Tumour Biological Factors Characterizing Metastasizing Serotonin-producing Ileocaecal Carcinoids

JANET LYNN CUNNINGHAM
Dissertation presented at Uppsala University to be publicly examined in Rudbeck Laboratory, Dag Hammarskjölds väg 20, Uppsala, Saturday, September 1, 2007 at 09:30 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

In this study, metastasizing serotonin-producing ileocaecal carcinoid tumours (MSPCs) were examined for biological characteristics that could be used to define clinically relevant subgroups within this patient population. Possible targets for new treatment options were also explored.

It was found that MSPCs share several biological characteristics such as expression of serotonin, tachykinins (TKs), chromogranin A, islet autoantigen-2 and connective tissue growth factor (CTGF). TKs and serotonin were demonstrated in the same endocrine tumours in the gut and lung. IA-2 expression was shown to be up-regulated in MSPCs, possibly in connection with active hormone secretion. CTGF expression was high in tumour areas adjacent to extensive stroma expressing alpha-smooth muscle actin. This indicated myofibroblast differentiation, which may be associated with fibrosis-related complications prevalent in patients with MSPCs. When compared with other endocrine tumours, MSPCs behaved as a relatively homogeneous group, though within the MSPC population several subgroups could be defined. Patients with tumours displaying either a solid growth pattern and/or a Ki67 index ≥1% had a less favourable prognosis than those who did not. Another group of patients, who had increased plasma TK concentrations, were more likely to suffer from severe diarrhea. This information should be considered when discussing clinical treatment and when undertaking tumour biological studies. New treatment possibilities, such as drugs that specifically target TK receptors and antibodies to CTGF, are also discussed.

In conclusion, MSPCs comprise a clinically relevant tumour group with similar biological features that are distinct from other endocrine tumours. Subgroups of patients within this patient category can be defined which may be relevant when establishing prognosis and when selecting future treatment modalities.

Keywords: endocrine tumour, midgut carcinoid, serotonin-producing neuroendocrine carcinoma, prognosis, morphology, tachykinin, connective tissue growth factor, islet autoantigen-2, morphology, carcinoid syndrome

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List of Papers Included in the Thesis

This thesis is based on the following papers\(^1\), which will be referred to in the text by their roman numerals:

I. Malignant Ileocaecal Serotonin-producing Carcinoid Tumours: The presence of a solid growth pattern and/or Ki67 index above 1% identifies patients with a poorer prognosis.
   **Cunningham JL**, Grimelius L, Sundin A, Agarwal S, Janson ET. *Acta Oncologica (in press)*

II. Tachykinins in Endocrine Tumours and the Carcinoid Syndrome
   **Cunningham JL**, Janson ET, Agarwal S, Grimelius L, Stridsberg M (*submitted for publ.)*

III. Connective Tissue Growth Factor (CTGF) Expression in Endocrine Tumours is Associated with High Stromal Expression of alpha-Smooth Muscle Actin.
   **Cunningham JL**, Jacobson A, Janson ET (*manuscript*)

IV. Transmembrane Protein Tyrosine Phosphatase IA-2 (ICA 512) is Expressed in Human Midgut Carcinoids but is not Detectable in Normal EC Cells.

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Introduction.....................................................................................................9
Analysis and Synthesis...............................................................................9
History........................................................................................................9

General Aspects ............................................................................................12
  Hormones and Symptoms - The Carcinoid Syndrome..............................13
  Regulated Hormone Secretion.............................................................14
  Treatment.............................................................................................15

Heterogeneity and Potential Prognostic Markers.........................................17
  Morphology .........................................................................................18
  Proliferation .........................................................................................18
  Apoptosis .............................................................................................19
  Growth factors .....................................................................................19
  Genetics ...............................................................................................19
  Tumour Stroma....................................................................................20

Aims of the Investigation..............................................................................22

Summary of the Investigation.......................................................................23
  Paper Ia: Solid Growth Pattern is Associated with a Shorter Survival...23
  Paper Ib: Ki67 Index ≥1% is Associated with Shorter Survival..............24
  Paper II: Tachykinin Expression in Endocrine Tumours and Elevated
            Plasma-Tachykinin Concentrations are Associated with Carcinoid
            Diarrhea and Flush ........................................................................25
  Paper III: Connective Tissue Growth Factor Expression in Endocrine
            Tumours and its Relation to alpha-Smooth Muscle Actin Expression in
            Tumour Stroma. ...........................................................................27
  Paper IV: Islet Autoantigen -2 Expression is Upregulated in Tumour
            Tissue Compared with Normal Enterochromaffin Cells...............29

Concluding Remarks.....................................................................................31

Main Findings of the Investigation ...............................................................33

Acknowledgements.......................................................................................34

References.....................................................................................................36
Abbreviations

GI  Gastrointestinal
NE  Neuroendocrine
EC  Enterochromaffin
5-HT  Serotonin
U-5HIAA  Urinary-5-Hydroxyindoleacetic Acid
CgA  Chromogranin A
P-CgA  Plasma Chromogranin A
TK  Tachykinin
P-TK  Plasma Tachykinin
NPK  Neuropeptide K
SP  Substance P
NKA  Neurokinin A
NKB  Neurokinin B
PBS  Phosphate-buffered Saline
HPF  High-power Field (x400)
IR  Immunoreactivity
NK 1-3  Neurokinin Receptors
DCV  Dense Core Vesicle
CTGF  Connective Tissue Growth Factor
TGF-α  Transforming Growth Factor-Alpha
TGF-β  Transforming Growth Factor-Beta
α-SMA  Alpha-Smooth Muscle Actin
IA-2  Islet Autoantigen-2 (ICA 512)
MSPC  Metastasizing Serotonin-Producing Ileocaecal Carcinoid
EGF  Epidermal Growth Factor
IGF I&II  Insulin-like Growth Factors I&II
PDGF  Platelet-derived Growth Factor
APUD  Amine Precursor Uptake and Decarboxylation
WHO  World Health Organisation
RT-PTP  Receptor Type - Protein Tyrosine Phosphatases
Introduction

Analysis and Synthesis

Analysis and synthesis are two important steps in any scientific process. Each alone is detrimental to research progress. The breaking down of complex phenomena into their elemental pieces, and then reconstructing them into an organized whole must be a continuously repeated process. Otherwise, dogma is blind and the true complexity of studied phenomena is underestimated.

