

Profiling of Small Intestine Neuroendocrine Tumors

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Till min familj

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ABSTRACT

Small intestine neuroendocrine tumors (SI-NETs) are malignant neoplasms usually presenting with disseminated disease and symptoms of hormone overproduction. Radical surgery is curative but can only be performed for localized disease. For most patients the disease follows a progressive and fatal course. New treatment strategies for cure and palliation are therefore needed. To explore the mechanisms of SI-NET formation and to define candidate therapeutic targets and biomarkers of prognosis, we examined the gene expression profiles and somatic copy number alterations (SCNAs) in SI-NETs by array-based techniques.

Tumors from forty-three patients with SI-NETs were investigated by high-resolution array-CGH. The number of SCNAs per tumor was low, indicating that SI-NETs are genetically relatively stable tumors. The most frequent SCNA was loss of chromosome 18 (74%), occurring in both primary tumors and metastases. In some tumors loss of chromosome 18 was the only SCNA, indicating an early event in tumor formation. Two separate groups of tumors with distinct patterns of SCNAs were observed: a major group of tumors with loss of chromosome 18 and a minor group of tumors with gain of whole chromosomes (chr. 4, 5, 14 and 20). Survival analysis showed that gain of chromosome 14, a characteristic event in the minor group, was a strong predictor of poor survival. Gene expression profiles of SI-NETs were analyzed in two different studies. In the first study we examined the expression profile of five SI-NETs and found amyloid precursor like protein 1 (APLP1), a member of the APP-family (APP, APLP1 and APLP2), to be differentially upregulated in SI-NETs. Higher expression of APLP1 in metastases compared to primary tumors indicated a role of APLP1 in tumor progression. Localization of the APP-family proteins in SI-NET cells (GOT1) by confocal laser microscopy showed partial co-localization with synaptophysin, Rab5 and FE65, suggesting a role in tumor cell adhesion and gene regulation. In the second study tumor tissue from thirty-three patients was subjected to expression profiling. We identified three different groups of SI-NETs by unsupervised hierarchical clustering with significant differences in patient survival. Genes related to patient survival included genes involved in cell cycle progression, apoptosis and DNA damage response. Genes involved in tumor invasion and immunity also correlated to patient survival. Cell cycle related genes were differentially expressed in tumors with gain of chromosome 14. Forkhead box M1 (*FOXM1*), a master regulator of cell cycle progression, was identified as an upstream regulator in these tumors by pathway analyzes. Analysis of upregulated genes in SI-NETs identified a number of candidate drug targets including SSTR2, receptor tyrosine kinases, transcriptional regulators and molecular chaperones. *In vitro* experiments on GOT1 cells demonstrated effective inhibition of tumor growth by multi-tyrosine kinase inhibitors as well as by inhibitors of AKT, HDAC and HSP90.

In conclusion, these studies have established the expression and SCNA profiles of SI-NETs. These data demonstrate a molecular heterogeneity among SI-NETs, and identifies a correlation between deregulation of cell cycle genes and patient survival. Furthermore, profiling of SI-NETs provides novel candidate therapeutic targets related to tumor subgroups and a platform for patient stratification in clinical trials.

Keywords: small intestine neuroendocrine tumor, somatic copy number alteration, expression profiling, APLP1, survival, targeted therapy

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är den vanligaste dödsorsaken under 85 år och ett stort hälsoproblem både i Sverige och i västvärlden. Cancer uppstår genom en flerstegsprocess varvid en normal cell transformeras till en cancercell. En cancercell utvecklar egenskaper som gör att den undgår kroppens normala reglering av tillväxt och därmed växer okontrollerat. Detta beror på att det uppstått genomiska förändringar i cellernas arvs massa (DNA), t.ex. kromosomförändringar eller förändringar som påverkar genernas uttryck. Neuroendokrinatumörer (NET) i tunntarmen upptäcks ofta då sjukdomen redan är spridd och symtom relaterade till överproduktion av hormoner finns. Den enda botande behandlingen som finns idag är radikal kirurgi som kan utföras vid lokaliserad sjukdom. Det finns därför ett stort behov av nya behandlingsstrategier för bot och lindring. I denna avhandling har vi undersökt potentiella genomiska förändringar, terapeutiska målgener och markörer för prognos i tunntarms NET. I samtliga studier har vi använt oss av microarray, en teknik som gör det möjligt att undersöka alla geners uttryck eller alla kromosomers kopienummer i en och samma analys. Vi undersökte tumörer från 43 patienter med tunntarms NET med avseende att identifiera kromosomförändringar. Vi fann att förlust av kromosom 18 var den mest förekommande förändringen och förekom i 74% av tumörerna. I ett antal tumörer var förlust av kromosom 18 den enda kopienummer förändringen som identifierades vilket tyder på att förlust av kromosom 18 är en tidig händelse i utvecklingen av tunntarms NET. Vi identifierade två separata grupper av tumörer med skilda mönster av kromosomförändringar. En större grupp med förlust av kromosom 18 och en mindre grupp av tumörer med förvärv av hela kromosomer (kromosom 4, 5, 14 och 20). En överlevnadsanalys visade att patienter med förvärv av en extra kromosom 14 hade en sämre överlevnad. Vi analyserade alla geners uttryck i två olika studier. I den första studien undersökte vi tumörvävnad från fem patienter och fann att genen APLP1, en medlem av APP-familjen (APP, APLP1 och APLP2), var högt uttryckt i tunntarms NET jämfört med normal tunntarmslemhinna. Levermetastaser hade ett högre uttryck av APLP1 jämfört med primärtumörer vilket tyder på att APLP1 kan ha betydelse för spridning av tumören. I den andra studien undersökte vi det totala genuttrycket i tumörvävnad från 33 patienter med tunntarms NET. Vi identifierade tre distinkta grupper av tunntarms NET med hjälp av en klusteranalys och fann signifikant skillnad i överlevnad mellan grupperna. Vi identifierade 168 gener som var relaterade till patientöverlevnad bland annat gener som reglerar celldelning, celldöd samt gener som reagerar om DNA skador uppkommer. I patienter med förvärv av kromosom 14 fann vi att FOXM1, en gen som reglerar celldelning, var högt uttryckt. Vid analys av högt uttryckta gener i tunntarms NET hittade vi ett antal kandidatgener där droger som prövas i kliniska studier skulle kunna ha en hämmande effekt. Vi testade ett urval av dessa droger *in vitro* på tunntarms NET celler (GOT1) och behandling med multi-tyrosinkinashämmare så väl som med hämmare av AKT, HDAC and HSP90 visade effektiv inhibition av tumörväxt. Sammanfattningsvis så har dessa studier kartlagt genuttrycksmönster och kromosomförändringar i ett stort antal patienter med tunntarms NET. Data visar att förändrat uttryck av gener som reglerar bland annat celldelning påverkar överlevnaden hos patienter. Dessutom så identifierades lovande kandidatgener för målriktad terapi.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. Arvidsson Y, **Andersson E**, Bergström A, Andersson M K, Altiparmak G, Illerskog A, Lamazhapova D, Ahlman H, Nilsson O. Amyloid precursor-like protein 1 is differentially upregulated in neuroendocrine tumours of the gastrointestinal tract. *Endocrine-Related Cancer* 2008; 15: 569-581.

- II. **Andersson E**, Swärd C, Stenman G, Ahlman H, Nilsson O. High-resolution genome profiling reveals gain of chromosome 14 as a predictor of poor outcome in ileal carcinoids. *Endocrine-Related Cancer* 2009; 16: 953-966.

- III. **Andersson E**, Kristiansson E, Arvidsson Y, Swärd C, Hofving T, Wängberg B, Nilsson O. Expression profiling of small intestine neuroendocrine tumors identifies subclasses of clinical relevance, therapeutic targets and biomarkers for prognosis. *Manuscript*.

