 20, LOH for chromosome 9 was found in two tumors, all chro-mosome 9 microsatellites were deleted in tumor 5216 while tumor 2762 showed LOH for one marker only (GATA 62F03), all chromosome 12 markers were deleted in one tumor (5216), only one chromosome 16 marker (D16S2624) at *16q22.1*, the e-cadherin locus, was lost in one tumor (2762). *3p23-3p22* harbours the *MLH1* gene causing the familial non-polyposis type of colonic cancer (FCC2) (Panariello, Scarano et al. 1998) as well as the *SCLC1* gene being involved in the carcinogenesis of small lung cell cancer (Hibi, Takahashi et al. 1992), the *HVBS6* gene maps to *4q32.1* and is rearranged in hepatocellular cancer (Blanquet, Garreau et al. 1988). Mutations of the *APC* gene located at *5q21* might be important in the evolvement of midgut carcinoid tumors, multiple colonic carcinoid tumors have been reported in one adenomatosis polyposis coli patient (July, Northcott et al. 1999) and *APC* is known to play a role in the development of a number of colorectal cancers (Kinzler, Nilbert et al. 1991). Down-regulation of the *DRA* (down-regulated in adenoma) gene on *7q22-7q31.1* is associated with the neoplastic transformation of normal colonic mucosa to polyps to adenocarcinoma (Antalis, Reeder et al. 1998) and *p16* at *9p21* is involved in various neogenetic events (Sun, Hildesheim et al. 1995). The *SRC* gene is located at *20q11.2* and is involved in the procession of advanced colonic cancers (http://www.gdb.org/). The small number of tumors, however, makes it impossible to draw any conclusions of these scattered allelic losses detected in three of eight analyzed carcinoids. They may be caused by random events in a genetic unstable cellular environment.

LOH on chromosomes 4, 5, 7, 9, 14 and 20 were detected in two of eight tumors whereas chromosomes 3, 16 and 19 were affected in only one tumor. Two lesions displayed allelic loss on seven and eight chromosomes respectively. One of the latter, tumor 5807, was the only tumor that had retained chromosome 18 but paradoxically displayed the highest extent of LOH on all chromosomes. In this tumor, all affected chromosomes presented with the same level of allelic retention (40-59%), in the other tumor, 5216, however, we detected markedly lower retention levels of 11-25% for chromosomes 5, 9, and 18 than for chromosomes 4, 7, 12, 14 and 20 (46-60%). This is suggestive of presence of intratumoral heterogeneity in tumor 5216 with chromo-some 5, 9 and 18 deletions in most tumor cells whereas the other chromosomal losses are present in tumor cell subclones only. Tumors displaying LOH are considered to be of monoclonal origin (Guo, Li et al. 2000). The genetic events on chromosomes 5, 9 and 18 might have developped earlier in the neoplastic process than those on the other chromosomes. Another hypothesis is that such deletions might give these mutated cells growth advantage over less altered tumor cells.

Some of the tumor samples contained a rather high amount of of fibroblasts. The normal DNA from these cells will dilute tumor DNA and interfere with the LOH analyses. However, we have been able to detect allelic losses in all lesions and the levels of retention of alleles of all but two tumors have been lower than 50%. One exception (5216) had lower levels of allelic retention in three of eight deleted chromosomes while sample 5807 displayed LOH on several chromosomes with allelic retention levels of approximately 50% for all affected chromosomes. We therefore believe that our figures are a true picture of the deletion patterns of these neoplasms.

Our findings of LOH on chromosome 18 in 88% of the tumors, however, suggests a model wherein the steps required for malignancy in midgut carcinoid tumors commonly involve the loss of genes on chromosome 18 that normally suppress tumorigenesis. Despite the alterations found on chromosome

18, genetic events in midgut carcinoids seem to differ from those found in gastrointestinal carcinomas with regard to the absence of *Smad4/DPC4* and *K-ras* and rare *p53* mutations in midgut carcinoid tumors. A more explorative and detailed analysis of loci deleted using a larger number of markers and a larger number of tumor specimens is warranted to clarify the comprehension of the unique neogenetic behaviour of classical midgut carcinoid tumors.