If we apply this model to cancer, analysis takes place within basic cancer research expanding the fund of information available to define each individual tumour. This information can lead to the development of new treatment options which, in turn, make new demands on tumour grouping. Synthesis is the formation of clinically relevant patient groups and sub-groups. Relevance is defined by the ability to amalgamate these groups into the correct choice for medical care. This process is enormously dynamic.

Rare tumours have characteristics which make it appropriate to sort them into a separate group. This collection of rare tumours share aspects that differ from the larger tumour groups. As a collection of rare tumours grows, it becomes apparent that several sub-groups or entirely new groups exist. Endocrine tumours form one such group.

History

The first description of a neuroendocrine tumour was by Lubarsch in 1888. (1) The term ‘Karzinoide’ was coined by Oberndorfer in 1907 (2). Gosset and Masson (3) later recognized that carcinoids are endocrine-related tumours. The collection of ‘carcinoids’ grew and in 1963 Williams and Sandler (4) proposed distinguishing carcinoid tumours by embryonic divisions in the gut: foregut carcinoids (stomach, pancreas, duodenum and upper jejunum), midgut carcinoids (lower jejunum, ileum, appendix and caecum and colon as far as the mid-transverse colon) and hindgut carcinoids (descending colon and rectum). This was the first attempt to categorize these tumours from clinical criteria.

The APUD (Amine content and/or Amine Precursor Uptake and Decarboxylation) concept, developed in 1969, describes cells in endocrine organs, stating that in theory each type of APUD cell could give rise to a specific
tumour, an “Apudoma” that contains certain amines and/or polypeptide product (5). The most debated aspect of the APUD system is the assumption that all APUD cells have a neuroectodermal origin. It has since been shown that pancreas endocrine cells and gut endocrine cells are derived from the endoderm (6-10).

The term “carcinoid” has been used both broadly to describe hormone-producing tumours as well as specifically, to denote serotonin-producing tumours in the gut. To reduce the risk of confusion, the term neuroendocrine tumour was proposed by Capella et al. (11) to encompass the entire neuroendocrine tumour spectrum, from classical carcinoid to malignant undifferentiated carcinomas. It has been debated that the term “endocrine tumours” is a more appropriate collective term as evidence indicates that most endocrine cells originate not in the neural crest, but from epithelial cells (12, 13).

The current WHO classification system distinguishes tumours by their site of origin. It then further classifies tumours using hormonal production, histological differentiation, angio-invasion, size and extension into surrounding tissues, and into the following grades of malignancy: benign, uncertain, low-grade malignant neoplasm, and highly malignant neoplasm. The term “functioning” is added to note the presence of a clinical syndrome of endocrine hyperfunction in addition to inappropriately increased circulating hormone concentrations.

The tumours studied in this thesis are metastasizing serotonin-producing carcinoids (MSPCs) arising from enterochromaffin cells (EC cells) in the ileum and caecum that have been included in the category midgut carcinoids. Appendix and hindgut (colon and rectum) carcinoids were excluded from

<table>
<thead>
<tr>
<th>1 Well-differentiated neuroendocrine tumour (carcinoid)</th>
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<tbody>
<tr>
<td>1.1 Benign non-functioning, confined to mucosa–submucosa,</td>
</tr>
<tr>
<td>non-angioinvasive, (ileum) &lt; 1cm or &lt; 2cm (colon-rectum)</td>
</tr>
<tr>
<td>1.1.1 Serotonin-producing tumour</td>
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<tr>
<td>1.1.2 Enteroglucagon-producing tumour</td>
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<tr>
<td>1.2 Benign or low-grade malignant tumour (uncertain malignant potential):</td>
</tr>
<tr>
<td>non-functioning, confined to mucosa–submucosa, non-angioinvasive, angioinvasive or &gt;1cm (ileum) or &gt; 2cm (colon-rectum)</td>
</tr>
<tr>
<td>1.2.1 Serotonin-producing tumour</td>
</tr>
<tr>
<td>1.2.2 Enteroglucagon-producing tumour</td>
</tr>
<tr>
<td>2 Well differentiated neuroendocrine carcinoma (malignant carcinoid)</td>
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<tr>
<td>2.1 Low-grade malignant: invasion of the muscularis propria or metastases</td>
</tr>
<tr>
<td>Non-functioning or functioning serotonin-producing carcinoma</td>
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<tr>
<td>(with carcinoid syndrome)</td>
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<tr>
<td>2.2 Non-functioning enterooglucagon-producing carcinoma</td>
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<tr>
<td>3 Poorly differentiated neuroendocrine carcinoma – high-grade malignant</td>
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<td>4 Mixed endocrine-exocrine carcinoma – moderate to high-grade malignant</td>
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</table>
this work. Though appendix carcinoids display a tumour cell pattern similar to the tumours included in this study, they, in contrast to the studied tumours, only rarely form metastases (14), often include S100 protein immunoreactive cells and, it has been speculated, derive from a different type of endocrine cell (15-17).
General Aspects

This section is a summary of the general characteristics that apply to MSPCs as a group.

MSPCs share many characteristics with EC cells in the jejunum, ileum, caecum and ascending colon of the gastrointestinal tract (GI-tract). They are characterized by serotonin (5-HT) and tachykinin (TK) production and secretion, which, in patients, cause clinical symptoms collectively called the carcinoid syndrome. Carcinoid tumours are very rare. About 100 new cases are diagnosed in Sweden every year. Usually, the tumour slowly grows and the patient is diagnosed with a tumour after hormone-related symptoms have occurred. These symptoms only develop in patients with metastases (18, 19). When tumour spread is limited, the purpose of radical surgery is to remove all visible tumours. Most patients relapse after a period of remission that varies considerably (20). With the exception of surgery at a very early stage, there is no curative treatment. Patients who receive palliative treatment, however, can live up to 10 years or more after diagnosis.