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ABBREVIATIONS

aCGH	array-based comparative genomic hybridization
CgA	chromogranin A
CGH	comparative genomic hybridization
CT	computed tomography
DNA	deoxyribonucleic acid
EC	enterochromaffin
GPCR	g-protein coupled receptor
HPF	high power field
LAR	long acting release
LINE	long interspersed nucleotide elements
MRI	magnetic resonance imaging
MPS	massively parallel sequencing
miRNA	micro RNA
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NEC	neuroendocrine carcinoma
NEN	neuroendocrine neoplasm
NET	neuroendocrine tumor
PDEC	poorly differentiated endocrine carcinoma
PET	positron emission tomography
PFS	progression free survival
qPCR	quantitative real-time polymerase chain reaction
RFA	radiofrequency ablation
RNA	ribonucleic acid
SCNA	somatic copy number alteration
SEER	Surveillance, Epidemiology, and End Results
SI-NET	small intestine neuroendocrine tumor
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SRS	somatostatin receptor scintigraphy
SSA	somatostatin analogues
SSTR	somatostatin receptors
SYP	synaptophysin
TACE	chemoembolization
TAE	transcatheter arterial embolization
TMA	tissue microarray
TNM	tumor-node-metastasis
TSG	tumor suppressor gene
VMAT1	vesicular monoamine transporter 1
WDEC	well differentiated endocrine carcinoma
WDET	well differentiated endocrine tumors
WHO	World health organization

INTRODUCTION

Epidemiology of small intestine neuroendocrine tumors (SI-NETs)

Neuroendocrine tumors (NET) of the small intestine are rare and occur most frequently in the ileum followed by the duodenum, jejunum and Meckel's diverticulum (Kim, et al. 2013; Lawrence, et al. 2011). The age-adjusted annual incidence for SI-NETs reported in the SEER (Surveillance, Epidemiology, and End Results) registry is 0.86/100,000 (duodenum: 0.19, ileum/jejunum: 0.67) (Yao, et al. 2008a). In a Swedish population-based study the annual age-adjusted incidence was 1.33/100,000 and similar incidence rates have also been reported in Norway and England (1.01 and 0.78 respectively) (Ellis, et al. 2010; Hauso, et al. 2008; Landerholm, et al. 2010). However, the highest incidence was reported in a Swedish autopsy study (5.4/100,000), indicating that a substantial proportion of tumors remains clinically unrecognized (Berge and Linell 1976). The incidence of SI-NETs has increased during the last decades and is still increasing in the US as well as in Europe (Hauso et al. 2008; Landerholm et al. 2010; Lawrence et al. 2011; Yao et al. 2008a). The median age at diagnosis is reported to be 66-69 years (Landerholm et al. 2010; Yao et al. 2008a). SI-NETs are more common in men and occur more frequently in whites and African Americans than in Asian/Pacific Islanders and American Indian/Alaskan natives (Yao et al. 2008a). The vast majority of SI-NETs are sporadic, but familial cases have been reported (Cunningham, et al. 2011b; Eschbach and Rinaldo 1962; Jarhult, et al. 2010; Moertel and Dockerty 1973; Pal, et al. 2001; Wale, et al. 1983). A slightly elevated risk of developing SI-NET in the offspring of patients with SI-NETs has been observed (Babovic-Vuksanovic, et al. 1999; Hassan, et al. 2008a; Hemminki and Li 2001; Hiripi, et al. 2009). Similarly, a family history of cancer is also a risk factor associated with development of SI-NETs (Hassan et al. 2008a; Hemminki and Li 2001; Hiripi et al. 2009). However, lifestyle factors such as smoking, alcohol consumption, diabetes mellitus or obesity are not associated with SI-NET susceptibility (Hassan, et al. 2008b).

Clinical presentation

SI-NETs are malignant tumors with localized (29%), regional (41%) or distant (30%) disease at diagnosis (Yao et al. 2008a). Tumors are often discovered during surgery for intestinal obstruction and ischemia. Alternatively, the diagnosis becomes evident due to disseminated disease with symptoms of hormone overproduction, known as the carcinoid syndrome. The syndrome was first described in 1954 by Thorson et al. and includes diarrhea, flush, bronchial

constriction and right-sided valvular disease (Thorson, et al. 1954). Hormonal symptoms develop exclusively in patients with distant metastases when hormones secreted by the tumor (*e.g.* serotonin and tachykinins) escape hepatic inactivation and reach the systemic circulation. In a Swedish population-based study the most common symptoms at presentation were chronic or acute abdominal pain (50%), intestinal obstruction (35%), diarrhea (26%), weight loss (24%), gastrointestinal hemorrhage (14%), flush (13%) and bronchial constriction (2%) (Landerholm et al. 2010).

Pathology of SI-NETs

Primary NETs of the small intestine frequently arise in the distal part of the ileum near the ileocecal valve but are also found in the duodenum, jejunum and Meckel's diverticulum. The diameter of the primary tumor is <1 cm in 13.5% of the tumors, 1-2 cm in 39.7% and ≥ 2 cm in 46.8% (Burke, et al. 1997). Multiple tumors occur in about 25-36% of patients (Burke et al. 1997; Norlen, et al. 2012; Yantiss, et al. 2003). The histopathological characteristics of SI-NETs include an insular growth pattern but other growth patterns such as trabecular, acinar, solid and small cell nest may occur. Tumor cell necrosis is rare (Cunningham, et al. 2007). The grading of SI-NETs is based on mitotic count and Ki67 index (see section below). The majority of the tumor cells are well differentiated with a low proliferation rate. Approximately 68% is grade 1 (G1) tumors, 30% is G2 and 2.3% is G3 tumors (Norlen et al. 2012). SI-NETs grow invasively in the wall of the small intestine and metastasize to regional lymph nodes and liver. Hematological spread to extra abdominal organs occurs late. At diagnosis a minority of patients have a primary tumor that invades lamina propria/submucosa with a size ≤ 1 cm (TNM stage I: 0.5%) or a primary tumor that invades subserosa (TNM stage II: 3%). Twenty-nine percent of the patients have a primary tumor that invades the serosa or other organs, or have spread to regional lymph nodes in the mesentery (TNM stage III). The majority of patients have distant metastases (TNM stage IV: 68%) (Strosberg, et al. 2013). The liver is the most frequent site (92%) of metastatic spread followed by mesentery (48%), retroperitoneum, bone, peritoneum and more seldom ovary, thorax and supraclavicular lymph nodes (Strosberg, et al. 2009). SI-NETs in the distal jejunum/ileum are thought to originate from enterochromaffin cells (EC-cells) of the gastrointestinal mucosa. EC-cells are the most common neuroendocrine cell type in the gastrointestinal tract. They are part of a system regulating intestinal blood flow, motility and secretion. EC-cells possess a neuroendocrine phenotype including production, storage and release of hormones and vesicle proteins. They produce >90% of the bodies output of the hormone serotonin (5-HT) which control gastrointestinal function (Ahlman and Nilsson 2001). Serotonin is

sequestered into secretory vesicles of the EC-cells by the vesicular monoamine transporter 1 (VMAT1) and is released into the blood circulation and gut lumen upon stimulation. Serotonin is degraded to 5-HIAA in the liver and lung and is excreted into the urine. EC-cells harbor two different types of storage organelles for vesicle proteins such as chromogranin A (CHGA) and synaptophysin (SYP): synaptic-like microvesicles where e.g. SYP is stored and large dense-core vesicles where e.g. CHGA is located. EC-cells express somatostatin receptors (SSTRs) on their surface. SSTRs are G protein-coupled receptors and five different subtypes have been described. The ligand of the SSTRs is the peptide hormone somatostatin which inhibits the release of serotonin from EC-cells. SI-NETs are composed of tumor cells with an enterochromaffin phenotype including expression of neuroendocrine markers such as serotonin, CHGA, SYP, VMAT1 and SSTRs. CHGA and SYP are biomarkers commonly used for the histopathological diagnosis. SI-NET cells express mainly SSTR subtype 2 and 5 and somatostatin analogues are used to diagnose and treat SI-NETs.

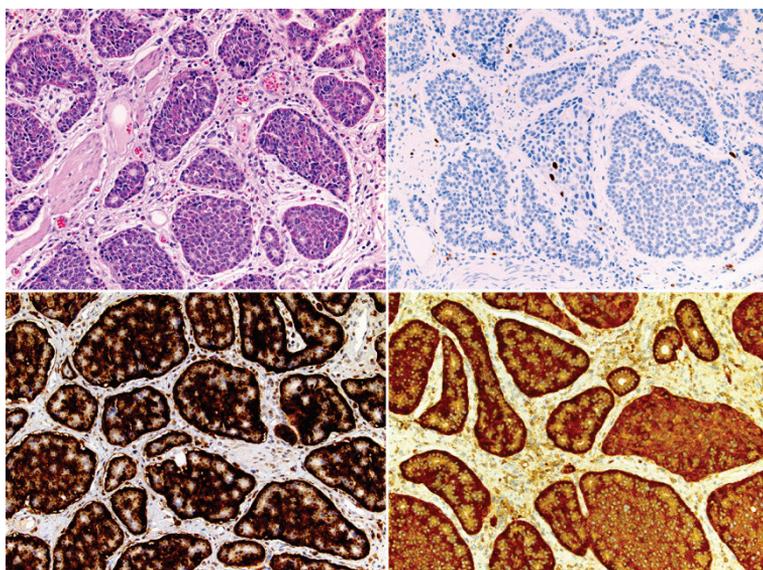


Figure 1. Hematoxylin and eosin staining, and immunohistochemical stainings of SI-NET.

