Improving the Diagnosis and Management of Neuroendocrine Tumors: Utilizing New Advances in Biomarker and Molecular Imaging Science

Valeria Giandomenico^a Irvin M. Modlin^b Fredrik Pontén^c Mats Nilsson^d
Ulf Landegren^d Jonas Bergqvist^e Mohid S. Khan^f Robert P. Millar^g Bengt Långström^h
Jürgen Borlakⁱ Barbro Eriksson^j Bengt Nielsen^k Lars Baltzer^h John C. Waterton^l
Håkan Ahlström^m Kjell Öbergⁿ

^aDepartment of Medical Sciences, Endocrine Oncology, University Hospital, Uppsala, Sweden; ^bGastrointestinal Pathobiology Research Group, School of Medicine, Yale University, New Haven, Conn., USA; ^cSwedish Human Proteome Resource Project, ^dRudbeck Laboratory, Department of Immunology, Genetics and Pathology, and ^eAnalytical Chemistry and Neurochemistry, Biomedical Center, Uppsala University, Uppsala, Sweden; ^fRoyal Free Hospital Neuroendocrine Tumor Unit, University College London, London, and ^gHuman Reproductive Sciences Unit, MRC, University of Edinburgh, Edinburgh, UK; ^hDepartment of Biochemistry and Organic Chemistry, Uppsala University, Uppsala, Sweden; ⁱCenter of Drug Research and Molecular Pharmacology, Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany; ^jDepartment of Endocrine Oncology, Center of Excellence, University Hospital, Uppsala, and ^kAcademic Program, GE Healthcare International Research Division, Stockholm, Sweden; ^lTranslational Sciences, AstraZeneca, and Biomedical Imaging Institute, University of Manchester, Manchester, UK; ^mDepartment of Oncology, Radiology and Clinical Immunology, and ⁿDepartment of Endocrine Oncology, Center of Excellence, Endocrine Tumors, Uppsala University, Uppsala, Sweden

Key Words

Biomarkers · Circulating tumor cells · Molecular imaging · Neuroendocrine tumors

Abstract

Neuroendocrine tumors (NET) are malignant solid tumors that arise in hormone-secreting tissue of the diffuse neuroendocrine system or endocrine glands. Although traditionally understood to be a rare disease, the incidence and prevalence of NET have increased greatly in the past 3 decades. However, during this time, progress in diagnosis and outcome of NET has generally been modest. In order to achieve improved outcome in NET, a better understanding of NET biology combined with more reliable serum markers and better techniques to identify tumor localization and small lesions are needed. Although some NET biomarkers exist, sensitive and specific markers that predict tumor growth and behavior are generally lacking. In addition, the integration of new mo-

lecular imaging technologies in patient diagnosis and follow-up has the potential to enhance care. To discuss developments and issues required to improve diagnostics and management of NET patients, with specific focus on the latest advances in molecular imaging and biomarker science, 17 global leaders in the fields of NET, molecular imaging and biomarker technology gathered to participate in a 2-day meeting hosted by Prof. Kjell Öberg at the University of Uppsala in Sweden. During this time, findings were presented regarding methods with potential prognostic and treatment applications in NET or other types of cancers. This paper describes the symposium presentations and resulting discussions.

Copyright © 2013 S. Karger AG, Basel

U.L. is one of the founders of and holds stock in Olink Bioscience AB, commercializing the proximity ligation and padlock technologies. K.Ö. has received speaker's honoraria from Pfizer, Novartis and IPSEN and consulting fees from their advisory boards.

Introduction

Neuroendocrine tumors (NET) are malignant solid tumors that arise in hormone-secreting tissues of the diffuse neuroendocrine system. During the early stages of disease, NET are generally slow growing and nonsymptomatic, but they have the potential to secrete excessive quantities of bioactive amines. Late in the disease course, typically following tumor metastasis to the liver, hormonal hypersecretion can lead to well-defined and debilitating clinical syndromes, such as flushing and diarrhea of carcinoid syndrome, and a variety of hormone-dependent syndromes from functioning pancreatic NET (pNET). Although several guidelines have been agreed upon to standardize diagnostics, diagnosis is typically only made after tumors produce clinical symptoms and are metastatic due to the insidious natural history of NET [1, 2].

Although traditionally understood as a rare disease, the incidence and prevalence of NET has increased greatly in the past 3 decades, and NET are now a more common form of gastrointestinal neoplasm than both gastric and esophageal cancers combined [3]. A dramatic improvement in survival time was observed among patients with metastatic NET diagnosed between 1988 and 2004, coincident with the introduction of the somatostatin analogue octreotide in 1987 for the control of carcinoid syndrome, a major cause of morbidity and mortality if untreated [3]. Although octreotide has had a substantial effect on relieving symptoms of functioning NET [4] and has shown antiproliferative effects in patients with well-differentiated metastatic NET of the small intestine [5], therapeutic interventions are usually noncurative once metastasis has occurred. Cytotoxic therapies have demonstrated some effect in controlling pNET growth and spread [6]. New targeted therapies such as mTOR (mammalian target of rapamycin) or tyrosine kinase inhibitors [7, 8] have demonstrated antitumor efficacy in pNET [8, 9]. An unmet need exists for additional therapies and especially for earlier disease detection and diagnosis.

Although advances in the past few years have been encouraging, progress in the diagnosis and outcome of NET has generally occurred at a modest rate. Many different variables may contribute to this situation, including the peculiarities of NET themselves. For example, the relative rarity and heterogeneity of the disease, the multiplicity of NET classification systems, and the historical lack of well-designed clinical trials have all impeded progress in this field. Additionally, as NET are a diverse set of tumors that can exhibit variable behavior, histological differentiation and clinical course, individualized therapy is required

that cannot be easily generalized to larger populations. It has previously been identified that in order to improve outcome in NET, a better understanding of NET biology combined with more reliable serum markers and better techniques to identify tumor localization and small lesions are needed [6, 9]. In general, there is a delay of 3–4 years before the correct diagnosis is made by the treating physician.

