Department of Pathology
University of Helsinki
Helsinki, Finland

PROGNOSTIC MARKERS IN RECTAL NEUROENDOCRINE TUMORS

Juha Jernman

ACADEMIC DISSERTATION

To be presented, with the permission of the Medicine Faculty of the University of Helsinki, for public examination at small auditorium, Haartman Institute, on 6 November 2015, at 12 noon.

Helsinki 2015

Supervised by
Docent Johanna Arola, M.D., Ph.D.
Department of Pathology, University of Helsinki,
HUSLAB and Haartman institute, Helsinki, Finland

Professor Caj Haglund, M.D., Ph.D.
Department of surgery, University of Helsinki and
Helsinki University Central Hospital, Helsinki, Finland

Reviewed by
Professor Tuomo Karttunen
Department of Pathology
University of Oulu, Oulu, Finland

Docent Johanna Laukkarinen
Department of Surgery
University of Tampere, Tampere, Finland

Official opponent
Docent Pasi Salmela,
Department of Internal Medicine
University of Oulu, Oulu, Finland

ISBN 978-951-51-1629-1 (Paperback)
ISBN 978-951-51-1630-7 (PDF)

Unigrafia
Helsinki 2015

ABSTRACT

Neuroendocrine tumors of the rectum were regarded as benign, when Oberndorfer originally described the entity in 1907. Later, he acknowledged that some neuroendocrine tumors (or carcinoids, the term at that time) behave in a more aggressive manner, and a few of them even had the potential to metastasize with poor outcome. In the novel World Health Organization (WHO) classification launched in 2010, all neuroendocrine tumors of the gastrointestinal (GI) tract are malignant. In this classification, tumors of every part of the GI tract are graded uniformly according to proliferation index and mitotic frequency, whereas the TNM-classification (tumor, node, metastasis) is specific for each site. Around 10% of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) occur in the rectum. The prognostic accuracy of the WHO 2010 classification has been sufficiently validated in the stomach and pancreas, but in the rest of the GI tract, including the rectum, its prognostic value is inadequately confirmed. What would be useful, if possible, would be to reliably stratify rectal NETs into categories based on their metastatic potential.

The tumor series comprised 73 rectal NETs, with the main objective being to study the prognostic value of the WHO 2010 classification in rectal NETs: additionally, as the WHO classification has been used for a rather short time, tumor markers were tested to find a good, reliable prognostic tool.

The WHO 2010 had excellent prognostic significance; none of the G1-NETs (grade 1) metastasized, whereas G2-NETs were often disseminated, some of them at initial presentation. Metastatic NETs have a poor prognosis. Cell-cycle antigen cyclin A also correlated with prognosis, and G2-NETs with high cyclin A expression were all metastatic. Transcription factor prospero homeobox 1 (PROX1) was immunohistochemically positive in a significant proportion of rectal NETs, and showed a correlation with metastatic potential and survival. It was also possible to conclude that the novel stem cell-associated factor HES77 (human embryonic stem cell factor 77) correlated well with rectal NETs metastatic potential and prognosis.

These results support the validity of the WHO 2010 classification in rectal NETs. In view of this study, for patients with a rectal G1-NET, one follow-up endoscopy to exclude local recurrence might suffice. Intensive follow-up does not seem indicated, as metastatic potential is very low. As to G2-NETs, a thorough work-up is recommended, since most of these tumors disseminate eventually, some after several years, and a standard 5-year follow-up may not suffice. In selected cases, adjuvant therapy even in the absence of metastatic lesions might be beneficial, although this was not the target of the study. PROX1-positivity suggests that colorectal

adenocarcinoma and rectal NET may, to some extent, share the same pathway in oncogenesis; this could lead to future therapeutic applications. The Ki-67 plays an established role as a prognostic marker in epithelial, hematolymphoid, and mesenchymal neoplasms, but its accuracy ought to be assessed separately in each tumor subtype. Furthermore, a selection of cell-cycle antigens may have enhanced prognostic value: the conclusion of this study was that cyclin A in combination with Ki-67 can recognize tumors with the highest propensity to metastasize.

ACKNOWLEDGEMENTS

The “father” of this project is Professor Matti Välimäki, whose idea it was to study rectal NETs, and the correlation of the WHO classification with their prognosis. He provided constructive comments on the manuscripts.

Professors Veli-Pekka Lehto and Tom Böhling deserve gratitude for providing excellent facilities for this project.

My supervisors, Docent Johanna Arola and Professor Caj Haglund deserve my most profound gratitude. Johanna is a very skillful pathologist and a leading colleague in her fields: pathology of the liver and endocrine organs. She knew when to appraise and encourage, and when to give one a small push. During difficult episodes, she managed to transmit to me her positive mental attitude. Caj is a renowned scientist whose enthusiasm was contagious: is was a pleasure to conduct this project under his supervision. His experience in revising manuscripts and in choosing the most suitable publications was of great value.

Professor Tuomo Karttunen and Docent Johanna Laukkarinen deserve thanks for revising this thesis. They conducted their work rapidly and expertly, and the thesis improved because of their comments.

Docent Jaana Hagström I thank for her assistance in analyzing the immunhistochemical stainings, and for her positive and supportive attitude, as well. It was very much needed during the less productive periods of this project. Johanna Louhimo, PhD, and Docent Hannu Haapasalo I sincerely acknowledge for their valuable assistance with the statistics. Professor Risto Sankila and Maarit Leinonen, PhD, from the Cancer Registry I warmly acknowledge for providing important information on the incidence of NETs in Finland. Docent Hanna Mäenpää delivered clinical data on NET – patients, and I thank her for that. The help of my Swedish collaborators Christian Fermér and Olle Nilsson is appreciated. Professor Kari Alitalo and Pauliina Kallio are warmly thanked for their contributions. James Thompson and Carol Norris are acknowledged for their expert and rapid English revision of the individual publications. Carol deserves special thanks for editing this thesis.

Päivi Peltokangas, Eija Heiliö and Tuire Koski are the laboratory technicians who performed the necessary immunohistochemical stainings, and their contribution is appreciated. I also want to express my thanks to Elina Aspiala who was of great help in many practical issues. She also gave me important mental guidance and support throughout this project. Päivi

Mulari-Matikainen aided me in arrangements concerning the actual defense, and I thank her wholeheartedly for that.

I thank all my colleagues, former and present, in Helsinki and Tampere.

My friends and my former fellow students in the medical school gave me important support during the thesis project.

My parents and sisters deserve thanks for the encouragement they gave me thoughout all these years.

Finally, I owe my deepest gratitude to those who make life worth living: my wife and children.

The study was financially supported by The Sigrid Jusélius Foundation,

The Finnish Cancer Foundation, Novartis Finland Oy, Helsinki University Hospital Research Funds, and Fimlab Laboratories.

Helsinki, October 2015

Juha Jernman

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Jernman, J., Välimaki, M.J., Louhimo, J., Haglund, C. & Arola, J. 2012, "The novel WHO 2010 classification for gastrointestinal neuroendocrine tumors correlates well with the metastatic potential of rectal neuroendocrine tumors," Neuroendocrinology. 2012;95(4):317-24.

II Jernman, J., Välimaki, M.J., Hagström, J., Louhimo, J.,Haapasalo, H., Arola, J. & Haglund, C. 2014, "Cyclin A predicts metastatic potential of rectal neuroendocrine tumors," Hum Pathol. 2014 Aug;45(8):1605-9.

III Jernman, J.,Kallio, P., Hagström, J., Välimäki, M.J., Haapasalo, H., Alitalo, K., Arola, J., Haglund, C. 2015 “PROX1 is involved in development of rectal neuroendocrine tumors, NETs,” Virchov’s Archiv 2015 Sep;467(3):279-84.

IV Jernman, J., Hagström, J., Mäenpää, H., Välimäki, M.J., Haapasalo, H., Nilsson, O., Fermér, C., Haglund, C., Arola, J. 2015 “Expression of stem cell associated marker HES77 in rectal neuroendocrine tumors,” Anticancer Res. 2015 Jul;35(7):3767-72.

ABBREVIATIONS

APUD	amine precursor uptake and decarboxylation
ATRX	alpha thalassemia/mental retardation syndrome X-linked protein
ABCG4	ATP-binding cassette sub-family G member 4
Bcl-2	B-cell lymphoma 2 protein
CD	cluster of differentiation
CDKN2A	cluster of differentiation 44 protein
CDX2	caudal type homeobox 2
CgA	chromogranin A
CIMP	CpG island methylator phenotype
COX2	cyclooxynase 2
CT	computed tomography
DAXX	death-domain associated protein
DES	diffuse endocrine system
DNA	deoxyribonucleic acid
DNMT	de novo methyltransferase
EC cell	enterochromaffin cell
ENETS	European Neuroendocrine Tumor Society
Ga	gallium
GEP-NET	gastroenteropancreatic neuroendocrine tumor
GI	gastrointestinal
GLI	glucagon-like immunoreactants
GLP-1	glucagon-like peptide-1
HE	hematoxylin-eosin
HES77	human embryonic stem cell factor 77
hESC	human embryonic stem cell
hMLH1	human mutL homolog 1
HPF	high-power field
HUCH	Helsinki University Central Hospital
IBD	inflammatory bowel disease
IMP3	insulin-like growth factor II mRNA-binding protein
Ki-67	Kiel-67
KLF4	Krüppel-like factor
Kras	Kirsten rat sarcoma viral oncogene
LDCV	large dense core vesicles
LKB1	liver kinase B1
MANEC	mixed adenoneuroendocrine carcinoma
Math1	mouse atonal homolog 1
MEN1	multiple endocrine neoplasia 1

MIB-1	molecular immunology Borstel 1
MRI	magnetic resonance imaging
NET	neuroendocrine tumor
NEC	neuroendocrine carcinoma
NF1	neurofibromatosis-type 1
Ngn3	neurogenin 3
NOTANOC	1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid -Nal3-octreotide
NSE	neuron-specific enolase
Oct4	octamer-binding transcription factor 4
OTP	orthopedia homeobox
PET	positron emission tomography
PGP 9.5	protein gene product 9.5
pNET	pancreatic neuroendocrine tumor
PROX1	prospero homeobox 1
PRRT	peptide receptor-targeted radiotherapy
SLMV	synaptic-like microvesicles
PYY	pancreatic polypeptide-like peptide
p21	protein 21
p53	protein 53
RASSF1	Ras association domain-containing protein 1
Rb	retinoblastoma protein
RFA	radiofrequency ablation
RUNX1T1	runt-related transcription factor 1
R0	radical resection
R1	resection margins microscopically positive
SALL4	spalt-like transcription factor 4
SEER	surveillance, epidemiology, and end results; a program of the National Cancer Institute, USA
Sox2	sex-determining region Y -box 2
SIRT	selective internal radiation therapy
SRI	somatostatin receptor imaging
SRS	somatostatin receptor scintigraphy
TAE	transcatheter arterial embolization
TACE	transcatheter arterial chemoembolization
TCF	T-cell factor
TIMP3	tissue inhibitor of metallopeptidase 3
TMN	tumor, node, metastasis classification
TTF-1	thyroid transcription factor
US	ultrasound
VIP	vasoactive intestinal peptide
WHO	World Health Organization
5-HT	5-hydroxytryptamine

INTRODUCTION

The study of neuroendocrine tumors (NETs) has been challenging due to their rarity: it is time-consuming to collect materials with sufficient number of cases in order to validate new classifications and diagnostic markers. Moreover, very long follow-up periods are necessary, since these tumors are known to metastasize even very late; this makes the task even harder. After the introduction of the "carcinoid" entity by Oberndorfer in his famous 1907 article, these tumors were considered altogether benign, or at least with much better prognosis than for conventional adenocarcinomas. Data accumulated, however, suggesting that some of these benign-appearing tumors had the ability to metastasize. For a long time, it remained unclear which tumors had this potential.

The nomenclature has undergone significant changes: the term "carcinoid" is no longer recommended due to its benign connotation. In certain countries, the term is still widely used in clinical practice, but in Europe, the recommended term "neuroendocrine tumor" has been extensively adopted by clinicians and pathologists. The old term frequently appears in research articles from both the old and new continents and Asia, however.

The present World health Organization (WHO) classification for neuroendocrine tumors of the gastrointestinal (GI) tract was launched in 2010 (Bosman et al. 2010). It most certainly will be replaced in the future, like all tumor classifications eventually, when enough information allows the field to discard the previous classification. In the WHO 2010 classification, all GI – NETs are considered malignant.

As said, neuroendocrine tumors are rare; in Finland, around 200 new gastroenteropancreatic (GEP) NETs are diagnosed annually, and of these, 20 (10%) occur in the rectum (unpublished data). Understandably, it has been difficult to gain vast experience in diagnostics or treatment of these patients in Finland because of its very small number of cases. In this study what became evident was that treatment and especially follow-up of these patients had been conducted in very many different ways, because national (and international), cohesive guidelines were lacking. The situation improved markedly after introduction of European and Nordic guidelines for their treatment and diagnosis (Caplin et al. 2012a, Janson et al. 2014).

In the novel WHO 2010 classification, the primary site of the tumor does not affect its grading, whereas there do exist specific TMN classifications (tumor, nodes, metastasis) for each anatomic region of the GI tract. When the classification was launched, evidence was sufficient to support the accuracy of the grading system mainly in NETs of the stomach and pancreas. In other parts of the GI tract, data were very limited, with additional reports needed to validate this classification's prognostic value

(Bosman et al. 2010). In this work, the intention was discovery of how well the WHO 2010 classification correlated with survival.

REVIEW OF THE LITERATURE

NEUROENDOCRINE SYSTEM OF THE GI TRACT

HISTORICAL ASPECTS

Neuroendocrine cells were discovered in 1897 by Kulchitsky. These cells were found to interact with chromium salts and thus carried the name enterochromaffin cell or Kulchitsky cell. In the first half of the twentieth century, the endocrine nature of these cells was revealed (Rindi et al. 2004). The endocrine capacity of the bowel was reported by Bayliss and Starling in 1902. In 1917, Myerson discovered, in the nerve cells, intracytoplasmic argyrophilic granules staining positive with a silver stain. This was most likely the first report of neurosecretory granules, although the exact nature of the granules was elucidated later. In neuroendocrine cells, Masson-Fontana stain could demonstrate the positive argentaffin reaction, meaning that neuroendocrine cells are capable of taking up silver and reducing it to a visible metallic state. For this purpose, Masson-Fontana staining has largely been replaced by immunohistochemistry, but it is still in use in clinical pathology to demonstrate the presence of melanin pigment in tissues. The Grimelius stain was another silver stain for identifying argyrophilic neuroendocrine cells. The exact chemical reaction on which this staining is based remains unclear (Grimelius 1968).

FUNCTIONING OF THE NEUROENDOCRINE SYSTEM AND CELL TYPES

The epithelium of the rectum and colon contains four main cell types: enterocytes, Paneth cells, goblet cells, and neuroendocrine cells. It was first thought that neuroendocrine cells were derived from the neural crest, but eventually they proved to be of endodermal origin (Pictet et al. 1976). Neuroendocrine cells occur in almost every organ of the human body, but particularly in the GI tract, lung, and skin. In the GI tract they occur as scattered single cells, with the exception of the pancreas, where neuroendocrine cells form islets, as first described by Langerhans in 1869. In the small intestine, colon and rectum, neuroendocrine cells reside in the crypts of Lieberkühn, 5 to 10 neuroendocrine cells per crypt. The term “neuroendocrine” refers to their having features of both neural and endocrine cells (Wiedenmann et al. 1998). In the intestine and stomach these

cells are also called enteroendocrine cells. Neuroendocrine cells can produce, store, and, upon stimulation, release hormones. Two kinds of secretory vesicles exist: large dense-core vesicles (LDCV) and synaptic-like microvesicles (SLMV) (Wiedenmann et al. 1998). Synaptophysin and chromogranins are integral constituents of those vesicles.

Defined by their hormonal products, 15 types of neuroendocrine cells are identifiable in the GI tract and pancreas (Rindi & Kloppel 2004); their distribution and frequency is site-specific: the rectum and colon harbor mainly serotonin-producing EC-cells and L-cells that produce glucagon-like immunoreactants (GLI) and pancreatic polypeptide-like peptide (PYY). Somatostatin-producing D-cells are very few in number in the colon and rectum (Solcia, Capella & Fiocca 1998). Other parts of the GI tract have a completely different distribution of neuroendocrine cells. The number of different cell types might be one explanation why NETs of the GI tract are a rather heterogeneous group, since a specific subtype of neuroendocrine cell is thought to give rise to a specific type of tumor (Kloppel 2011).

Neuroendocrine cells form the diffuse endocrine system (DES) that plays an important role in regulating GI tract function by secreting hormones into the bloodstream, and also by exerting local control on gut motility, and controlling secretion and proliferation of mucosal cells (Solcia, Capella & Fiocca 1998). The first to propose the concept of a diffuse endocrine gland was Masson in 1928, and the idea was further developed by Feyrther in publications in 1938 and 1956.

DIFFERENTIATION OF NEUROENDOCRINE CELLS

Pluripotent stem cells are the precursor for enterocyte stem cells and secretory stem cells, from which Paneth cells, goblet cells, and neuroendocrine cells are derived (Yang et al. 2001, Jenny et al. 2002). This is controlled by transcription factors such as caudal type homeobox 2 (CDX2), mouse atonal homolog 1 (Math1), neurogenin 3 (Ngn3), NeuroD and prospero homeobox 1 (PROX1) (Silberg et al. 2000, Yang et al. 2001, Beck 2002, Petrova et al. 2008). As a result of Notch signaling, in normal epithelium, two neuroendocrine cells are never adjacent (Apelqvist et al. 1999, Jensen et al. 2000). The neuroendocrine system is capable of responding to different stimuli and of adapting its function accordingly (Karam & Leblond 1995). For example, chronic inflammation, as in chronic inflammatory bowel diseases, causes an increase in number of neuroendocrine cells (Miller & Sumner 1982, Gledhill, Enticott & Howe 1986, Bishop et al. 1987). This is achieved, probably not by proliferation of terminally differentiated neuroendocrine cells, but by entry of stem cells into the differentiation trail (Barrett et al. 1995).

NEUROENDOCRINE TUMORS

HISTORY OF NETS

The German pathologist Siegfried Oberndorfer (1876-1944) was the first to describe carcinoid tumors in 1907. He had encountered several cases in which this tumor had some features in common with adenocarcinoma, but found also differences in the growth pattern and, importantly, in its clinical behavior and prognosis, since none of the patients had any symptoms. Oberndorfer inferred that he had discovered a new tumor subtype, and also gave this tumor entity its name, which, although a bit obsolete today, still sometimes appears. Oberndorfer concluded that these new tumors, although resembling carcinomas, showed major differences. Hence the name in German: "karzinoide," carcinoma-like. In his famous article he stated that these tumors are small, often multiple, do not infiltrate into surrounding tissues, have no metastatic potential, and are slow-growing and harmless (Oberndorfer 1907). He later admitted, when cases with metastases came to his knowledge that "malignant carcinoids" exist (Oberndorfer 1929).

In 1929 Masson published his observations on appendiceal carcinoids; the series consisted of 50 tumors. He described very precise morphological details of tumor cells and discovered cytoplasmic vacuoles and granules absent from conventional adenocarcinomas. He also detected similarities between carcinoid tumor cells and cells of the adrenal cortex. In silver stains, Kulchitsky cells, and carcinoid tumor cells had common features: he concluded that both were of endocrine nature, and that Kulchitsky cells or enterochromaffin cells constitute a diffuse endocrine gland (Masson 1928). These observations have withstood the test of time and are still valid. Moreover, he postulated that intraneural argentaffin cells are the cells of origin in carcinoids. In the earliest articles on carcinoids, these novel tumors were thought to have a benign clinical course, but over time, metastatic cases with poor outcome were reported, raising doubts as to whether this was an entirely benign entity, after all.

The earliest reports of NETs, or carcinoids as they were called at that time, concerned mainly lesions of the small bowel and appendix, and NETs were treated more or less as a homogeneous group; not much emphasis was placed on tumor localization. Later, the primary site of the tumor began to attract interest, and articles appeared taking this aspect into consideration.

The first report on rectal carcinoid was by Saltykow in 1912. Stout reported a series of six rectal carcinoids in 1942. None of them showed metastases. He observed morphological differences and weaker positivity in silver staining compared to that of carcinoids at other sites, concluding that in these rectal carcinoids, the cell of origin was a pre-enterochrome cell of the rectal mucosa, where argentaffin (secretory) granules had not yet been formed (Stout 1942). Due to abnormal silver-staining properties of some rectal carcinoids, the term "atypical carcinoid" was introduced by Morson in

1958. At that time, this term did not refer to the potentially malignant nature of a tumor. This term was re-adopted later with the connotation of potentially malignant tumor behavior. In 1948, Pearson reported three rectal carcinoids, two with metastases (Pearson & Fitzgerald 1948). Prior to this, 29 rectal carcinoids were reported in the literature, but only 2 of these were associated with distant metastases. Thus, up to that time, 32 rectal carcinoids had received mention in the literature, of these, 4 with distant metastases. It became evident that not all rectal carcinoids behave in an indolent manner, and some tumors have the potential to metastasize. It was unclear which features would predict poor outcome, however. Cruickshank and Cunningham had, in 1949, observed in their series of 17 carcinoids that the number of mitotic figures varied between tumors; they thought that tumors with increased mitoses indicated rapid growth, but they did not speculate on increased metastatic potential. Around the same time, Haynes and Pearson estimated that the rate of malignancy in rectal carcinoid is 12% (Pearson & Fitzgerald 1949, Haynes, Shirley & Hume 1953). Raven stated in his review article in 1950 that the occurrence of metastatic lesions in patients with a rectal carcinoid is only "a matter of time." Nevertheless, he considered the risk smaller than in rectal adenocarcinomas. It is notable that by that time, no author had made an attempt to distinguish indolent cases from more aggressive ones prone to metastasize. Johnson et al reported in 1983 that certain growth patterns are associated with more aggressive behavior, the undifferentiated pattern being the worst. In the context of GI-tract tumors, the terms "neuroendocrine tumor" and "neuroendocrine carcinoma" appeared in the literature in the late 1980s.