Figure 1. EC cells (left) are individually distributed in the intestine. The cells are polarized and hormone-containing granules are released towards the basal membrane. MSPCs (right) are thought to be derived from these cells as they maintain the ability to produce and secrete the same hormones. Photos, taken by confocal microscopy, show CgA immunoreactivity; this protein is located in the secretory granules.
Hormones and Symptoms - The Carcinoid Syndrome

The carcinoid syndrome is a combination of symptoms thought to be caused by the hormones released by the tumours into the blood stream, usually after the development of metastases. The symptoms of the carcinoid syndrome vary, depending on the hormone combinations and circulating concentrations. The commonly discussed hormones released are 5-HT, bradykinin, and members of the TK and chromogranin families. The carcinoid syndrome includes symptoms of flush, diarrhea, carcinoid heart disease and bronchial constriction. Diarrhea may also have causes such as mechanical obstruction, ischaemia and short bowel syndrome. Patients can have all or some of these symptoms.

<table>
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<tr>
<th>Table 1. Symptoms in patients with MSPC</th>
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<tbody>
<tr>
<td>Symptom</td>
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<tr>
<td>Flush</td>
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<tr>
<td>Diarrhea</td>
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<tr>
<td>Carcinoid Heart Disease</td>
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</table>

Serotonin and Urinary-5-Hydroxyindoleacetic Acid

Carcinoid tumour cells convert tryptophan to 5-HT. 5-HT is stored in the tumour cell secretory granules and secretion is regulated. Free 5-HT is generally removed from circulation by the liver and lungs where it is metabolized to 5-HIAA. Urinary concentrations of U-5HIAA are used to diagnose and monitor treatment response (21). Healthy individuals typically excrete less than 80 µmol of U-5HIAA in 24 hours. Patients with the carcinoid syndrome can excrete more than 3000 µmol of U-5HIAA in 24 hours (22).

5-HT is now thought to cause carcinoid heart disease. The strongest evidence for this is that daily 5-HT administered to rats over a 3-month period led to the development of fibrotic plaques on the heart valves (23).

Chromogranin A

CgA, a member of the granin family, is thought to play a role in regulated secretion. Recently, a knock-out mouse null for the Chga gene that codes for CgA was developed. Phenotypical changes in the appearance and function of neuroendocrine cells were not observed, though, other members of the granin family were up-regulated and likely compensating for the CgA deficiency (24).

Plasma-CgA (P-CgA) correlated with tumour burden both in animal studies (25) and in patients with MSPCs (22). In patients with limited tumour disease, P-CgA has been shown to be a better biochemical marker with which to monitor tumour growth and treatment response, than is U-5HIAA (26).
Tachykinins
The members of the TK family include substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), neurokinin B (NKB), and hemokinin-1 (HK-1); these hormones are encoded on three genes: TAC1, TAC3 and TAC4 (27-29). TKs are expressed in normal EC cells in the gut (30, 31) and in tumour tissue from patients with MSPCs (32-34). Norheim et al. demonstrated a correlation between high plasma concentrations of TKs and flush (33) while Lundin et al. have shown that MSPC patients with pronounced right heart disease have higher plasma SP and NPK concentrations as well as higher excretion of the 5-HT metabolite U-5HIAA than those without (35). Apart from potentially causing some of the symptoms in the carcinoid syndrome, TKs can have other important roles in the biology of MSPCs. Signalling via neuropeptide receptors can have autocrine and paracrine mitogenic effects on normal and cancer cells (36, 37). TKs can, via activation of neurokinin and purinergic receptors, stimulate growth in normal and tumour cell lines (36, 38, 39).

Regulated Hormone Secretion
In general, there are two different patterns of secretion: constitutive and regulated. In all cells, proteins are continuously secreted from the cell, regardless of environmental factors (40). No external signals are needed to initiate this process. Proteins are packaged in vesicles in the Golgi apparatus and are secreted via exocytosis, all around the cell. In contrast, substances such as hormones, neurotransmitters and digestive enzymes are only secreted in response to a specific signal, such as neural or hormonal stimulation. This allows a rapid release on demand. While awaiting release, these substances are stored in secretory granules. 5-HT is found in both dense core vesicles (DCVs) and outside of the DCV, suggesting alternative storage mechanisms (41-43). Secretory granule release involves calcium signalling, soluble N-ethylmaleimide-sensitive factor attachment (SNARE) proteins and synaptosome-associated protein-25 (SNAP-25). Calcium-dependent activator proteins (CAPS) are thought to be required for DCV exocytosis (43, 44). The control mechanisms in regulated secretion are not completely understood, but several potential mechanisms have been proposed (45): cells may be able to control vesicle maturation and the number of secretion competent vesicles (46), fusion pores in the plasma membrane may exhibit dynamic behaviour where pore size regulates the amount of vesicle content released, a process termed ‘cavicide’ exocytosis (47), and finally, the solubility of the packaged signalling substance may influence release and signalling.

Islet autoantigen-2
Islet autoantigen-2 (IA-2/ICA 512) is present in DCV membranes in nearly all neuroendocrine tissues (48). IA-2 is also a major autoantigen in Type I
diabetes mellitus (49) where auto antibodies are detectable in about 70% of newly diagnosed patients but in fewer than 1% of control subjects. IA-2 encodes a transmembrane protein of 979 amino acids and features a region homologous to the catalytic domain of receptor type protein tyrosine phosphatases (RT-PTPs) (50). It does not demonstrate phosphatase activity in assays with common PTP substrates due to two sequence deviations in the PTP core domain. IA-2 is located in regulated secretory granules of neuroendocrine tissues and is suggested to be a marker for neuroendocrine differentiation in lung cancer (51). Furthermore, it has been shown that stimulation of GH4C1 cells, a rat pituitary tumour cell line, with estradiol, insulin and epidermal growth factor (EGF), which leads to increased granulogenesis and regulated secretion of prolactin (52), also results in increased amounts of IA-2 protein (53). Based on these findings, IA-2 has been proposed to play a part in or to be affected by the regulated secretory process in either the granulogenesis or later secretory stages of neuroendocrine cells (48, 50, 53).