The publication of well-designed clinical trials in patients with NET during the last few years, including the PROMID study, which established the antitumor effect of octreotide LAR 30 mg/month in patients with functioning or nonfunctioning metastatic NET of the midgut (small intestine) [5], the RADIANT series of trials evaluating the mTOR inhibitor everolimus alone or in combination with octreotide LAR [7, 10, 11], late-stage trials of sunitinib for patients with metastatic well-differentiated pNETs [8] and evaluation of bevacizumab (in combination or alone) in patients with NETs or neuroendocrine carcinomas [12, 13], will undoubtedly provide new insights into patient care and perhaps serve to hasten the pace of further biomarker development [12]. Identified markers for therapeutic response, such as the presence of sst (somatostatin subtype receptors) [14], the absence of O⁶-methylguanine DNA methyltransferase with temozolomide [15] and loss of heterozygosity on chromosome 18 [16], or the expression of PTEN or TC1 protein in pNET [17], may enable more personalized and targeted therapy [18].

Novel Biomarker Applications in NET

Biomarkers are physical, functional or biochemical indicators of disease that can provide vital information for assessing disease development and prognosis, predicting response to therapy and estimating the likelihood of adverse events associated with therapy [19]. For many diseases, the desire to achieve early diagnosis and curative treatment has led to the current drive to identify new clinically relevant biomarkers with prognostic and/or predictive value.

Status of NET Biomarkers and Unmet Need

Sensitive and specific biomarkers are still largely lacking, and current assays fail to identify biomarkers at an early stage in disease progression in most conditions, including the rare malignancy NET. Although some NET biomarkers do exist (table 1), sensitive and specific markers that predict tumor growth and behavior are generally

Table 1. Serum markers with diagnostic and prognostic use in NET

Serum markers		Specificity
<i>General diagnostic use</i>		
Various	chromogranin A/B	high
	pancreatic polypeptide,	inter-
	neuron-specific enolase	mediate
	choriogonadotropin α -/ β -chain	low
<i>Specific diagnostic use</i>		
Thymus	adrenocorticotrophic hormone	inter-
Bronchus	adrenocorticotrophic hormone,	mediate
	antidiuretic hormone, serotonin	low
	(urinary 5-hydroxyindoleacetic acid)	
	Histamine (urinary-methylimidazole	
Stomach	acetic acid), gastrin-releasing peptide,	high
	growth-hormone-releasing hormone,	
	vasoactive intestinal peptide,	
	parathyroid-hormone-related protein	
Pancreas	histamine, gastrin	high
	ghrelin	low
Duodenum	gastrin, insulin, proinsulin, glucagon,	high
	somatostatin	inter-
	proinsulin C peptide, neurotensin,	
	vasoactive intestinal peptide,	
Ileum	parathyroid-hormone-related protein	mediate
	calcitonin	high
Colorectum	somatostatin, gastrin	
	serotonin (urinary 5-hydroxy-	high
Colorectum	indoleacetic acid), neurokinin A,	inter-
	neuropeptide K, substance P	mediate
Colorectum	peptide YY, somatostatin	inter-
		mediate

scarce [6]. NET differ from other types of neoplasias as they synthesize, store and secrete characteristic peptides, providing an opportunity for their use as biomarkers in diagnosis. One of these peptides is chromogranin A (CgA), the plasma levels of which are elevated in approximately 80–90% of patients with NET of the gut, and CgA levels also correlate with tumor burden and recurrence [20]. Although CgA is the best available biomarker and accessible via minimally invasive blood sampling, CgA only serves as a general NET marker and cannot distinguish among different forms of NET. Furthermore, CgA testing is usually performed when symptoms present, which is often associated with cancer metastasis. Although CgA monitoring provides information that is helpful in delineating tumor burden, rate of growth and subsequent treatment therapies, additional more sensitive tumor-specific biomarkers are required. Similarly,

the urinary 5-hydroxyindoleacetic acid test is generally performed too late in the disease course, i.e. when liver metastases occur, and it does not warrant serious consideration as a diagnostic screening test [21].

Genetic Analysis

Recent data from exome sequencing in pNET have shown significant mutations in DAXX/ATRX and MEN1, which has to be translated into clinical practice [17].

Using biomathematical tools, genes with potential diagnostic or therapeutic relevance might be identified via mRNA measurements. For example, NET of different tumor grades present a different set of transcripts, which provides information regarding tumor status and prognostic capacity. Analysis with quantitative real-time PCR has identified five genetic markers that separate genetic appendiceal carcinoids into benign or malignant and mixed cell forms. A mitosis-regulatory gene *NAP1L1*, an adhesin *MAGE-D2*, an estrogen antagonist and metastasis marker *MTA1*, the apoptotic marker *NALP* and *CgA* were identified from 42 appendiceal samples [22]. Using tools such as perceptron classification and principal component analysis, it is hoped that models could be used to discriminate tumors, predict how tumors might behave and estimate the probability of metastasis [22, 23].

Supervised machine learning can be used to classify small-intestine NET subtypes and accurately predict metastasis on the basis of gene expression measurements [23]. The advent of machine learning within the context of gene expression relies on designing algorithms that delineate gene expression patterns within tumor cells. The effectiveness of such mathematical tools relies on the selection of appropriate housekeeping genes. A predictive gene-based model constructed for small-intestinal NET identified a panel of molecular markers related to tumorigenicity, metastasis and hormone production. Other than those previously identified in appendiceal carcinoids, identified markers included: Ki-67, a protein associated with cell proliferation; *Survivin*, an inhibitor of apoptosis [24]; *FZD7*, known to be highly expressed in well-differentiated colorectal cancer tumor cells; *Kiss1*, a metastasis-suppressing gene, and *NRP2*, a protein that interacts with vascular endothelial growth factor [23]. Furthermore, gene expression profiling and supervised machine learning could be used to classify NET subtypes and accurately predict metastasis.

A majority of small-intestinal NETs (carcinoids) show losses on chromosome 18. However, no genotype-phenotype correlation has been found. Sometime losses of the whole chromosome 18 occur [23].