CLASSIFICATION OF GI – NETS

The classification, terminology, and perception of NETs as a tumor entity have undergone considerable changes over the past years, causing confusion. After introduction of the carcinoid entity by Oberndorfer, these tumors were considered benign when compared to conventional adenocarcinomas. Quite soon, information and experience accumulated suggesting that some carcinoids failed to behave in an indolent manner after all. Tumors were divided into benign and malignant. In the latest WHO 2010 classification, all NETs are considered potentially malignant. It is necessary that all health-care providers adopt and implement the latest WHO 2010 classification and the terminology therein. All pathology reports must contain the essential information needed by clinicians to determine the accurate treatment for each patient.

ERA OF THE CARCINOID TUMOR

When Oberndorfer described the carcinoid entity in 1907, he considered carcinoid a benign tumor. Later, it became evident that some carcinoids do metastasize and are clearly malignant with poor prognosis. Even with the potential to metastasize acknowledged, the prognosis seemed better than in conventional GI tract adenocarcinomas.

In 1961, Williams and Sandler classified GI - NETs according to their primary site: tumors were divided into carcinoids of the foregut (respiratory tract, thymus, and stomach), midgut (small intestine, appendix, and proximal colon) and hindgut (distal colon, and the rectum). Some morphological features characteristic of each region were observable, but this classification failed to predict patients' clinical outcome and had little prognostic value (Williams & Sandler 1963).

Subsequently, carcinoids were divided into typical and atypical (In early reports, the term "atypical carcinoid" referred to cases in which the silver staining was only weakly positive or negative in a tumor that was morphologically an obvious carcinoid). This classification was hardly clear-cut: carcinoids with cellular atypia, elevated mitotic count, poorer differentiation, or focal necrosis received the diagnosis of atypical carcinoids. Few reports exist on whether this classification correlated with metastatic potential or prognosis. A study by Soga classified 156 pancreatic carcinoids: 144 were typical carcinoids and 12 atypical carcinoids; this classification did not predict clinical outcome (Soga 2005a). Small-cell carcinoma of the lung is an old entity, but as to the GI tract, a report of an oat-cell carcinoma (small-cell carcinoma) in the esophagus appeared in 1952 (McKeown 1952) and in the pancreas in 1971, with oat-cell carcinoma regarded as a poorly differentiated carcinoid arising from the endocrine cells (Corrin et al. 1971).

In the lung, the term "carcinoid" is still valid and commonly used, and "carcinoid syndrome" is an appropriate term. In the appendix, a special subtype of NET with both endocrine and exocrine function is called goblet cell carcinoid (Bosman et al. 2010), but otherwise in the GI tract, the term "carcinoid" is becoming obsolete; its use thus should probably be discouraged, not least because of its erroneous association with benign behavior.

WHO 1980 AND WHO 2000 CLASSIFICATIONS

In the first WHO classification of endocrine tumors in 1980, the term carcinoid applied to most endocrine tumors: carcinoids were tumors of the diffuse neuroendocrine system, ones either benign or being neoplasms with a better prognosis than carcinomas. These comprised enterochromaffin, gastrin and unspecific carcinoids. Neuroendocrine tumors of the pancreas and thyroid, small-cell carcinoma of the lungs and skin (Merkel cell carcinoma), and paragangliomas were excluded from the carcinoid group.

In 2000, the WHO launched the second classification for endocrine tumors (Solcia, Klöppel & Sobin 2000) with a significant change in terminology, with the terms "neuroendocrine tumor" and "neuroendocrine carcinoma" encouraged, instead of "carcinoid," which served as a synonym for a well-differentiated neuroendocrine tumor. The WHO 2000 classification for tumors of the gastrointestinal tract referred to this classification, when covering neuroendocrine tumors of the GI tract (Hamilton & Aaltonen 2000). The term "carcinoid" was, however, not entirely abandoned as yet. Well-differentiated neuroendocrine tumors were usually small (≤ 1cm) and considered benign. Tumors that were 1 to 2 cm in diameter or showed angioinvasion were thought to exhibit uncertain malignant potential. Well-differentiated neuroendocrine carcinomas (malignant carcinoids) showed invasion of the muscularis propria or had metastasized and were thus considered of low-grade malignancy. Poorly differentiated neuroendocrine carcinomas were high-grade malignancies with poor prognosis. The proliferation index and mitotic count were regarded as prognostic factors and were not actually included in the WHO 2000 classification (Kloppel et al. 2007).

WHO 2010 CLASSIFICATION

The present classification for GI-NETs was launched in 2010. It includes grading for NETs that is applied to all NETs regardless of their primary site in the GI tract, as well as a TNM classification for each site. All NETs are considered malignant with the potential to metastasize, although the potential differs a great deal between individual tumors and sites. The diagnostic criteria for grading of GEP-NETs are in Table 1 and the TNM classification for rectal NETs in Table 2.

This grading is based on mitotic count and proliferation index by Ki-67 immunostaining. In cases of discrepancy between the two parameters, the higher grade is to be assumed. Compared to previous classifications, major differences appear. All tumors are potentially malignant, and, grading and TNM classification was introduced. Tumor size or depth of infiltration does not affect grade, but are considered in the TNM classification. Use of the term "carcinoid" is to be avoided, with the exception of carcinoid syndrome and goblet-cell carcinoid.

The prognostic accuracy of the WHO 2010 classification has been appropriately validated in NETs of the stomach and pancreas, but in the rectum, its prognostic value should be confirmed. It is also of interest, whether the new WHO 2010 classification is superior to its predecessor, WHO 2000 classification.

Table 1. *In the World Health Organization (WHO) 2010 classification for gastroenteropancreatic neuroendocrine tumors (GEP-NETs), this grading is applied regardless of the primary site of the tumor. All GEP-NETs are considered malignant (Bosman et al. 2010).*

WHO 2010 grade	Ki-67	Mitoses /10 HPF	Tumor diameter	Invasion into muscularis propria	Invasion into vascular structures
G1	≤ 2%	< 2	Not included	Not included	Not included
G2	3-20%	2-20			
G3	> 20%	> 20			

Table 2 *TMN-classification of rectal neuroendocrine tumors (Bosman et al. 2010).*

TNM classification
T – Primary tumor
TX Primary tumor cannot be assessed
T0 No evidence of primary tumor
T1 Tumor invades lamina propria or submucosa and is no grater than 2 cm in size
T1a Tumor less than 1 cm in size
T1b Tumor 1 to 2 cm in size
T2 Tumor invades muscularis propria or is greater than 2 cm in size
T3 Tumor invades subserosa, or non-peritonealized perirectal tissue
T4 Tumor perforates peritoneum or invades other organs
N – Regional lymph nodes
NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Regional lymph node metastasis
M – Distant metastasis
M0 No distant metastasis
M1 Distant metastasis

ENETS PROPOSALS AND GUIDELINES

In 2007 the European neuroendocrine tumor society (ENETS) published a proposal for grading and staging of midgut and hindgut neuroendocrine tumors (Rindi et al. 2007), acknowledging the possibility of malignant

behavior in all NETs. Grading of GEP-NETs according to that ENETS proposal and WHO 2010 are congruent, but in the TNM staging of GEP-NETs, differences emerge between the two systems in the appendix and pancreas.

GASTROENTEROPANCREATIC NEUROENDOCRINE TUMORS

INCIDENCE

NETs are rare tumors arising from neuroendocrine cells of the GI tract and pancreas. Based on the hormones that these cells produce, the cell types thus far identified number 15 (Rindi & Kloppel 2004), but with only 8 hormones recognized in GEP-NETs, thus far (Kloppel 2011). In pancreatic tumors, hormonal activity (insulin, glucagon, somatostatin, pancreatic polypeptide, gastrin, or vasoactive intestinal peptide VIP) detected in serum and tissues is associated with less aggressive behaviour (Morin et al. 2013).

In an analysis of 35 825 NETs, 27% occurred in the lungs, 51% in the GI tract, 6% in the pancreas, and 16% at other sites (Yao et al. 2008). In an autopsy series from Sweden, 1.22% had a carcinoid tumor, 90% of which were considered incidental findings (Berge & Linell 1976). In the USA, the incidence in 2008 of all GEP-NETs was 3.26/100 000 in men and 2.62 in women (Yao et al. 2008). In a German series, the annual incidence of GEP-NETs was 2.27/100 000 in men and 2.38/100 000 in women (Scherubl et al. 2013), and in a Swedish study the incidence of GI-NETs (pancreatic NETs not included) was 2.0/100 000 for men and 2.4/100 000 for women (Hemminki & Li 2001). In Finland, according to the Finnish Cancer Registry, 200 to 300 GEP-NETs are diagnosed annually (unpublished data).

The increase in incidence has been marked: Scherübl and colleagues detected, in Germany between 1976 and 2006, an increase in annual incidence of GEP-NETs from 0.31 per 100 000 inhabitants up to 2.27 in men, and from 0.57 to 2.38 in women. The greatest absolute increase was in NETs of the small bowel, and a relative increase in rectal NETs. The authors speculate that this increase, at least in part, can be attributed to colorectal cancer screening by colonoscopy and enhanced availability of radiological imaging (Scherubl et al. 2013), leading to detection of small (1cm or less in diameter), asymptomatic tumors. As a consequence, the proportion of small tumors has been on the rise (Scherubl 2009, 2011).

GENETICS

High-frequency mutations that would be characteristic of a special NET subtype, are as yet unknown. Mutations involving classical promoters and oncogenes such as protein 53, retinoblastoma, and Kirsten rat sarcoma viral oncogene (*p53, RB,* and *KRAS*) that are commonly encountered in many solid and epithelial tumors, are absent from NETs. In epigenetic changes, the deoxyribonucleic acid (DNA) sequence itself remains unaltered. Instead, gene expression is influenced by heritable changes that include DNA methylation, histone modification, and the expression of microribonucleic acid (miRNA). These features have been reported in NETs, particularly in pancreatic NETs, which have attracted the most attention among GI-NETs in terms of genetic studies (Karpathakis, Dibra & Thirlwell 2013).

In pancreatic NETs, DNA methylation has emerged in many genes such as Ras association domain-containing protein 1, cyclin-dependant kinase 2A (*RASSF1* and *CDKN2A*), the latter may even be of prognostic value (House et al. 2003). Alterations in chromatin remodelers (histone proteins) are common in pancreatic NETs and involve multiple endocrine neoplasia 1, death-domain associated protein, and alpha thalassemia/mental retardation syndrome X-linked protein (*MEN1, DAXX, and ATRX*) (Jiao et al. 2011). A distinctive microRNA expression pattern of possible prognostic value was detectable in pNETs (Roldo et al. 2006).

NEUROENDOCRINE TUMOR SYNDROMES

Multiple endocrine neoplasia syndrome-type 1 (MEN1) is a rare tumor syndrome with a genetic background. Patients with this syndrome develop tumors in several organs of the endocrine system: the adrenal and parathyroid gland, the pituitary gland, and the diffuse endocrine system of the GI tract. These patients are not exceptionally prone to develop rectal tumors, however (Yamaguchi et al. 1980, Salmela 2012). In MEN syndromes 2A and 2B, a neuroendocrine tumor of the GI tract is not a typical feature.

In von Hippel-Lindau disease. another tumor syndrome, patients develop hemangioblastomas of the central nervous system and retina, renal cell carcinomas, phaeochromocytomas, and neuroendocrine tumors of the pancreas. Rectal NETs are not, however, commonly associated with this rare syndrome (Maher, Neumann & Richard 2011).

INFLAMMATORY BOWEL DISEASE AND NET

Patients with Crohn´s disease are at increased risk for GI-NET. Crohn's is a chronic inflammatory bowel disease typically with segmental involvement of

the GI tract. Interestingly, NETs were discovered in the areas of the bowel that showed no inflammation at the time of diagnosis. West and colleagues speculated in their 2007 study that inflammation (by cytokines and other mediators) stimulates enteroendocrine cells and thus promotes hyperplasia and, eventually, neoplasia, but the observation was based on four patients only. Increased risk for NET in the ileum and other locations (not the rectum in particular) is also associated with ulcerative colitis, but authors speculated that this may, at least in part, be due to increased medical attention (Hemminki et al. 2008). In patients with ulcerative colitis, colorectal NETs are uncommon (Nascimbeni et al. 2005, Fu et al. 2008). Quinn et al. reported a patient who had ulcerative colitis and several microcarcinoids in the bowel. When colitis subsided as a result of treatment, the tumors resolved. The authors in that particular case speculated the role of an inflammatory stimulus as the cause of the NETs (Quinn & Platell 2004).

MANEC INCLUDING GOBLET CELL CARCINOID

Mixed adenoneuroendocrine carcinomas (MANEC) are exceedingly rare tumors, ones having both exocrine (adenocarcinoma) and endocrine components. The endocrine component is usually of high grade (G3 NET). In these tumors, the two separate components are not sharply demarcated, but rather intertwined in close juxtaposition. They occur, although infrequently, throughout the GI tract (Klimstra et al. 2010).

Another peculiar tumor with neuroendocrine features, the goblet cell carcinoid, presenting almost exclusively in the appendix, was first described by Gagné et al. in 1969. Subbuswamy and colleagues in 1974 launched the term “goblet cell carcinoid”, which has persisted and is still widely used – a term, however, causing discomfort in many authors. Morphologically, this tumor consists of small groups of tumor cells that include intracellular mucin (resembling goblet cells) and that express neuroendocrine markers at least focally. In most cases, the classical goblet cell growth pattern is the sole component, but a combined tumor with a component resembling conventional NET has been described (Chetty et al. 2010). What has been under debate is whether this is an unusual subtype of neuroendocrine tumor, or a variant of adenocarcinoma that undergoes neuroendocrine differentiation (Roy & Chetty 2010). The TNM classification of the appendiceal adenocarcinoma, not the neuroendocrine tumor, is applied, reflecting the aggressiveness of this neoplasm. Goblet cell carcinoid has never been reported in the rectum.

RECTAL NETS

Of all neoplasms in the rectum, rectal NETs comprise less than 1%. Among the most common are benign hyperplastic polyps and other serrated lesions. Adenomas, with dysplastic changes in the epithelium, are also common. Although considered benign, they are potentially precursor lesions for adenocarcinoma, which is the most common malignant tumor in the rectum. Melanoma, lymphomas, and benign and malignant mesenchymal tumors also occur, although rarely, in the rectum.

Rectal NETs originate from the rectal endocrine cells. These cells are part of the diffuse endocrine system and are dispersed throughout the GI tract. The rectum harbors mainly serotonin-producing EC-cells, and the L-cells that produce GLI and PYY. Somatostatin-producing D-cells are very few in number in the colon and rectum (Solcia, Capella & Fiocca 1998).

GENETIC BACKGROUND AND OTHER PREDISPOSING FACTORS

No high-frequency mutations involving DNA have been discovered in rectal NETs, but some epigenetic changes do occur. CpG island methylator phenotype (CIMP) positivity is more common in poorly differentiated colorectal NECs, and expression of DNMT1 (de novo methyltransferase), -3A and 3B is associated with advanced stage. Promoter methylation of human mutL homolog 1, tissue inhibitor of metallopeptidase 3 (*CDKN2A, hMLH1,* and *TIMP3*) occur exclusively in colorectal NETs (Arnold et al. 2008, La Rosa et al. 2012).

Patients with genetic syndromes such as von Hippel-Lindau syndrome (VHL), multiple endocrine neoplasia syndrome-type 1 (MEN1), and neurofibromatosis-type 1 (NF1), sometimes develop GI-NETs. However, these patients are not particularly prone to rectal NET. In sporadic GEP-NETs, a number of different genetic changes arise, but none of them specific for rectal NET.

INCIDENCE

According to the SEER 17 (surveillance, epidemiology, and end results; a program of the National Cancer Institute, USA) report, of all NETs (including NETs in lungs, pancreas and GI tract), in the USA rectal tumors comprise 18%, and their proportion of GI-NETs is 27% (Yao et al. 2008). In Europe, of all NETs, the proportion of rectal NETs is lower, 5 to 14% (Ploeckinger et al. 2009, Niederle et al. 2010, Garcia-Carbonero et al. 2010). It is possible that small, benign-appearing tumors are incompletely reported, and that true numbers would be higher. In a study from Japan, the rectal NETs represented 55.7% of GI-NETs (Ito et al. 2010). Such a high proportion may

be attributed to meticulous reporting of even small, polypoid, incidentally detected lesions, and to frequency of health checks including colonoscopy (Ito et al. 2007). Several countries have screening programs for occult blood in stool samples in order to find early-stage colorectal carcinomas: 1 to 2 % of screened subjects return a positive sample leading to endoscopy, which may also raise the incidence of NETs among the screened population (Hardcastle et al. 1996). In Finland, 20 to 30 rectal NETs are diagnosed each year, according to the Finnish Cancer Registry (unpublished data), but the exact incidence is unknown.

In patients with rectal NET, the average age at diagnosis is 48 to 56 years (Jetmore et al. 1992, Matsui, Iwase & Kitagawa 1993, Modlin, Lye & Kidd 2003, Yao et al. 2008, Korse et al. 2013), compared to 70 years for adenocarcinoma (Siegel et al. 2012). No significant gender predominance exists, but black and Asian populations in the USA are more commonly affected (Modlin, Lye & Kidd 2003, Yao et al. 2008).

Well-differentiated G1/G2-tumors (grade) predominate in the rectum, whereas in other parts of the colon, well-differentiated NETs are less common than are poorly differentiated G3-NECs (neuroendocrine carcinoma) (Anthony et al. 2010, Ito et al. 2010). Poorly differentiated NECs are high-grade tumors with an unfavorable prognosis and are often disseminated at initial presentation (Bernick et al. 2004, Brenner et al. 2004, 2007).

SYMPTOMS

Half the patients diagnosed with rectal NET are asymptomatic (Jetmore et al. 1992). Endoscopy is readily available in many countries enabling early diagnosis of small, asymptomatic rectal NETs (Scherubl 2009, 2011). When present, symptoms are caused either by the tumor mass of the primary tumor or by metastasis, by biogenic substances secreted by the tumor (carcinoid syndrome), or by tumor-induced fibrosis. Symptoms include abdominal pain, weight-loss, GI bleeding, diarrhea, discomfort, change in bowel habits, and constipation (Jetmore et al. 1992, Shebani et al. 1999).

At diagnosis, 75 to 85% of rectal NETs are local (Modlin, Lye & Kidd 2003, Fahy et al. 2007, Yao et al. 2008). In an autopsy series including all primary sites, the most frequent sites of metastases were regional lymph nodes (89.8%), liver (44.1%), lung (13.6%), peritoneum (13.6%) and pancreas (6.8%) (Berge & Linell 1976). Fahy and colleagues in 2007 reported the liver to be the most common site of metastases. Symptoms caused by metastatic disease include right upper-quadrant abdominal pain, hepatomegaly, lethargy, weight loss, symptoms due to carcinomatosis, and bowel obstruction due to fibrosis caused by widespread intra-abdominal disease (Caplin et al. 2012a).

CARCINOID SYNDROME

Carcinoid syndrome occurs in around 10% of NET patients, and in 48% of patients with liver metastases, but in patients with rectal NET, even when metastatic, the carcinoid syndrome is rare, since serotonin-producing tumors are uncommon at this site (Shebani et al. 1999).

Some NETs produce bioactive substances such as serotonin, prostaglandins, histamine, and tachykinins in amounts sufficiently high to cause carcinoid syndrome. Classical symptoms include cutaneous flushing and diarrhea. Cardiac manifestations, bronchospasm, myopathy, artropathy, edema, and skin pigmentation occur less frequently (Modlin et al. 2005).

Of patients with carcinoid syndrome, 20 to 50% develop carcinoid heart disease (Pellikka et al. 1993, Bhattacharyya et al. 2008). Vasoactive substances lead to fibrinoid depositions mainly on the tricuspidal valve and pulmonary valve on the right side of the heart, followed by regurgitation and stenosis of the valves which may eventually progress to right-sided heart failure (Bernheim et al. 2007, Palaniswamy, Frishman & Aronow 2012). The lungs have the capacity to degrade the vasoactive substances thus protecting the left side of the heart and as a consequence, left-sided carcinoid heart disease is observable in cases with a bronchial NET or with a metastatic NET producing vasoactive peptides in massive amounts that surpass the capacity of the lungs, or with a NET patient's having a patent foramen ovale (Gustafsson et al. 2008).

DIAGNOSTICS

Endoscopy

Many asymptomatic rectal NETs are discovered incidentally at endoscopy. They can be polypoid, or frequently submucosal, causing a subtle bulge in the mucosa. They may be yellowish or reddish, or have color similar to that of the surrounding mucosa. Endoscopic ultrasound is useful in determining tumor size, sharpness of the tumor edges, and depth of infiltration (Matsumoto et al. 1991, Liu et al. 2013). High-grade tumors may present as large, circumscribed, ulcerated, and even obstructing tumors.

Imaging

In completely removed G1-NETs, further imaging is not considered mandatory, whereas in G2/G3 lesions, scanning of the liver, thorax, and pelvis is recommended, with computed tomography (CT) preferable (Figure

2). In evaluation of local stage and depth of invasion, magnetic resonance imaging (MRI) is accurate. Transabdominal ultrasound (US) can be useful in the search for liver metastasis. Metastatic lesions are sometimes available for biopsy with ultrasound guidance (Pelage et al. 1999, Burton et al. 2008, Taylor et al. 2011, Caplin et al. 2012a). Positron emission tomography-computed tomography (PET-CT, Figure 1) is superior to PET and somatostatin reseptor scintigraphy (SRS) in planning treatment of disseminated NET (Gabriel et al. 2007, Krausz et al. 2011, Ruf et al. 2011). Additional, synchronous tumors must be excluded, since multicentric NETs have been described in up to 10% even the in absence of NET syndromes, and synchronous non-neuroendocrine tumors in up to 22% indicating a thorough radiological work-up of NET patients (Shebani et al. 1999).