Treatment

There are several options for the treatment of carcinoid tumours:

- Surgery
- Radiofrequency ablation
- Hepatic artery embolization
- Interferon
- Somatostatin analogues
- Tumour targetting with radiolabelled somatostatin analogues

The primary treatment for midgut carcinoid tumours is surgery. Surgery is seldom curative and most patients will develop a recurrence of their disease; however, in patients with limited disease, recurrence may be delayed for many years (54). In patients with spread disease, surgery aims to alleviate present and prevent future symptoms due to intestinal obstruction and intestinal ischaemia and excessive hormone production. It has been shown that aggressive surgery can prolong survival and liver resection is recommended in cases with uni-lobar disease or when 90% of the tumour volume can be excised (55). Today it is also common to combine surgery with radiofrequency ablation to reduce tumour volume (56). Hepatic artery embolization is also used to reduce tumour mass and hormone production in patients with inoperable disseminated disease and multiple liver metastases.

Biotherapy with interferon-alpha and/or somatostatin analogues is used to control both tumour proliferation and hormone-related symptoms (19, 57, 58). Objective biochemical responses are seen in up to 47% of patients (59-61). Radiological responses vary between 5 and 20% in most studies. The
side-effects of IFN treatment include fever and flu-like symptoms at treatment start and myalgia, fatigue, anorexia and autoimmune diseases.

Somatostatin analogues inhibit the secretion of bioactive substances that cause the carcinoid syndrome. Treatment with somatostatin analogues, octreotide or lanreotide, relieves symptoms in more than 70% of patients (62-64). The most common side-effects of somatostatin analogues are nausea, transient abdominal pain, flatulence, diarrhea and gallstone development (65, 66).

Radiolabelled somatostatin analogues are being developed and results from initial studies are promising. Complete and partial response in patients with gastroenteropancreatic tumours is reported to lie between 0 and 33% and for GI carcinoids, 5 to 35% (67).
Heterogeneity and Potential Prognostic Markers

While the previous section outlines general aspects of MSPCs, this section focuses on variation within the tumour group. Treatment response in patients with MSPCs ranges from 0 to 80%, depending on the drug, dosage and response criteria used (68). A substantial proportion of patients do not respond satisfactorily to any available treatment. These patients differ in some important way from others in the group. Variation in survival is one of the most striking aspects of MSPCs. For patients with metastases, the 5-year survival rate is about 50%. Although survival can range from weeks to decades (22, 69, 70), there are surprisingly very few reliable prognostic markers. The WHO classification includes some general prognostic recommendations for all neuroendocrine tumours of the GI tract (71):

<table>
<thead>
<tr>
<th>1- Well differentiated endocrine tumour (carcinoid)</th>
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<tbody>
<tr>
<td>As a rule, the behaviour of non-angioinvasive tumours measuring &lt;1 cm in size, localized to the mucosa or sub-mucosa and showing &lt;2 mitoses per 10 HPF is benign. The others are at increased risk of malignancy</td>
</tr>
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<table>
<thead>
<tr>
<th>2- Well differentiated endocrine carcinoma (malignant carcinoma)</th>
</tr>
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<tbody>
<tr>
<td>The tumour is as a rule &gt;1cm in size and may have a moderately elevated mitotic index (&gt;2/10 HPF) or proliferation index (&gt;2% Ki67 immunoreactive cells)</td>
</tr>
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</table>

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<tr>
<th>3- Poorly differentiated endocrine carcinoma - high-grade malignant</th>
</tr>
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<tbody>
<tr>
<td>The tumour usually shows a highly increased mitotic rate (at least 10/10HPF) and high proliferation index (&gt;15% Ki67 immunoreactive cells), p53 immunostaining and both local and distant (abdominal and extra-abdominal) metastases</td>
</tr>
</tbody>
</table>

Basic science is currently trying to understand the biological processes underlying the development of MSPCs. In this process, tumour markers are being discovered that may have clinical significance or prognostic value. The following section gives a very brief overview of research areas where such candidates may arise.
Morphology
Pathology textbooks and review articles generalize that midgut carcinoids display a typical insular growth pattern. Support for this is commonly taken from a study by Soga where it was concluded that morphology correlates with primary tumour site of origin. Of 20 midgut carcinoid tumours included in the Soga study, most originated in the appendix. These tumours displayed type A (insular) growth pattern in either a pure form or mixed with type B (trabecular) or C (acinar and rosette), though those with mixed morphology all originated from either the ileum or colon (72). Jones and Dawson, in a morphological study (73), included 15 argentaffin carcinoids from the small intestine and one from the proximal colon and categorized all of these tumours as either insulin (type A1) or insulin-acinar/glandular (type A1/A2). Johnson et al. have examined growth patterns in neuroendocrine tumours from different localizations in relation to survival but did not report the outcome specifically for MSPCs (74). Other studies published on the histopathological characteristics of midgut carcinoid tumours in relation to clinical data have been based on neuroendocrine tumour material from different regions of the GI-tract and in some cases included tumours from other organs such as the pancreas and lung (75, 76).

There is a general belief that MSPCs share a common morphology that is associated with relatively good prognosis. While this is perhaps true for the MSPC population when it is compared with other endocrine tumours, the prognostic value of growth pattern within the MSPC population has not been studied.