Hematoxylin and eosin staining of a primary tumor with a mixed acinar and insular growth pattern (*upper left*). Ki67 (*upper right*), serotonin (*lower left*), and chromogranin A (*lower right*) immunohistochemical stainings of the same tumor.

The classification of neuroendocrine neoplasms (NENs)

In 1907, Siegfried Oberndorfer gave the tumors we now designate SI-NETs the name ‘carcinoid’ (carcinoma-like) to discern this entity from adenocarcinomas (Oberndorfer 1907). Since then the classification of NETs has been revised several times. They have been classified after their embryological origin by Williams and Sandler into foregut (bronchus, stomach, duodenum, pancreas), midgut (jejunum, ileum, appendix, ascending colon) and hindgut (colon, rectum) tumors (Williams and Sandler 1963). In the WHO classification of year 2000 NETs were classified based on differentiation and stage into well-differentiated endocrine tumor (WDET), well-differentiated endocrine carcinoma (WDEC) and poorly differentiated endocrine carcinoma (PDEC). The present WHO classification of 2010 classifies NENs of the gastrointestinal tract according to their grade into neuroendocrine tumors (NETs) or neuroendocrine carcinomas (NECs). Grade 1 (G1) and 2 (G2) tumors are designated NETs and grade 3 (G3) tumors NECs. The grading system is based on Ki67 index (the percentage of Ki67 positive tumor cells) and mitotic count (number of mitoses per 10 high-power fields (HPF)), as follows: G1: mitotic count <2 per 10 HPF and/or $\leq 2\%$ Ki67 index, G2: mitotic count 2-20 per 10 HPF and/or 3-20% Ki67 index and G3: mitotic count >20 per 10 HPF and/or >20% Ki67 index.

TNM -staging of NETs

The extent of the disease is categorized according to the TNM (tumor-node-metastasis) system. The 7th edition of TNM classification 2009 included SI-NETs for the first time (Sobin, et al. 2009). These tumors are classified as in table 1 and 2.

Table 1. TNM system.

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor invades lamina propria or submucosa and size ≤ 1 cm
T2	Tumor invades muscularis propria or size > 1 cm
T3	Tumor invades subserosa
T4	Tumor perforates visceral peritoneum (serosa) or invades other organs or adjacent structures
For any T add (m) for multiple tumors	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M0	No distant metastases
M1	Distant metastases

Table 2. Disease stage.

Disease stage	T	N	M
Stage I	T1	N0	M0
Stage IIA	T2	N0	M0
Stage IIB	T3	N0	M0
Stage IIIA	T4	N0	M0
Stage IIIB	Any T	N1	M0
Stage IV	Any T	Any N	M1

Biomarkers for diagnosis and follow-up

Chromogranin A in plasma (P-CgA) and urinary 5-HIAA are two widely used biomarkers for diagnosis and follow-up. Measurement of P-CgA is more sensitive and can be used to detect small tumor burden while measurement of 5-HIAA is used to monitor the carcinoid syndrome (Modlin, et al. 2010a; Welin, et al. 2009). Several different imaging methods can be used to diagnose and follow SI-NETs. These methods include computed tomography (CT), magnetic resonance imaging (MRI) and somatostatin receptor imaging by ¹¹¹Indium-scintigraphy (SRS), ⁶⁸Gallium- or ¹¹C-5-hydroxytryptophane-positron emission tomography (PET) (Orlefors, et al. 2006; Pape, et al. 2012). Echocardiography is performed to detect carcinoid heart disease in patients with carcinoid syndrome (Dobson, et al. 2014).

Treatment for patients with SI-NETs

Surgery is the primary treatment for patients with SI-NET and should be performed whenever possible (Pape et al. 2012; Pavel, et al. 2012). Radical surgery is the only curative treatment and can be performed for localized disease (stage I and II) (Jann, et al. 2011). Radical resection of liver metastases (surgery with curative intent) increases long-term and symptom-free survival (Elias, et al. 2003; Sarmiento, et al. 2003). Palliative surgery is recommended if approximately 90% of the tumor burden can safely be removed (Pavel et al. 2012). Liver transplantation can be considered in carefully selected cases, when metastases are limited to the liver (Olausson, et al. 2007). Palliation can also be achieved by interventional treatments such as radiofrequency ablation (RFA), transcatheter arterial embolization (TAE) or chemoembolization (TACE) (Pavel et al. 2012). Somatostatin analogues (SSAs) are first-line medical therapy in functioning NETs and were introduced during the late 1980 to control hypersecretion of hormones from tumor cells and to alleviate symptoms (Modlin, et al. 2010c). Treatment with octreotide LAR, a long acting release somatostatin analogue, induces symptomatic relief and delays tumor progression from 6 to

14.3 months in patients with SI-NETs (PROMID study) (Rinke, et al. 2009). Recently, another SSAs, lanreotide, was also shown to prolong progression free survival (PFS) (Caplin, et al. 2014) The immune modulator interferon- α (IFN) has been used as second-line therapy or as an add-on therapy to SSAs (Eriksson, et al. 2008). Conventional chemotherapy is rarely used because of poor response rates. SI-NETs are relatively insensitive to radiation therapy. However, peptide receptor radionuclide therapy (PRRT) with ^{177}Lu - or ^{90}Y -labelled somatostatin analogues has been shown to be effective in patients with inoperable or metastatic disease (Kwekkeboom, et al. 2008; Kwekkeboom, et al. 2009; Vinjamuri, et al. 2013). Targeted therapy with small molecule inhibitors is a new therapeutic approach in SI-NETs. There are only a few published studies on small molecule inhibitors but there are several ongoing clinical trials. Available studies are often small due to the rarity of the disease and/or a heterogeneous study population and not all of them are randomized control studies (Walter, et al. 2012; Walter and Krzyzanowska 2012). A clinical trial (RADIANT-2) of a mammalian target of rapamycin (mTOR) inhibitor, everolimus, demonstrate increased PFS from 14 to 18.6 months in SI-NETs treated with everolimus plus octreotide LAR compared to patients treated with placebo plus octreotide LAR (Pavel, et al. 2011). There are also a few published studies on angiogenesis inhibitors and SI-NETs. Kulke et al. enrolled 19 patients with midgut carcinoid in a non-randomized study of sunitinib, a multi-tyrosine kinase inhibitor, but reported no antitumor effect (Kulke, et al. 2008b). A random assignment phase II study of the anti-VEGF monoclonal antibody (bevacizumab) and IFN demonstrate longer PFS with bevacizumab and a larger confirmatory study is ongoing (Yao, et al. 2008b). In summary, surgery is the only curative treatment for patients with SI-NETs. Palliation can be offered through a variety of medical and interventional treatment options, but has limited effect and do not cure the patient. Development of novel treatment strategies for systemic disease is therefore needed.

Survival and prognostic factors for SI-NETs

The overall 5-year survival reported for SI-NETs in the SEER registry is 68.1% (Lawrence et al. 2011). In a German study the 5- and 10-year disease-specific survival was 88.9% and 69.2% respectively and in a Swedish study 75.0% and 63.4% (Jann et al. 2011; Landerholm, et al. 2011). Survival rates are similar in men and women and has increased during the last decades in the US as well as in Europe (Landerholm et al. 2011; Lawrence et al. 2011; Scherubl, et al. 2013; Yao et al. 2008a; Zar, et al. 2004). Factors influencing survival in patients with SI-NETs have been reported. Resection of the primary tumor is a positive

prognostic factor for patients with SI-NETs (Ahmed, et al. 2009; Norlen et al. 2012). Old age at diagnosis, elevated 5-HIAA and carcinoid heart disease are shown to be negative prognostic factors (Landerholm et al. 2011; Moller, et al. 2005; Norlen et al. 2012; Srirajaskanthan, et al. 2013; Strosberg et al. 2013; Westberg, et al. 2001; Zar et al. 2004). TNM stage is also influencing survival. The overall 5-year survival for stage I to IV are 100%, 100%, 91% and 72% and in another study 100%, 100%, 86% and 57% respectively (Norlen et al. 2012; Strosberg et al. 2013). The same studies also reported that high WHO grade (G2-3) influence outcomes and is an unfavorable factor. A nomogram, which includes several prognostic factors, may be a useful tool for prognostication and personalized treatment (Modlin, et al. 2010b). Although prognostic biomarkers have been identified for patients with SI-NETs the outcome for individual patients remains difficult to predict. New biomarkers that more accurately can predict disease development and response to therapy are needed.