Laboratory tests

Neuroendocrine tumors contain chromogranin A (CgA) protein in their neurosecretory granules. The plasma CgA level is measurable and reflects tumor burden at time of diagnosis. The plasma content of CgA decreases when the tumor diminishes as a result of the treatment. CgA level is often increased in relapses, making CgA a useful marker for follow-up (Pirker et al. 1998b, Ardill, Erikkson 2003b, Kolby et al. 2004b). Increased CgA is associated with some non-neuroendocrine tumors, and elevated levels of plasma CgA are encountered in chronic inflammatory bowel disease such as Crohn´s disease or ulcerative colitis, reflecting the increased activity of neuroendocrine cells in these conditions (Tropea et al. 2006, Sciola et al. 2009).

Histology

On routine HE sections (haematoxylin-eosin), rectal NETs can be frankly polypoid, or be merely slightly elevated from the surrounding mucosa. The neoplastic tissue is usually covered by intact mucosa. Tumor tissue is visible in the lamina propria of the mucosa, sometimes as solitary islets or grandular formations between normal intestinal glands. Accurate histological diagnosis is sometimes challenging from small biopsies. In specimens containing the entire tumor, it is easier to determine whether infiltration into the muscularis propria or angioinvasion has occurred. Four distinctive growth patterns are recognizable: glandular, insular, trabecular, and diffuse (Pilichowska et al. 1999). Tumor size is assessed from tissue specimens, as well as the frequency of mitotic figures and the presence or absence of significant cytological atypia. Grading and staging should be according to the WHO 2010 criteria, Tables 1 and 2.

Immunohistochemistry

Although in most cases, the morphology of the tumor is suggestive of its neuroendocrine nature, this must always be confirmed by immunohistochemistry by chromogranin A and synaptophysin; CD56 (cluster of differentiation 56) is not recommended, as it is considered less specific than other neuroendocrine markers. The proliferation index is determined by Ki-67 (Caplin et al. 2012a).

Chromogranin A (CgA) is one of the peptides of the chromogranin family characterized in 1966. CgA appears in the secretory granules of neurons and neuroendocrine cells (Ferrari et al. 1999). Its function of is not yet entirely discovered; it may act as a precursor peptide for various hormones (Louthan 2011). Most NETs are positive for CgA by immunohistochemistry. Poorly differentiated NECs may be only weakly positive, or even negative for CgA (Bussolati, Volante & Papotti 2001, Lloyd 2003, Arnold et al. 2009). CgA level in serum can be measured and can serve as a tumor marker: if a neuroendocrine tumor is clinically suspected, a high concentration of CgA in serum corroborates this. If the level of CgA is elevated at diagnosis, it may reflect tumor burden. During follow-up, elevated CgA levels can be a sign of tumor recurrence or metastasis (Pirker et al. 1998a, Ardill & Erikkson 2003a, Kolby et al. 2004a).

Synaptophysin, characterized in 1985, is a protein found in the membrane of the small synaptic vesicles (Wiedenmann & Franke 1985) occurring in neural cells and in neuroendocrine cells. When the vesicles release their content, synaptophysin is involved (Valtorta et al. 2004). By immunohistochemistry with a monoclonal antibody, the presence of synaptophysin-containing vesicles in NET tumor cells was a finding in 1986 (Wiedenmann et al. 1986). Almost all NETs express synaptophysin. Poorly differentiated tumors are often negative for chromogranin A, but nearly always positive for synaptophysin (Bussolati, Volante & Papotti 2001, Lloyd 2003, Arnold et al. 2009).

Ki-67 antigen (see below) is recognized by the MIB-1 antibody used in routine diagnostics to evaluate the proliferation index. In the diagnosis of a NET, determination of the percentage of MIB-1 positive cells is essential and tumor grade is based on the mitotic activity and proliferation index by MIB-1.

METASTATIC NET WITH UNKNOWN PRIMARY

In clinical practice, metastatic lesions are occasionally discovered prior to discovery of the primary tumor. In cases with a metastatic NET, the site of the primary tumor is unknown at initial presentation in up to 30% (Morris et

al. 2010, Stoyianni, Pentheroudakis & Pavlidis 2011, Balaker et al. 2012). With conventional CT, the primary tumor has been detectable in 20% of cases compared to 59% with ^{68}Ga-DOTA-NOC (Gallium; 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid - Nal3-octreotide) by PET/CT and 39% with ^{111}In-Octreoscan (Prasad et al. 2010). It is notable that even with such sophisticated imaging techniques, a significant proportion of primary tumors remain occult.

If tissue material from a metastasis is available, gene expression analysis may provide valuable information in terms of primary tumor site. Primaries of the stomach, small intestine, and pancreas have in some series had genetic signatures (Capurso et al. 2006, Posorski et al. 2011, Hainsworth et al. 2013). Immunohistochemistry is also helpful: positivity of thyroid transcription factor (TTF-1) points towards pulmonary, and CDX2 towards gastrointestinal and, more specifically, midgut origin (Erickson et al. 2004, Jaffee et al. 2006, Lin et al. 2007). No specific marker exists for rectal origin.

TREATMENT

Surgical or endoscopic removal is the only curative treatment for localized rectal NET. Small lesions are often encountered incidentally at endoscopy, and in most cases diagnosis is confirmed at histological examination. Sometimes lesions are biopsied, allowing more careful planning of the surgery. When choosing the appropriate method, tumor size is important (Caplin et al. 2012b).

Endoscopic and surgical treatment

Endoscopic removal is the treatment of choice in G1-NETs of the rectum smaller than 1 cm in size and without infiltration of the muscularis propria. (Scherubl et al. 2011, Kwaan, Goldberg & Bleday 2008, Onozato et al. 2010). If a small tumor invades the muscularis propria, or is of grade G2 to G3, transanal excision should be considered. In tumors 1 to 2 cm in diameter with no evidence of muscularis propria invasion or lymph node involvement, endoscopic removal is recommended for G1 tumors and transanal excision for G2 tumors. However, if a 1- to 2-cm G2 tumor has invaded the muscularis propria or beyond, anterior resection of the rectum is recommended, and the same applies for G3 tumors (Shields et al. 2010). Even in the presence of metastatic lesions, surgical treatment of the primary tumor may be beneficial by providing alleviation of local symptoms (Pavel et al. 2012a).

Surgical and ablative treatment of metastases

In patients with disseminated NET at any primary location, distant metastases occur most commonly in the liver (Berge & Linell 1976). Three patterns of liver metastasis are shown in Figure 1: in 20 to 25%, the infiltration has a simple pattern (Type A), in which the metastatic lesions are within one liver lobe or in two adjacent segments, and if extrahepatic metastases are excluded, liver resection is usually available. In the complex pattern (Type B, 10 to 15% of cases), mainly one lobe is affected with minor lesions in the contralateral lobe (Figure 1). With this pattern, liver resection still may be feasible. In the diffuse Type C pattern (60 to 70% of cases) multifocal metastatic tumors cannot be treated surgically (Pavel et al. 2012a). In cases with metastatic G3 NEC, liver resection is usually not recommended, but in selected cases with only a few metastatic lesions, it may be considered as one option.

Ablative treatments including radiofrequency ablation (RFA), transcatether arterial embolization (TAE), and transcatether arterial chemoembolization (TACE), can serve as the sole treatment in metastatic disease, or in conjunction with liver resection. With RFA, complete or significant local control has been observed for several months in 80% (Berber, Flesher & Siperstein 2002). When RFA is combined with liver resection, even total removal of metastatic tumor tissue can be achievable even in conventionally unresectable cases, but with tumors less than 3 cm in diameter best suitable for this treatment option (Pawlik et al. 2003). Significant symptom improvement is achievable in a majority of cases (Eriksson et al. 2008b). In a more recent study with metastatic NETs of the small intestine, however, no difference was detectable in patients treated with RFA or liver resection, and non-surgical therapy (Norlen et al. 2013).

In TAE and TACE peripheral liver arteries are selectively embolized causing ischemia in the metastatic tumor tissue, and in TACE, a chemotherapeutic agent, usually doxorubicin or streptozotocin, is also injected into the tumor tissue (Ruszniewski et al. 1993, Marrache et al. 2007). By TACE, the 5-year survival rates have been 50 to 83% and by TAE 40 to 67%, (Vogl et al. 2009). TAE and TACE were of equal effectiveness, but with TAE, fewer complications occurred (Fiore et al. 2014) Rather similar results have resulted from selective internal radiation therapy (SIRT) (Engelman et al. 2014). In a recent consensus conference, all ablative treatments yielded comparable results, and no method was found superior to be others (Kennedy et al. 2014).

Since histological data from the primary tumor is useful in planning the treatment of the metastases, resection of the primary tumor is recommended first. A complete resection of liver metastases leads to survival rates of 60 to 80% in 5 years (Chamberlain et al. 2000, Sarmiento et al. 2003, Elias et al. 2003) in contrast to only 30% in patients without complete liver resection (Touzios et al. 2005, Kianmanesh et al. 2005) implying, that in disseminated cases the liver resection may be more effective than the

resection of the primary tumor. A larger liver resection is usually not combined to resection of the primary tumor, and a two-step approach is preferable in these cases (Pavel et al. 2012a).

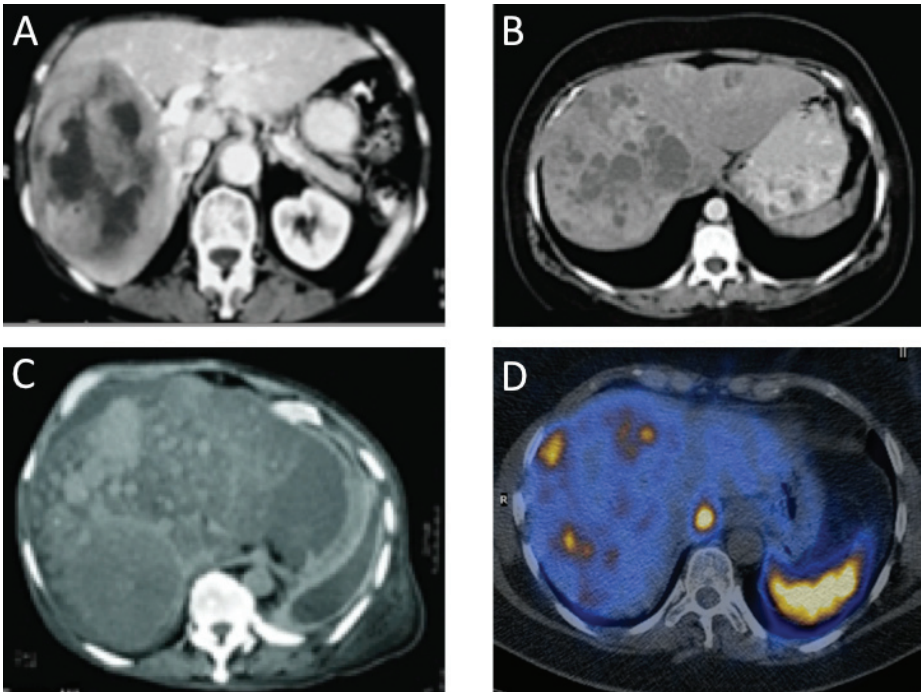


Figure 1. Three different patterns of liver metastases are recognized: Type A (A), Type B (B) and Type (C). Metastatic lesion seen in PET/CT (D). (Images with permission of John Wiley and Sons).

Liver transplantation

A malignant tumor in the liver is only in exceptional cases an indication for liver transplantation. Patients with a slowly progressing G1 or G2 NET which fails to respond to any other treatment are thought to benefit most. The tumor may be hormonally functioning or non-functioning. A candidate

patient for liver transplantation should be followed for at least 6 months prior to the operation in order to rule out aggressive tumor behavior and small extrahepatic metastatic lesions. The number of patients treated with liver transplantation is limited, with no generally applied consensus criteria for patient selection (Pavel et al. 2012b). In Finland, only three patients have undergone liver transplantation for liver metastasis of GEP-NET: in one of these patients the primary tumor was in the rectum (personal communication).

Somatostatin analogs and interferon

The somatostatin analogs octreotide and lanreotide have antisecretory properties and are effective in treatment of symptoms of carcinoid syndrome (a rare event in case of rectal NET) (Eriksson et al. 2008a, Modlin et al. 2010). Interferon may be combined with somatostatin analogs (Oberg 2000, Pavel et al. 2006). These drugs also have a weak antiproliferative effect: reduction of metastatic lesions occurs in fewer than 10% of patients (Faiss et al. 2003, Welin et al. 2004, Arnold et al. 2005, Modlin et al. 2010). In the PROMID study, metastases treated with octreotide LAR were stabilized in 67% vs. 37% in the placebo group. The progression-free period was 14.3 months with octreotide LAR, and 6.0 months with placebo (Rinke et al. 2009). Thus, somatostatin analogs may be useful (with or without interferon) in cases of disseminated G1-G2 NET with unresectable liver metastases, whereas in G3 tumors this is not recommended (Pavel et al. 2012b).

Systemic chemotherapy

In localized rectal G1-G2 tumors, adjuvant chemotherapy is not a recommendation (Caplin et al. 2012b). In metastatic cases, chemotherapy as a treatment option is not well established, but may be considered in rapidly progressing cases. G3 tumors should be treated with systemic chemotherapy, even in the absence of disseminated disease (Pavel et al. 2012a). No publications are available on neoadjuvant therapy (chemotherapy and radiotherapy) prior to operation in high-grade cases.

PROGNOSIS OF RECTAL NETS

At discovery, 2 to 8% of cases have distant metastases (Yao et al. 2008, Modlin, Lye & Kidd 2003) and 5% regional metastases (Yao et al. 2008). In a Japanese study, distant metastases were present in 8%, and regional

metastases in 30% (Ito et al. 2010). Due to increased availability of endoscopy, rectal NETs are discovered earlier than previously, and the prognosis has improved (Scherubl 2009). The 5-year survival in all rectal NETs is 75.2 to 88.3% (Modlin, Lye & Kidd 2003), in patients with distant metastases, at 15 to 30% (Soga 2005b, Konishi et al. 2007), and in nodal-positive disease 34% (Konishi et al. 2007). Small tumors (< 1cm) without angioinvasion or infiltration to the muscularis propria have a favorable 5-year prognosis of 98 to 100% (Soga 2005b, Konishi et al. 2007). Although the rectum is anatomically part of the colon, colonic NETs have a poorer prognosis than rectal NETs (Murray et al. 2013). The proportion of metastatic tumors among all rectal NETs ought to be assessed in additional studies.

Proliferation index by Ki-67 and mitotic rate

When mice were immunized against nuclei of Hodgkin lymphoma cells in 1983 in Kiel, Germany, the Ki-67 antibody was the result. The original clone was in well number 67 in the 96-well plate (Gerdes et al. 1983). The protein recognized by this antibody was named after the antibody: Ki-67. The polyclonal antibody was replaced by the MIB-1 antibody (molecular immunology Borstel 1).

The Ki-67 antibody stains proliferative cells in the G_1, S, or G_2 phases or in mitosis. It does not stain resting or quiescent cells in the G_0 phase. The nature of the antigen recognized by the Ki-67 antibody was revealed in 1991 (Gerdes et al. 1991) and the complete structure reported in 1993 (Schluter et al. 1993). It serves widely in clinical pathology as a marker of a proliferative, dividing cell, as it is non-specific to any cell type, but readily stains all types of proliferative cells. Cellular localization and staining intensity varies during the cell cycle, with highest intensity during metaphase (Starborg et al. 1996). The proliferation index by Ki-67 alone does not determine tumor growth, because it is not entirely certain whether every cell in the G_1, S, or G_2 phases is eventually going to divide, and because the duration of the intermitotic phase of a cell cycle varies, and other antigens involved in the cell cycle need study, as well.

The proliferation index may also reflect the effect of antineoplastic drugs on a certain tumor cell population; in tumors with a high proliferation index, administration of an antineoplastic drug will lead to destruction of more tumor cells when compared to a tumor with a low proliferation index. The index predicts the prognosis of various tumor types: probably the most extensive data on Ki-67 and its prognosis is for breast carcinoma, in which the correlation has been confirmed by several large studies. Prognostic significance should be separately studied in each tumor type, and use of a combination of cell cycle parameters might prove beneficial (Scholzen & Gerdes 2000).

Function of the Ki-67 in cell division remained unclear for a long time, and only very recently was it clear that Ki-67 is involved in perichromosomal compartment coating, which plays a role in nucleolar reassembly and organization after completion of mitosis (Booth et al. 2014).

Reports on how well the Ki-67 index (WHO 2010 classification) correlates with metastatic potential and prognosis of GEP-NETs in general and rectal NETs in particular are few. Yamaguchi and colleagues in 2013 reported on a series of 45 GEP-NETs in which of 29 rectal NETs, 5 were either metastatic or recurred. The conclusion followed that for rectal NETs, division into G1/G2 NETs based on the Ki-67 cut-point of the WHO 2010 classification is appropriate. In duodenal NETs, the predictive value of Ki-67 is not optimal. Another series included 184 NETs, of which 9 were rectal. Interobserver reproducibility of determining the Ki-67 index was high. In the whole series, the Ki-67 index correlated with distant metastasis, but not with lymph node metastasis. Rectal NETs were not analyzed as a separate group (Nadler et al. 2013). A study of 39 pancreatic NETs included 14 metastatic cases: all disseminated tumors had Ki-67 index of al least 5% (Jorda et al. 2003).

Rectal NETs with a low mitotic rate (<2/50 HPF) are metastasized in 3%, compared to tumors with an elevated mitotic rate which at least 65% metastasize (≥2/ 50 HPF) (Fahy et al. 2007).

Size

Tumor size of rectal NETs is associated with prognosis. Tumors larger than 2 cm showed regional metastasis in 58 to 59% and distant metastases in 24 to 27%, whereas small tumors (1 cm or less in diameter) metastasized to regional lymph nodes in 7 to 8%, and never to distant sites (Konishi et al. 2007, Shields et al. 2010). In two studies that did not separate regional and distant metastases, large tumors (>2 cm) were disseminated in 57 to 64%, whereas of small tumors (1 cm or less in diameter) 3 to 10% had metastasized (Soga 2005b, Fahy et al. 2007).

Angioinvasion

When lymphatic invasion occurred in the primary tumor, lymph node metastases were detectable in 70%, compared to 4% with no lymphatic invasion. As to venous invasion, 73% of positive tumors and 4% of negative tumors had metastasized to lymph nodes. Distant metastases occurred in 31% of positive cases and 3% of negative (Konishi et al. 2007). In another study, tumors with lymphovascular invasion metastasized to regional lymph nodes in 65% and to distant sites in 17%, compared to 5% and 0% in cases

without lymphovascular invasion, but lymphatic and venous invasion were not separated (Shields et al. 2010).

Depth of invasion

Tumors that invade the muscularis propria or beyond have metastasized to regional lymph nodes in 43 to 67% and to distant sites in 14 to 29%, whereas tumors restricted to the mucosa and submucosa have metastasized to regional lymph nodes in 13 to 16%, and to distant sites in 0 to 1% (Konishi et al. 2007, Shields et al. 2010). In a study not separating regional and distant metastases, of tumors restricted to the mucosa and submucosa 3% had metastasized compared to 56% of tumors invading the muscularis propria (Fahy et al. 2007). Depth of invasion correlated with prognosis: 5-year survival rate for patients without muscularis propria invasion was 100% as opposed to 57% in patients with invasion (Wang et al. 2011).

Age

Older patients (>55 years) had regional lymph node metastases at the rate of 40%, but younger patients (≤55 years) at only in 20%. Distant metastases occurred in 12% in the older age group compared to only 2% in younger patients (Konishi et al. 2007). According to Shields and colleagues (2010) the metastatic potential of tumors shows no association with age.

FOLLOW-UP

Local tumors

In small (<1 cm) G1-G2 NETs without lymph node involvement or invasion to the muscularis propria, regular follow-up is not considered necessary. For G3 tumors smaller than 2 cm, and G1-G2 NETs of 1 to 2 cm, annual follow-up is recommended, notably by endoscopy. Large G1-G3 tumors (> 2 cm) are followed by endoscopy, and liver scanning (preferably with MRI or CT), as well as by serum markers (e.g. chromogranin A); G1-G2 tumors annually and G3 tumors every 4 to 6 months the first year and then annually (Caplin et al. 2012a). Local recurrence is rare. The role of repeated colonoscopy as the mode of follow-up is questionable (Sauven et al. 1990).

Disseminated tumors

R0/R1 resected liver metastases of G1-G2 NETs are followed by serum markers (CgA and/or NSE) and imaging every 3 to 6 months, and G3 NECs every 2 to 3 months, and unresectable liver metastases at 3-month intervals at first, and at 6- to 12-month intervals in cases of stable disease. SRI should be performed after 18 to 24 months or even earlier, if serum markers are elevated and conventional imaging shows no enlargement in metastatic tumors. If metastases progress rapidly, the proliferation index should be evaluated, and biopsy is indicated (Pavel et al. 2012a).

NET patients with second malignancies

The risk of developing second malignancies is increased: 16.7% of NET patients had another malignant tumor either synchronously or metachronously (Scherubl et al. 2013). Patients without a second malignancy had better prognosis. Common sites of second malignant tumor include: the GI tract, female and male genital organs, skin, and breast (Scherubl et al. 2013). As a consequence in the follow-up, risk of other neoplasms must be considered.

TUMOR MARKERS OF THIS STUDY

Tumor markers are not very extensively studied in neuroendocrine neoplasms. The literature has reports on tumor markers mostly in lung carcinoids and in pancreatic NETs, but few reports on markers in the GI-NETs (see Table 3). The search was performed in Pubmed with the keywords: rectum/rectal, neuroendocrine tumor/carcinoid, immunohistochemistry, tumor marker and prognosis. Thus, markers should be studied in rectal NETs, as studies are, to the best of knowledge, non-existent. Below are presented in detail the markers that were chosen for publications due to their significant correlation with metastatic potential and prognosis.