Proliferation
General recommendations for NE tumour management suggest that a cut-off Ki67 index of 2% can be used to differentiate “well differentiated tumours” (presumably benign) from the more malignant carcinomas (57, 77). Studies on tumour cell proliferation in MSPCs have, however, been based on small numbers of tumours and often included several different neuroendocrine tumour entities. Furthermore, the use of counting mitoses as well as different methods for Ki67 index calculation makes study comparison difficult (75-84). A cut-off of 2 mitoses/mm² showed a non-significant trend to identify patients with a poor prognosis in low-grade-malignant midgut carcinoids (82). An earlier pilot study (83), showed that Ki67 expression in more than 1 cell/mm² correlated with a shorter survival when examined in biopsies from liver metastases from 14 patients with untreated MSPCs. Canzveses and others report that in a set of 17 MSPCs, Ki67 expression ranged from nil to 1.76% (84). It is possible that the current recommended Ki67 index cut-off of 2% is too high and that a cut-off of 1% might be more appropriately scaled to differentiate between high and low proliferation in MSPCs.
Several mechanisms may be contributing to the low proliferation index of this tumour entity. For example, tissue growth factor-beta (TGF-β) is highly expressed in MPSCs and has been proposed to act as a paracrine/autocrine inhibitor of neuroendocrine tumour cell proliferation. This inhibition can be mediated by the TGF-β activated transcription factors, SMADs, which decrease expression of c-myc and induce of cyclin kinase inhibitor, p21(WAF1) and p15INK4B (85). Another work has, however, shown that virtually all tumour cells in MSPCs express another cyclin kinase inhibitor, p27 and none p21 (84).

Apoptosis
MSPCs seldom display areas of necrosis or signs of apoptosis. Extensive analysis of apoptosis in these tumours has not been undertaken. Using the TUNEL method (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling) to detect DNA fragmentation apoptosis index has been estimated to lie between 0 and 8% (86, 87).

Growth factors
The expression of growth factors and their receptors also varies between tumours. Platelet-derived growth factor (PDGF), insulin-like growth factors I and II (IGF-I and II), EGF and tissue growth factor alpha and beta (TGF-α and β) and their receptors have been demonstrated in MSPCs (88-92). These proteins stimulate tumour and stroma cell growth in other tumour types but their prognostic value in MSPCs is unknown.

Genetics
The genetics underlying carcinoid tumor development are largely unknown. The advances that have been made in elucidating the genetics of other tumour types are rarely applicable to MSPCs. For example: signs of microsatellite instability (91, 93), v-raf murine sarcoma viral oncogene homologue B1 (BRAF), p53 and Kirsten rat sarcoma viral oncogene homologue (K-ras) mutations are rarely found in MSPCs (94-98). Somatic multiple endocrine neoplasia type 1 gene (MEN1) mutations, common in patients with the MEN1 syndrome and foregut carcinoids, are seldom found in MSPCs (99, 100).

An Uppsala study of MSPC tissue has shown that loss of heterozygosity on chromosome 18 is a common event in primary tumours (101). This was confirmed by another group who narrowed the loss to the region 18q22qter. Other alterations described include a loss on 11q22-23, a loss on 16q21 and a gain on 4p14 (102).
Methylation studies have shown that RAS-association domain family 1, isoform A (RASSF1A) and, cell cycle inhibitor, p14, are methylated in 11/16 ileal carcinoids. Cell cycle inhibitor, p16, is methylated 4/16. RASSF1A gene is a tumour suppressor gene in the RAS pathway that can regulate proliferation, induce apoptosis, and bind to and stabilize microtubules (103).

Tumour Stroma

Fibrosis

MSPCs have a pronounced association with fibrosis development (104-106). Fibrosis caused by tumour-associated factors is emerging as a major issue in the morbidity and mortality of the disease (107, 108). The following are examples of fibrosis-associated complications that arise in patients with MSPCs (35, 108-117):

- Mesenterial fibrosis
- Intestinal obstruction
- Vascular occlusion and ischaemia
- Retroperitoneal fibrosis
- Stenosis of the ureters
- Fibrosis in distant organs
- Carcinoid heart disease
- Pulmonary and pleural fibrosis

Fibrotic complications are reported to arise in 16-48% of patients (106). The list of factors that may contribute to carcinoid-associated fibrosis is long. Tumour-produced hormones, 5-HT (23, 112, 118, 119) and TKs (120-122), have been shown to be related to fibrosis. Growth factors such as PDGF, IGF-I, IGF-II, EGF, TGF-α and TGF-β and their receptors have also been discussed in the context of carcinoid fibrosis (88-90, 92, 106, 123, 124). TGF-β has been shown to have several roles in various fibrotic conditions (125-128) and it is highly expressed in MSPCs (92, 129). TGF-β induced fibroblast proliferation, collagen synthesis and myofibroblast differentiation have been shown to be mediated by connective tissue growth factor (CTGF) dependent pathways (130-132). These processes are mutually independent and in vitro experiments have demonstrated that one CTGF domain stimulates myofibroblast differentiation and collagen production while another stimulates fibroblast cell proliferation (133). The presence of other growth factors, such as EGF, IGF-II and TGF-β, also seems to influence the degree to which these different fibrotic processes are active (131).

Myofibroblasts

Myofibroblasts are fibroblast cells that have partially differentiated toward a smooth muscle phenotype that can contract by using alpha-smooth muscle actin (α-SMA). These cells are then capable of speeding wound repair by
contracting the edges of the wound. After healing is complete, these cells are lost through apoptosis (134). In fibrotic diseases, however (for example liver cirrhosis, kidney fibrosis) this mechanism fails to work, leading to persistence of the myofibroblasts, and consequently expansion of the extracellular matrix (fibrosis) and contraction (135). Myofibroblasts in tumours may be involved in invasiveness and early metastases formation (135-137). Modlin et al. have reported that connective tissue growth factor (CTGF/CCN2) is expressed in MSPCs (106).
Aims of the Investigation

In this thesis, factors common to MSPCs that distinguish them from other endocrine tumours and from mucosal EC cells are examined. Variations in these and other factors that can be used to define subgroups of patients are also described. The objective is to increase our understanding of the biological mechanisms in these tumours and ultimately define clinically relevant groups that may be helped by new treatment forms or have specific treatment needs.

The specific aims are as follows:

1. to establish histopathological criteria to identify patients with MSPCs with worse prognosis,

2. to screen endocrine tumours for TK expression,

3. to test for correlation between plasma TK concentrations and symptoms in patients with MSPCs,

4. to screen endocrine tumours for CTGF expression in relation to α-SMA expression,

5. to characterize the expression of IA-2 in MSPCs in comparison with mucosal EC cells.
Summary of the Investigation

**Paper Ia: Solid Growth Pattern is Associated with a Shorter Survival**

The aim of this study was to ascertain if morphological characteristics determined at the time of operation correlate with survival. Tumour tissues from 81 patients were included in the study. All patients had metastases and survival ranged from 1-223 months. Five main morphological patterns, insular, insular-trabecular, insular-acinar, solid growth pattern and small cell-nest, were identified and described (Fig. 2).