Genetics and epigenetics of cancer

Cancer formation is a multistep process, transforming a normal cell into a cancer cell. Through genetic and epigenetic alterations the normal cell acquires new capabilities ultimately leading to uncontrolled growth. Genetic alterations include mutations and chromosomal aberrations. Three classes of genes are involved: oncogenes, tumor suppressor genes and DNA repair genes. Proto-oncogenes are genes that control cell proliferation and apoptosis. They encode proteins functioning as transcription factors (e.g. *MYC*), growth factors (e.g. *PDGFB*), growth factor receptors (e.g. *EGFR*), signal transducers (e.g. *SRC*), inhibitors of apoptosis (e.g. *BCL2*) and chromatin remodelers (e.g. *ALL1*) (Croce 2008). In cancer cells proto-oncogenes can be activated and become oncogenes due to point mutations, gene amplification and translocations. The activation leads to cell proliferation and inhibited apoptosis. Tumor suppressor genes (TSGs) are genes that are sufficient to constrain cell proliferation (Weinberg 2014). TSGs are involved both in sporadic and hereditary tumors and some of the most well-known TSGs are *TP53*, *RB*, *NF1*, *NF2*, *APC*, and *VHL*. Through mutations, deletions and promoter hypermethylation TSGs are inactivated causing uncontrolled growth. DNA repair genes (e.g. *BRCA1*) are important for maintaining the integrity of the genome. Alterations in these genes cause genomic instability including chromosomal instability (CIN), microsatellite instability and increased mutation rates (Negrini, et al. 2010). Epigenetic alterations comprise DNA methylation, histone modifications (acetylation, methylation, phosphorylation) and microRNA (miRNA) expression. DNA methylation is important for normal development. Aberrant methylation in cancer cells include global hypomethylation and hypermethylation in CpG

islands of TSGs which give rise to genomic instability and cell proliferation respectively (Cheung, et al. 2009). miRNAs are small noncoding RNA molecules regulating mRNA translation. Aberrant expression of miRNAs occur in cancer cells and may result in suppression of TSGs alternatively overexpression of oncogenes. miRNA expression can be altered by several mechanisms including chromosomal aberrations, epigenetic alterations, mutations and polymorphisms and defects in the miRNA biogenesis (Iorio and Croce 2012). The consequence of genetic and epigenetic alterations is development of the cancer cell. The hallmarks of the cancer cell have been summarized by Hanahan and Weinberg and include: i) self-sufficiency in growth signals, ii) insensitivity to growth-inhibitory signals, iii) evasion of programmed cell death, iv) limitless replicative potential, v) sustained angiogenesis, vi) tissue invasion and metastasis, vii) deregulating cellular energetics and viii) avoiding immune destruction (Hanahan and Weinberg 2000, 2011). The molecular alterations leading to the development of SI-NETs have not been fully characterized and our knowledge of genetic and epigenetic alterations in SI-NETs is therefore limited. More detailed knowledge is needed to understand the mechanisms of the disease and to develop better prognostic and predictive biomarkers and therapy. However, some information is available and is summarized in the following sections.

Somatic copy number alterations (SCNA) in SI-NETs

Genetic alterations in malignant tumors often include loss or gain of DNA material. Such alterations are commonly known as SCNA and can be analyzed by a wide range of techniques. SCNAs in SI-NETs have been studied under the last decades using microsatellite markers, chromosome-based comparative genomic hybridization (CGH), array-based comparative genomic hybridization (aCGH) with BAC clones or oligonucleotides, single nucleotide polymorphism analysis (SNP) and next-generation sequencing (Banck, et al. 2013; Cunningham et al. 2011b; Francis, et al. 2013; Hashemi, et al. 2013; Kim do, et al. 2008; Kulke, et al. 2008a; Kytola, et al. 2001; Löllgen, et al. 2001; Stancu, et al. 2003; Terris, et al. 1998; Tonnies, et al. 2001; Wang, et al. 2005). The most common SCNA in SI-NETs is loss of whole chromosome 18 which occurs in >60% of tumors (Banck et al. 2013; Cunningham et al. 2011b; Francis et al. 2013; Kim do et al. 2008; Kulke et al. 2008a). Loss of chromosome 18 is identified in both primary tumors and metastases and is in some SI-NETs the only SCNA reported. In a few cases segmental deletions in the telomeric part of the 18q-arm have been identified allowing identification of candidate TSGs (Cunningham, et al. 2011a; Hashemi et al. 2013; Kulke et al. 2008a; Kytola et al. 2001; Löllgen et al. 2001). Kulke et al. identified one case with loss of a 1.39 Mb region at 18q21.1,

covering 8 genes. One of the genes, *SMAD2*, was sequenced but revealed no mutations (Kulke et al. 2008a). *SMAD4* located in the same region has also been sequenced without finding any mutations (Löllgen et al. 2001). In another study four segmental deletions on the q-arm of chromosome 18 was reported. Three minimal common regions were identified: 18q21.1-q21.31, 18q22.1-q22.2 and 18q22.3-q23. They performed mutation analysis of 18 genes from these regions including: *SMAD7*, *ACAA2*, *ST8SIA3*, *ONECUT2*, *FECH*, *NARS*, *ATP8B1*, *NEDD4L*, *ALPK2*, *CDH7*, *CDH19*, *DSEL*, *TXNDC10*, *CCDC102B*, *DOK6*, *CD226*, *SOCS6* and *CBLN2* but no mutations were found (Cunningham et al. 2011b). Other recurrent deletions in SI-NETs comprise deletions on 3p, 9p, 11q and 16q (Cunningham et al. 2011b; Hashemi et al. 2013; Kim do et al. 2008; Kulke et al. 2008a). Gain of whole chromosomes is frequently observed in SI-NETs especially gain of chromosome 4, 5, 7, 14 and 20 (Banck et al. 2013; Cunningham et al. 2011a; Kim do et al. 2008; Kulke et al. 2008a). Kulke et al. performed hierarchical clustering on recurrent alterations and found that tumors with gain on chromosome 4, 5, 7 and 14 clustered together, indicating that there may exist two different genetic pathways in SI-NETs (Kulke et al. 2008a). High level gains or homozygous losses are rarely seen in SI-NETs (Kulke et al. 2008a).

Massively parallel sequencing (MPS) of SI-NETs

MPS is a powerful tool to detect small insertions and deletions, amplifications and translocations in tumors. This technique has only recently been applied to SI-NETs. Banck et al. analyzed SCNA and somatic single nucleotide variants (SNVs) in 48 SI-NETs by using MPS. They detected a total of 1013 SCNA with an average of 21.3 SCNA/tumor and 0.1 SNV/10⁶ nucleotides showing that SI-NETs are genetically relatively stable tumors. 197 SNVs were identified in e.g. *FGFR2*, *MEN1*, *E2H2*, *VHL* and *SMAD1* but no recurrent SNVs were detected. Combining the identified SCNAs and SNVs recurrent well-known cancer related mechanisms were identified including TGFβ/Wnt signaling, chromatin remodeling, mitosis/spindle formation and PI3K-AKT-mTOR signaling. In 72% of the patients several candidate therapeutic targets were defined (Banck et al. 2013). Francis et al. performed MPS on 55 tumors from 50 patients with SI-NETs. They found 1230 genes with somatic mutations but the majority of mutations occurred only in one individual. *CDKN1B* was the only gene harboring recurrent frame shift mutations and hemizygous deletions which were identified in 7.8% and 14% SI-NETs respectively. Genomic rearrangements was studied in 24 SI-NETs and 7 rearrangements per tumor in median (range 0-45) were detected, but none of the rearrangements were recurrent (Francis et al. 2013).