Table 2. Potential use of established and novel gastrointestinal NET markers [26]

Symbol	Description	Potential use
<i>MAGE-D2</i>	melanoma antigen family D2	SI NET subtype classification and metastasis prediction
<i>MTA1</i>	metastasis-associated protein 1	SI NET subtype classification and metastasis prediction
<i>NAP1L1</i>	nucleosome assembly protein 1-like 1	SI NET subtype classification and metastasis prediction
<i>Ki-67</i>	markers of proliferation	SI NET subtype classification and metastasis prediction
<i>Kiss1</i>	metastasis suppressor	SI NET subtype classification and metastasis prediction
<i>Survivin</i>	implicated in inhibition of apoptosis	SI NET subtype classification and metastasis prediction
<i>FZD7</i>	frizzled homologue 7, a frizzled receptor, is a receptor for Wnt signaling proteins	SI NET subtype classification and metastasis prediction
<i>NRP2</i>	neuropilin 2	SI NET subtype classification and metastasis prediction
<i>CgA</i>	chromogranin A	SI NET subtype classification and metastasis prediction
<i>APLP1</i>	amyloid precursor-like protein 1	tumor progression
<i>GRIA2</i>	glutamate receptor, ionotropic, AMPA2	diagnostic mRNA marker
<i>SPOCK1</i>	sparc, osteonectin (proteoglycan)	tumor invasion
<i>PNMA2</i>	paraneoplastic antigen MA2	diagnostic and prognostic marker
<i>SERPINA10</i>	α_1 -antiproteinase antitrypsin	diagnostic and prognostic marker
<i>CXCL14</i>	chemokine (CXC motif) ligand 14	tumor progression
<i>Nkx2-3</i>	NkX2-3 transcription factor related, locus 3	tumor progression
<i>GPCR112</i>	G-protein-coupled receptor 112	antibody-based therapy
<i>OR51E1</i>	olfactory receptor, family 51, subfamily E, member 1	novel cancer biomarker

SI = Small intestine.

While the molecular pathologies that lead to tumorigenesis and metastatic processes are relatively unknown in NET, elucidating these molecular triggers will provide targets for pharmacological intervention. A recent study by Leja et al. [25], using Affymetrix microarrays and advanced bioinformatic analysis to recognize differentially and specifically expressed genes, identified six novel marker genes for neuroendocrine small intestinal tumors (carcinoids): paraneoplastic antigen Ma2 (*PNMA2*), testican-1 precursor (*SPOCK1*), serpin A10 (*SERPINA10*), glutamate receptor ionotropic AMPA 2 (*GRIA2*), G-protein-coupled receptor 112 (*GPCR112*) and olfactory receptor family 51 subfamily E member 1 (*OR51E1*). All markers were expressed in both NET and normal neuroendocrine cells except for *GRIA2*, which was specifically expressed in NET cells. The authors suggested that *GPCR112* and *OR51E1* may be potential targets for antibody-based diagnosis and therapy as these genes encode proteins associated with the plasma membrane [26]. To date, studies using gene expression have led to the inference of potential uses of established and novel gastrointestinal NET markers (table 2) [27].

Novel Methods for Genetic Analysis

Cancer companion diagnostics is a pathway-oriented approach to cancer analysis that integrates mutational

Table 3. Examples of technologies and their potential applications (from Nilsson et al. [28])

Padlock probes and molecular inversion probes in solution

Parallel SNP genotyping
Gene expression profiling
Multiplex microbial analysis
Relative quantification of DNA sequences
Epigenetic profiling

Selector-guided circularization of genomic sequences

Template generation for resequencing
Relative quantification of DNA sequences
Epigenetic profiling

Circle-to-circle amplification

Multiplex amplification

Padlock probes in situ

Single-cell SNP genotyping
Single-cell gene expression analysis
Nucleic acid localization

analyses with protein activity biomarkers in clinical samples from patients treated with molecularly directed drugs. The goal is to develop diagnostics that better predict patient response to a drug and to create improved methods for monitoring therapy. A mutational analysis will be conducted in all genes involved in the pathway

Fig. 1. Progression- and recurrence-free survival of patients with primary small-intestine NET after surgery with curative intent depend on Ma2 autoantibody titer [32].

targeted by the drug of interest, as well as evaluation of protein activation markers such as phosphorylation status in the pathway of interest. Although genome sequencing is now available, newer and less expensive approaches and enrichment strategies are needed in situations where analysis of minority cell populations (such as solid tumors) are essential. Recent technological developments based on targeted circularization reactions of genomic DNA and amplification now allow high-throughput sample preparation for next-generation sequencing and related methods permit single-cell genotyping even in the preserved context of tumor tissue sections (table 3) [28].

The padlock probe technique enables detection of single nucleotide variants and other tumor- or cell-specific transcriptomic markers in tissue specimens *in situ* at the single-cell level. The assay could be of great value for clinical pathology [29]. The selector probes, by contrast, are used to direct the circularization of genomic DNA sequences, targeted for sequence analysis by next-generation sequencing techniques [30, 31]. The selector technique allows for highly specific and cost-effective amplification of sets of 20–500 genes, focusing the sequencing power of the next-generation sequencing instruments to part of the genome that is most relevant for diagnostics, affording deeper sequencing of genetically heterogeneous tumor tissues.

Blood-Based Analysis

PNMA2 Autoantibodies

Building on results derived from genetic analysis, PNMA2 has been evaluated as a potential biomarker. Specifically, the analyses have centered on the prognostic significance of PNMA2 autoantibodies in blood. A novel indirect ELISA was set up to detect PNMA2 autoantibodies in serum and plasma samples from 124 patients with NET of the small intestine at different stages of disease. Among patients with a primary tumor ($n = 36$), lymph node metastasis ($n = 38$) and liver metastasis ($n = 50$), the novel PNMA2 autoantibody ELISA showed high sensitivity, specificity and accuracy, with receiver-operating characteristics curve analysis demonstrating an area of 0.734 for liver metastasis, 0.802 for lymph node metastasis and 0.816 for a primary NET of the small intestine [32]. As such, the assay was able to stratify between patients with a primary NET and healthy controls (area under the curve >0.730). Patients with an NET of the small intestine who expressed low levels of Ma2 autoantibodies exhibited significantly longer progression- and recurrence-free survival (fig. 1) [32].

This analysis was further expanded to patient samples with typical and atypical lung carcinoids to determine if this prognostic screen could be used in the diagnosis of other NET. Higher levels of Ma2 autoantibodies were

measured in samples of patients with typical and atypical lung carcinoids compared with control samples. This study illustrates an early potential translational success as Ma2 autoantibodies may be novel, sensitive biomarkers for small-intestine NET that may allow for early detection of uncontrolled cellular proliferation prior to tumor metastasis with the concomitant possibility of curative therapy.

Identification of Circulating Tumor Cells

Circulating tumor cells (CTC) are cells that have been dislodged from the tumor mass and circulate in the body through the blood. The fact that CTC can be found in patients before primary tumors are detected point to their potential role in early diagnosis and prognostication. The presence of tumor cells in blood also provides ready, minimally invasive access to tumor tissue for molecular analyses, e.g. for monitoring responses to therapy. CTC are currently used as an independent prognostic marker in metastatic breast cancer, with high CTC levels during therapy associated with a poor outcome [33]. As it is unknown if NET release CTC and/or if potential CTC from NET express epithelial cell adhesion molecule (EpCAM), an antigen commonly expressed on carcinomas, the first systematic analysis of EpCAM expression in NET was performed using CellSearch, the only FDA-approved method to detect CTC in whole blood [34].