![Microscopic images of growth patterns](image)

*Figure 2. MSPCs growth patterns in the mucosa/submucosa stained with Grimelius silver stain. (A) Insular growth pattern: some of the insular nodules show peripheral palisading. Scale bar = 50 µm. (B) Insular-trabecular growth pattern. (C) Insular-acinar growth pattern: insular cell nests with glandular or rosette formations. (D) Solid growth pattern: solid aggregations of tumour cells. (E) Small cell nest growth pattern: irregular small cell nests together with slender trabeculae and abundance of fibrous stroma.*
A Cox regression analysis was used to test if morphological appearance facilitates identification of patients with shorter than expected survival. When each growth pattern was tested against the other patterns pooled into one group, only the solid growth pattern was associated with a higher risk of death per time fraction (shown in Table 2).

Table 2. The solid growth pattern can be used to identify patients with a poorer prognosis. The relative hazard ratio for each risk factor obtained in Cox proportional hazard models of overall survival in patients with MSPCs is shown with 95% confidence intervals (95% CI). Models are adjusted for age and sex.

<table>
<thead>
<tr>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid growth pattern in primary tumour</td>
<td>2.9 (1.3-6.3)</td>
</tr>
<tr>
<td>Solid growth pattern in metastasis</td>
<td>2.3 (1.2-4.6)</td>
</tr>
</tbody>
</table>

This result was significant for both primary tumours and metastases, and was especially prominent for the mesentery metastases (hazard ratio 3.6, p=0.005).

This study is the first to identify the solid growth pattern as an independent risk factor for shorter survival in both primary tumours and metastases. This was true even when this pattern was present in the tumour, though not dominant. The solid growth pattern was more frequently associated with a higher Ki67 index than was the other growth patterns.

**Paper Ib: Ki67 Index ≥1% is Associated with Shorter Survival**

In addition, this study aimed to determine if Ki67 and apoptotic indices measured peroperatively correlate with survival. Ki67 expression was evaluated in tumour material from 66 patients, using immunohistochemistry. Ki67 index in 10 randomly selected tumour areas as well as “hot spots” (tumour area with the highest Ki67 index) was calculated. Ki67 index ≥1% could be used to identify patients with a higher mortality rate. This was found for both primary tumours and metastases, using a Cox proportional hazard model of overall survival adjusted for age and sex.

The apoptotic index in both primary tumours and metastases was under 0.01% in both treated and untreated patients regardless of their Ki67 index, and necrosis could not be identified in any of the tumour sections studied.
Table 3. Ki67 index ≥1% can be used to identify patients with a poorer prognosis. The relative hazard ratio for each risk factor obtained in Cox proportional hazard models of overall survival in patients with MSPCs is shown with 95% confidence intervals (95% CI). Models are adjusted for age and sex.

<table>
<thead>
<tr>
<th></th>
<th>n/N</th>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary tumour</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Hot spot’ Ki67 index ≥1%</td>
<td>11/36</td>
<td>2.4 (1.1-5.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Average Ki67 index ≥1%</td>
<td>5/36</td>
<td>5.4 (1.7-17.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Metastasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Hot spot’ Ki67 index ≥1%</td>
<td>21/49</td>
<td>2.0 (1.0-3.7)</td>
<td>0.03</td>
</tr>
<tr>
<td>Average Ki67 index ≥1%</td>
<td>13/49</td>
<td>2.5 (1.2-5.1)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The group of patients with an average Ki67 index ≥1% had a median survival only half that of those with Ki67 index <1%. Most tumours in the study displayed a Ki67 index clearly under or above 1% which in clinical practice simplifies the prognostic evaluation. This report shows that Ki67 index <2% is seemingly not useful as a criterion for benign tumour behaviour and that Ki67 index ≥1% in tumour tissue specimens characterizes patients with MSPCs having a poorer prognosis.

**Paper II: Tachykinin Expression in Endocrine Tumours and Elevated Plasma-Tachykinin Concentrations are Associated with Carcinoid Diarrhea and Flush**

This study aimed to evaluate the association between plasma-TK (P-TK) concentrations and clinical symptoms in MSPCs. A new polyclonal antibody to the common C-terminal of the TK family was designed and created by Mats Stridsberg at the department of clinical chemistry and then used in a radioimmunoassay (RIA) and in immunohistochemical analysis (IHC). In this study, the antibody to TK was used in immunohistochemical studies on 24 patients with MSPCs and 35 patients with various types of endocrine tumours from other locations in the GI tract and other organs: ECLoma type 1 (n=1), ECLoma type III (n=1), lung carcinoid (n=5), endocrine pancreatic tumour (n=6), appendix carcinoid (n=6), rectal carcinoid (n=1), medullary thyroid cancer (n=5), adreocortical cancer (n=5), pheochromocytoma (n=1), neuroblastoma (n=2), follicular thyroid adenoma (n=2) and parathyroid adenoma (n=2). P-TK was measured in 42 patients diagnosed with MSPCs before treatment. Plasma-Cga (P-CgA) and U-5HIAA were also analysed. A Spearman rank test was used to test each marker for association with the
symptoms of diarrhea and flush. A partial Spearman rank test was used to
test each marker while adjusting for the other two markers.

TK immunoreactivity (IR) was only seen in tumours that also produce 5-HT. This finding agrees with earlier results and with speculations on an as-
association between 5-HT and TK expression (17, 30, 138). In MSPCs, the
generally high tumour TK IR can be used neither to predict the diversity nor
the severity of symptoms in patients.

Figure 3. Immunohistochemical staining of metastases from MSPC, using antibod-
ies to C-terminal TK.

All tumour markers were correlated with the presence of diarrhea and
flush (Table 3). P-TK was the only tumour marker independently correlated
with the presence of diarrhea (p<0.01). Basal P-TK was high and was raised
further during a cutaneous flush test in individuals later shown to have
MSPCs.