Expression profiling of SI-NETs

Expression profiling has been successfully introduced in cancer biology to obtain a molecular characterization of tumor subgroups as well as to identify prognostic markers and therapeutic targets. Genome wide expression profiling of SI-NETs has been performed in several studies with different approaches (Carr, et al. 2012; Duerr, et al. 2008; Edfeldt, et al. 2011; Kidd, et al. 2006; Leja, et al. 2009). Kidd et al. profiled 8 SI-NETs (liver and lymph node metastases) and by comparison with normal small intestine mucosa they revealed 1709 differentially expressed genes and found *NAPILI*, *MAGE-D2* and *MTA1* to be overexpressed in SI-NETs. In further analyses they focused on identifying genes responsible for the transformation of a primary tumor to a primary tumor with metastatic properties. Protein levels of *MTA1* (metastasis associated 1) was found to be increased in primary tumors in patients that develop lymph node and/or liver metastases (Kidd et al. 2006). Duerr et al. found *ECM1*, *VMAT1*, *LGALS4* and *RET* to be highly up-regulated in 6 gastrointestinal NETs (3 primary tumors and 2 liver metastases from small intestine and 1 primary tumor from colon) compared to pancreatic NETs. Gene ontology analyses identified ‘transport’, ‘motor activity’ and ‘binding’ to be important molecular functions in SI-NETs (Duerr et al. 2008). Leja et al. profiled 3 primary tumors and 3 liver metastases from 6 patients with SI-NETs. Differential expression of 370 genes was found in SI-NETs compared to normal small intestine mucosa. Six novel biomarkers for diagnosis and/or therapy in SI-NETs were found including *GRIA2*, *SPOCK1*, *PNMA2*, *SERPINA10*, *GPR112* and *OR51E1*. *GPR112* and *OR51E1* encode for cell membrane receptors and may be targetable with antibody-therapy. Two genes, *CXCL14* and *NKX2-3*, were differentially expressed between primary tumors and metastases and were less expressed in metastases indicating involvement in tumor progression (Leja et al. 2009). Edfeldt et al. studied 18 primary tumors, 17 lymph node metastases and seven liver metastases from 19 patients with SI-NETs. They focused on genes related to tumor progression by comparing primary tumors and metastases. Differential expression of 7130 genes was identified between primary tumors and lymph node metastases. *ACTG2*, *REG3A* and *GREM2* were expressed at a higher level in primary tumors than lymph node metastases. Gene ontology analyses found functions related to “extracellular matrix” in highly expressed genes in the primary tumors and to “the organelle and cell cycle” in lymph nodes. Hierarchical clustering revealed three clusters of tumors: one cluster with only primary tumors (n=11), one with only lymph node metastases (n=7) and one cluster with tumors from all sites harboring the majority of tumors (n=26). Differentially expressed genes between tumors in the three cluster groups were *CDH6*, *RUNX1*, *TGFBR2*, *THPH1* and *TUSC2* (Edfeldt et al. 2011). Carr et al. used exon arrays to identify differential expressed genes in 11 SI-NETs (primary tumors) compared to normal small

intestine mucosa. They found 173 genes with more than a fivefold change in expression e.g. *TAC1*, *VMAT1*, *TPH1*, *CGHB*, *OR51E1* and *GPR112*. In further analyses they focused on genes associated with the G-protein coupled receptor (GPCR) pathway that could discriminate between SI-NETs and pancreas-NETs and found *OXTR* and *GPR113* to be up-regulated in SI-NETs but not in pancreas-NETs (Carr et al. 2012).

Epigenetic alterations in SI-NETs

Epigenetic alterations change the gene expression without altering the DNA sequence. DNA methylation, histone modifications and miRNA expression are examples of such alterations. Epigenetic alterations are common in cancer and have been studied in SI-NETs. Global hypomethylation of long interspersed nucleotide elements 1 (LINE1) has been reported in SI-NETs (Choi, et al. 2007; Fotouhi, et al. 2014; Stricker, et al. 2012). In one study hypomethylation was found in 82% of the tumors and low LINE1 methylation in the primary tumor was associated with lymph node metastases (Stricker et al. 2012). In another study low LINE1 methylation was correlated with loss of chromosome 18 (Fotouhi et al. 2014). Global profiling of DNA methylation has been performed in SI-NETs. Comparisons between primary tumors (n=10) and their corresponding lymph node metastases identified 2697 differentially methylated CpG promoter islands. Metastases had less methylated sites than the primary tumors. Highly methylated genes included *RUNX3*, *TP73*, *CHFR*, *MAPK4*, *RBI*, and *BTG4*. In the 18q21-qter region frequently affected by hemizygous loss, five methylated genes were identified, *ELAC1*, *MAPK4*, *MBD1*, *SETBP1* and *TCEB3C*, occurring in all tumors (Verdugo, et al. 2014). *TCEB3C*, which is the only imprinted gene on chromosome 18, was further investigated and suggested as a candidate TSG in SI-NETs (Edfeldt, et al. 2014). Promoter methylation in other genes have also been reported e.g. *CDH1*, *CTNNB1*, *CXCL14*, *LAMAI*, *NKX2-3*, *P16*, *RASSF1A* and *WIFI* (Chan, et al. 2003; Fotouhi et al. 2014; Liu, et al. 2005; Zhang, et al. 2006). Ruebel et al. studied miRNA expression in 8 primary tumors and corresponding metastases of SI-NETs. Ninety-five miRNAs were examined. Down-regulation of miRNA-133a, -145, -146, -222 and -10b and up-regulation of miRNA-183, -488, -19a and -19b were demonstrated in metastases compared to primary tumors (Ruebel, et al. 2010). Li et al. performed a genome-wide profiling of miRNAs in 15 SI-NETs (5 primary tumors, 5 lymph node metastases and 5 liver metastases). Five miRNAs were identified to be up-regulated during tumor progression, miRNA-96, 182, -183, -196a and -200a and four miRNAs were down-regulated: miRNA-31, 129-5p, -133a and 215 (Li, et al. 2013). Two miRNAs were overlapping in the two studies, down-regulation of

miRNA-133a and up-regulation of miRNA-183 in metastatic tumors compared to primary tumors.

OBJECTIVES OF THE THESIS

The general aim of this thesis was to characterize SI-NETs at the molecular level to identify candidate therapeutic targets and biomarkers for prognosis.

The specific aims were:

- to characterize the copy number profiles of SI-NETs in order to identify genetic changes important for tumor initiation, progression and survival.
- to define the gene expression profiles of SI-NETs in order to classify tumors based on expression patterns.
- to search gene expression profiles of SI-NETs for targetable genes.
- to analyze gene expression of SI-NETs with respect to genes associated with patient survival.

MATERIAL AND METHODS

Tumor material

Fresh frozen biopsies (Paper I, II, III)

Table 3. Fresh frozen biopsies from SI-NETs (unless indicated otherwise) used in paper I-III:

Method	Patients	Biopsies	Primary tumors	Liver metastases	Lymph node metastases
<i>Paper I</i>					
Expression microarray	5	5		5	
qPCR ^a	11	11	6	5	
qPCR ^b	32	32	12 SI-NETs, 10 GISTs and 10 colorectal cancers		
Western blot ^c	8	8	4	4	
<i>Paper II</i>					
SCNA analysis ^d	43	52	24	28	
<i>Paper III</i>					
Expression microarray ^e	33	33	10	21	2
Validation experiments ^f	4	4	2	1	1

Abbreviation: GIST, Gastrointestinal stromal tumor.

^aAnalysis of expression levels of *APP*, *APLP1* and *APLP2* during tumor progression. The five liver metastases are the same as for expression microarray analysis. Normal tissue from brain (n=1) and normal intestine mucosa (n=3) were used as controls.

^bAnalysis of expression levels of *APP*, *APLP1* and *APLP2* in different gastrointestinal tumor types.

^cNormal tissue from liver (n=1) and small intestine mucosa (n=1) were used as controls.

^dIn 9 cases both primary tumor and liver metastases were analyzed from the same patient.

^eIn paper III, the same cohort as in paper II. However, as a result of no tumor material left (n=2), <60% tumor purity (n=2), low RNA quality (n=2), weak labelling (n=1) and not fulfilling the microarray quality control (n=3) ten patients were excluded. Normal small intestine mucosa (n= 10) was used as reference.

^fTumor biopsies from an additional four patients were included in the validation experiments. Nineteen tumor biopsies from the extended cohort were analyzed by qPCR.

Formalin-fixed and paraffin-embedded (FFPE) tumor tissues (Papers I, II and III).