Table 3. *Tumor markers studied in neuroendocrine tumors of the GI-tract and the lung.*

Marker	Localization/tumor type	Result	Reference
p21	GI-NET	Over expression = poor prognosis	(Kawahara et al. 2002)
E-Cadherin	GI-NET	Reduced expression = poor prognosis	(Kawahara et al. 2002)
p53, cyclin D1, Rb, bcl-2	GI-NET	No correlation with prognosis	(Kawahara et al. 2002)
E-Cadherin	Goblet cell carcinoid of appendix	No correlation with prognosis	(Li et al. 2002)
Fascin	Lung carcinoid	High expression = poor prognosis	(Pelosi et al. 2003)
Beta-catenin	Lung carcinoid	Altered expression = poor prognosis	(Pelosi et al. 2005)
COX-2	Midgut NET	Over expression = poor prognosis	(Cadden et al. 2007)
p53	Gastric NET	High expression = poor prognosis	(Safatle-Ribeiro et al. 2007)
LKB1	Lung carcinoid	Low expression = poor prognosis	(Amin et al. 2008)
RUNX1T1	Pancreas NET	Under expression = poor prognosis	(Nasir et al. 2011)
CD44, OTP	Lung carcinoid	Low expression = poor prognosis	(Swarts et al. 2013)
PGP 9.5	Pancreas NET	Low expression = poor prognosis	(Tomita 2013)
CDX2, Oct4	Ileum NET	No correlation with prognosis	(Heverhagen et al. 2013)
IMP3	Lung carcinoid	Positivity = poor prognosis	(Del Gobbo et al. 2014)
KLF4, p21	Lung carcinoid	Lack of expression = poor prognosis	(Naranjo Gomez et al. 2014)
GLP-1	Pancreas NET	No correlation with prognosis	(Cases et al. 2014)
DAXX, ATRX	Pancreas NET	Negativity = poor prognosis	(Marinoni et al. 2014)

CYCLIN A

Cyclin A controls the cell cycle by activating the cyclin-dependent kinases CDK1 and CDK2. The phosphorylated cyclin A – CDK complex is important in the initiation of DNA replication in the S phase. In the early S-phase the amount of cyclin A increases, whereas in mid M-phase it falls. A higher amount of cyclin A and a following dysregulation of the cyclin A – CDK complex is encountered in several tumors. The exact role of cyclin A in mitosis is not completely explained. It may stabilize and prevent the degradation of other cyclins (Sherr 1996, Yam, Fung & Poon 2002, Obaya & Sedivy 2002).

Cyclin A correlates with poor prognosis in gastric adenocarcinoma (Mrena et al. 2006). It also correlates with prognosis in squamous cell carcinoma of the esophagus and in adenocarcinomas of the endometrium and breast (Furihata et al. 1996, Kyushima et al. 2002, Bostrom et al. 2009, Santala et al. 2014). High cyclin A level is a predictor of complete response to therapy in acute leukemia (Dzietczenia et al. 2011). In neuroendocrine carcinoma of the skin (Merkel cell carcinoma), pituitary adenoma, or in non-small cell lung carcinoma, cyclin A expression has no prognostic significance (Turner et al. 2000, Koljonen et al. 2004, Cooper et al. 2009).

PROX1

The embryonic development of various organs such as the liver, retina, lymphatic endothelium, and lens is controlled by a homeobox transcription factor encoded by PROX1 (Oliver et al. 1993, Sosa-Pineda, Wigle & Oliver 2000, Dyer et al. 2003). In the APC mouse model, PROX1 has been implicated in the development and progression of intestinal adenocarcinoma (Petrova et al. 2008). The protein product of the adenomatous polyposis gene, APC, associates with a protein complex containing cytoplasmic beta-catenin, and degradation is induced in normal cells. Inhibition by Wnt signalling leads to accumulation of beta-catenin in the nucleus as a beta-catenin/TCF (T-cell factor) complex that has the capacity to control the expression of numerous genes. PROX1 is upregulated during colorectal cancer development, being a target of the beta-catenin/TCF pathway. PROX1 modulates cell adhesion and extracellular matrix interactions when normal intestinal mucosa transforms into an adenoma and invasive carcinoma (Petrova et al. 2008, Kinzler, Vogelstein 1996). In non-neoplastic mucosa, neuroendocrine epithelial cells and lymphatic endothelial cells express PROX1 (Skog et al. 2011). At least 15 types of neuroendocrine cells have been identified in the gastrointestinal tract, and PROX1 is expressed in only some of them. (Petrova et al. 2008). PROX1 predicts an aggressive clinical course

in colorectal adenocarcinoma, and Kaposiform hemangioendothelioma. Gliomas are found to express PROX1 (Elsir et al. 2011, Skog et al. 2011, Miettinen, Wang 2012).

HES77

In recent years, a new generation of antibodies, stem cell markers, have attracted attention in tumor pathology. The monoclonal, stem cell-associated antibody HES77 (human embryonic stem-cell factor 77) was produced by immunization of mice against the undifferentiated human embryonic stem cell (hESC) line SA167. The antigen epitope of this antibody remains unknown thus far.

AIMS OF THE STUDY

It has long been accepted that some rectal NETs have an aggressive clinical course but it has been unclear whether the WHO 2010 classification has prognostic significance in rectal NETs. Moreover, tumor markers have not been studied in rectal NETs. The detailed aims of this study were:

1. To estimate the incidence of rectal NETs in Finland (I).

2. To evaluate, among rectal NETs, the proportion of metastatic tumors (I).

3. To validate the prognostic value of the WHO 2010 classification in rectal NETs and to compare the novel WHO 2010 and previous WHO 2000 classifications (I).

4. To test new markers for rectal NETs able to reliably identify tumors prone to metastasize (II-IV).

MATERIALS AND METHODS

PATIENTS, CLINICAL DATA, AND ETHICAL ASPECTS

Patients with a rectal NET were identified from the database (Qpati) of the Department of Pathology, Helsinki University Central Hospital (HUCH) between 1980 and 2005. Tumors found numbered 73, in patients who were 28 male and 45 female: the mean age at diagnosis was 54 years. Average follow-up was 124 months (23 days to 314 months). During that period of time, 118 rectal NETs were registered in the Finnish Cancer Registry representing all cases reported in the Uusimaa region. Among these 73 patients, metastases had occurred in 10 patients, with tissue material available in 6. A number of cases were initially diagnosed in private clinics, but it is likely that this series includes all overtly malignant cases, since treatment of these cases is centralized in public hospitals. Non-metastasized cases diagnosed in private clinics were excluded from this tumor series.

Clinical and follow-up data came from patient records of Departments of Surgery and Oncology, HUCH, and from its pathology laboratory database (Qpati) of the Department of Pathology. Survival data came from the Finnish Population Register Center, and cause of death from Statistics Finland. Of particular interest were age at diagnosis, gender, method of surgery (biopsy, polypectomy, mucosal resection, or bowel resection) and follow-up, clinical symptoms, and local recurrences. In metastatic cases, the time elapsed from primary diagnosis to metastasis and treatment were registered.

The research procedures were approved by the National Authority for Medicolegal Affairs (predecessor of Valvira) and The Ethics Committee of the Helsinki University Hospital.

PREPARATION OF TISSUE MATERIAL

Archived tissue material was available for 73 rectal NETs. Standard procedure in diagnostic histopathology has been fixation in formalin (aqueous solution containing 37% formaldehyde). The material is washed and dehydrated in an ascending alcohol series and treated with an organic solvent. The hardened tissue is subsequently embedded in paraffin. From archived paraffin blocks, thin slices are cut, paraffin is removed with a solvent, and the tissue is rehydrated in descending alcohol series.

RE-ANALYSIS OF TUMORS

All tumor samples were re-evaluated from HE slides by two pathologists (Juha Jernman and Johanna Arola) in order to confirm the diagnosis and to estimate whether the material was sufficient for immunohistochemistry, with tumor size, growth pattern (insular, trabecular, glandular, or diffuse), depth of infiltration, mitotic frequency, and vascular invasion recorded.

WHO 2010 CLASSIFICATION

Tumors were re-classified according to the WHO 2010 classification for neuroendocrine tumors of the GI tract. GI-NETs usually exhibit typical, recognizable morphology: tumor cells have moderate amounts of cytoplasm and a round nucleus with coarse chromatin. Variation in nuclear size and shape is seldom considerable. Differing growth patterns are observable, and one tumor may show a combination of growth patterns. The neuroendocrine nature was confirmed by immunohistochemistry and grade determined by proliferation index (Ki-67) and mitotic frequency (Table 1); TNM status was determined as shown in Table 2.

TUMOR SIZE AND OTHER FEATURES

Tumor size was measured on a microscope slide. Larger tumors were measured with a ruler and smaller ones in the microscope field. The predominant growth pattern was registered for each tumor: insular, trabecular, glandular, or diffuse (Kloppel 2004). Some tumors showed a combination of growth patterns. The tumor tissue may be limited to the lamina propria, but it may extend to the submucosa and the muscularis propria. It may perforate the peritoneum or invade adjacent tissue. Invasion into the muscularis propria was classified as invasive growth. Mitotic figures were registered per 10 high power fields and invasion of tumor cells into vascular spaces. Endothelial marker CD31 served to highlight the vascular structures.

IMMUNOHISTOCHEMISTRY AND ANTIBODIES

In routine pathology, immunohistochemistry is a common method to demonstrate the presence of specific antigen in tissues. When tissue material is fixed using formalin, cross-linking of protein amino acids prevents tissue from deterioration, also masking antigen epitopes, and antigen retrieval is needed to expose the immunogenic molecules. This purpose required use of

trishydroxymethylaminomethanehydrochloride (tris-HCl), or citrate buffer, depending on the antibody, for 20 min at 98°C. Since many tissues and cells show endogenous peroxidase activity that may react with the substrate, leading to non-specific background staining and making interpretation difficult, a peroxidase-blocking solution was necessary. In order to obtain a recognizable staining pattern, the choice was EnVision detection system by Dako. Slides were counterstained with Meyer's hematoxylin, enabling identification of tissue anatomic structures.

Monoclonal antibodies have high affinity for a specific antigen epitope. They are produced by the hybridoma technique, in which mice are immunized against the chosen antigen. The most specific cell line is then selected and fused with a myeloma cell line. Polyclonal antibody raised in a laboratory animal is able to detect different epitopes of the antigen and is, in that sense, more sensitive but less specific. As many tumors included in the series were very small, tumor markers were first underwent study in a test series of 18 larger tumors (1 G3, 7 G2 and 10 G1 tumors). With positive staining result and possible correlation with metastatic potential, the staining was subsequently carried out on the entire series: these antibodies are shown in Table 4. Several antibodies that either showed no immunoreactivity at all nor had any correlation with metastatic potential or overall survival were tested in the limited series only. These antibodies are listed in Table 5.

Table 4. Antibodies reported in a publication or that qualified for the full series of 73 tumors.

Antibody	Clone	Study/ Why discarded	Manu-facturer	Dilution	Antibody type
Ki-67	Mib-1	I	Dako	1:100	Mouse mAb
Chromogranin A	5H7	I	Novocastra	1:2000	Mouse mAb
CD31	JC70A	I	Dako	1:20	Mouse mAb
Pancreatic polypeptide		I	NeoMarkers	1:600	Rabbit mAb
CDX-2	EPR2764Y	I	CellMarque	Ready-to-use	Rabbit mAb
Serotonin	5HT-H209	I	Dako	1:10	Mouse mAb
Cyclin A	6E6	II	Novocastra	1:50	Mouse mAb
PROX1		III	R&D Systems	1:2000	Goat pAb
HES77		IV	Fujirebio	1:150	Mouse mAb
MMP9		No correlation	Maerck	1:1000	Rabbit mAb
MMP7	141-7B2	No correlation	Chemicon	1:1500	Mouse mAb
Cyclin D1	SP4	No correlation	NeoMarkers	1:20	Rabbit mAb
VeGF-R3		No correlation	SantaCruz	1:1500	Rabbit pAb

Table 5. *Antibodies stained on the test series only. All with no correlation except for negative staining for Bcl-2 and p53.*

Antibody	Clone	Manu-facturer	Dilution	Antibody type
Bcl-2	124	Dako	1:40	Mouse mAb
MMP2	17B11	Novocastra	1:75	Mouse mAb
MMP8		Neomarkers	1:200	Rabbit pAb
MMP25		Sigma-Aldrich	1:300	Rabbit pAb
GATA3		SantaCruz	1:100	Goat pAb
GATA4		SantaCruz	1:100	Goat pAb
GATA6		SantaCruz	1:100	Rabbit pAb
VeGF-C		Zymed	1:100	Rabbit pAb
Cip2A		Novus	1:500	Rabbit pAb
p53	DO-7	Dako	1:1500	Mouse mAb
p27	Kip1	BD Biosciences	1:100	Mouse mAb
Hur3A2	3A2	SantaCruz	1:1500	Mouse mAb
Hur19f12	19f12	SantaCruz	1:30000	Mouse mAb
COX-2	CX229	Cayman Chemical	1:100	Mouse mAb
Endostatin	91318	R&D System	1:75	Mouse mAb
c-myc	9E10	SantaCruz	1:400	Mouse mAb
HES5		Fujirebio	1:300	Mouse mAb
SNAIL		Abcam	1:2000	Rabbit pAb
BMI-1	1.T.21	Abcam	1:400	Mouse mAb

EVALUATION OF IMMUNOHISTOCHEMICAL STAININGS

In all tumors, whole sections were independently analyzed by two pathologists blinded to the clinicopathological data. Any cases with disagreement required a consensus score. Staining intensity - interpreted as a positive result - as well as the localization of the positive staining varied between antibodies.

STATISTICAL ANALYSIS

Correlations between individual clinicopathological characteristics and immunohistochemical staining results were assessed with univariate analyses, the chi-square test, and Fischer's exact test. Kaplan-Meier life tables were computed by the log-rank test. Survival time was calculated starting from the primary diagnosis; follow-up ended either at the last follow-up date (or at death from cause other than metastatic NET) or at

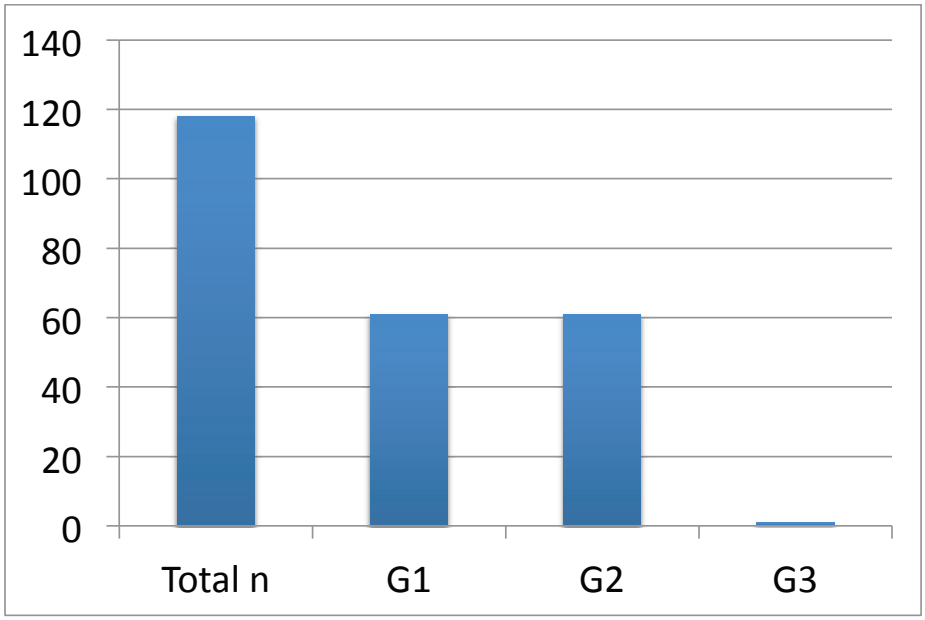
occurrence of metastasis (disease-free survival), or death from the disease (disease-specific survival). Variables emerging from univariate analysis as having a significant correlation were tested in Cox regression analysis to assess whether a variable would qualify as an independent prognostic factor. P-values less than 0.05 were considered statistically significant.

RESULTS

RE-CLASSIFICATION OF TUMORS AND FOLLOW-UP

Each of the 73 tumors' neuroendocrine nature was confirmed by positivity for chromogranin A. According to the WHO 2010 classification, 61 tumors were classified as G1, 11 as G2, and one as G3 (Figure 2). In this study, the classification was based on the Ki-67 index, as mitoses were too few to fulfill G2 or G3 criteria in any tumor. Of the 61 G1 tumors, none had metastasized during follow-up (Table 6), but 3 recurred locally.

Figure 2. In the Uusimaa province of Finland, 118 rectal neuroendocrine tumors were diagnosed according to Cancer registry, and 73 of these were included in the study. Distribution of grades is shown here.



The 11 G2 tumors were considerably larger than the G1 tumors; 2 of the patients (18%) were male, 9 (82%) were female (Table 7). The metastatic rate for G2 tumors was high, with nine (82%) patients having metastatic disease. Metastatic lesions were detected at initial presentation in three patients, and six developed metastases during a follow-up of 4 to 151 months after primary diagnosis. Five patients with a G2 tumor died of metastatic rectal NET, and

at the end of the follow-up, four patients with disseminated disease were still alive.

This series included only one G3 patient with distant metastases (liver) at initial presentation. According to her records, the tumor was "large," but its exact diameter went unreported. Only small biopsies were available for histology, making it impossible to assess tumor size. Growth pattern was diffuse with a proliferation index of 25%. This patient died of metastatic NET 22 months after diagnosis.

In the chi-square test, the WHO 2010 grade, tumor size, vascular invasion, and muscularis propria invasion correlated with metastatic potential. In the Kaplan-Meier life-tables and the log-rank test, the WHO 2010 grade was a clear prognostic factor. When the distant metastasis was the end-point, tumor diameter, muscularis propria invasion, and angioinvasion also correlated with prognosis. In Cox multivariate analysis, WHO 2010 classification emerged as an independent prognostic factor.

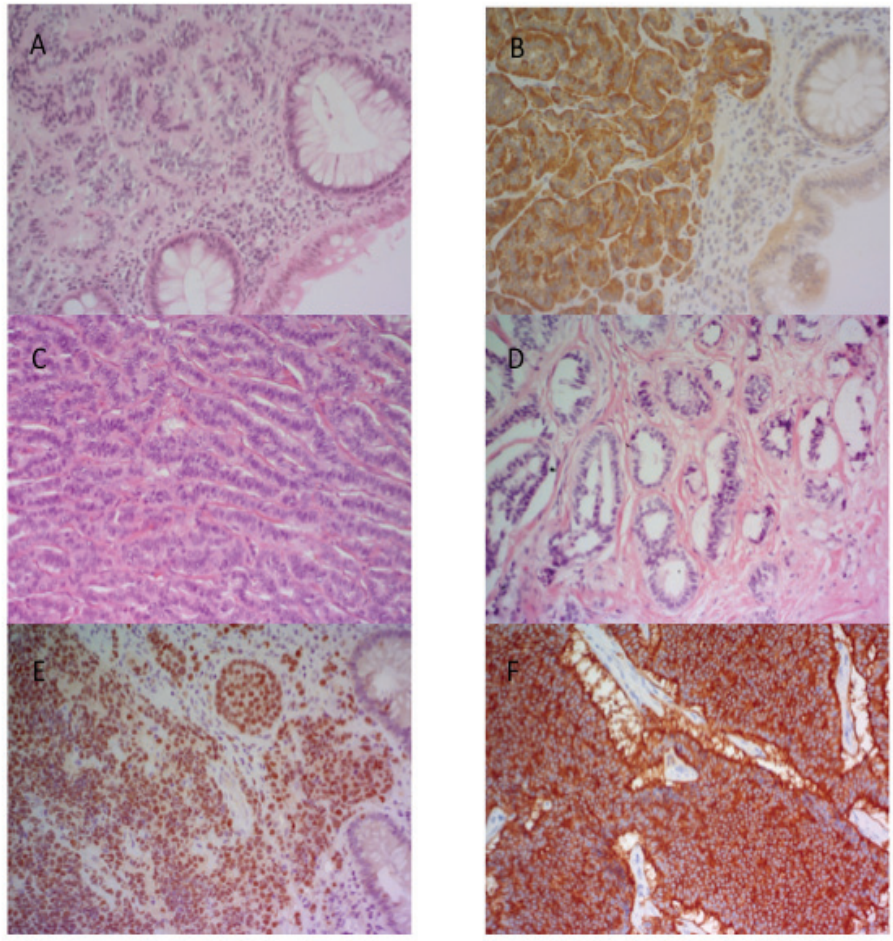
Table 6. *Comparison of present and previous WHO classifications for GI-NETs. Both classifications have high correlation with metastatic potential, but in WHO 2000 classification, two G1 tumors turned out metastatic, whereas in WHO 2010, all G1 tumors remained local. Both classifications with high p-values (p<0.001).*

	Metastatic disease	Local disease
WHO 2010 Grade		
G1	0	61
G2	9	2
WHO 2000		
G1	2	63
G1b	2	0
G2	5	0

Table 7. *Some clinicopathological characteristics of the tumor series. Female gender was over-represented in the G2-group, and G2-tumors were considerably larger than G1 tumors.*

WHO 2010 Grade	Number of patients	Gender: males females	Tumor size (mm): mean size range median
G1	61	26 (43%) 35 (57%)	3.6 1 – 9 3
G2	11	2 (18%) 9 (82%)	19 4 – 40 17
G3	1	1 (100%) 0 (0%)	Tumor size not available

Figure 3. Islets of tumor cells of a G1 NET are visible in the lamina propria (A) and positive staining for chromogranin A confirms the neuroendocrine nature of the tumor (B). Trabecular (C) and glandular (D) growth pattern in a G2 NET. Strong positive staining result for PROX1 in a G2 NET with diffuse and insular growth patterns (E). HES77 is strongly positive in a G2 NET.



SIZE AND INVASION INTO MUSCLE WALL AND VASCULAR STRUCTURES

Most tumors were less than 1 cm in diameter; of 63 tumors, only one G2 tumor was metastatic. Of 1- to 2-cm tumors, four of five were metastatic, and the five large tumors were all disseminated. Size showed strong correlation with metastatic potential.