In this study, EpCAM protein expression was evaluated in 74 samples of formalin-fixed, paraffin-embedded NET tissue by immunohistochemistry [34]. Seventy-nine patients with metastatic NET, 42 midgut, 5 unknown primary, 19 pancreatic and 13 bronchopulmonary tumors, had blood samples drawn for CTC isolation and enumeration, utilizing the CellSearch platform. Patients were classified as having progressive or nonprogressive disease on the basis of serial imaging. Strong homogeneous membranous EpCAM expression was observed in all ileal NET ($n = 26$) and pNET ($n = 16$), whereas variable EpCAM expression was observed in bronchopulmonary NET ($n = 13$). Forty-three percent of midgut and 21% of pNET had CTC detected, with a range of 0–62 and 0–11, respectively. The absence of CTC was strongly associated with stable disease ($p < 0.001$). There were moderate correlations between CTC levels and urinary 5-hydroxyindoleacetic acid ($p = 0.007$), and between CTC levels and burden of liver metastases ($p < 0.001$). There was no or only a low correlation between CTC levels and Ki-67 ($p = 0.59$) and serum CgA ($p = 0.03$; fig. 2) [34].

Fig. 2. Levels of CTCs in NET [34].

Protein Analysis

Human Protein Atlas Initiative

The Swedish Human Protein Atlas (HPA) project has been set up for the systematic generation of specific antibodies on a global scale and to utilize these antibodies for studies of the corresponding proteins [35]. This effort relies on an antibody-based proteomics strategy [36] applied to combine high-throughput generation of affinity-purified polyclonal antibodies with (i) immunohistochemistry-based protein profiling on tissue microarrays containing human normal and cancer tissues, and (ii) immunofluorescence-based protein profiling of the subcellular proteome. The ultimate goal of the HPA is threefold: (i) to generate and validate antibodies against all human proteins (a major isoform for each protein-encoding gene); (ii) to create an information database including a map of protein expression patterns in cells and human tissues, and (iii) to utilize affinity reagents and protein expression data to identify and explore biomarkers of clinical relevance. This large-scale project hopes to provide new diagnostic, prognostic and treatment-predictive markers not only for NET but also for many other diseases. Antibody-based proteomics appears as an advantageous route to further develop diagnostics and find new therapeutic approaches in oncology [37], as this strategy allows numerous functional assays to be performed on

Fig. 3. Schematic of the HPA project (based on Berglund et al. [39]). IF = Immunofluorescence; IHC = immunohistochemistry.

sample tissue, ranging from a simple ELISA to detailed localization studies using fluorescent probes and immunoprecipitation pull-down assays.

Protein expression patterns in normal human tissue from 144 individuals and cancer tissue, corresponding to the 20 most common forms of human cancer, from 216 patients were determined for over 15,500 antibodies, corresponding to over 12,200 unique genes (approximately 60% of all human protein-coding genes). Moreover, all antibodies were tested on over 40 cell lines. All data including underlying images are freely available at the HPA web portal (www.proteinatlas.org) [38–40]. The HPA can also be used as a tool for pathology with opportunities to identify different types of biomarkers with in silico-based methods [41]. The HPA can also be utilized for finding clinically useful biomarkers for other diseases than cancer, exemplified by the recent identification of novel surface markers expressed on β -cells in Langerhans islets of the pancreas, which could be used to develop tools for positron emission tomography (PET) [42]. As various endocrine cells are represented in normal tissue and 4 different endocrine tumors are also used for protein profiling, it is hoped that similar insights based on the HPA could be identified in NET. An overview of the HPA process is provided in figure 3.

Novel Methods for Sensitive and Specific Protein Detection

Improvements in sensitivity and throughput of protein detection methods will be needed to identify novel biomarkers to observe complex protein modifications and interactions. The proximity ligation assay (PLA) is an immunoassay for protein analysis for the measurement and characterization of proteins with high specificity and sensitivity via DNA ligation and amplification reactions. The method relies on converting detection reactions to DNA reporter sequences. PLA probes with attached oligonucleotides are designed to bind pair-wise to target proteins to form amplifiable tag sequences. Upon proximal binding of a pair of probes to the target molecule, the DNA strands are brought into close proximity and allowed to hybridize to a connector oligonucleotide. The DNA strands can then be joined by enzymatic ligation to form a reporter DNA molecule. Subsequent amplification of the DNA molecule generates a signal that can be amplified using PCR, allowing for the detection of minute amounts of protein in serum (fig. 4). The technique allows the specificity of detection to be enhanced by requiring simultaneous target detection via three or more antibodies [43]. Importantly, this enhanced specificity can be obtained without problems of cross reactivity when more

Fig. 4. Schematic of the solid-phase PLA method [43]. A = Samples are incubated with antibodies preimmobilized on microparticles; B = microparticles are washed and incubated with pairs of PLA probes; C = oligonucleotides on PLA probes are ligated upon proximal binding of a common antigen and addition of a connector oligonucleotide. This is followed by amplification and detection of the ligated products by quantitative real-time PCR, the primers of which are indicated by arrows.

proteins are targeted in the same reaction, allowing multiplexed protein detection [44]. The sensitivity of the PLA technique for plasma protein measurement renders the technique suitable for detection and identification of rare protein molecules in blood, potentially allowing for new classes of promising biomarkers to be identified from bio-banked samples. The attraction of serum or plasma biomarkers lies in their potential to reveal disease processes throughout the body and guide selection of therapy and follow-up using minimally invasive blood sampling.

The PLA method can also be combined with standard fluorescent microscopy for the detection of proteins or protein-protein interactions and posttranslational protein modifications as activation markers in tissue sections using *in situ* PLA [45]. A major application of *in situ* PLA is to demonstrate the dynamic interplay between signaling molecules [46]. The technique provides an insight into protein complex association during cellular transformation events and cellular signal transmission.