In paper I, tissue microarrays (TMAs) were constructed using core biopsies from 214 patients with neuroendocrine tumors and non-neuroendocrine tumors which are described in detail in paper I. Briefly, the neuroendocrine tumors included gastric-, small intestinal-, appendiceal-, rectal- and pancreatic-NETs and the non-neuroendocrine tumors: gastric-, colorectal- and pancreatic- adenocarcinomas, and gastrointestinal stromal tumors. In paper II, tumor tissues from 7 patients with SI-NETs were used to verify gain of chromosome 14 by FISH analysis. In paper III, tumor tissues from 19 patients with SI-NETs were used to verify protein expression (the same cohort as for qPCR validation in paper III) by immunohistochemistry (IHC).

Neuroendocrine cell lines (Papers I, III).

Two neuroendocrine cell lines, GOT1 and BON, were used for gene expression analysis (microarray and qPCR), western blot analysis and confocal laser scanning microscopy analysis (only GOT1) in paper I. GOT1 was also used in the *in vitro* experiments in paper III. The GOT1 cell line was established from a liver metastasis of a SI-NET and the BON cell line was derived from a metastasis of a malignant pancreatic-NET (Evers, et al. 1991; Kolby, et al. 2001).

Methods

Methods used in this thesis are well established and described in detail in their respective papers. Methods used include gene expression analyses, qPCR, western blot, immunohistochemistry, immunofluorescence and confocal microscopy, somatic copy number analyses, fluorescence in situ hybridization (FISH) analysis, cell culture and drug testing. A short summary is found in the following section.

Genome-wide expression analysis (Papers I, III)

Expression profiling was performed using microarrays in papers I and III. High quality RNA and high tumor cell purity are important factors in microarray analyses. In our studies RNA quality was verified by gel electrophoresis using the 2100 Bioanalyzer (Agilent). High tumor purity was controlled by taking a section from the biopsy used for RNA preparation. The sections were FFPE, stained with hematoxylin and eosin, and the level of tumor cell purity was examined microscopically. In paper I, expression profiling was performed on 27k whole genome microarrays (Swegene DNA Microarray Resource Center,

Lund University, Sweden). The universal human reference RNA (URR) was used as reference. In paper III, expression profiling was performed on 44k Whole Human Genome Microarrays (G4112F, Agilent Technologies). Normal small intestine mucosa was used as reference.

qPCR (Papers I, III)

In papers I and III, qPCR analysis was used to validate gene expression using pre-designed TaqMan® Gene Expression Assays (Life Technologies). In paper I, mRNA expression was reported relative to *GAPDH* and in paper III relative to *GAPDH* and *HPRT1*.

Western blot (Paper I)

Western blot was used to determine protein expression in paper I. Proteins were separated by electrophoresis using NuPAGE 10 % polyacrylamide gels and running buffer (Novex). Proteins were then transferred to a PVDF membrane using NOVEX blotting system. Membranes were incubated with primary antibodies overnight followed by alkaline phosphatase-conjugated secondary antibodies and CDP-star (Tropix) was used as substrate. Membranes were exposed to ECL film and developed

Immunohistochemistry (Papers I-III)

To explore the expression of proteins in tumor cells we performed immunohistochemistry and immunofluorescence analyses. These analyses reveal also the localization of the protein in the tumor cell *e.g.* cytoplasmic, nuclear or membrane localization.

Immunohistochemistry analyses on sections from FFPE tumor tissue were performed in papers I and III. Briefly, antigen retrieval with a Tris/EDTA buffer pH 9.0 was followed by blocking of endogenous peroxidase. Incubation with primary antibodies and thereafter with a dextran polymer coupled with multiple HRP (horseradish peroxidase) enzyme molecules and anti-mouse/anti-rabbit secondary antibodies (EnVision system, DAKO) were performed. Diaminobenzidine was used as a chromogen, giving a brown end product and nuclei were counterstained with hematoxylin (blue). In paper III, an additional signal amplification step (2-5 times) with EnVision FLEX+ (LINKER) was used. The staining procedure was manually performed in paper I and in paper III we used the Autostainer Link instrument.

Immunofluorescence and confocal laser microscopy (Paper I)

Immunofluorescence analyses on tumor cells were performed in paper I. Chamber slides with GOT1 cells were fixed with formalin and incubated with primary antibodies. Thereafter the cells were incubated with a mixture of secondary antibodies conjugated with fluorescent molecules (Alexa Fluor

594/488) and Hoechst 33258 (nuclear staining). A confocal laser scanning microscope was used to detect the fluorescence signal.

Genome-wide SCNA analysis (Papers II, III)

In papers II and III, somatic copy number alterations (SCNAs) were analyzed by using array-based comparative genomic hybridization (CGH). The oligonucleotide arrays contained 44 000 probes distributed over all chromosomes (Agilent). Array-based CGH can detect genomic imbalances including losses and gains but are not able to identify copy-neutral loss of heterozygosity or balanced rearrangements.

FISH analysis (Paper II)

Fluorescence *in situ* hybridization (FISH) analysis was performed on paraffin sections to detect copy numbers of chromosome 14 (Paper II). A telomere-specific probe for chromosome 14 (TelVysion) was used and the numbers of fluorescence signals were evaluated in at least 300 tumor cell nuclei.

Cell culture and drug testing (Paper III)

GOT1 cells were cultured as previously described (Kolby et al. 2001). GOT1 cells were treated with cabozantinib, MK-2206, P276-00, regorafenib, sorafenib, sunitinib, veliparib, vorinostat or alvespimycin for 4 days. The viability was estimated using AlamarBlue® (Molecular Probes).

Statistical analysis (Paper I-III)

In paper I, statistical analyses were performed in the Genespring software (Agilent Technologies) and GraphPad. In Genespring, fluorescence ratios from microarrays were normalized using the LOWESS algorithm. Differentially expressed genes related to the URR reference passed a Student's t-test (P -value <0.02) and Benjamini and Hochberg false discovery rate. In paper II, statistical analyses were performed in the CGH Analytics 3.4 software using the moving average and z-score algorithm. Survival analyses were performed using programs developed by the statistical consultant Anders Odén. In paper III, the microarrays were analyzed in the statistical language R 2.13.0 (www.r-project.org). Pathway analyses were performed using Ingenuity Pathway Analysis (IPA®, Qiagen), see section below.

Ingenuity pathway analysis (IPA) (Paper III)

We used the commercially available web-based program IPA® (Qiagen) to identify biological functions, canonical pathways, upstream-regulators and predicted drug targets. The program uses the Ingenuity® Knowledge Base where over 5 million relationships between molecules, diseases and biological functions are collected. The upstream regulator analysis identifies molecules

upstream of the genes in the dataset that potentially can explain the observed gene expression changes. Two statistical measures are calculated for each upstream-regulator: an overlapping P -value and an activation Z -score. The overlapping P -value measures overlap of observed and predicted regulated gene sets using Fisher's exact test ($P < 0.01$). Activation z -score identify the match of observed and predicted up/down regulation patterns and indicate if the predicted upstream regulator is activated (≥ 2) or inhibited (≤ -2) (Kramer, et al. 2014). Canonical pathway analysis identifies pathways that are significant to the input dataset. The significance of the association is measured in two ways: i) a ratio is calculated by dividing the number of genes in the input dataset that participate in a specific canonical pathway by the total number of genes included in the canonical pathway and ii) a P -value is calculated using Fisher's exact test (right-tailed) to measure the probability that the association between the genes in the input dataset and the canonical pathway is due to random chance. Fisher's exact test (right-tailed) is also used to calculate the P -value associated with a biological function.

Ethical approval (Papers I, II, III)

These studies were approved by the Regional Ethical Review Board in Gothenburg, Sweden.