Four tumors had invaded the muscularis propria, all of them metastatic. In 69 tumors, no invasion into the muscularis propria was evident, but metastases were detectable in 6 patients. Invasion correlated strongly with metastatic potential at $p<0.001$ in the chi-square test.

Tumor tissue invaded vascular structures in four cases, all metastatic; in 69 tumors, there appeared no histological evidence of angioanvasion. Angioinvasion correlated with metastatic potential at $p<0.001$ in the chi-square test.

It is of note that especially smaller tumors were often – in 48 cases - removed in pieces, often making it difficult to exclude invasive growth and to determine exact size. However, in only three G2-G3 tumors was this was the surgical method.

TUMORS MARKERS BY IMMUNOHISTOCHEMISTRY

CYCLIN A

In this series, 44 tumors were immunohistochemically positive for cyclin A, whereas 27 were negative with no expression detectable. Positivity of 0 to 2% of tumor cells was regarded as of low expression and 5% or more as of high expression. None of the tumors showed intermediate expression of 3 to 4%. All tumors with high cyclin A positivity were metastatic (Table 8). Tumors with cyclin A positivity of 1% or less had remained local.

Cyclin A expression strongly correlated with WHO 2010 classification. Tumor size correlated with cyclin A positivity, but neither age nor gender did. High expression of cyclin A correlated strongly with metastatic potential. In Cox regression analysis, both WHO 2010 and cyclin A were independent prognostic factors, but the WHO 2010 classification was stronger than the cyclin A expression.

Table 8. Correlation of cyclin A positivity with metastasis, grade, and size.

Cyclin A expression	n	metastasis	G1	G2-G3	Size, average (range)
Low 0-2%	65	4 (6%)	59	6	5 mm, (1 – 40 mm)
High ≥ 5%	6	6 (100%)	0	6	19 mm (1 – 30 mm)

PROX1

Normal mucosa showed PROX1-positive neuroendocrine cells. Most epithelial cells were negative for PROX1, although some cells in mucosal crypts showed positivity for PROX1. Nuclei of endothelial cells in lymphatic veins were positive, but nuclei of blood vessel endothelial cells were negative for PROX1. Tumors with strong nuclear positivity of ≤50 were classified as low expression, and tumors with (positivity of) >50% as high expression. Immunohistochemical expression of PROX1 correlated with disease-free and disease-specific survival at $p<0.001$ in chi-square and log-rank tests (Table 9).

Table 9. *Correlation of PROX1-expression with metastatic potential and grade.*

PROX1	Number	Metastasis	G1	G2 - G3
Low expression	59	4 (7%)	55	4
High expression	13	6 (46%)	5	8

HES77, CDX2, SEROTONIN AND PANCREATIC POLYPEPTIDE

The novel stem cell-associated marker HES77 showed significant correlation with metastatic potential and prognosis of rectal NETs at $p<0.001$ in chi-square and log-rank tests, when tumors were divided into two categories: tumors positive and tumors negative for HES77. Its decreased expression in metastasis, when compared to that in the primary tumor, predicted poor outcome, but the number of cases was too small for statistical analyses.

CDX2 is a transcription marker expressed by epithelial cells of the GI tract and commonly serves as a marker of intestinal origin in tumor pathology. Only two tumors were positive for CDX2: both were G2 tumors: one was metastatic, and the other remained local. Five tumors were positive for serotonin with no association with metastatic potential.

Pancreatic polypeptide was detectable immunohistochemically in 24 local tumors and in one disseminated G2 tumor. In statistical analysis, neither CDX2, serotonin, nor pancreatic polypeptide had value as prognostic marker.

DISCUSSION

A subset of rectal NETs have been known to behave in an aggressive manner, but predicting this malignant potential has been difficult, with the occurrence of metastatic lesions often being the first sign of malignant behavior. In the WHO 2010 classification, all GI-NETs are considered malignant, and their grading system showed itself, in this study, to be of excellent prognostic value.

AGE AND GENDER DISTRIBUTION IN PATIENTS

Female patients predominated, at 62 of 73. Of patients with a high-grade tumor (G2-G3), of 12, 10 were female (83%). Such striking female over-representation is a novel finding considering earlier gender percentages (Modlin, Lye & Kidd 2003, Yao et al. 2008). The number of high-grade tumors was quite small: 12, allowing for a possibility for bias.

The general belief is that women are more concerned about their health than are men, which may lead to higher rates of colonoscopies, during which many small NETs unlikely to cause symptoms are discovered incidentally (Ito et al. 2007). Their greater concern about their health fails, however, as a plausible explanation for female predominance for high-grade tumors, since metastatic cases would be diagnosed eventually regardless of gender.

Mean age at diagnosis was 54. Others have similarly reported a mean of 48 to 56 years (Jetmore et al. 1992, Matsui, Iwase & Kitagawa 1993, Modlin, Lye & Kidd 2003, Yao et al. 2008). Patients with colorectal adenocarcinoma are at diagnosis a mean 70 years old (Siegel et al. 2012); that rectal NETs occur younger may imply differences in tumorigenesis, although hyperplastic polyps and adenomas are occasionally encountered in considerably younger age groups.

TUMOR SIZE AND HISTOPATHOLOGICAL FEATURES

In this tumor series, G2 tumors were considerably larger than G1 (mean 4 mm vs. 17 mm), and tumor size correlated with metastatic potential and prognosis. Reports state that tumors larger than 2 cm were disseminated in 24 to 64% of cases. Tumors smaller than 1 cm metastasized at 0 to 10% (Soga 2005b, Fahy et al. 2007, Konishi et al. 2007, Shields et al. 2010, Weinstock et

al. 2013). In the present study, among the 10 metastatic rectal NETs, only one tumor was smaller than 1 cm, whereas of large tumors (> 2 cm), all had disseminated. What remains unknown is how rectal NETs develop. In colorectal adenocarcinoma, the adenoma-carcinoma sequence is established, but in NETs, no pre-malignant lesions have as yet been recognized. Small tumors have a low proliferation index and very low metastatic potential. If high-grade tumors develop de novo, one would expect to see more small high-grade tumors, as well. High-grade tumors were histologically monotonous with no discernible low-grade areas.

Depth of invasion correlated here with metastatic potential. All four tumors that invaded the muscularis propria had metastasized. Other authors have reached similar conclusions: tumors invading the muscularis propria or beyond often metastasize: to regional lymph nodes in 43 to 67% and to distant sites in 14 to 29%, whereas in tumors restricted to the mucosa and submucosa, metastases occur in regional lymph nodes in 13 to 16%, and at distant sites in 0 to 1% (Konishi et al. 2007, Shields et al. 2010). In our study, six G2 tumors with no detectable invasion into the muscularis propria had metastasized. Some of these tumors were exophytic polypoid lesions, some removed by polypectomy. It is important to recognize the risk for aggressive behavior in some of these tumors, even in the absence of invasion into the muscularis propria. In polypoid adenomas of the colon, malignant transformation does occur in cases with high-grade dysplasia. Invasive glandular structures in the submucosa can be pointed out, the so-called carcinoma in adenoma, with an acknowledged risk for metastasis. Unlike in epithelial neoplasms, in polypoid rectal NETs, histologically proven invasion into the muscularis propria is not a prerequisite for aggressive metastatic disease.

All four tumors with angioinvasion were metastatic, but in six metastatic cases angioinvasion was undetectable even with CD31 staining of vascular structures; indeed angioinvasion must have occurred, metastasis being its indirect evidence. In one study, tumors with lymphovascular invasion had a high frequency of dissemination, which is in line with our findings (Shields et al. 2010).

WHO 2010 CLASSIFICATION

Tumor grade according to the WHO 2010 classification had excellent prognostic accuracy for rectal NETs: none of the G1 tumors had metastasized during follow-up, whereas of 11 G2 tumors, 9 had disseminated. Local recurrence of three G1-NETs occurred; the primary lesions were removed in pieces, not by polypectomy.

The WHO 2010 classification considers all GEP-NETs malignant with the potential to metastasize, which is a major difference from older

classifications. The multi-step journey has been long, from the benign carcinoids as described by Oberndorfer to the neuroendocrine neoplasms of today that are all thought to be malignant but still have a more favorable prognosis than does conventional adenocarcinoma, which has a 5-year survival rate of around 60% (Brenner et al. 2012). The 5-year survival rate for rectal NET with liver metastasis is only 24%, as opposed to an overall survival for all rectal NETs of 88% (Weinstock et al. 2013), justifying efforts to identify tumors with the capability to metastasize.

In the present study, WHO 2010 grade was based on the proliferation index by Ki-67, since mitotic frequency was very low in all tumors not fulfilling G2 criteria. Studies on the prognostic value of WHO 2010 or Ki-67 in GI-NETs according to primary site are limited. Yamaguchi and colleagues concluded in their small series (total 29, with 5 metastatic) that WHO 2010 grading was of good prognostic value in rectal NETs. Their Ki-67 index was higher in metastatic cases, and in their work, the optimal cut-off point dividing tumors into G1 and G2 groups was 2.8%. Nadler and colleagues studied 184 neuroendocrine tumors including some pulmonary tumors; Ki-67 correlated with distant metastasis, but no rectal NETs were subjected to study separately. A Japanese study that included 43 rectal NETs revealed an average Ki-67 index of 3.9% in metastatic cases and 1.0% in local tumors (Hotta et al. 2006, Nadler et al. 2013, Yamaguchi et al. 2013). In a recent systematic review article covering 4,575 patients diagnosed with rectal NET, size emerged as a key feature in tumors with metastatic potential. Risk factors were size more than 10 mm, atypical surface, high age (> 60 years), and muscularis propria, perineural, or lymphovascular invasion. The WHO 2010 classification system received a brief mention, but tumor grade was discussed no further (McDermott et al. 2014). As a prognostic factor, in a study of 141 rectal NETs, tumor grade was only second to tumor stage. That study included 10 G2-G3 tumors, and grade was unavailable in as many as 57 cases (Weinstock et al. 2013).

Reports are few on the impact of tumor grade on metastatic potential. Results of the present study are congruent with the few existing findings suggesting that grade by WHO 2010 classification is a good prognostic factor and reliably recognizes tumors with substantial risk for aggressive behavior.

In the light of this study, patients with G1 rectal NET may not need years-long follow-up: none showed metastases, and only three patients with a G1 NET had developed a local recurrence, probably due to incomplete removal of the primary tumor. None of the G1 tumors invaded into the muscularis propria. Thus, one follow-up endoscopy to exclude local recurrence would seem appropriate; G2 tumors, however, are another matter, because in most cases they eventually disseminate. After primary diagnosis, metastases can occur years later. One endoscopy to exclude local recurrence is adequate, but early detection of metastatic lesions is more essential. The WHO 2010 classification had better prognostic value than the previous WHO 2000

classification in rectal NETs. Although WHO 2010 classification is of excellent prognostic value, when metastatic propensity is being assessed, tumor size remains an important parameter.

TUMOR MARKERS BY IMMUNOHISTOCHEMISTRY

CYCLIN A AND KI-67

Cyclin A expression correlated with rectal NETs' metastatic potential, since all tumors with high expression of cyclin A were metastatic (II). Cyclin A can facilitate the differentiation of dysplastic nevi from thin melanoma, and Spitz nevi from melanoma (Stefanaki et al. 2007, Kiszner et al. 2014). In superficial spreading melanoma, cyclin A is an independent prognostic factor (Florenes et al. 2001). Few reports exist concerning cyclin A expression in epithelial neoplasms, but in GI-NETs, to the best of knowledge, it has not yet been described. When evaluated in neuroendocrine carcinoma of the skin (Merkel cell carcinoma), no correlation with prognosis was detectable (Koljonen et al. 2004).

Ki-67 is a strong marker of prognosis in a plethora of tumor types. It is expressed extensively during the cell cycle in dividing, proliferative cells and is probably the most widely used and best validated proliferation marker in clinical pathology. However, cell-cycle markers need testing separately in every tumor type, because duration of each cell-cycle phase varies between cell types, and what remains uncertain is whether all cells that have entered the cell cycle eventually divide. Additionally, researchers have been encouraged to study the prognostic significance of a combination of cell-cycle-associated markers (Scholzen & Gerdes 2000). We investigated the expression of two proliferation markers; G2 tumors with high cyclin A expression were all metastatic, contrasting with G2 tumors with low cyclin A expression, where 67% were metastatic, so such a combination might prove useful in identifying tumors at highest risk for metastasis. These patients need the most intensive follow-up to detect metastatic lesions at an early stage, and may benefit from adjuvant therapy even without evidence of metastatic disease.

PROX1

Colorectal adenomas and carcinomas harbor tumor stem cells with the ability to self-renew and differentiate. They play a role in tumor recurrence, in metastasis, and in development of resistance to antiangiogenic drugs. Some of these tumor stem cells express PROX1, thus contributing to their tumor stem-cell characteristics. Moreover, PROX1 is not involved in maintaining

the homeostasis of the normal epithelium, making it an interesting target for antineoplastic therapy (Wiener et al. 2014).

When adenoma of the colon transforms into invasive carcinoma, PROX1 is involved, and its expression correlates with prognosis (Kinzler & Vogelstein 1996, Petrova et al. 2008). In gastric cancer, cytoplasmic PROX1-positivity correlates with prognosis (Taban et al. 2014). In renal cell carcinoma, high PROX1 expression indicates poor prognosis (Lv et al. 2014). It has value in differential diagnostics of vascular neoplasms (Le Huu et al. 2010, Wang et al. 2013). A subset of enteroendocrine cells express PROX1 (Petrova et al. 2008), but reports are very few on PROX1 expression in neuroendocrine tumors of the GI tract. In the present study, immunohistochemical positivity for PROX1 was demonstrated in a significant proportion of rectal NETs, as well as its increased expression in metastases. PROX1 correlated with metastatic potential and prognosis, although not as strongly as did WHO 2010 grade. These results suggest that oncogenesis of rectal NETs and oncogenesis of colonic adenocarcinoma may, at least in part, share the same pathways. Although the prognostic value of the WHO 2010 classification exceeds that of PROX1, therapeutic applications may emerge in the future (Wiener 2014).

HES77

Study IV showed that the stem-cell associated marker HES77 was expressed in 28% of rectal NETs. It correlated with metastatic potential and prognosis of rectal NETs. This marker is novel: the antibody emerged when mice were immunized against a human embryonic stem-cell line. As the exact antigen epitope has not yet been revealed, its function and role in the cell remain unclear. Reports of HES77 expression are lacking, with only a few reports on the expression of other stem-cell markers in neuroendocrine neoplasms. Insulin-like growth factor II mRNA-binding protein 3 (IMP3) correlates with poor prognosis in the neuroendocrine tumors of the lung (Del Gobbo et al. 2014). Spalt-like transcription factor 4 (SALL4) and sex-determining region Y-box 2 (Sox2) are transcription factors associated with embryonic stem-cell pluripotency, immunohistochemical expression has been detected in lung small-cell neuroendocrine carcinoma, and Sox2 expression is associated with poor prognosis (Sholl, Long & Hornick 2010, Miettinen et al. 2014). In non-small-cell lung carcinoma with neuroendocrine differentiation, expression of the stem-cell markers CD117, CD133 (cluster of differentiation), and ATP-binding cassette sub-family G member 4 (ABCG4) have had no prognostic significance (Gottschling et al. 2013). Positivity of CD117 has been detectable with prognostic value in colorectal poorly differentiated NEC and MANEC (La Rosa et al. 2012). Few reports cover expression of stem-cell markers in neuroendocrine tumors, and most concern lung NETs.

In view of the present study, investigation of expression of other stem-cell markers in GI-NETs is encouraged, since it might supply important information on GI-NETs, particularly on their tumorigenesis.

STUDY LIMITATIONS

Neuroendocrine tumors tumors are very rare; approximately 20 to 30 new rectal cases occur annually in Finland (personal communication), and collecting extensive tumor series with a large number of cases is time-consuming. During the study period of 25 years, 118 from the Uusimaa region were reported to the Cancer Registry, of which 73 were included here. All of the overtly malignant, metastatic cases are probably included in the series, but it is possible that some non-metastasized G2 tumors are missing because systematic classification and grading has been common practice only in recent years, raising the proportion of metastatic tumors among G2-NETs. And of course, a higher number of tumors might have enhanced the accuracy of this study.

The present study of 73 rectal NETs included merely one G3-NEC, which is a weak point; this may in fact be due to pathologists' imperfect recognition of these tumors. If the pathologist is unaware of the entity, G3-NECs can be confused with poorly differentiated adenocarcinoma. In cases of poorly differentiated tumor without the glandular formations characteristic of adenocarcinoma, their morphology should raise suspicion of a poorly differentiated neuroendocrine tumor. This should then lead to immunohistological staining of neuroendocrine markers. Re-evaluation of poorly differentiated adenocarcinomas in order to find more NECs was beyond the resources and scope of this study. Besides, a fact already known is that poorly differentiated NECs are very aggressive neoplasms with a dismal prognosis. Whether any differences existed in metastatic potential and prognosis between G1 and G2 tumors was of more interest.

Diagnostic procedures have evolved since the 1980s, when the tumor series started: radiological work-up of patients with a recently diagnosed rectal NET was an uncommon practice, since these tumors were then considered benign. With current follow-up practices, some metastases might have been detected earlier. Follow-up of patients was more or less arbitrary. Because guidelines for treatment have existed for only a few years, surgical treatment was variable. Many tumors were removed in pieces rather than by polypectomy, occasionally resulting in incomplete removal and local recurrence. From small, fragmented pieces obtained for histology, it was sometimes impossible to reliably confirm or exclude invasion to the muscularis propria. As a result, it was impossible to determine the TNM status retrospectively, clearly a study shortcoming.

Immunohistochemistry as a delicate technique has an in-built possibility of false-positive and false-negative staining results for several reasons. We had tissue sample from only six metastases (III and IV), and a very specific conclusion cannot emerge from such a small number of cases. In the actual tumor series, whole tumors and whole sections were analyzed, but of the metastases, some of the samples were biopsies and thus represented only a small portion of the metastasis.

A number of markers failed to show a significant correlation, or had no correlation at all with the metastatic potential, or were completely negative (Tables 4 and 5), and they may not be among the most promising markers of neuroendocrine differentiation or prognosis in NETs of other parts of the GI tract. Nevertheless, one must bear in mind that NETs of different regions of the GI tract must be studied separately, and it is not entirely excluded that useful markers will eventually emerge from the group of markers which showed no prognostic value in this study.

Treatment of disseminated rectal NETs has undergone significant changes over time. As guidelines have been available only for a short period, treatment has followed differing treatment regimens, perhaps affecting patient survival.

FUTURE PROJECTS

The prognostic accuracy of the WHO classification and proliferation index are best validated in NETs of the pancreas. As the same classification is applied to all parts of the GI tract, prognostic significance ought to be studied in all of them separately. In terms of rectal tumors, it would be interesting to re-assess tumors that were diagnosed as poorly differentiated or undifferentiated adenocarcinomas, in order to discover how many of them are in fact neuroendocrine carcinomas. It would be beneficial to gradually expand the tumor series, and to thus enlarge the number of G2 tumors: this would allow verification of the proportions of different grades among rectal NETs. Follow-up periods should be longer since these tumors often metastasize very late. Although the WHO 2010 reliably predicts the prognosis of rectal NETs, additional new markers need testing in the quest for an even better marker.

CONCLUSIONS

The present studies on rectal NETs allow following conclusions:

1) Rectal neuroendocrine tumors are rare with 20 to 30 new cases diagnosed in Finland annually.

2) Around 10% of rectal NETs will develop metastases eventually. G2-NETs of the rectum have a high metastatic potential. Long follow-up is recommended, since neuroendocrine tumors could metastasize even very late. Repeated colonoscopies may not be the most effective method of follow-up; instead, early detection of metastasis should have the highest priority.

3) The WHO 2010 classification for rectal NETs has excellent prognostic accuracy and is superior to its predecessor, the WHO 2000 classification.

4) All G2-tumors with high cyclin A expression metastasized. HES77 and PROX1 correlated with prognosis and PROX1 may be involved in oncogenesis of rectal NETs.

REFERENCES

Amin, R.M., Hiroshima, K., Iyoda, A., Hoshi, K., Honma, K., Kuroki, M., Kokubo, T., Fujisawa, T., Miyagi, Y. & Nakatani, Y. 2008, "LKB1 protein expression in neuroendocrine tumors of the lung", *Pathology international,* vol. 58, no. 2, pp. 84-88.

Anthony, L.B., Strosberg, J.R., Klimstra, D.S., Maples, W.J., O'Dorisio, T.M., Warner, R.R., Wiseman, G.A., Benson, A.B.,3rd, Pommier, R.F. & North American Neuroendocrine Tumor Society (NANETS) 2010, "The NANETS consensus guidelines for the diagnosis and management of gastrointestinal neuroendocrine tumors (nets): well-differentiated nets of the distal colon and rectum", *Pancreas,* vol. 39, no. 6, pp. 767-774.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D.J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. & Edlund, H. 1999, "Notch signalling controls pancreatic cell differentiation", *Nature,* vol. 400, no. 6747, pp. 877-881.

Ardill, J.E. & Erikkson, B. 2003a, "The importance of the measurement of circulating markers in patients with neuroendocrine tumours of the pancreas and gut", *Endocrine-related cancer,* vol. 10, no. 4, pp. 459-462.

Ardill, J.E. & Erikkson, B. 2003b, "The importance of the measurement of circulating markers in patients with neuroendocrine tumours of the pancreas and gut", *Endocrine-related cancer,* vol. 10, no. 4, pp. 459-462.

Arnold, C.N., Nagasaka, T., Goel, A., Scharf, I., Grabowski, P., Sosnowski, A., Schmitt-Graff, A., Boland, C.R., Arnold, R. & Blum, H.E. 2008, "Molecular characteristics and predictors of survival in patients with malignant neuroendocrine tumors", *International journal of cancer.Journal international du cancer,* vol. 123, no. 7, pp. 1556-1564.

Arnold, R., Chen, Y.J., Costa, F., Falconi, M., Gross, D., Grossman, A.B., Hyrdel, R., Kos-Kudla, B., Salazar, R., Plockinger, U., Mallorca Consensus Conference participants & European Neuroendocrine Tumor Society 2009, "ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: follow-up and documentation", *Neuroendocrinology,* vol. 90, no. 2, pp. 227-233.