Aptamers and Other Affinity Regents

Although monoclonal antibodies have provided a rich source of ligands for detecting the location and activity of target proteins, the number of new targets outstrips the capacity of the methodology for generating and screening cancer biomarkers. Alternative approaches to new ligand discovery include ('aptamers') acid that can be used to target a range of molecules via specific affinity reactions [47, 48]. The term aptamers is derived from the Latin term *ap-utus* meaning 'to fit'. Aptamers are usually 15–60 nucleotides in length that form intramolecular interactions in solution, allowing the formation of tight interaction with the surface of target molecules. Because of the extraordinary diversity of shapes that aptamers can form these agents can be developed to bind to a vast array of molecular targets. The specificity is such that closely related proteins can be distinguished, including nonidentical members of a protein family, and even between different conformation

states of the same protein. In addition to their remarkable specificity, aptamers typically bind to targets with high affinity and are developed entirely in the test tube (an advantage compared with monoclonal antibodies). Typical binders have molecular weights of around 5,000 kDa, can be readily modified by chemical methods to incorporate radionuclides, fluorophores and other functional groups site specifically and stoichiometrically, and can be immobilized on various surfaces, beads and nanoparticles without loss of binding performance. This technology offers unique possibilities in drug development, for example in target validation and in the evaluation of binding properties of early-stage leads as well as in the identification and quantification of biomarkers [48–50].

Mass Spectrometry Analysis

Detection and characterization of protein biomarkers in any tissue or body fluid, e.g. human CSF, can be performed using modern mass spectrometry (MS) methods, such as MALDI TOF MS (matrix-assisted laser desorption ionization tandem time-of-flight MS), and high-resolution nanoelectrospray MS, such as ESI-FTICR (Fourier Transform Ion Cyclotron Resonance) MS or Orbitrap MS, in conjunction with multidimensional capillary electrophoresis, capillary electrochromatography, nano- or microliquid chromatography and microchip-based devices for preclinical as well as clinical studies (fig. 5) [51–53].

MS analysis has the potential to identify and distinguish between modified proteins within the tissue extract or body fluid (e.g. CSF) and can be used for the discovery of valid biomarkers, complementing other diagnostic methods such as immunoassay and therapeutic imaging technologies.

It is also possible that miniaturized mass spectrometers (possibly hand-held devices) may soon be available at the point of care, although appropriate sample introduction systems are needed for optimal performance [52].

Fig. 5. The approach for biomarker identification: shotgun proteomics. LC = Liquid chromatography.

Novel Imaging Applications in NET

Molecular imaging technologies have undergone an explosion in growth over the past few decades and now play a central role in clinical oncology. The primary role of imaging in cancer diagnosis has traditionally been in diagnosis and staging, to precisely locate the primary tumor and metastatic lesions. However, new imaging techniques provide biomarkers of specific molecules (e.g. protein kinases) and cellular processes (perfusion, hypoxia, apoptosis, cell death, proliferation and metastasis) that influence tumor behavior and response to therapy. This functional information can lead to a greater understanding of how cancer arise, how tumors progress and metastasize, and as a guide to therapy. Importantly, functional or molecular imaging measurements with PET, single-photon emission computed tomography (SPECT) or magnetic resonance imaging (MRI) often provide pharmacodynamic biomarkers which can assist in identifying which therapies benefit which patients.

PET and SPECT Biomarkers

Rapid developments in synthetic chemistry have provided new potential tracers for molecular imaging in oncology. The function of specific metabolic pathways or expression levels of receptors and enzymes can now be measured selectively using an array of various tracers with different properties. These imaging techniques can now be used to measure metabolic changes within the tumor during and after treatment.

To date, there are a number of tracer molecules for use in PET, which have reached a level of clinical use, although some require further validation. The four preferred positron-emitting radioisotopes are fluorine-18, carbon-11, nitrogen-13 and oxygen-15. The reason why these are most common center on the ease in which they can be substituted into biomolecules without affecting function. The relatively short half-life of ^{11}C , which was previously considered to be a disadvantage, has rekindled the interest in ^{11}C tracer molecules as the short half-life permits repeat imaging, providing real-time data on a number of cellular processes. Repetitive studies can be performed using tracers limited by short half-lives (2–20 min) and to collect data for ‘time dimension’ analyses [53–55].

A variety of PET and SPECT imaging biomarkers is currently applicable to NET. The majority of all NET express the sst_2 protein, which can be tightly bound by radioactively labeled octreotide (somatostatin receptor scintigraphy) and this is now routinely used [56] in the diagnosis and staging of NET. Other functional imaging techniques used in NET include metaiodobenzylguanidine scintigraphy, PET using ^{18}F -FDG (fluorodeoxyglucose), a glucose analogue. PET combined with specific tracers [^{11}C -5-hydroxytryptophan (HTP) and ^{18}F -DOPA] provide excellent visualization of endocrine tumors and may be useful for the identification or small primary tumors, organ metastases and staging [57–60]. Currently available tracers for NET imaging are summarized in table 4. Whole-body (WB) ^{11}C -5-HTP PET can be used as

Fig. 6. Examples of clinical tracers in oncology, including NET in which ^{11}C is utilized.

Table 4. Specific isotopes for NET

^{11}C -5-HTP
^{11}C -dopamine
^{18}F -dopamine
^{68}Ga -DOTA-octreotide
^{99}Tc EDDA-HYNIC-octreotide
$[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$ -Exendin-4 (GLP-1)

a universal imaging technique to visualize the primary tumor in a range of different NET (fig. 6). A study comparing diagnostic sensitivity of PET scanning in carcinoid and islet cell tumor patients with ^{11}C -5-HTP- and ^{18}F -DOPA-based tracers revealed ^{18}F -DOPA PET/computed tomography (CT) is the optimal imaging modality for staging in carcinoid patients and ^{11}C -5-HTP PET/CT for islet cell tumor patients [59]. A drawback of using ^{11}C -5-HTP is that the high concentrations of radioactivity in the urine can produce image reconstruction artifacts that reduce quality, although carbidopa pretreatment im-

proves the detection capacity of ^{11}C -5-HTP PET [61]. Another problem is the poor availability of the technique; only two centers in the world (Uppsala, Sweden, and Groningen, The Netherlands) can produce ^{11}C -5-HTP.

Future developmental aims are to coordinate gene transcript analysis with imaging techniques such as PET to allow for a more accurate and early diagnosis of specific indicators of NET. PET/CT with ^{68}Ga -DOTA-GOC or DOTATATE is likely to replace somatostatin receptor scintigraphy in the future as it offers better resolution [57, 62] and provides a quantitative method for peptide receptor radionuclide therapy [63] as well as being less expensive than the other PET tracers (fig. 7). PET/CT triphasic imaging using ^{68}Ga DOTA-GOC for the therapeutic management of patients with NET improves anatomical localization (fig. 8). These methods are similarly sensitive, deliver complementary information and contribute equally to therapeutic decision-making [64]. FDG-PET is useful in patients with poorly differentiated tumors. New PET tracers marking apoptosis and proliferation require further study [65].