Table 4. Biochemical markers associated with diarrhea in patients with MSPCs
before treatment start. All markers are elevated in patients with daily episodes of
flush and diarrhea when compared with those experiencing occasional or no symp-
toms. P-TK association with the severity of diarrhea is independent of U-5HIAA and
P-CgA effects.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Severity of diarrhea*</th>
<th>Severity of diarrhea§</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-TK</td>
<td>0.6 (p&lt;0.001)</td>
<td>0.4 (p&lt;0.01)</td>
</tr>
<tr>
<td>U-5HIAA</td>
<td>0.4 (p&lt;0.05)</td>
<td>−0.1 (p=0.5)</td>
</tr>
<tr>
<td>CgA</td>
<td>0.4 (p&lt;0.01)</td>
<td>0.2 (p=0.2)</td>
</tr>
</tbody>
</table>

* Spearman's rank correlation (r) between measured concentrations and severity of diarrhea.
§ Partial Spearman correlation co-efficient adjusted for the other two biochemical markers

P-TK was, unexpectedly, the only tumour marker to be independently asso-
ciated with diarrhea in newly diagnosed patients with MSPCs. The mecha-
nisms behind the characteristic carcinoid flush and diarrhea are unknown.
Results from studies on the role of tachykinins in other GI disorders could
help explain this association between TK and carcinoid diarrhea. P-TKs are
involved in other diarrheic conditions such as the irritable bowel syndrome
In the normal gut, SP and NKA, via TK receptors on muscle cells and secretomotor neurons, have stimulatory effects on gut motility (139, 142). There is also evidence that TK can induce 5-HT release from colonic mucosa via NK2 and NK3 receptors (143). P-SP concentrations have been shown to correlate with the severity of diarrhea in cryptosporidiosis (144). Earlier studies have not shown any significant correlation between individual members in the TK family and carcinoid diarrhea (34, 145, 146). In this study, total P-TK concentrations were independently associated with diarrhea, which tallies with results of the studies mentioned above, demonstrating overlapping effects of the members of the TK family that may have a combined effect on intestinal motility.

Figure 4. Plasma-tachykinins in relation to diarrhea severity

**Paper III: Connective Tissue Growth Factor**

**Expression in Endocrine Tumours and its Relation to alpha-Smooth Muscle Actin Expression in Tumour Stroma**

Immunohistochemical studies were performed to determine the extent of CTGF expression in MSPCs (from 42 patients) in relation to other endocrine tumour types (from 34 patients): ECLoma type 1 (n=1), ECLoma type III (n=3), lung carcinoid (n=3), endocrine pancreatic tumour (n=6), appendix carcinoid (n=6), rectal carcinoid (n=2), medullary thyroid cancer (n=3),
adrenocortical cancer (n=4), pheochromocytoma (n=1), neuroblastoma (n=2), follicular thyroid adenoma (n=2) and parathyroid adenoma (n=2) as well as in the endocrine tumour cell line BON1. Further characterization of CTGF in MSPC and BON1 cell protein lysate was done using Western immunoblotting technique. To study the possible relationship between CTGF and fibrosis in endocrine tumours, CTGF expression was compared with the immunohistochemical expression of α-SMA, a marker for myofibroblast differentiation.

Intensive CTGF IR, in more than half of the tumour cells, was detected in all tumour tissue from MSPCs included in this study and Western blot analysis revealed protein bands corresponding to full length and fragments of CTGF. With exception for two endocrine pancreatic tumours, CTGF IR in the other endocrine tumours studied was less or absent. α-SMA IR was highest in tumours with greater numbers of CTGF IR tumour cells. 34/42 MSPCs and one endocrine pancreatic tumour expressed α-SMA in more than half of fibroblast-like tumour stromal cells.

**Figure 5.** MSPC tissue immunostained with antibodies to CTGF, α-SMA and CD31/CD34 (identifies vascular endothelial cells) demonstrating typical tumour cell IR for CTGF and stromal expression of α-SMA detected both in myofibroblasts and in vascular smooth muscle cells.

CTGF expression was detected, using immunoﬂuorescence in a subpopulation of BON1 cells; minimal overlap of CTGF and CgA immunoreactivity was present. Protein bands migrating at 38, 36 and 20 kD were also detected using Western blot. BON1 cells could be a model for future study of CTGF function in endocrine tumours.

CTGF is known to mediate TGF-β-induced myofibroblast differentiation and collagen production which are both key mechanisms in fibrosis development (125, 131, 133, 147). In a large series, this study confirms that MSPCs generally express high concentrations of CTGF. Additionally, most fibroblast-like cells surrounding tumour cell aggregations express α-SMA, indicating myofibroblastic differentiation. Myofibroblasts probably contribute to the excessive fibrosis that is often present in these tumours and CTGF may be involved in stimulating this process.
**Paper IV: Islet Autoantigen-2 Expression is Up-regulated in Tumour Tissue Compared with Normal Enterochromaffin Cells**

To characterize gene expression differences in MSPCs compared with intestinal mucosa, the differential display method was used. One band was distinctly up-regulated in primary and metastatic tumours. This band was reamplified and subsequently cloned into plasmid. DNA sequencing of several clones showed that the band was Islet autoantigen-2 (IA-2/ICA 512).

Distinct expression of IA-2 mRNA was detected by *in situ* hybridization in all tumour tissue samples, including primary tumours and metastases from 13 patients. However, IA-2 mRNA expression could not be detected in normal small intestinal mucosa. This finding was confirmed using indirect immunofluorescence and confocal laser microscopy; a distinct and intensive expression of IA-2 in primary and metastatic tumours was seen whereas normal EC cells failed to show detectable IA-2 protein expression. The same EC cells were clearly immunoreactive when using antibodies to CgA and 5-HT.

These results suggest that IA-2 is not essential for the basal production of these hormones. It is more likely that IA-2 expression is necessary for distal events in the secretory pathway. Differences in IA-2 expression between normal EC cells and tumour cells may be due to differences in the amount of secretion stimulus they are receiving, or could represent other tumour-related changes.