Arnold, R., Rinke, A., Klose, K.J., Muller, H.H., Wied, M., Zamzow, K., Schmidt, C., Schade-Brittinger, C., Barth, P., Moll, R., Koller, M., Unterhalt, M., Hiddemann, W., Schmidt-Lauber, M., Pavel, M. & Arnold, C.N. 2005, "Octreotide versus octreotide plus interferon-alpha in endocrine gastroenteropancreatic tumors: a randomized trial", *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association,* vol. 3, no. 8, pp. 761-771.

Balaker, A.E., Abemayor, E., Elashoff, D. & St John, M.A. 2012, "Cancer of unknown primary: does treatment modality make a difference?", *The Laryngoscope,* vol. 122, no. 6, pp. 1279-1282.

Barrett, P., Hobbs, R.C., Coates, P.J., Risdon, R.A., Wright, N.A. & Hall, P.A. 1995, "Endocrine cells of the human gastrointestinal tract have no proliferative capacity", *The Histochemical journal,* vol. 27, no. 6, pp. 482-486.

Beck, F. 2002, "Homeobox genes in gut development", *Gut,* vol. 51, no. 3, pp. 450-454.

Berber, E., Flesher, N. & Siperstein, A.E. 2002, "Laparoscopic radiofrequency ablation of neuroendocrine liver metastases", *World journal of surgery,* vol. 26, no. 8, pp. 985-990.

Berge, T. & Linell, F. 1976, "Carcinoid tumours. Frequency in a defined population during a 12-year period", *Acta pathologica et microbiologica Scandinavica.Section A, Pathology,* vol. 84, no. 4, pp. 322-330.

Bernheim, A.M., Connolly, H.M., Hobday, T.J., Abel, M.D. & Pellikka, P.A. 2007, "Carcinoid heart disease", *Progress in cardiovascular diseases,* vol. 49, no. 6, pp. 439-451.

Bernick, P.E., Klimstra, D.S., Shia, J., Minsky, B., Saltz, L., Shi, W., Thaler, H., Guillem, J., Paty, P., Cohen, A.M. & Wong, W.D. 2004, "Neuroendocrine carcinomas of the colon and rectum", *Diseases of the colon and rectum,* vol. 47, no. 2, pp. 163-169.

Bhattacharyya, S., Toumpanakis, C., Caplin, M.E. & Davar, J. 2008, "Usefulness of N-terminal pro-brain natriuretic peptide as a biomarker of the presence of carcinoid heart disease", *The American Journal of Cardiology,* vol. 102, no. 7, pp. 938-942.

Bishop, A.E., Pietroletti, R., Taat, C.W., Brummelkamp, W.H. & Polak, J.M. 1987, "Increased populations of endocrine cells in Crohn's ileitis", *Virchows Archiv.A, Pathological anatomy and histopathology,* vol. 410, no. 5, pp. 391-396.

Booth, D.G., Takagi, M., Sanchez-Pulido, L., Petfalski, E., Vargiu, G., Samejima, K., Imamoto, N., Ponting, C.P., Tollervey, D., Earnshaw, W.C. & Vagnarelli, P. 2014, "Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery", *eLife,* vol. 3, pp. e01641.

Bosman, F.T., Carneiro, F., Hruban, R. & Theise, N. 2010, *WHO Classification of Tumours of the Digestive System*, 4th Edition edn, International Agency for Research on Cancer (IARC), Lyon.

Bostrom, P., Soderstrom, M., Palokangas, T., Vahlberg, T., Collan, Y., Carpen, O. & Hirsimaki, P. 2009, "Analysis of cyclins A, B1, D1 and E in

breast cancer in relation to tumour grade and other prognostic factors", *BMC research notes,* vol. 2, pp. 140.

Brenner, B., Tang, L.H., Klimstra, D.S. & Kelsen, D.P. 2004, "Small-cell carcinomas of the gastrointestinal tract: a review", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 22, no. 13, pp. 2730-2739.

Brenner, B., Tang, L.H., Shia, J., Klimstra, D.S. & Kelsen, D.P. 2007, "Small cell carcinomas of the gastrointestinal tract: clinicopathological features and treatment approach", *Seminars in oncology,* vol. 34, no. 1, pp. 43-50.

Brenner, H., Bouvier, A.M., Foschi, R., Hackl, M., Larsen, I.K., Lemmens, V., Mangone, L., Francisci, S. & EUROCARE Working Group 2012, "Progress in colorectal cancer survival in Europe from the late 1980s to the early 21st century: the EUROCARE study", *International journal of cancer.Journal international du cancer,* vol. 131, no. 7, pp. 1649-1658.

Burton, S., Brown, G., Bees, N., Norman, A., Biedrzycki, O., Arnaout, A., Abulafi, A.M. & Swift, R.I. 2008, "Accuracy of CT prediction of poor prognostic features in colonic cancer", *The British journal of radiology,* vol. 81, no. 961, pp. 10-19.

Bushnell, D.L.,Jr, O'Dorisio, T.M., O'Dorisio, M.S., Menda, Y., Hicks, R.J., Van Cutsem, E., Baulieu, J.L., Borson-Chazot, F., Anthony, L., Benson, A.B., Oberg, K., Grossman, A.B., Connolly, M., Bouterfa, H., Li, Y., Kacena, K.A., LaFrance, N. & Pauwels, S.A. 2010, "90Y-edotreotide for metastatic carcinoid refractory to octreotide", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 28, no. 10, pp. 1652-1659.

Bussolati, G., Volante, M. & Papotti, M. 2001, "Classic and recent special stains used in differential diagnosis of endocrine tumors", *Endocrine pathology,* vol. 12, no. 4, pp. 379-387.

Cadden, I., Johnston, B.T., Turner, G., McCance, D., Ardill, J. & McGinty, A. 2007, "An evaluation of cyclooxygenase-2 as a prognostic biomarker in mid-gut carcinoid tumours", *Neuroendocrinology,* vol. 86, no. 2, pp. 104-111.

Capella, C., Solcia, E., Sobin, L.H. & Arnold, R. 2000, "Endocrine tumours of the colon and rectum" in *World Health Organization classification of tumours, pathology and genetics of tumours of the digestive system*, eds. S.R. Hamilton & L.A. Aaltonen, 1st edition edn, IARC Press, Lyon, pp. 137-139.

Caplin, M., Sundin, A., Nillson, O., Baum, R.P., Klose, K.J., Kelestimur, F., Plockinger, U., Papotti, M., Salazar, R., Pascher, A. & Barcelona Consensus Conference participants 2012a, "ENETS Consensus Guidelines for the management of patients with digestive neuroendocrine neoplasms: colorectal neuroendocrine neoplasms", *Neuroendocrinology,* vol. 95, no. 2, pp. 88-97.

Caplin, M., Sundin, A., Nillson, O., Baum, R.P., Klose, K.J., Kelestimur, F., Plockinger, U., Papotti, M., Salazar, R., Pascher, A. & Barcelona Consensus Conference participants 2012b, "ENETS Consensus Guidelines for the management of patients with digestive neuroendocrine neoplasms: colorectal neuroendocrine neoplasms", *Neuroendocrinology,* vol. 95, no. 2, pp. 88-97.

Capurso, G., Lattimore, S., Crnogorac-Jurcevic, T., Panzuto, F., Milione, M., Bhakta, V., Campanini, N., Swift, S.M., Bordi, C., Delle Fave, G. & Lemoine, N.R. 2006, "Gene expression profiles of progressive pancreatic endocrine tumours and their liver metastases reveal potential novel markers and therapeutic targets", *Endocrine-related cancer,* vol. 13, no. 2, pp. 541-558.

Cases, A.I., Ohtsuka, T., Fujino, M., Ideno, N., Kozono, S., Zhao, M., Ohuchida, K., Aishima, S., Nomura, M., Oda, Y., Mizumoto, K. & Tanaka, M. 2014, "Expression of glucagon-like Peptide 1 receptor and its effects on biologic behavior in pancreatic neuroendocrine tumors", *Pancreas,* vol. 43, no. 1, pp. 1-6.

Chamberlain, R.S., Canes, D., Brown, K.T., Saltz, L., Jarnagin, W., Fong, Y. & Blumgart, L.H. 2000, "Hepatic neuroendocrine metastases: does intervention alter outcomes?", *Journal of the American College of Surgeons,* vol. 190, no. 4, pp. 432-445.

Chetty, R., Klimstra, D.S., Henson, D.E. & Albores-Saavedra, J. 2010, "Combined classical carcinoid and goblet cell carcinoid tumor: a new morphologic variant of carcinoid tumor of the appendix", *The American Journal of Surgical Pathology,* vol. 34, no. 8, pp. 1163-1167.

Cooper, W.A., Kohonen-Corish, M.R., McCaughan, B., Kennedy, C., Sutherland, R.L. & Lee, C.S. 2009, "Expression and prognostic significance of cyclin B1 and cyclin A in non-small cell lung cancer", *Histopathology,* vol. 55, no. 1, pp. 28-36.

Corrin, B., Gilby, E.D., McMillan, M., Patrick, J. & Spencer, H. 1971, "Ultrastructural evidence of secretion in hormonally active oat-cell carcinomas: origin of one such tumour in the pancreas", *Journal of clinical pathology,* vol. 24, no. 5, pp. 481.

Cruickshank, B. & Cunningham, A.W. 1949, "The carcinoid tumour; a review of 17 cases", *Edinburgh medical journal,* vol. 56, no. 5, pp. 196-205.

Del Gobbo, A., Vaira, V., Rocco, E.G., Palleschi, A., Bulfamante, G., Ricca, D., Fiori, S., Bosari, S. & Ferrero, S. 2014, "The Oncofetal Protein IMP3: A Useful Marker to Predict Poor Clinical Outcome in Neuroendocrine Tumors of the Lung", *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer,* .

Dyer, M.A., Livesey, F.J., Cepko, C.L. & Oliver, G. 2003, "Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina", *Nature genetics,* vol. 34, no. 1, pp. 53-58.

Dzietczenia, J., Wrobel, T., Jazwiec, B., Mazur, G., Butrym, A. & Kuliczkowski, K. 2011, "Expression of cyclin A and bone morphogenetic protein receptors and response to induction therapy in patients with acute leukemias", *Leukemia & lymphoma,* vol. 52, no. 12, pp. 2336-2341.

Elias, D., Lasser, P., Ducreux, M., Duvillard, P., Ouellet, J.F., Dromain, C., Schlumberger, M., Pocard, M., Boige, V., Miquel, C. & Baudin, E. 2003, "Liver resection (and associated extrahepatic resections) for metastatic well-differentiated endocrine tumors: a 15-year single center prospective study", *Surgery,* vol. 133, no. 4, pp. 375-382.

Elsir, T., Qu, M., Berntsson, S.G., Orrego, A., Olofsson, T., Lindstrom, M.S., Nister, M., von Deimling, A., Hartmann, C., Ribom, D. & Smits, A. 2011, "PROX1 is a predictor of survival for gliomas WHO grade II", *British journal of cancer,* vol. 104, no. 11, pp. 1747-1754.

Engelman, E.S., Leon-Ferre, R., Naraev, B.G., Sharma, N., Sun, S., O'Dorisio, T.M., Howe, J., Button, A., Zamba, G. & Halfdanarson, T.R. 2014, "Comparison of transarterial liver-directed therapies for low-grade metastatic neuroendocrine tumors in a single institution", *Pancreas,* vol. 43, no. 2, pp. 219-225.

Erickson, L.A., Papouchado, B., Dimashkieh, H., Zhang, S., Nakamura, N. & Lloyd, R.V. 2004, "Cdx2 as a marker for neuroendocrine tumors of unknown primary sites", *Endocrine pathology,* vol. 15, no. 3, pp. 247-252.

Eriksson, B., Kloppel, G., Krenning, E., Ahlman, H., Plockinger, U., Wiedenmann, B., Arnold, R., Auernhammer, C., Korner, M., Rindi, G., Wildi, S. & Frascati Consensus Conference participants 2008a, "Consensus guidelines for the management of patients with digestive neuroendocrine tumors--well-differentiated jejunal-ileal tumor/carcinoma", *Neuroendocrinology,* vol. 87, no. 1, pp. 8-19.

Eriksson, J., Stalberg, P., Nilsson, A., Krause, J., Lundberg, C., Skogseid, B., Granberg, D., Eriksson, B., Akerstrom, G. & Hellman, P. 2008b, "Surgery and radiofrequency ablation for treatment of liver metastases from midgut and foregut carcinoids and endocrine pancreatic tumors", *World journal of surgery,* vol. 32, no. 5, pp. 930-938.

Ezziddin, S., Opitz, M., Attassi, M., Biermann, K., Sabet, A., Guhlke, S., Brockmann, H., Willinek, W., Wardelmann, E., Biersack, H.J. & Ahmadzadehfar, H. 2011, "Impact of the Ki-67 proliferation index on response to peptide receptor radionuclide therapy", *European journal of nuclear medicine and molecular imaging,* vol. 38, no. 3, pp. 459-466.

Fahy, B.N., Tang, L.H., Klimstra, D., Wong, W.D., Guillem, J.G., Paty, P.B., Temple, L.K., Shia, J. & Weiser, M.R. 2007, "Carcinoid of the rectum risk stratification (CaRRS): a strategy for preoperative outcome assessment", *Annals of surgical oncology,* vol. 14, no. 2, pp. 396-404.

Faiss, S., Pape, U.F., Bohmig, M., Dorffel, Y., Mansmann, U., Golder, W., Riecken, E.O., Wiedenmann, B. & International Lanreotide and Interferon Alfa Study Group 2003, "Prospective, randomized, multicenter trial on the antiproliferative effect of lanreotide, interferon alfa, and their combination for therapy of metastatic neuroendocrine gastroenteropancreatic tumors--the International Lanreotide and Interferon Alfa Study Group", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 21, no. 14, pp. 2689-2696.

Ferrari, L., Seregni, E., Bajetta, E., Martinetti, A. & Bombardieri, E. 1999, "The biological characteristics of chromogranin A and its role as a circulating marker in neuroendocrine tumours", *Anticancer Research,* vol. 19, no. 4C, pp. 3415-3427.

Feyrter, F. 1956, "Zur Lehre von den periferen endocrinen (parakrinen) Drüsen des Menschen. Neue Erkentnisse.", *Wien Med Wochenschau,* .

Feyrter, F. 1938, "Ûber diffuse endocrine epitheliale Organe.", *Leipzig Zentr Inn Med,* vol. 29, pp. 545-571.

Fiore, F., Del Prete, M., Franco, R., Marotta, V., Ramundo, V., Marciello, F., Di Sarno, A., Carratu, A.C., de Luca di Roseto, C., Colao, A. & Faggiano, A. 2014, "Transarterial embolization (TAE) is equally effective and slightly safer than transarterial chemoembolization (TACE) to manage liver metastases in neuroendocrine tumors", *Endocrine,* vol. 47, no. 1, pp. 177-182.

Florenes, V.A., Maelandsmo, G.M., Faye, R., Nesland, J.M. & Holm, R. 2001, "Cyclin A expression in superficial spreading malignant melanomas correlates with clinical outcome", *The Journal of pathology,* vol. 195, no. 5, pp. 530-536.

Fu, K., Igarashi, S., Jindo, O., Ishikawa, T., Hirabayashi, K., Kotake, K., Matsui, T., Azuma, H. & Kaji, Y. 2008, "Synchronous colitic cancers and microcarcinoids in a patient with long-standing and extensive ulcerative colitis: a case report and review of the literature", *Surgical laparoscopy, endoscopy & percutaneous techniques,* vol. 18, no. 3, pp. 304-307.

Furihata, M., Ishikawa, T., Inoue, A., Yoshikawa, C., Sonobe, H., Ohtsuki, Y., Araki, K. & Ogoshi, S. 1996, "Determination of the prognostic significance of unscheduled cyclin A overexpression in patients with esophageal squamous cell carcinoma", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 2, no. 10, pp. 1781-1785.

Gabriel, M., Decristoforo, C., Kendler, D., Dobrozemsky, G., Heute, D., Uprimny, C., Kovacs, P., Von Guggenberg, E., Bale, R. & Virgolini, I.J. 2007, "68Ga-DOTA-Tyr3-octreotide PET in neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and CT", *Journal of nuclear medicine : official publication, Society of Nuclear Medicine,* vol. 48, no. 4, pp. 508-518.

Gagne, F., Fortin, P., Dufour, V. & Delage, C. 1969, "Tumors of the appendix associating histologic features of carcinoid and adenocarcinoma", *Annales d'Anatomie Pathologique,* vol. 14, no. 4, pp. 393-406.

Garcia-Carbonero, R., Capdevila, J., Crespo-Herrero, G., Diaz-Perez, J.A., Martinez Del Prado, M.P., Alonso Orduna, V., Sevilla-Garcia, I., Villabona-Artero, C., Beguiristain-Gomez, A., Llanos-Munoz, M., Marazuela, M., Alvarez-Escola, C., Castellano, D., Vilar, E., Jimenez-Fonseca, P., Teule, A., Sastre-Valera, J., Benavent-Vinuelas, M., Monleon, A. & Salazar, R. 2010, "Incidence, patterns of care and prognostic factors for outcome of gastroenteropancreatic neuroendocrine tumors (GEP-NETs): results from the National Cancer Registry of Spain (RGETNE)", *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO,* vol. 21, no. 9, pp. 1794-1803.

Gerdes, J., Li, L., Schlueter, C., Duchrow, M., Wohlenberg, C., Gerlach, C., Stahmer, I., Kloth, S., Brandt, E. & Flad, H.D. 1991, "Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67", *The American journal of pathology,* vol. 138, no. 4, pp. 867-873.

Gerdes, J., Schwab, U., Lemke, H. & Stein, H. 1983, "Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation", *International journal of cancer.Journal international du cancer,* vol. 31, no. 1, pp. 13-20.

Gledhill, A., Enticott, M.E. & Howe, S. 1986, "Variation in the argyrophil cell population of the rectum in ulcerative colitis and adenocarcinoma", *The Journal of pathology,* vol. 149, no. 4, pp. 287-291.

Gottschling, S., Jensen, K., Herth, F.J., Thomas, M., Schnabel, P.A. & Herpel, E. 2013, "Lack of prognostic significance of neuroendocrine differentiation and stem cell antigen co-expression in resected early-stage non-small cell lung cancer", *Anticancer Research,* vol. 33, no. 3, pp. 981-990.

Grimelius, L. 1968, "The argyrophil reaction in islet cells of adult human pancreas studies with a new silver nitrate procedure", *Acta Societatis Medicorum Upsaliensis,* vol. 73, no. 5-6, pp. 271-294.

Gustafsson, B.I., Hauso, O., Drozdov, I., Kidd, M. & Modlin, I.M. 2008, "Carcinoid heart disease", *International journal of cardiology,* vol. 129, no. 3, pp. 318-324.

Hainsworth, J.D., Rubin, M.S., Spigel, D.R., Boccia, R.V., Raby, S., Quinn, R. & Greco, F.A. 2013, "Molecular gene expression profiling to predict the tissue of origin and direct site-specific therapy in patients with carcinoma of unknown primary site: a prospective trial of the Sarah Cannon research institute", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 31, no. 2, pp. 217-223.

Hamilton, S.R. & Aaltonen, L.A. (eds) 2000, *Pathology and Genetics of tumours of the Digestive system*, 3rd edn, IARC Press, Lyon.

Hardcastle, J.D., Chamberlain, J.O., Robinson, M.H., Moss, S.M., Amar, S.S., Balfour, T.W., James, P.D. & Mangham, C.M. 1996, "Randomised controlled trial of faecal-occult-blood screening for colorectal cancer", *Lancet,* vol. 348, no. 9040, pp. 1472-1477.

Haynes, L.L., Shirley, L.E. & Hume, W.I.,Jr 1953, "Carcinoid tumors of the rectum; a report of three cases", *Surgery,* vol. 33, no. 4, pp. 567-578.

Hemminki, K. & Li, X. 2001, "Incidence trends and risk factors of carcinoid tumors: a nationwide epidemiologic study from Sweden", *Cancer,* vol. 92, no. 8, pp. 2204-2210.

Hemminki, K., Li, X., Sundquist, J. & Sundquist, K. 2008, "Cancer risks in ulcerative colitis patients", *International journal of cancer.Journal international du cancer,* vol. 123, no. 6, pp. 1417-1421.

Heverhagen, A.E., Geis, C., Fendrich, V., Ramaswamy, A., Montalbano, R., Di Fazio, P., Bartsch, D.K., Ocker, M. & Quint, K. 2013, "Embryonic transcription factors CDX2 and Oct4 are overexpressed in neuroendocrine tumors of the ileum: a pilot study", *European surgical research.Europaische chirurgische Forschung.Recherches chirurgicales europeennes,* vol. 51, no. 1-2, pp. 14-20.

Hotta, K., Shimoda, T., Nakanishi, Y. & Saito, D. 2006, "Usefulness of Ki-67 for predicting the metastatic potential of rectal carcinoids", *Pathology international,* vol. 56, no. 10, pp. 591-596.

House, M.G., Herman, J.G., Guo, M.Z., Hooker, C.M., Schulick, R.D., Lillemoe, K.D.,
Cameron, J.L., Hruban, R.H., Maitra, A. & Yeo, C.J. 2003, "Aberrant hypermethylation of tumor suppressor genes in pancreatic endocrine neoplasms", *Annals of Surgery,* vol. 238, no. 3, pp. 423-31; discussion 431-2.

Ito, T., Sasano, H., Tanaka, M., Osamura, R.Y., Sasaki, I., Kimura, W., Takano, K., Obara, T., Ishibashi, M., Nakao, K., Doi, R., Shimatsu, A., Nishida, T., Komoto, I., Hirata, Y., Nakamura, K., Igarashi, H., Jensen, R.T., Wiedenmann, B. & Imamura, M. 2010, "Epidemiological study of gastroenteropancreatic neuroendocrine tumors in Japan", *Journal of gastroenterology,* vol. 45, no. 2, pp. 234-243.