Fig. 7. PET/CT with ^{11}C -5-HTP: illustration of spinal bone metastases in a patient with carcinoid tumors. Other imaging techniques were negative.

Fig. 8. PET/CT with ^{68}Ga -DOTA-octreotide.

WB-PET-MRI

Whereas in the past imaging was focused on specific organs, today disease-specific WB, multimodality imaging is possible and is especially attractive in the case of systemic disease [65, 66]. Multimodality WB approaches revealing functional information from PET as well as anatomical information from CT or MRI are particularly

appealing. Although PET/CT has been described as the imaging modality of choice for working up most common tumor entities as well as some of the rare malignancies [67], a major drawback of CT is that soft tissue is imaged with little contrast and significant radiation is applied to the patient. Additionally, PET and CT scans cannot occur simultaneously. They potentially may cre-

ate image artifacts that could hinder interpretation of the two overlaid scans. Thus, researchers have attempted to develop a single machine incorporating both applications. Additionally, since MRI can also be used to obtain anatomical images, other researchers have envisaged the PET-MRI combination. The goal is to include simultaneous data acquisition from both modalities, resulting in combined functional and morphological images with an excellent soft-tissue contrast, good spatial resolution of the anatomy, accurate temporal and spatial image fusion and lower costs [68]. A potential PET-MRI may have application in tumor identification, staging and grading, therapy evaluation and tumor surveillance, thus improving patient management [69].

A potential treatment monitoring application of this method is also possible as, following tumor necrosis or apoptosis, tumor signals decrease commensurate with the increased mobility of water molecules. A second technique uses apparent diffusion coefficient (ADC). ADC is a measure of the diffusion path length for a water proton over the timescale of a few tens of milliseconds: since viable cells impose barriers to diffusion, the ADC may be a proxy for the amount of nonviable or dead material in the tumor, with a low ADC corresponding to a highly proliferative mass of packed cells. ADC is relatively easy to measure, and since it is an intrinsic biophysical property of the water protons rather than an MR parameter, it should be fairly robust. In a recent study by Liapi et al. [70], patients with hepatic metastasis from a primary NET were treated with transcatheter arterial chemoembolization. Treatment response was evaluated by contrast-enhanced and diffusion-weighted MRI. Following therapy, mean tumor size and percentage enhancement in the arterial and portal venous phases decreased significantly ($p < 0.0001$). The authors concluded that contrast-enhanced and diffusion-weighted imaging showed significant changes (1.51 vs. $1.79 \times 10^{-3} \text{ mm}^2/\text{s}$, $p < 0.0001$) after transcatheter arterial chemoembolization in NET and that ADC can be used to assess response of targeted tumors [70]. Ongoing projects in the US and Europe are aiming at qualifying ADC as a biomarker in drug trials. In addition, WB-MRI may have a role in tumor surveillance when repeated evaluations are required.

Several 'biomarkers' are used in MRI evaluation, the most familiar of which are vascular measurements. Such an example is the transfer constant for a gadolinium chelate contrast agent into the tumor, k^{trans} , derived from dynamic contrast-enhanced MRI [71, 72]. This MRI method is currently still under investigation as a prognostic technique. Emerging MR biomarkers measure oxygen deliv-

ery and consumption in tumors, such as deoxyhemoglobin concentrations in the tumor measured by the MRI R_2 and R_2^* relaxation rates, while another group of biomarkers uses the effect of changes in the concentration of dissolved paramagnetic dioxygen molecules on the water protons measured through the R_1 relaxation rate, a proxy for changes in the free oxygen tension in tumor voxels.

^1H -MR spectroscopy (MRS) provides biomarkers of proliferation through choline metabolites as measures of the choline pathway, while ^{13}C -MRS can provide measurements of abundant metabolites giving insights into tumor metabolism. These approaches have been limited by the very poor sensitivity of MRS. However, emerging techniques of hyperpolarized MRS are likely to lead to a resurgence of interest in this topic, although hyperpolarized MRS has not been evaluated clinically. Finally, MRS can provide measurements of drug concentrations and metabolism, particularly where the drug contains fluorine. In contrast to PET, MRS is very insensitive, and only practical for drugs of very low potency that approach millimolar concentrations in the tissue. Regarding patients with NET, currently available techniques can be used for the evaluation of liver metastases from NET primaries, but methods may require further development before application to lung metastases or primary NET [73].

Expert Opinion and Conclusions

Challenges for practitioners of NET include detection of disease before metastasis, detection of tumor remnants after surgery, reliable monitoring of therapeutic efficacy and translating findings into clinical practice. Assays at the point of care, early diagnosis and personalized therapy are all healthcare goals. It is hoped that integrating the latest techniques in capturing, enriching and visualizing cancer cells in the evaluation of NET can improve patient outcome in the clinic.

In general, the lack of a clear therapeutic target in patients with NET is a point of frustration for researchers. However, the fact that most hormonally related symptoms from functioning NET can be controlled by somatostatin analogues, particularly octreotide, is very encouraging, and it was suggested that this success may contribute a slightly more optimistic tune as further difficult research is undertaken. The view that NET are a sort of 'Rosetta stone' was also articulated, whereby the study of proliferative and secretory pathways in NET could provide insight into deciphering the mechanism for tumor growth in other cell types. The following outlines a few

broad unmet needs in the field of NET and general actions for further investigation:

- *Identify Key Tumor Pathways.* Transcription patterns within tumor samples provide an opportunity to determine a molecular signature that can distinguish not only between healthy and neoplastic cells but also between subtypes of malignant and benign NET.
- *Investigate a 'Cocktail Approach' to Targeting G-Protein-Coupled Receptors.* Initially identify all G-protein-coupled receptors and ligands that are expressed on the NET cell surface and utilize high-throughput screening and labeling in vitro to explore the effects of binding on cell growth and/or potentially target radionuclides, chemoagents and nanoparticles on the tumor site.

- *Apply a Multidisciplinary Approach to Develop New Biomarkers.*
- *Qualify Potential Biomarkers Early.* Poorly evaluated biomarkers may generate unexpected false-positive or false-negative results, as seen with prostate-specific antigen in prostate cancer.

Acknowledgment

We thank Helene Darmofal for medical editorial assistance with the manuscript. The expert meeting and manuscript development was supported by an unrestricted grant from Novartis Pharmaceuticals.