Support for a secretory role was also inferred from differences in IA-2 expression following tumour biotherapy. IA-2 expression was lower in patients who had received a combination of α-interferon and octreotide prior to operation, perhaps reflecting reduced secretory activity in the tumour cells.

More recent studies, focused mainly on the beta-cell population, produced results that have lead to speculation about a key role for IA-2 in the regulation of insulin secretion. Glucose stimulation rapidly up-regulates IA-2 biosynthesis in resting INS-1 cells, a highly differentiated rat insulinoma cell line (148). IA-2 knockout mice show increased blood glucose concentrations and diminished insulin release in glucose tolerance tests (149). Knockdown of endogenous IA-2 by short interfering RNA resulted in a nearly-total loss of glucose-induced secretion and a 50% decrease in basal insulin release. Over-expression of IA-2 resulted in a 6-fold increase in glucose or K+ induced insulin secretion and a nearly 3-fold increase in the number of insulin-containing secretory granules (150). The half-life of insulin in cells over-expressing IA-2 was nearly twice as great as that in mock transfected cells, suggesting that IA-2 was stabilizing the insulin-containing vesicles. Finally, one interesting study provided evidence of a feedback mechanism. In INS-1 cells, exocytosis and insertion of IA-2 in the cell membrane promoted a Ca2+ dependent cleavage of human IA-2 by the protease µ-capain,
releasing a fragment that translocates to the nucleus and promotes expression of the insulin gene (151).

These studies corroborate our conclusion that IA-2 is an integral component of late-stage hormone secretion regulation. IA-2 is richly expressed in untreated tumours, reflecting their intense hormone secretion activity, while IA-2 expression appears to some extent to be reduced by somatostatin analogue and interferon treatment.
Concluding Remarks

The purpose of this thesis was to analyse MSPCs in order to improve our understanding of their biology by examining both similarities and disparities in the biological characteristics of this tumour group. Using the information gathered, clinically relevant groups can be formed that may be helped by new treatment forms or have specific treatment needs.

It is demonstrated in this thesis that MSPCs share several biological characteristics such as production of 5-HT, TKs, CgA, CTGF, and IA-2. These tumours also display few Ki67 immunoreactive cells, indicating a low proliferation and few apoptotic cells. Additionally, abundant myofibroblasts in the tumour stroma may account for the high prevalence of fibrotic complications in patients with these tumours as well as contribute to tumour invasion and vascular development. IA-2 expression is greater in MSPCs than in EC cells in the mucosa and may be related to increased activity in the later stages of regulated secretion. These factors can be used in future studies to define these tumours as a group within the endocrine tumour population.

In these studies, MSPCs behave as a relatively homogeneous group when compared with other endocrine tumours. Recent genetic (101, 102, 152-154) and expression studies (155) indicate that MSPCs probably have a distinct tumour biology when compared with other NETs. It may therefore no longer be appropriate to extrapolate results from heterogeneous populations of NETs that originate from different regions in the GI tract and other organs and assume them to be valid for MSPCs.

Within this ‘homogeneous’ MSPC group, several subgroups can be defined. Patients with tumours displaying either a solid growth pattern and/or a Ki67 index $\geq 1\%$ had less favourable prognosis than those who did not. This information should be considered both in discussions concerning clinical treatment and in studies on the biology of these tumours. Subgroups of patients that suffer from symptoms of flush and/or diarrhea can also be defined. These symptoms are associated with increased concentrations of P-TK, U-5HIAA and P-CgA.

New treatment possibilities for patients with these tumours are introduced in this thesis. Carcinoid diarrhea can often be controlled with somatostatin analogues; however, for patients whose symptoms persist, new drugs that specifically target TK receptors may be useful. Lecci et al. speculate that secretory diarrhea in irritable bowel syndrome can be alleviated by treatment with NK2 receptor antagonists acting through a mechanism of pre-junctional...
modulation of cholinergic motor neurone activity (139). In a phase III trial, NK2 receptor antagonists reduced NKA-induced gut motility in healthy volunteers – but not NKA-induced flush (156) which is probably mediated by NK1 receptors (157). Kordasti et al. have shown that SP receptor antagonists can shorten the duration of diarrhea in mice (158).

Tumour stroma is no longer considered a passive component in tumour development. The dominance of myofibroblasts in MSPC stroma certainly warrants further investigation. Another possible target for future therapy is CTGF, which may be involved in the development of MSPCs through paracrine processes such as myofibroblast activation. Future studies may show that circulating concentrations of CTGF may be also be related to fibrosis and/or tumour development in these patients. A recent study reported that human CTGF-specific monoclonal antibody reduced tumour growth and metastases and attenuated tumour angiogenesis and cancer cell proliferation in an orthotopic mouse model of pancreatic cancer (159).

In conclusion, MSPCs comprise a clinically relevant group of patients with similar biological features that are distinct from other endocrine tumours. Subgroups of patients within this group can be defined which may be relevant for establishing prognosis and future treatment modalities for patients with these tumours. A more thorough description of the genetic and molecular changes in tumour development is the next step.
Main Findings of the Investigation

- Primary tumours and metastases from well characterized MSPCs were studied with the aim of improving the prognostic power of histopathology. This work defined two factors that may help to identify patients with a less favourable prognosis: solid growth pattern and a Ki67 index ≥1%.

- TKs are produced in association with 5-HT in endocrine tumours in the gut and lung. The presence of both flush and diarrhea in patients with MSPCs is related to higher P-TK concentrations. P-TK association with diarrhea is independent of U-5HIAA concentrations.

- CTGF expression is generally high in MSPCs, especially in tumour areas adjacent to extensive α-SMA-expressing stroma. This supports a role for CTGF in carcinoid fibrosis. CTGF is also expressed in other neuroendocrine tumours, though, most often at lower levels. BON1 cells may be a useful in vitro system to study CTGF in an endocrine tumour setting.

- IA-2 expression is greater in MSPCs than in EC cells in the mucosa possibly related to differences in the later stages in regulated secretion.
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TACK!!!


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