RESULTS AND DISCUSSION

Amyloid precursor-like protein 1 (APLP1) is upregulated in neuroendocrine tumors of the gastrointestinal tract (Paper I)

We examined the global gene expression profile of five liver metastases from SI-NETs. High expression of neuroendocrine- and neural-related genes including secretagogin (*SCGN*), synaptotagmin XIII (*SYT13*), DOPA decarboxylase (*DDC*), chromogranin A (*CHGA*) and amyloid precursor-like protein 1 (*APLP1*) was found. The hierarchical clustering of the five liver metastases and two neuroendocrine cell lines, GOT1 and BON, revealed that GOT1 had a more similar expression profile to SI-NETs than BON, which is in line with the fact that GOT1 is derived from a SI-NET and BON from a pancreatic-NET. One of the highest expressed genes, *APLP1*, was further studied. *APLP1* belongs to the APP family which consists of three homologous proteins, the amyloid precursor protein (APP) and the APP-like protein 1 and 2 (*APLP1*, *APLP2*) (Paliga, et al. 1997; Sprecher, et al. 1993; Wasco, et al. 1992; Wasco, et al. 1993). APP and *APLP2* are ubiquitously expressed, while *APLP1* is expressed in neural tissue (Lorent, et al. 1995; Slunt, et al. 1994; Tanzi, et al. 1988). The APP family members are reported to be involved in several tumor types such as brain, colon, pancreatic, breast, thyroid, and prostate cancer (Baldus, et al. 2004; Hansel, et al. 2003; Ko, et al. 2004; Mauri, et al. 2005; Meng, et al. 2001; Nakagawa, et al. 1999; Quast, et al. 2003). Expression of *APLP1* in tumors is only reported for neuroblastoma cells (Beckman and Iverfeldt 1997; Wasco et al. 1992). To explore the expression of *APLP1* in a wide range of non-gastrointestinal normal and tumor tissues a meta-analysis was performed using 35 datasets from the ONCOMINE database. In 8/35 datasets we found differential expression of *APLP1*. Upregulation of *APLP1* was seen in 3 of those 8 datasets, all of which were lung-NETs. To examine protein expression and localization of the APP-family members in neuroendocrine and non-neuroendocrine tumors of the gastrointestinal tract we performed immunohistochemistry on our constructed tissue microarrays. Eighty-four percent of the neuroendocrine tumors showed positive staining for *APLP1* while 26% of the non-neuroendocrine tumors were positive. *APLP2* was frequently expressed (81-100%) in all types of tumors except for pancreas-NETs (24%) and APP was expressed in the majority of all tumors (64-100%). APP, *APLP1* and *APLP2* are type I transmembrane proteins with an extracellular domain (n-terminal), a transmembrane domain and an intracellular domain (c-terminal). The membrane proteins are proteolytically processed by α -, β - and γ -secretases. The most well-known cleavage product is

the amyloid β (A β) peptide, an essential part of amyloid plaques causing Alzheimer's disease (Jacobsen and Iverfeldt 2009). Cleavage with γ -secretase reveals c-terminal fragments of APP, APLP1 and APLP2: APP intracellular domain (AID/AICD) and APP-like intracellular domains 1 and 2 (ALID1, ALID2) respectively (De Strooper and Annaert 2000; Scheinfeld, et al. 2002). We found cytoplasmic localization of APP, APLP1 and APLP2 in the tumor cells when using antibodies directed to the c-terminal domain. Nuclear labelling was identified using the c-terminal antibody to APLP2 in pancreatic adenocarcinoma, gastric adenocarcinoma and gastric-NET. The APP members have also been reported to have a function in cell adhesion (Kaden, et al. 2009; Soba, et al. 2005). N-terminal directed antibodies showed membrane labelling in small intestine-NETs and appendix-NETs which suggests a role of the APP family members in cell adhesion in these tumors influencing migration and invasive growth. The cellular localization of APP, APLP1 and APLP2 in GOT1 cells were visualized by using c-terminal directed antibodies and confocal laser scanning microscopy. Above all a cytoplasmic localization was seen but also a nuclear related localization was determined. All three APP family proteins partly co-localized with synaptic-like vesicles, early endosomes and FE65. Co-localization with FE65 indicates a role in gene regulation. It is shown that FE65 forms a complex with AID and together with Tip60 regulate transcription (Baek, et al. 2002; Cao and Sudhof 2001, 2004). Co-localization of the APP family members with synaptic-like vesicles and early endosomes suggests that they may be transported to the cell membrane by vesicles. To examine the APP-family members' role in tumor progression we studied RNA and protein expression in primary tumors and liver metastases using qPCR and western blot. APLP1 was expressed at a higher level in liver metastases compared to primary tumors, indicating a role of APLP1 in tumor progression.

In summary, we found that the amyloid precursor family (APP, APLP1 and APLP2) is expressed in gastrointestinal tumors. APLP1 is highly expressed in neuroendocrine gastrointestinal tumors compared to non-neuroendocrine gastrointestinal cancers. In two subsequent studies APLP1 have been reported to be highly expressed in SI-NETs supporting our finding (Kidd, et al. 2014; Leja et al. 2009). The expression of APLP1 in SI-NETs was higher in metastases than in primary tumors suggesting a role in tumor progression.

High-resolution genomic profiling reveals gain of chromosome 14 as a predictor of poor outcome in SI-NETs (Paper II)

The purpose of this study was to analyze a large clinically well characterized cohort of patients by high-resolution array-based CGH to identify copy-number changes important for tumor initiation, progression and survival. In this study we analyzed biopsies from 43 patients with SI-NETs. A total of 370 SCNAs were identified in the 24 primary tumors and 28 liver metastases (n=52). The average number of SCNA per tumor was 7.1 (range 1-22), indicating that SI-NETs are genomically relatively stable. The average number of SCNAs was twice as high in liver metastases compared to primary tumors. Losses were more frequent than gains, with a ratio of 1.4. Forty-one percent of all SCNAs were losses or gains of whole chromosomes. High-level amplifications were not observed and only in one case a homozygous loss was detected. Recurrent SCNAs, defined as an alteration occurring in >3 patients, represented 59% of all SCNAs. The most frequent alteration was loss of a whole chromosome 18 occurring in 74% of all patients. This is in line with previous and subsequent studies showing loss of chromosome 18 in 61-100% (Cunningham et al. 2011b; Francis et al. 2013; Hashemi et al. 2013; Kim do et al. 2008; Kulke et al. 2008a). The frequency of loss of chromosome 18 was similar in primary tumors (79%) and liver metastases (61%). In six cases loss of chromosome 18 was the only SCNA found, indicating that loss of chromosome 18 is an early event in tumor formation. Only one tumor in our cohort displayed a partial loss of 18q22.2-qter. The second most common loss was detected on chromosome 11q. Three minimal common regions spanning: 11q22.1-q22.2, 11q22.3-q23.1 and 11q23.3, were observed in 21% of all patients. Four candidate TSGs were found in these regions including *RFX*, *BTG4*, *PPP2R1B* and *TAGLN*. We also found two recurrently deleted minimal common regions on chromosome 16, 16q12.2-q22.1 and 16q23.2-qter, in 16% of the patients. These regions harbored several candidate TSGs including *CDH 1, 3, 5, 8, 11, 13, 15, TRADD, E2F4, CTCF, OSGIN1, WFDC1, IRF8, FBXO31, BANP, CBFA2T3, ZNF276, FANCA* and *GAS8*. In 14% of the patients loss of 3p13 was detected and in this region *RYBP* was identified as a candidate TSG. Recurrent segmental gains were not observed, however we found recurrent gains of whole chromosomes including gain of chromosome 20 (33%), 4 (30%), 5 (28%), 14 (23%), 7 (11%) and 10 (9%). Gain of whole chromosomes was found in a higher frequency in liver metastases than in primary tumors. Survival analysis was performed with respect to all recurrent SCNAs. Significant correlation was found in a multivariate analysis between three variables including i) loss of 3p13, ii) gain of chromosome 14 and iii) carcinoid heart disease, and overall survival. Patients with 3p13 had a significantly reduced risk of death (hazard ratio (HR) of 0.14) while patients with

gain of chromosome 14 and carcinoid heart disease had a significantly increased risk of death (HR of 8.39 and HR of 2.89 respectively). We identified two patients with segmental gains on chromosome 14, 14q11.2-q13.1, 14q23.3-q24.1 and 14q24.3-qter. The regions were not overlapping and due to the large regions involved we were not able to identify specific genes that could contribute to the poor survival. However, Kulke et al. found gain of a minimal common region in 7/18 patients on 14q11.2, a region harboring the gene, defender against cell death 1 (*DADI*) (Kulke et al. 2008a). Amplifications in the same region was also detected in another study (Cunningham et al. 2011a). Gain of chromosome 14 was found in 23% of the patients and gain of whole chromosomes 4, 5, 7, 10 and 20 was frequently seen in these patients. In our study the majority of patients with gain of chromosome 14 had an intact chromosome 18, but numerical alterations on chromosome 14 and chromosome 18 were not mutually exclusive. We therefore suggested that tumors with gain of chromosome 14 follow a different genetic pathway than the majority of tumors. However, in subsequent studies the occurrence of both loss of chromosome 18 and gain of chromosome 14 have been reported in substantial proportions (Cunningham et al. 2011b; Hashemi et al. 2013). In a contemporary study, Kulke et al. performed hierarchical clustering on recurrent alterations and found that tumors with gain on chromosome 4, 5, 7 and 14 clustered together. They defined two separate groups of tumors, one group harboring ≥ 2 of these clustered gains and one group without. There is a close similarity between the two cytogenetically defined groups in Kulke's and our study. We both found a subgroup characterized by a high frequency of gain of whole chromosomes. A subsequent study have also demonstrated two separate groups of tumors, one of them characterized by gain on chromosomes 4, 5, 14 and 20. In that study gain of 20pter-p11.21 was associated with shorter survival (Hashemi et al. 2013).