References

- 1 Vinik AI, Woltering EA, Warner RR, Caplin M, O'Dorisio TM, Wiseman GA, Coppola D, Go VL: NANETS consensus guidelines for the diagnosis of neuroendocrine tumor. *Pancreas* 2010;39:713–734.
- 2 Kloppel G, Couvelard A, Perren A, Komminoth P, McNicol AM, Nilsson O, Scarpa A, Scoazec JY, Wiedenmann B, Papotti M, Rindi G, Plockinger U: ENETS Consensus Guidelines for the Standards of Care in Neuroendocrine Tumors: towards a standardized approach to the diagnosis of gastroenteropancreatic neuroendocrine tumors and their prognostic stratification. *Neuroendocrinology* 2009;90:162–166.
- 3 Yao JC, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, Abdalla EK, Fleming JB, Vauthey JN, Rashid A, Evans DB: One hundred years after 'carcinoid': epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J Clin Oncol* 2008;26:3063–3072.
- 4 Anthony L, Freda PU: From somatostatin to octreotide LAR: evolution of a somatostatin analogue. *Curr Med Res Opin* 2009;25:2989–2999.
- 5 Rinke A, Muller HH, Schade-Brittinger C, Klose KJ, Barth P, Wied M, Mayer C, Aminossadati B, Pape UF, Blaker M, Harder J, Arnold C, Gress T, Arnold R: 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. *J Clin Oncol* 2009;27:4656–4663.
- 6 Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruzsniowski P, Sundin A: Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008;9:61–72.
- 7 Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevila J, de Vries EG, Tomassetti P, Pavel ME, Hoosen S, Haas T, Lincy J, Lebowitz D, Oberg K: Everolimus for advanced pancreatic neuroendocrine tumors. *N Engl J Med* 2011;364:514–523.
- 8 Raymond E, Dahan L, Raoul JL, Bang YJ, Borbath I, Lombard-Bohas C, Valle J, Metrakos P, Smith D, Vinik A, Chen JS, Horsch D, Hammel P, Wiedenmann B, Van Cutsem E, Patyna S, Lu DR, Blaukmeister C, Chao R, Ruzsniowski P: Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. *N Engl J Med* 2011;364:501–513.
- 9 Modlin IM, Moss SF, Chung DC, Jensen RT, Snyderwine E: Priorities for improving the management of gastroenteropancreatic neuroendocrine tumors. *J Natl Cancer Inst* 2008;100:1282–1289.
- 10 Yao JC, Lombard-Bohas C, Baudin E, Kvols LK, Rougier P, Ruzsniowski P, Hoosen S, St Peter J, Haas T, Lebowitz D, Van Cutsem E, Kulke MH, Hobday TJ, O'Dorisio TM, Shah MH, Cadiot G, Luppi G, Posey JA, Wiedenmann B: Daily oral everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. *J Clin Oncol* 2010;28:69–76.
- 11 Pavel ME, Hainsworth JD, Baudin E, Peeters M, Horsch D, Winkler RE, Klimovsky J, Lebowitz D, Jehl V, Wolin EM, Oberg K, Van Cutsem E, Yao JC, RADIANT-2 Study Group: Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. *Lancet* 2011;378:2005–2012.
- 12 Yao J, Phan A, Fogelman DR, et al: Randomized run-in study of bevacizumab (B) and everolimus (E) in low- to intermediate-grade neuroendocrine tumors (LGNETS) using perfusion CT as functional biomarker (abstract). *J Clin Oncol* 2010;28:4002.
- 13 Yao JC, Phan A, Hoff PM, Chen HX, Charnsangavej C, Yeung SC, Hess K, Ng C, Abbruzzese JL, Ajani JA: Targeting vascular endothelial growth factor in advanced carcinoid tumor: a random assignment phase II study of depot octreotide with bevacizumab and pegylated interferon alpha-2b. *J Clin Oncol* 2008;26:1316–1323.
- 14 Oberg K: Somatostatin-receptor mediated diagnosis and treatment in gastrointestinal neuroendocrine tumours (GEP-NET's). *Rocz Akad Med Bialymst* 2005;50:62–68.
- 15 Kulke MH, Hornick JL, Fraumeni C, Hooshmand S, Ryan DP, Enzinger PC, Meyerhardt JA, Clark JW, Stuart K, Fuchs CS, Redston MS: O⁶-methylguanine DNA methyltransferase deficiency and response to temozolomide-based therapy in patients with neuroendocrine tumors. *Clin Cancer Res* 2009;15:338–345.
- 16 Kulke MH, Freed E, Chiang DY, Philips J, Zahrieh D, Glickman JN, Shivdasani RA: High-resolution analysis of genetic alterations in small bowel carcinoid tumors reveals areas of recurrent amplification and loss. *Genes Chromosomes Cancer* 2008;47:591–603.
- 17 Jiao Y, Shi C, Edil BH, de Wilde RF, Klimstra DS, Maitra A, Schulick RD, Tang LH, Wolfgang CL, Choti MA, Velculescu VE, Diaz LA Jr, Vogelstein B, Kinzler KW, Hruban RH, Papadopoulos N: DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science* 2011;331:1199–1203.
- 18 Missiaglia E, Dalai I, Barbi S, Beghelli S, Falconi M, della Peruta M, Piemonti L, Capurso G, Di Florio A, delle Fave G, Pederzoli P, Croce CM, Scarpa A: Pancreatic endocrine tumors: expression profiling evidences a role for AKT-mTOR pathway. *J Clin Oncol* 2010;28:245–255.