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Acknowledgements

This work, supported by the Swedish Medical Research Council, The Swedish Cancer Society, Lions Cancer Research Fund, The Swedish Society of Medicine and Thorsten and Ragnar Söderbergs Foundation, was performed at the Department of Surgery, Uppsala, Sweden. I would like to express my sincere gratitude to the following people for their invaluable help.

Ola Hessman, my supervisor, for introducing me into the exciting field of laboratory work and teaching me a good deal of methods and lab smartness, knowhow in data processing, for always being so patient with me and always having a spare moment to help and advise me on my questions and for the nice good-bye come-together on his boat.

Göran Åkerström, professor and “Doktorvater”, head of our research group, for offering this project to me when I first had the idea of doing research for my German doctoral thesis in Sweden, for welcoming me so warmly into his group, for believing in me and complimenting me on my „ well-done first try in research „ .

Daniel Lindberg, for being an enormous help with all kinds of computer catastrophies and for invaluable talks about interhuman matters !

Pamela Correa, for taking me along to the social side of Swedish life and for helping me with her knowhow at the lab.

Gunnar Westin, the genetic expert in our group, for always being helpful in all matters.

Eva Szabo, for introducing me to PCR robots and Genetic Analyzers, great working-together and for many good talks when not feeling like working.

All other members of our wonderful lab group, Birgitta Bondesson, Ulrika Segersten, Per Hellman, Peter Lillhager and Tobias Carling.

All of the above-mentioned, for welcoming me so warmly into their group and being very patient in teaching me Swedish, for helping me getting into the field of science, I definitely picked the best lab in the world to start research at.

Bertram Wiedenmann, professor and “Doktorvater”, for taking over the supervision of my doctoral thesis in Berlin.

My friends Sibylle and Franziska for keeping up my humour over the phone when life was being unfair.

My mother Ingeborg and my father Herbert for constantly encouraging me from back home and reminding me that there are more moments when everything goes wrong but that this wouldn´t last. Thank you so much for making my wonderful years in Sweden possible !

My sisters Deborah, Noemi and Eva for reminding me that there is always a bright side of life, my grandmother and aunt, for making us a great family !

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Lollgen RM, Hessman O, Szabo E, Westin G, Akerstrom G: Chromosome 18 deletions are common events in human midgut carcinoid tumors. Int J Cancer 2001 Jun 15;92(6):812-815.

107. Kongress Deutsche Gesellschaft für Innere Medizin 23.-26.04.2001, Wiesbaden:

Posterpräsentation „Chromosom 18-Deletionen als häufiger Befund bei Karzinoidtumoren des Dünndarms, R.Löllgen, O.Hessman, E. Szabo, G. Westin und G. Åkerström

XXI. Jahrestagung der CAEK (Chirurgische Arbeitsgemeinschaft für Endokrine Chirurgie)

15./16.11.2002, Potsdam: Vortrag „Chromosom 18 Deletionen: Ein häufiger Befund bei Neuroendokrinen Tumoren des Mitteldarms“

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Sonstige Kenntnisse/Interessen

Anwendungsorientierte Kenntnisse EDV

Sprachen: Fließend in Wort und Schrift **Englisch, Französisch, Schwedisch, Italienisch**

Gut Latein, Spanisch, Grundkenntnisse Chinesisch

Skifahren, Reisen, Musik, Theater, Literatur

Eidestattliche Erklärung

Diese Dissertation wurde von mir selbst und ohne die (zulässige) Hilfe Dritter verfasst, sie stellt auch in Teilen keine Kopie anderer Arbeiten dar und die benutzten Hilfsmittel sowie die Literatur sind vollständig angegeben.