We conclude that loss of chromosome 18 is an early event in SI-NET tumorigenesis. We were not able find a minimal common region of deletion on chromosome 18 that could potentially harbor genes important for tumor formation. However, in recent studies massive parallel sequencing have not revealed any recurrent mutations on chromosome 18 (Banck et al. 2013; Francis et al. 2013). In our study we found a significant increased risk of death in patients with gain of chromosome 14. However, two subsequent studies have not been able to find correlation between gain of chromosome 14 and survival in their cohorts (Cunningham et al. 2011b; Hashemi et al. 2013).

Expression profiling of small intestine neuroendocrine tumors identifies subclasses with clinical relevance, therapeutic targets and biomarkers for prognosis (Paper III)

In this study we examined the global gene expression profiles of SI-NETs from 33 patients. We found 10,522 differentially expressed genes when comparing tumors to normal small intestine mucosa. Unsupervised hierarchical clustering revealed three clusters of tumors (Figure 2).

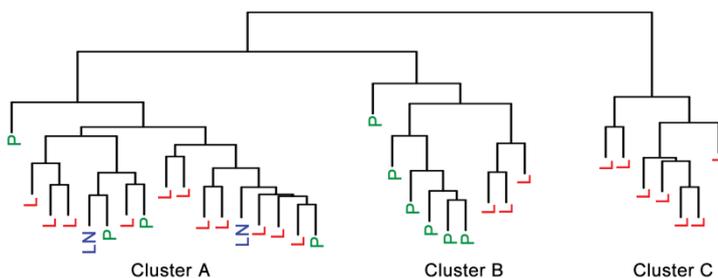


Figure 2. Clustering analysis based on genome-wide expression profiling.

Highest expression of marker genes, *CHGA*, *CHGB*, *SYP*, *VMAT1*, *TPHI*, *SSTR2* and *FEV*, were demonstrated in cluster A compared to cluster B and C. Age, gender and tumor stage were similar in the three clusters. We found a tendency to higher tumor grade and higher frequency of gain of whole chromosomes in cluster B and C. Survival analysis revealed significant shorter survival in patients in cluster B compared to patients in cluster A. To further characterize the three clusters we compared their gene expression to normal small intestine mucosa. Analysis of the most upregulated genes in each cluster revealed a high proportion of genes associated with neurosecretory function and formation of cellular protrusions to be highly expressed in clusters A and B respectively. In cluster C, a high proportion of genes were related to angiogenesis and cytoskeletal organization. When comparing cluster B to cluster A, *TGFB1* was predicted to be the most activated upstream regulator in cluster B. *TGFB* signaling has been shown to play an important role in epithelial to mesenchymal transition during invasive growth of tumor cells (Fuxe and Karlsson 2012; Heldin, et al. 2012). *NURP1*, a transcription factor promoting cell survival by regulating stress response and resistance to chemotherapy, was an activated predicted upstream regulator when comparing cluster C to cluster A. Taken together, based on the three cluster's expression profiles, we propose tentative descriptors for the identified clusters: "neurosecretory" (cluster A),

“invasive” (cluster B) and “angiogenic” (cluster C). The knowledge base in IPA was used to identify candidate therapeutic targets in SI-NETs. Several drugable genes were found to be highly expressed including receptor tyrosine kinases (RET, FGFR1/3, PDGFRB, FLT1, VEGFB), nuclear kinases (CDK4/9), transcriptional regulators (HDAC5), molecular chaperones (HSP90AA1, HSP90AB1), G-protein coupled receptors (SSTR2), signal transduction molecules (AKT3) and enzymes involved in DNA repair mechanisms (PARP1). SSTR2 was shown to be a therapeutic target in all subgroups. Highest expression of SSTR2 was found in the “neurosecretory” group, suggesting better response to PRRT for these tumors. We also identified anticancer drugs to the candidate targets currently in clinical trials. A selection of anticancer drugs was tested on the SI-NET cell line GOT1. Multi-tyrosine kinase inhibitors which inhibits RET, FGFR1, PDGFRB and FLT1, and inhibitors of AKT, HDAC, HSP90 and CDK4/9, effectively inhibited growth of GOT1 cells. PARP1 inhibition had on the other hand no effect on tumor growth. We also compared the gene expression of primary tumors and metastases. Upstream-regulator analysis using IPA predicted highest activation of TGFB1 in primary tumors and INHA in metastases. In paper II we found that patients with gain of chromosome 14 in our cohort have a poorer survival. In order to characterize this group of tumors we compared the expression profiles of tumors with gain of chromosome 14 to tumors without gain of chromosome 14. We found a total of 181 differentially regulated genes and among the top 10 up- and down-regulated genes we found genes related to cell cycle progression, mitotic spindle formation, apoptosis, DNA damage, cell mobility, and neuroendocrine function. Pathway analysis identified alterations in several canonical pathways related to cell cycle regulation. FOXM1 was the most significant upstream regulator activated in tumors with gain of chromosome 14. FOXM1 is a transcription factor that regulates cell cycle progression and DNA damage response. In order to find genes related to patient survival we applied a Cox proportional hazards model to individual gene expression values from all SI-NETs. We identified 168 genes associated with patient survival including genes associated with cell cycle progression, apoptosis, DNA damage response, invasive growth, immune system, neural differentiation and biogenesis of ribosomes. We further investigated if some of these genes could account for the poorer survival in patients with gain of chromosome 14 and patients in the “invasive” cluster (B). We found that half of the top survival related genes also were differentially expressed in tumors from cluster B and tumors with gain of chromosome 14. Thirteen genes related to survival were represented in both groups of tumors and included genes associated with cell cycle progression and apoptosis, suggesting alterations in these genes as the cause of poor survival for these patients.

In summary, we identified three distinct groups of SI-NETs based on their genome-wide expression profiles. One of the subgroups, the “invasive” group, showed significant shorter survival. This classification of SI-NETs provided prognostic information in addition to that obtained by histopathological grading and staging of tumors. Several genes related to patient survival were involved in cell cycle progression, apoptosis and DNA damage response. Cell cycle related genes were also differentially expressed in tumors with gain of chromosome 14. FOXM1, a regulator of cell cycle progression, was identified as an upstream regulator in these tumors by pathway analysis. Analysis of upregulated genes in SI-NETs identified a number of promising therapeutic targets including receptor tyrosine kinases, HDAC and HSP90. *In vitro* experiments on GOT1 cells demonstrated inhibition of tumor growth by multi-tyrosine kinase inhibitors as well as by inhibitors of HDAC and HSP90, suggesting that these targeted therapies should be further evaluated in SI-NETs.

CONCLUSION

- The amyloid precursor family (APP, APLP1 and APLP2) is expressed in gastrointestinal tumors. APLP1 is highly expressed in neuroendocrine gastrointestinal tumors compared to non-neuroendocrine cancers. The expression of APLP1 in SI-NETs was higher in metastases than in primary tumors suggesting a role in tumor progression.
- Loss of chromosome 18 is the most common SCNA in SI-NETs. Loss of chromosome 18 may be an early event in SI-NET formation. Two different groups of SI-NETS can be identified based on SCNAs. Copy number alterations (gain of chromosome 14) may influence patient survival.
- Genome-wide expression profiling of SI-NETs identifies three distinct groups of tumors. Subgroups of tumors identified by expression profiling carries prognostic significance. Survival related genes in SI-NETs frequently relate to cell cycle progression, apoptosis and DNA damage response. The poor prognosis in patients with tumors carrying gain of chromosome 14 may be due to alterations in cell cycle progression and apoptosis. Receptor tyrosine kinases, HDAC and HSP90 are promising therapeutic targets in SI-NETs.

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