Ito, T., Tanaka, M., Sasano, H., Osamura, Y.R., Sasaki, I., Kimura, W., Takano, K., Obara, T., Ishibashi, M., Nakao, K., Doi, R., Shimatsu, A., Nishida, T., Komoto, I., Hirata, Y., Imamura, M., Kawabe, K., Nakamura, K. & Neuroendocrine Tumor Workshop of Japan 2007, "Preliminary results of a Japanese nationwide survey of neuroendocrine

gastrointestinal tumors", *Journal of gastroenterology,* vol. 42, no. 6, pp. 497-500.

Jaffee, I.M., Rahmani, M., Singhal, M.G. & Younes, M. 2006, "Expression of the intestinal transcription factor CDX2 in carcinoid tumors is a marker of midgut origin", *Archives of Pathology & Laboratory Medicine,* vol. 130, no. 10, pp. 1522-1526.

Janson, E.T., Sorbye, H., Welin, S., Federspiel, B., Gronbaek, H., Hellman, P., Ladekarl, M., Langer, S.W., Mortensen, J., Schalin-Jantti, C., Sundin, A., Sundlov, A., Thiis-Evensen, E. & Knigge, U. 2014, "Nordic guidelines 2014 for diagnosis and treatment of gastroenteropancreatic neuroendocrine neoplasms", *Acta Oncologica (Stockholm, Sweden),* vol. 53, no. 10, pp. 1284-1297.

Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Jensen, J., Kedinger, M. & Gradwohl, G. 2002, "Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium", *The EMBO journal,* vol. 21, no. 23, pp. 6338-6347.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P. & Madsen, O.D. 2000, "Control of endodermal endocrine development by Hes-1", *Nature genetics,* vol. 24, no. 1, pp. 36-44.

Jetmore, A.B., Ray, J.E., Gathright, J.B.,Jr, McMullen, K.M., Hicks, T.C. & Timmcke, A.E. 1992, "Rectal carcinoids: the most frequent carcinoid tumor", *Diseases of the colon and rectum,* vol. 35, no. 8, pp. 717-725.

Jiao, Y., Shi, C., Edil, B.H., de Wilde, R.F., Klimstra, D.S., Maitra, A., Schulick, R.D., Tang, L.H., Wolfgang, C.L., Choti, M.A., Velculescu, V.E., Diaz, L.A.,Jr, Vogelstein, B., Kinzler, K.W., Hruban, R.H. & Papadopoulos, N. 2011, "DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors", *Science (New York, N.Y.),* vol. 331, no. 6021, pp. 1199-1203.

Johnson, L.A., Lavin, P., Moertel, C.G., Weiland, L., Dayal, Y., Doos, W.G., Geller, S.A., Cooper, H.S., Nime, F., Masse, S., Simson, I.W., Sumner, H., Folsch, E. & Engstrom, P. 1983, "Carcinoids: the association of histologic growth pattern and survival", *Cancer,* vol. 51, no. 5, pp. 882-889.

Jorda, M., Ghorab, Z., Fernandez, G., Nassiri, M., Hanly, A. & Nadji, M. 2003, "Low nuclear proliferative activity is associated with nonmetastatic islet cell tumors", *Archives of Pathology & Laboratory Medicine,* vol. 127, no. 2, pp. 196-199.

Karam, S. & Leblond, C.P. 1995, "Origin and migratory pathways of the eleven epithelial cell types present in the body of the mouse stomach", *Microscopy research and technique,* vol. 31, no. 3, pp. 193-214.

Karpathakis, A., Dibra, H. & Thirlwell, C. 2013, "Neuroendocrine tumours: cracking the epigenetic code", *Endocrine-related cancer,* vol. 20, no. 3, pp. R65-82.

Kawahara, M., Kammori, M., Kanauchi, H., Noguchi, C., Kuramoto, S., Kaminishi, M., Endo, H. & Takubo, K. 2002, "Immunohistochemical prognostic indicators of gastrointestinal carcinoid tumours", *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology,* vol. 28, no. 2, pp. 140-146.

Kennedy, A., Bester, L., Salem, R., Sharma, R.A., Parks, R.W. & Ruszniewski, P. 2014, "Role of hepatic intra-arterial therapies in metastatic neuroendocrine tumours (NET): guidelines from the NET-Liver-Metastases Consensus Conference", *HPB : the official journal of the International Hepato Pancreato Biliary Association, .*

Kianmanesh, R., O'toole, D., Sauvanet, A., Ruszniewski, P. & Belghiti, J. 2005, "Surgical treatment of gastric, enteric, and pancreatic endocrine tumors Part 1. Treatment of primary endocrine tumors", *Journal de chirurgie,* vol. 142, no. 3, pp. 132-149.

Kinzler, K.W. & Vogelstein, B. 1996, "Lessons from hereditary colorectal cancer", *Cell,* vol. 87, no. 2, pp. 159-170.

Kiszner, G., Wichmann, B., Nemeth, I.B., Varga, E., Meggyeshazi, N., Teleki, I., Balla, P., Maros, M.E., Penksza, K. & Krenacs, T. 2014, "Cell cycle analysis can differentiate thin melanomas from dysplastic nevi and reveals accelerated replication in thick melanomas", *Virchows Archiv : an international journal of pathology,* vol. 464, no. 5, pp. 603-612.

Klimstra, D.S., Arnold, R., Capella, C., Klöppel, G., Komminoth, P., Solcia, E. & Rindi, G. 2010, "Neuroendocrine neoplasms of the colon and rectum" in *WHO Classification of Tumours of the Digestive System,* eds. F.T. Bosman, F. Carneiro, R. Hruban & N. Theise, 4th edn, IARC, Lyon, pp. 174-177.

Kloppel, G. 2011, "Classification and pathology of gastroenteropancreatic neuroendocrine neoplasms", *Endocrine-related cancer,* vol. 18 Suppl 1, pp. S1-16.

Kloppel, G., Dege, K., Remmele, W., Kapran, Y., Tuzlali, S. & Modlin, I.M. 2007, "Siegfried Oberndorfer: a tribute to his work and life between Munich, Kiel, Geneva, and Istanbul", *Virchows Archiv : an international journal of pathology,* vol. 451 Suppl 1, pp. S3-7.

Kloppel, G., Perren, A. & Heitz, P.U. 2004, "The gastroenteropancreatic neuroendocrine cell system and its tumors: the WHO classification", *Annals of the New York Academy of Sciences,* vol. 1014, pp. 13-27.

Kolby, L., Bernhardt, P., Sward, C., Johanson, V., Ahlman, H., Forssell-Aronsson, E., Stridsberg, M., Wangberg, B. & Nilsson, O. 2004a, "Chromogranin A as a determinant of midgut carcinoid tumour volume", *Regulatory peptides,* vol. 120, no. 1-3, pp. 269-273.

Kolby, L., Bernhardt, P., Sward, C., Johanson, V., Ahlman, H., Forssell-Aronsson, E., Stridsberg, M., Wangberg, B. & Nilsson, O. 2004b, "Chromogranin A as a determinant of midgut carcinoid tumour volume", *Regulatory peptides,* vol. 120, no. 1-3, pp. 269-273.

Koljonen, V., Tukiainen, E., Haglund, C. & Bohling, T. 2004, "Expression of cyclin A in Merkel cell carcinoma", *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica,* vol. 112, no. 1, pp. 39-44.

Konishi, T., Watanabe, T., Kishimoto, J., Kotake, K., Muto, T., Nagawa, H. & Japanese Society for Cancer of the Colon and Rectum 2007, "Prognosis and risk factors of metastasis in colorectal carcinoids: results of a nationwide registry over 15 years", *Gut,* vol. 56, no. 6, pp. 863-868.

Korse, C.M., Taal, B.G., van Velthuysen, M.L. & Visser, O. 2013, "Incidence and survival of neuroendocrine tumours in the Netherlands according to histological grade: experience of two decades of cancer registry", *European journal of cancer (Oxford, England : 1990),* vol. 49, no. 8, pp. 1975-1983.

Krausz, Y., Freedman, N., Rubinstein, R., Lavie, E., Orevi, M., Tshori, S., Salmon, A., Glaser, B., Chisin, R., Mishani, E. & J Gross, D. 2011, "68Ga-DOTA-NOC PET/CT imaging of neuroendocrine tumors: comparison with (1)(1)(1)In-DTPA-octreotide (OctreoScan(R))", *Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging,* vol. 13, no. 3, pp. 583-593.

Kulchitsky, N. 1897, "Zur Frage über den Bau des Darmkanals", *Arch Mikr Anat,* , pp. 49.

Kwaan, M.R., Goldberg, J.E. & Bleday, R. 2008, "Rectal carcinoid tumors: review of results after endoscopic and surgical therapy", *Archives of surgery (Chicago, Ill.: 1960),* vol. 143, no. 5, pp. 471-475.

Kwekkeboom, D.J., de Herder, W.W., Kam, B.L., van Eijck, C.H., van Essen, M., Kooij, P.P., Feelders, R.A., van Aken, M.O. & Krenning, E.P. 2008, "Treatment with the radiolabeled somatostatin analog [177 Lu-DOTA 0,Tyr3]octreotate: toxicity, efficacy, and survival", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 26, no. 13, pp. 2124-2130.

Kwekkeboom, D.J., de Herder, W.W., van Eijck, C.H., Kam, B.L., van Essen, M., Teunissen, J.J. & Krenning, E.P. 2010, "Peptide receptor radionuclide therapy in patients with gastroenteropancreatic neuroendocrine tumors", *Seminars in nuclear medicine,* vol. 40, no. 2, pp. 78-88.

Kyushima, N., Watanabe, J., Hata, H., Jobo, T., Kameya, T. & Kuramoto, H. 2002, "Expression of cyclin A in endometrial adenocarcinoma and its correlation with proliferative activity and clinicopathological variables", *Journal of cancer research and clinical oncology,* vol. 128, no. 6, pp. 307-312.

La Rosa, S., Marando, A., Furlan, D., Sahnane, N. & Capella, C. 2012, "Colorectal poorly differentiated neuroendocrine carcinomas and mixed adenoneuroendocrine carcinomas: insights into the diagnostic immunophenotype, assessment of methylation profile, and search for prognostic markers", *The American Journal of Surgical Pathology,* vol. 36, no. 4, pp. 601-611.

Le Huu, A.R., Jokinen, C.H., Rubin, B.P., Mihm, M.C., Weiss, S.W., North, P.E. & Dadras, S.S. 2010, "Expression of prox1, lymphatic endothelial nuclear transcription factor, in Kaposiform hemangioendothelioma and tufted angioma", *The American Journal of Surgical Pathology,* vol. 34, no. 11, pp. 1563-1573.

Li, C.C., Hirowaka, M., Qian, Z.R., Xu, B. & Sano, T. 2002, "Expression of E-cadherin, b-catenin, and Ki-67 in goblet cell carcinoids of the appendix: an immunohistochemical study with clinical correlation", *Endocrine pathology,* vol. 13, no. 1, pp. 47-58.

Lin, X., Saad, R.S., Luckasevic, T.M., Silverman, J.F. & Liu, Y. 2007, "Diagnostic value of CDX-2 and TTF-1 expressions in separating metastatic neuroendocrine neoplasms of unknown origin", *Applied Immunohistochemistry & Molecular Morphology : AIMM / Official Publication of the Society for Applied Immunohistochemistry,* vol. 15, no. 4, pp. 407-414.

Liu, J., Wang, Z.Q., Zhang, Z.Q., Chen, X. & Zhang, Y. 2013, "Evaluation of colonoscopy in the diagnosis and treatment of rectal carcinoid tumors with diameter less than 1 cm in 21 patients", *Oncology letters,* vol. 5, no. 5, pp. 1667-1671.

Lloyd, R.V. 2003, "Practical markers used in the diagnosis of neuroendocrine tumors", *Endocrine pathology,* vol. 14, no. 4, pp. 293-301.

Louthan, O. 2011, "Chromogranin a in physiology and oncology", *Folia biologica,* vol. 57, no. 5, pp. 173-181.

Lv, T., Liu, Y., Zhang, J., Xu, L., Zhu, Y., Yin, H., An, H., Lin, Z., Xie, Y. & Chen, L. 2014, "Impact of an altered PROX1 expression on clinicopathology, prognosis and progression in renal cell carcinoma", *PloS one,* vol. 9, no. 5, pp. e95996.

Maher, E.R., Neumann, H.P. & Richard, S. 2011, "von Hippel-Lindau disease: a clinical and scientific review", *European journal of human genetics : EJHG,* vol. 19, no. 6, pp. 617-623.

Marinoni, I., Kurrer, A.S., Vassella, E., Dettmer, M., Rudolph, T., Banz, V., Hunger, F., Pasquinelli, S., Speel, E.J. & Perren, A. 2014, "Loss of DAXX and ATRX are associated with chromosome instability and reduced survival of patients with pancreatic neuroendocrine tumors", *Gastroenterology,* vol. 146, no. 2, pp. 453-60.e5.

Marrache, F., Vullierme, M.P., Roy, C., El Assoued, Y., Couvelard, A., O'Toole, D., Mitry, E., Hentic, O., Hammel, P., Levy, P., Ravaud, P., Rougier, P. & Ruszniewski, P. 2007, "Arterial phase enhancement and body mass index are predictors of response to chemoembolisation for liver metastases of endocrine tumours", *British journal of cancer,* vol. 96, no. 1, pp. 49-55.

Masson, P. 1928, "Carcinoids (Argentaffin-Cell Tumors) and Nerve Hyperplasia of the Appendicular Mucosa", *The American journal of pathology,* vol. 4, no. 3, pp. 181-212.19.

Matsui, K., Iwase, T. & Kitagawa, M. 1993, "Small, polypoid-appearing carcinoid tumors of the rectum: clinicopathologic study of 16 cases and effectiveness of endoscopic treatment", *The American Journal of Gastroenterology,* vol. 88, no. 11, pp. 1949-1953.

Matsumoto, T., Iida, M., Suekane, H., Tominaga, M., Yao, T. & Fujishima, M. 1991, "Endoscopic ultrasonography in rectal carcinoid tumors: contribution to selection of therapy", *Gastrointestinal endoscopy,* vol. 37, no. 5, pp. 539-542.

McDermott, F.D., Heeney, A., Courtney, D., Mohan, H. & Winter, D. 2014, "Rectal carcinoids: a systematic review", *Surgical endoscopy,* vol. 28, no. 7, pp. 2020-2026.

McKeown, F. 1952, "Oat-cell carcinoma of the oesophagus", *The Journal of pathology and bacteriology,* vol. 64, no. 4, pp. 889-891.

Miettinen, M., Wang, Z., McCue, P.A., Sarlomo-Rikala, M., Rys, J., Biernat, W., Lasota, J. & Lee, Y.S. 2014, "SALL4 expression in germ cell and non-germ cell tumors: a systematic immunohistochemical study of 3215 cases", *The American Journal of Surgical Pathology,* vol. 38, no. 3, pp. 410-420.

Miettinen, M. & Wang, Z.F. 2012, "Prox1 transcription factor as a marker for vascular tumors-evaluation of 314 vascular endothelial and 1086 nonvascular tumors", *The American Journal of Surgical Pathology,* vol. 36, no. 3, pp. 351-359.

Miller, R.R. & Sumner, H.W. 1982, "Argyrophilic cell hyperplasia and an atypical carcinoid tumor in chronic ulcerative colitis", *Cancer,* vol. 50, no. 12, pp. 2920-2925.

Modlin, I.M., Kidd, M., Latich, I., Zikusoka, M.N. & Shapiro, M.D. 2005, "Current status of gastrointestinal carcinoids", *Gastroenterology,* vol. 128, no. 6, pp. 1717-1751.

Modlin, I.M., Lye, K.D. & Kidd, M. 2003, "A 5-decade analysis of 13,715 carcinoid tumors", *Cancer,* vol. 97, no. 4, pp. 934-959.

Modlin, I.M., Pavel, M., Kidd, M. & Gustafsson, B.I. 2010, "Review article: somatostatin analogues in the treatment of gastroenteropancreatic neuroendocrine (carcinoid) tumours", *Alimentary Pharmacology & Therapeutics,* vol. 31, no. 2, pp. 169-188.

Morin, E., Cheng, S., Mete, O., Serra, S., Araujo, P.B., Temple, S., Cleary, S., Gallinger, S., Greig, P.D., McGilvray, I., Wei, A., Asa, S.L. & Ezzat, S. 2013, "Hormone profiling, WHO 2010 grading, and AJCC/UICC staging in pancreatic neuroendocrine tumor behavior", *Cancer medicine,* vol. 2, no. 5, pp. 701-711.

Morris, G.J., Greco, F.A., Hainsworth, J.D., Engstrom, P.F., Scialla, S., Jordan, W.E.,3rd & Thomas, L.C. 2010, "Cancer of unknown primary site", *Seminars in oncology,* vol. 37, no. 2, pp. 71-79.

Mrena, J., Wiksten, J.P., Kokkola, A., Nordling, S., Haglund, C. & Ristimaki, A. 2006, "Prognostic significance of cyclin A in gastric cancer", *International journal of cancer.Journal international du cancer,* vol. 119, no. 8, pp. 1897-1901.

Murray, S.E., Lloyd, R.V., Sippel, R.S. & Chen, H. 2013, "Clinicopathologic characteristics of colonic carcinoid tumors", *The Journal of surgical research,* vol. 184, no. 1, pp. 183-188.

Myerson, A. 1917, "The Argyrophilic Granules of the sympathetic Nervous System", *The Journal of medical research,* vol. 35, no. 3, pp. 459-463.

Nadler, A., Cukier, M., Rowsell, C., Kamali, S., Feinberg, Y., Singh, S. & Law, C.H. 2013, "Ki-67 is a reliable pathological grading marker for neuroendocrine tumors", *Virchows Archiv : an international journal of pathology,* vol. 462, no. 5, pp. 501-505.

Naranjo Gomez, J.M., Bernal, J.F., Arranz, P.G., Fernandez, S.L. & Roman, J.J. 2014, "Alterations in the expression of p53, KLF4, and p21 in neuroendocrine lung tumors", *Archives of Pathology & Laboratory Medicine,* vol. 138, no. 7, pp. 936-942.

Nascimbeni, R., Villanacci, V., Di Fabio, F., Gavazzi, E., Fellegara, G. & Rindi, G. 2005, "Solitary microcarcinoid of the rectal stump in ulcerative colitis", *Neuroendocrinology,* vol. 81, no. 6, pp. 400-404.

Nasir, A., Helm, J., Turner, L., Chen, D.T., Strosberg, J., Hafez, N., Henderson-Jackson, E.B., Hodul, P., Bui, M.M., Nasir, N.A., Hakam, A., Malafa, M.P., Yeatman, T.J., Coppola, D. & Kvols, L.K. 2011, "RUNX1T1: a novel predictor of liver metastasis in primary pancreatic endocrine neoplasms", *Pancreas,* vol. 40, no. 4, pp. 627-633.

Niederle, M.B., Hackl, M., Kaserer, K. & Niederle, B. 2010, "Gastroenteropancreatic neuroendocrine tumours: the current incidence and staging based on the WHO and European Neuroendocrine Tumour Society classification: an analysis based on prospectively collected parameters", *Endocrine-related cancer,* vol. 17, no. 4, pp. 909-918.

Norlen, O., Stalberg, P., Zedenius, J. & Hellman, P. 2013, "Outcome after resection and radiofrequency ablation of liver metastases from small intestinal neuroendocrine tumours", *The British journal of surgery,* vol. 100, no. 11, pp. 1505-1514.

Obaya, A.J. & Sedivy, J.M. 2002, "Regulation of cyclin-Cdk activity in mammalian cells", *Cellular and molecular life sciences : CMLS,* vol. 59, no. 1, pp. 126-142.

Oberg, K. 2000, "Interferon in the management of neuroendocrine GEP-tumors: a review", *Digestion,* vol. 62 Suppl 1, pp. 92-97.

Oberndorfer, S. 1929, "Die Geschwülste des Darms." in *Handbuch der speziellen pathologischen Anatomie und Histologie*, eds. F. Henke & O. Lubarsch, Springer, Berlin, pp. 717-953.

Oberndorfer, S. 1907, "Karzinoide Tumoren des Dünndarms.", *Frankf Z Pathol,* vol. 1, pp. 425-432.

Oliver, G., Sosa-Pineda, B., Geisendorf, S., Spana, E.P., Doe, C.Q. & Gruss, P. 1993, "Prox 1, a prospero-related homeobox gene expressed during mouse development", *Mechanisms of development,* vol. 44, no. 1, pp. 3-16.

Onozato, Y., Kakizaki, S., Iizuka, H., Sohara, N., Mori, M. & Itoh, H. 2010, "Endoscopic treatment of rectal carcinoid tumors", *Diseases of the colon and rectum,* vol. 53, no. 2, pp. 169-176.

Palaniswamy, C., Frishman, W.H. & Aronow, W.S. 2012, "Carcinoid heart disease", *Cardiology in review,* vol. 20, no. 4, pp. 167-176.

Pavel, M., Baudin, E., Couvelard, A., Krenning, E., Oberg, K., Steinmuller, T., Anlauf, M., Wiedenmann, B., Salazar, R. & Barcelona Consensus Conference participants 2012a, "ENETS Consensus Guidelines for the management of patients with liver and other distant metastases from neuroendocrine neoplasms of foregut, midgut, hindgut, and unknown primary", *Neuroendocrinology,* vol. 95, no. 2, pp. 157-176.

Pavel, M., Baudin, E., Couvelard, A., Krenning, E., Oberg, K., Steinmuller, T., Anlauf, M., Wiedenmann, B., Salazar, R. & Barcelona Consensus Conference participants 2012b, "ENETS Consensus Guidelines for the management of patients with liver and other distant metastases from neuroendocrine neoplasms of foregut, midgut, hindgut, and unknown primary", *Neuroendocrinology,* vol. 95, no. 2, pp. 157-176.

Pavel, M.E., Baum, U., Hahn, E.G., Schuppan, D. & Lohmann, T. 2006, "Efficacy and tolerability of pegylated IFN-alpha in patients with neuroendocrine gastroenteropancreatic carcinomas", *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research,* vol. 26, no. 1, pp. 8-13.

Pawlik, T.M., Izzo, F., Cohen, D.S., Morris, J.S. & Curley, S.A. 2003, "Combined resection and radiofrequency ablation for advanced hepatic malignancies: results in 172 patients", *Annals of surgical oncology,* vol. 10, no. 9, pp. 1059-1069.

Pearson, C. & Fitzgerald, P.J. 1948, "Carcinoid Tumors of the Rectum-Report of Three Cases, Two with Metastases", *Annals of Surgery,* vol. 128, no. 1, pp. 128-143.

Pearson, C.M. & Fitzgerald, P.J. 1949, "Carcinoid tumors; a re-emphasis of their malignant nature; review of 140 cases", *Cancer,* vol. 2, no. 6, pp. 1005-26, illust.

Pelage, J.P., Soyer, P., Boudiaf, M., Brocheriou-Spelle, I., Dufresne, A.C., Coumbaras, J. & Rymer, R. 1999, "Carcinoid tumors of the abdomen: CT features", *Abdominal Imaging,* vol. 24, no. 3, pp. 240-245.

Pellikka, P.A., Tajik, A.J., Khandheria, B.K., Seward, J.B., Callahan, J.A., Pitot, H.C. & Kvols, L.K. 1993, "Carcinoid heart disease. Clinical and echocardiographic spectrum in 74 patients", *Circulation,* vol. 87, no. 4, pp. 1188-1196.

Pelosi, G., Pasini, F., Fraggetta, F., Pastorino, U., Iannucci, A., Maisonneuve, P., Arrigoni, G., De Manzoni, G., Bresaola, E. & Viale, G. 2003, "Independent value of fascin immunoreactivity for predicting lymph node metastases in typical and atypical pulmonary carcinoids", *Lung cancer (Amsterdam, Netherlands),* vol. 42, no. 2, pp. 203-213.

Pelosi, G., Scarpa, A., Puppa, G., Veronesi, G., Spaggiari, L., Pasini, F., Maisonneuve, P., Iannucci, A., Arrigoni, G. & Viale, G. 2005, "Alteration of the E-cadherin/beta-catenin cell adhesion system is common in pulmonary neuroendocrine tumors and is an independent predictor of lymph node metastasis in atypical carcinoids", *Cancer,* vol. 103, no. 6, pp. 1154-1164.

Petrova, T.V., Nykanen, A., Norrmen, C., Ivanov, K.I., Andersson, L.C., Haglund, C., Puolakkainen, P., Wempe, F., von Melchner, H., Gradwohl, G., Vanharanta, S., Aaltonen, L.A., Saharinen, J., Gentile, M., Clarke, A., Taipale, J., Oliver, G. & Alitalo, K. 2008, "Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype", *Cancer cell,* vol. 13, no. 5, pp. 407-419.

Pictet, R.L., Rall, L.B., Phelps, P. & Rutter, W.J. 1976, "The neural crest and the origin of the insulin-producing and other gastrointestinal hormone-producing cells", *Science (New York, N.Y.),* vol. 191, no. 4223, pp. 191-192.

Pilichowska, M., Kimura, N., Ouchi, A., Lin, H., Mizuno, Y. & Nagura, H. 1999, "Primary hepatic carcinoid and neuroendocrine carcinoma:

clinicopathological and immunohistochemical study of five cases", *Pathology international,* vol. 49, no. 4, pp. 318-324.

Pirker, R.A., Pont, J., Pohnl, R., Schutz, W., Griesmacher, A. & Muller, M.M. 1998a, "Usefulness of chromogranin A as a marker for detection of relapses of carcinoid tumours", *Clinical chemistry and laboratory medicine : CCLM / FESCC,* vol. 36, no. 11, pp. 837-840.

Pirker, R.A., Pont, J., Pohnl, R., Schutz, W., Griesmacher, A. & Muller, M.M. 1998b, "Usefulness of chromogranin A as a marker for detection of relapses of carcinoid tumours", *Clinical chemistry and laboratory medicine : CCLM / FESCC,* vol. 36, no. 11, pp. 837-840.

Ploeckinger, U., Kloeppel, G., Wiedenmann, B., Lohmann, R. & representatives of 21 German NET Centers 2009, "The German NET-registry: an audit on the diagnosis and therapy of neuroendocrine tumors", *Neuroendocrinology,* vol. 90, no. 4, pp. 349-363.

Posorski, N., Kaemmerer, D., Ernst, G., Grabowski, P., Hoersch, D., Hommann, M. & von Eggeling, F. 2011, "Localization of sporadic neuroendocrine tumors by gene expression analysis of their metastases", *Clinical & experimental metastasis,* vol. 28, no. 7, pp. 637-647.

Prasad, V., Ambrosini, V., Hommann, M., Hoersch, D., Fanti, S. & Baum, R.P. 2010, "Detection of unknown primary neuroendocrine tumours (CUP-NET) using (68)Ga-DOTA-NOC receptor PET/CT", *European journal of nuclear medicine and molecular imaging,* vol. 37, no. 1, pp. 67-77.

Quinn, P. & Platell, C.F. 2004, "Resolving microcarcinoids in ulcerative colitis: report of a case", *Diseases of the colon and rectum,* vol. 47, no. 3, pp. 387-391.

Raven, R.W. 1950, "Carcinoid tumours of the rectum", *Proceedings of the Royal Society of Medicine,* vol. 43, no. 8, pp. 675-677.

Rindi, G. & Kloppel, G. 2004, "Endocrine tumors of the gut and pancreas tumor biology and classification", *Neuroendocrinology,* vol. 80 Suppl 1, pp. 12-15.

Rindi, G., Kloppel, G., Couvelard, A., Komminoth, P., Korner, M., Lopes, J.M., McNicol, A.M., Nilsson, O., Perren, A., Scarpa, A., Scoazec, J.Y. & Wiedenmann, B. 2007, "TNM staging of midgut and hindgut (neuro) endocrine tumors: a consensus proposal including a grading system", *Virchows Archiv : an international journal of pathology,* vol. 451, no. 4, pp. 757-762.

Rindi, G., Leiter, A.B., Kopin, A.S., Bordi, C. & Solcia, E. 2004, "The "normal" endocrine cell of the gut: changing concepts and new evidences", *Annals of the New York Academy of Sciences,* vol. 1014, pp. 1-12.

Rinke, A., Muller, H.H., Schade-Brittinger, C., Klose, K.J., Barth, P., Wied, M., Mayer, C., Aminossadati, B., Pape, U.F., Blaker, M., Harder, J., Arnold, C., Gress, T., Arnold, R. & PROMID Study Group 2009, "Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID Study Group", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 27, no. 28, pp. 4656-4663.

Roldo, C., Missiaglia, E., Hagan, J.P., Falconi, M., Capelli, P., Bersani, S., Calin, G.A., Volinia, S., Liu, C.G., Scarpa, A. & Croce, C.M. 2006, "MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 24, no. 29, pp. 4677-4684.

Roy, P. & Chetty, R. 2010, "Goblet cell carcinoid tumors of the appendix: An overview", *World journal of gastrointestinal oncology,* vol. 2, no. 6, pp. 251-258.

Ruf, J., Schiefer, J., Furth, C., Kosiek, O., Kropf, S., Heuck, F., Denecke, T., Pavel, M., Pascher, A., Wiedenmann, B. & Amthauer, H. 2011, "68Ga-DOTATOC PET/CT of neuroendocrine tumors: spotlight on the CT phases of a triple-phase protocol", *Journal of nuclear medicine : official publication, Society of Nuclear Medicine,* vol. 52, no. 5, pp. 697-704.

Ruszniewski, P., Rougier, P., Roche, A., Legmann, P., Sibert, A., Hochlaf, S., Ychou, M. & Mignon, M. 1993, "Hepatic arterial chemoembolization in patients with liver metastases of endocrine tumors. A prospective phase II study in 24 patients", *Cancer,* vol. 71, no. 8, pp. 2624-2630.

Safatle-Ribeiro, A.V., Ribeiro, U.,Jr, Corbett, C.E., Iriya, K., Kobata, C.H., Sakai, P., Yagi, O.K., Pinto, P.E.,Jr, Zilberstein, B. & Gama-Rodrigues, J. 2007, "Prognostic value of immunohistochemistry in gastric neuroendocrine (carcinoid) tumors", *European journal of gastroenterology & hepatology,* vol. 19, no. 1, pp. 21-28.

Salmela, P.I. 2012, "Multiple endocrine neoplasia type 1", *Duodecim; laaketieteellinen aikakauskirja,* vol. 128, no. 22, pp. 2345-2354.

Santala, S., Talvensaari-Mattila, A., Soini, Y., Honkavuori-Toivola, M. & Santala, M. 2014, "High expression of cyclin A is associated with poor prognosis in endometrial endometrioid adenocarcinoma", *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine,* vol. 35, no. 6, pp. 5395-5399.

Sarmiento, J.M., Heywood, G., Rubin, J., Ilstrup, D.M., Nagorney, D.M. & Que, F.G. 2003, "Surgical treatment of neuroendocrine metastases to the liver: a plea for resection to increase survival", *Journal of the American College of Surgeons,* vol. 197, no. 1, pp. 29-37.

Sauven, P., Ridge, J.A., Quan, S.H. & Sigurdson, E.R. 1990, "Anorectal carcinoid tumors. Is aggressive surgery warranted?", *Annals of Surgery,* vol. 211, no. 1, pp. 67-71.

Scherubl, H. 2009, "Rectal carcinoids are on the rise: early detection by screening endoscopy", *Endoscopy,* vol. 41, no. 2, pp. 162-165.

Scherubl, H., Jensen, R.T., Cadiot, G., Stolzel, U. & Kloppel, G. 2011, "Management of early gastrointestinal neuroendocrine neoplasms", *World journal of gastrointestinal endoscopy,* vol. 3, no. 7, pp. 133-139.

Scherubl, H., Streller, B., Stabenow, R., Herbst, H., Hopfner, M., Schwertner, C., Steinberg, J., Eick, J., Ring, W., Tiwari, K. & Zappe, S.M. 2013, "Clinically detected gastroenteropancreatic neuroendocrine tumors are on the rise: epidemiological changes in Germany", *World journal of gastroenterology : WJG,* vol. 19, no. 47, pp. 9012-9019.

Schluter, C., Duchrow, M., Wohlenberg, C., Becker, M.H., Key, G., Flad, H.D. & Gerdes, J. 1993, "The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins", *The Journal of cell biology,* vol. 123, no. 3, pp. 513-522.

Scholzen, T. & Gerdes, J. 2000, "The Ki-67 protein: from the known and the unknown", *Journal of cellular physiology,* vol. 182, no. 3, pp. 311-322.

Sciola, V., Massironi, S., Conte, D., Caprioli, F., Ferrero, S., Ciafardini, C., Peracchi, M., Bardella, M.T. & Piodi, L. 2009, "Plasma chromogranin a in patients with inflammatory bowel disease", *Inflammatory bowel diseases,* vol. 15, no. 6, pp. 867-871.

Shebani, K.O., Souba, W.W., Finkelstein, D.M., Stark, P.C., Elgadi, K.M., Tanabe, K.K. & Ott, M.J. 1999, "Prognosis and survival in patients with gastrointestinal tract carcinoid tumors", *Annals of Surgery,* vol. 229, no. 6, pp. 815-21; discussion 822-3.

Sherr, C.J. 1996, "Cancer cell cycles", *Science (New York, N.Y.),* vol. 274, no. 5293, pp. 1672-1677.

Shields, C.J., Tiret, E., Winter, D.C. & International Rectal Carcinoid Study Group 2010, "Carcinoid tumors of the rectum: a multi-institutional international collaboration", *Annals of Surgery,* vol. 252, no. 5, pp. 750-755.

Sholl, L.M., Long, K.B. & Hornick, J.L. 2010, "Sox2 expression in pulmonary non-small cell and neuroendocrine carcinomas", *Applied Immunohistochemistry & Molecular Morphology : AIMM / Official Publication of the Society for Applied Immunohistochemistry,* vol. 18, no. 1, pp. 55-61.

Siegel, R., DeSantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T., Cooper, D., Gansler, T., Lerro, C., Fedewa, S., Lin, C., Leach, C., Cannady, R.S., Cho, H., Scoppa, S., Hachey, M., Kirch, R., Jemal, A. & Ward, E. 2012, "Cancer treatment and survivorship statistics, 2012", *CA: a cancer journal for clinicians,* vol. 62, no. 4, pp. 220-241.

Silberg, D.G., Swain, G.P., Suh, E.R. & Traber, P.G. 2000, "Cdx1 and cdx2 expression during intestinal development", *Gastroenterology,* vol. 119, no. 4, pp. 961-971.

Skog, M., Bono, P., Lundin, M., Lundin, J., Louhimo, J., Linder, N., Petrova, T.V., Andersson, L.C., Joensuu, H., Alitalo, K. & Haglund, C.H. 2011, "Expression and prognostic value of transcription factor PROX1 in colorectal cancer", *British journal of cancer,* vol. 105, no. 9, pp. 1346-1351.

Soga, J. 2005a, "Carcinoids of the pancreas: an analysis of 156 cases", *Cancer,* vol. 104, no. 6, pp. 1180-1187.

Soga, J. 2005b, "Early-stage carcinoids of the gastrointestinal tract: an analysis of 1914 reported cases", *Cancer,* vol. 103, no. 8, pp. 1587-1595.

Solcia, E., Klöppel, G. & Sobin, L.H.e. 2000, *Histological typing of endocrine tumours,* 2nd edn, Springer, Berlin.

Solcia, E., Capella, C. & Fiocca, R. 1998, "Disorders of the endocrine system" in *Pathology of the gastrointestinal tract*, ed. G.H. Ming S.C., Williams & Wilkins, Philadelphia, pp. 295-332.

Sosa-Pineda, B., Wigle, J.T. & Oliver, G. 2000, "Hepatocyte migration during liver development requires Prox1", *Nature genetics,* vol. 25, no. 3, pp. 254-255.

Starborg, M., Gell, K., Brundell, E. & Hoog, C. 1996, "The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression", *Journal of cell science,* vol. 109 (Pt 1), no. Pt 1, pp. 143-153.

Stefanaki, C., Stefanaki, K., Antoniou, C., Argyrakos, T., Patereli, A., Stratigos, A. & Katsambas, A. 2007, "Cell cycle and apoptosis regulators in Spitz nevi: comparison with melanomas and common nevi", *Journal of the American Academy of Dermatology,* vol. 56, no. 5, pp. 815-824.

Stout, A.P. 1942, "Carcinoid Tumors of the Rectum Derived from Erspamer's Pre-enterochrome Cells", *The American journal of pathology,* vol. 18, no. 6, pp. 993-1009.

Stoyianni, A., Pentheroudakis, G. & Pavlidis, N. 2011, "Neuroendocrine carcinoma of unknown primary: a systematic review of the literature and

a comparative study with other neuroendocrine tumors", *Cancer treatment reviews,* vol. 37, no. 5, pp. 358-365.

Subbuswamy, S.G., Gibbs, N.M., Ross, C.F. & Morson, B.C. 1974, "Goblet cell carcinoid of the appendix", *Cancer,* vol. 34, no. 2, pp. 338-344.

Swarts, D.R., Henfling, M.E., Van Neste, L., van Suylen, R.J., Dingemans, A.M., Dinjens, W.N., Haesevoets, A., Rudelius, M., Thunnissen, E., Volante, M., Van Criekinge, W., van Engeland, M., Ramaekers, F.C. & Speel, E.J. 2013, "CD44 and OTP are strong prognostic markers for pulmonary carcinoids", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 19, no. 8, pp. 2197-2207.

Taban, O., Cimpean, A.M., Raica, M. & Olariu, S. 2014, "PROX1 expression in gastric cancer: from hypothesis to evidence", *Anticancer Research,* vol. 34, no. 7, pp. 3439-3446.

Taylor, F.G., Quirke, P., Heald, R.J., Moran, B., Blomqvist, L., Swift, I., Sebag-Montefiore, D.J., Tekkis, P., Brown, G. & MERCURY study group 2011, "Preoperative high-resolution magnetic resonance imaging can identify good prognosis stage I, II, and III rectal cancer best managed by surgery alone: a prospective, multicenter, European study", *Annals of Surgery,* vol. 253, no. 4, pp. 711-719.

Tomita, T. 2013, "PGP 9.5 immunocytochemical staining for pancreatic endocrine tumors", *Islets,* vol. 5, no. 3, pp. 122-128.

Touzios, J.G., Kiely, J.M., Pitt, S.C., Rilling, W.S., Quebbeman, E.J., Wilson, S.D. & Pitt, H.A. 2005, "Neuroendocrine hepatic metastases: does aggressive management improve survival?", *Annals of Surgery,* vol. 241, no. 5, pp. 776-83; discussion 783-5.

Tropea, F., Baldari, S., Restifo, G., Fiorillo, M.T., Surace, P. & Herberg, A. 2006, "Evaluation of chromogranin A expression in patients with non-neuroendocrine tumours", *Clinical drug investigation,* vol. 26, no. 12, pp. 715-722.

Turner, H.E., Nagy, Z., Sullivan, N., Esiri, M.M. & Wass, J.A. 2000, "Expression analysis of cyclins in pituitary adenomas and the normal pituitary gland", *Clinical endocrinology,* vol. 53, no. 3, pp. 337-344.

Valtorta, F., Pennuto, M., Bonanomi, D. & Benfenati, F. 2004, "Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis?", *BioEssays : news and reviews in molecular, cellular and developmental biology,* vol. 26, no. 4, pp. 445-453.

Vogl, T.J., Naguib, N.N., Zangos, S., Eichler, K., Hedayati, A. & Nour-Eldin, N.E. 2009, "Liver metastases of neuroendocrine carcinomas: interventional treatment via transarterial embolization,

chemoembolization and thermal ablation", *European Journal of Radiology,* vol. 72, no. 3, pp. 517-528.

Wang, L., Chen, L., Yang, X., Gao, T. & Wang, G. 2013, "Benign lymphangioendothelioma: a clinical, histopathologic and immunohistochemical analysis of four cases", *Journal of cutaneous pathology,* vol. 40, no. 11, pp. 945-949.

Wang, M., Peng, J., Yang, W., Chen, W., Mo, S. & Cai, S. 2011, "Prognostic analysis for carcinoid tumours of the rectum: a single institutional analysis of 106 patients", *Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland,* vol. 13, no. 2, pp. 150-153.

Weinstock, B., Ward, S.C., Harpaz, N., Warner, R.R., Itzkowitz, S. & Kim, M.K. 2013, "Clinical and prognostic features of rectal neuroendocrine tumors", *Neuroendocrinology,* vol. 98, no. 3, pp. 180-187.

Welin, S.V., Janson, E.T., Sundin, A., Stridsberg, M., Lavenius, E., Granberg, D., Skogseid, B., Oberg, K.E. & Eriksson, B.K. 2004, "High-dose treatment with a long-acting somatostatin analogue in patients with advanced midgut carcinoid tumours", *European journal of endocrinology / European Federation of Endocrine Societies,* vol. 151, no. 1, pp. 107-112.

West, N.E., Wise, P.E., Herline, A.J., Muldoon, R.L., Chopp, W.V. & Schwartz, D.A. 2007, "Carcinoid tumors are 15 times more common in patients with Crohn's disease", *Inflammatory bowel diseases,* vol. 13, no. 9, pp. 1129-1134.

Wiedenmann, B. & Franke, W.W. 1985, "Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles", *Cell,* vol. 41, no. 3, pp. 1017-1028.

Wiedenmann, B., Franke, W.W., Kuhn, C., Moll, R. & Gould, V.E. 1986, "Synaptophysin: a marker protein for neuroendocrine cells and neoplasms", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 83, no. 10, pp. 3500-3504.

Wiedenmann, B., John, M., Ahnert-Hilger, G. & Riecken, E.O. 1998, "Molecular and cell biological aspects of neuroendocrine tumors of the gastroenteropancreatic system", *Journal of Molecular Medicine (Berlin, Germany),* vol. 76, no. 9, pp. 637-647.

Wiener, Z., Hogstrom, J., Hyvonen, V., Band, A.M., Kallio, P., Holopainen, T., Dufva, O., Haglund, C., Kruuna, O., Oliver, G., Ben-Neriah, Y. & Alitalo, K. 2014, "Prox1 promotes expansion of the colorectal cancer stem cell population to fuel tumor growth and ischemia resistance", *Cell reports,* vol. 8, no. 6, pp. 1943-1956.

Williams, E.D. & Sandler, M. 1963, "The classification of carcinoid tum ours", *Lancet,* vol. 1, no. 7275, pp. 238-239.

Yam, C.H., Fung, T.K. & Poon, R.Y. 2002, "Cyclin A in cell cycle control and cancer", *Cellular and molecular life sciences : CMLS,* vol. 59, no. 8, pp. 1317-1326.

Yamaguchi, K., Kameya, T. & Abe, K. 1980, "Multiple endocrine neoplasia type 1", *Clinics in endocrinology and metabolism,* vol. 9, no. 2, pp. 261-284.

Yamaguchi, T., Fujimori, T., Tomita, S., Ichikawa, K., Mitomi, H., Ohno, K., Shida, Y. & Kato, H. 2013, "Clinical validation of the gastrointestinal NET grading system: Ki67 index criteria of the WHO 2010 classification is appropriate to predict metastasis or recurrence", *Diagnostic pathology,* vol. 8, pp. 65-1596-8-65.

Yang, Q., Bermingham, N.A., Finegold, M.J. & Zoghbi, H.Y. 2001, "Requirement of Math1 for secretory cell lineage commitment in the mouse intestine", *Science (New York, N.Y.),* vol. 294, no. 5549, pp. 2155-2158.

Yao, J.C., Hassan, M., Phan, A., Dagohoy, C., Leary, C., Mares, J.E., Abdalla, E.K., Fleming, J.B., Vauthey, J.N., Rashid, A. & Evans, D.B. 2008, "One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States", *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* vol. 26, no. 18, pp. 3063-3072.