- 19 Biomarkers Definitions Working Group: Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001;69:89–95.
- 20 Modlin IM, Gustafsson BI, Moss SF, Pavel M, Tsolakis AV, Kidd M: Chromogranin A – biological function and clinical utility in neuroendocrine tumor disease. *Ann Surg Oncol* 2010;17:2427–2443.
- 21 Borbath I, Jamar F, Delaunoit T, Demetter P, Demolin G, Hendlisz A, Pattyn P, Peeters M, Roeyen G, Van Cutsem E, Van Hootegem P, Van Laethem JL, Verslype C, Pauwels S: Diagnostic pitfalls in digestive neuroendocrine tumours. *Acta Gastroenterol Belg* 2009;72:29–33.
- 22 Modlin IM, Kidd M, Latich I, Zikusoka MN, Eick GN, Mane SM, Camp RL: Genetic differentiation of appendiceal tumor malignancy: a guide for the perplexed. *Ann Surg* 2006;244:52–60.
- 23 Drozdzov I, Kidd M, Nadler B, Camp RL, Mane SM, Hauso O, Gustafsson BI, Modlin IM: Predicting neuroendocrine tumor (carcinoid) neoplasia using gene expression profiling and supervised machine learning. *Cancer* 2009;115:1638–1650.
- 24 Grabowski P, Griss S, Arnold CN, Horsch D, Goke R, Arnold R, Heine B, Stein H, Zeitl M, Scherubl H: Nuclear survivin is a powerful novel prognostic marker in gastroenteropancreatic neuroendocrine tumor disease. *Neuroendocrinology* 2005;81:1–9.
- 25 Leja J, Essaghir A, Essand M, Wester K, Oberg K, Totterman TH, Lloyd R, Vasmatzis G, Demoulin JB, Giandomenico V: Novel markers for enterochromaffin cells and gastrointestinal neuroendocrine carcinomas. *Mod Pathol* 2009;22:261–272.
- 26 Cunningham JL, Diaz de Stahl T, Sjoblom T, Westin G, Dumanski JP, Janson ET: Common pathogenetic mechanism involving human chromosome 18 in familial and sporadic ileal carcinoid tumors. *Genes Chromosomes Cancer* 2011;50:82–94.
- 27 Giandomenico V: Molecular pathology of gastrointestinal neuroendocrine tumours – selected topics. *Diagn Histopathol* 2010;16:243–250.
- 28 Nilsson M, Dahl F, Larsson C, Gullberg M, Stenberg J: Analyzing genes using cloning and replicating circles. *Trends Biotechnol* 2006;24:83–88.
- 29 Dahl F, Stenberg J, Fredriksson S, Welch K, Zhang M, Nilsson M, Bicknell D, Bodmer WF, Davis RW, Ji H: Multigene amplification and massively parallel sequencing for cancer mutation discovery. *Proc Natl Acad Sci USA* 2007;104:9387–9392.
- 30 Johansson H, Isaksson M, Sorqvist EF, Roos F, Stenberg J, Sjoblom T, Botling J, Micke P, Edlund K, Fredriksson S, Kulthra HG, Ericsson O, Nilsson M: Targeted resequencing of candidate genes using selector probes. *Nucleic Acids Res* 2011;39:e8.
- 31 Larsson C, Grundberg I, Soderberg O, Nilsson M: In situ detection and genotyping of individual mRNA molecules. *Nat Methods* 2010;7:395–397.
- 32 Cui T, Hurtig M, Elgue G, Li SC, Veronesi G, Essaghir A, Demoulin JB, Pelosi G, Alimohammadi M, Oberg K, Giandomenico V: Paraneoplastic antigen Ma2 autoantibodies as specific blood biomarkers for detection of early recurrence of small intestine neuroendocrine tumors. *PLoS One* 2010;5:e16010.
- 33 Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004;351:781–791.
- 34 Khan MS, Tsigani T, Rashid M, Rabouhans JS, Yu D, Luong TV, Caplin M, Meyer T: Circulating tumor cells and EpCAM expression in neuroendocrine tumors. *Clin Cancer Res* 2011;17:337–345.
- 35 Ponten F, Schwenk JM, Asplund A, Edqvist PH: The Human Protein Atlas as a proteomic resource for biomarker discovery. *J Intern Med* 2011;270:428–446.
- 36 Uhlen M, Ponten F: Antibody-based proteomics for human tissue profiling. *Mol Cell Proteomics* 2005;4:384–393.
- 37 Brennan DJ, O'Connor DP, Rexhepaj E, Ponten F, Gallagher WM: Antibody-based proteomics: fast-tracking molecular diagnostics in oncology. *Nat Rev Cancer* 2010;10:605–617.
- 38 Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwahlen M, Kampf C, Wester K, Hober S, Wernerus H, Bjorling L, Ponten F: Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol* 2010;28:1248–1250.
- 39 Berglund L, Bjorling E, Oksvold P, Fagerberg L, Asplund A, Szgyarto CA, Persson A, Ottosson J, Wernerus H, Nilsson P, Lundberg E, Sivertsson A, Navani S, Wester K, Kampf C, Hober S, Ponten F, Uhlen M: A gene-centric Human Protein Atlas for expression profiles based on antibodies. *Mol Cell Proteomics* 2008;7:2019–2027.
- 40 Uhlen M, Bjorling E, Agaton C, Szgyarto CA, Amini B, Andersen E, Andersson AC, Angelidou P, Asplund A, Asplund C, Berglund L, Bergstrom K, Brumer H, Cerjan D, Ekstrom M, Eloheid A, Eriksson C, Fagerberg L, Falk R, Fall J, Forsberg M, Bjorklund MG, Gumbel K, Halimi A, Hallin I, Hamsten C, Hansson M, Hedhammar M, Hercules G, Kampf C, Larsson K, Lindskog M, Lodewyckx W, Lund J, Lundeberg J, Magnusson K, Malm E, Nilsson P, Odling J, Oksvold P, Olsson I, Oster E, Ottosson J, Paaivilainen L, Persson A, Rimini R, Rockberg J, Runeson M, Sivertsson A, Skolleremo A, Steen J, Stenvall M, Sterky F, Stromberg S, Sundberg M, Tegel H, Tourle S, Wahlund E, Walden A, Wan J, Wernerus H, Westberg J, Wester K, Wrethagen U, Xu LL, Hober S, Ponten F: A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol Cell Proteomics* 2005;4:1920–1932.
- 41 Ponten F, Jirstrom K, Uhlen M: The Human Protein Atlas – a tool for pathology. *J Pathol* 2008;216:387–393.
- 42 Lindskog C, Korsgren O, Ponten F, Eriksson JW, Johansson L, Danielsson A: Novel pancreatic beta cell-specific proteins: antibody-based proteomics for identification of new biomarker candidates. *J Proteomics* 2012;75:2611–2620.
- 43 Darmanis S, Nong RY, Hammond M, Gu J, Alderborn A, Vanelid J, Siegbahn A, Gustafsdottir S, Ericsson O, Landegren U, Kamali-Moghaddam M: Sensitive plasma protein analysis by microparticle-based proximity ligation assays. *Mol Cell Proteomics* 2010;9:327–335.
- 44 Darmanis S, Nong RY, Vanelid J, Siegbahn A, Ericsson O, Fredriksson S, Backlin C, Gut M, Heath S, Gut IG, Wallentin L, Gustafsson MG, Kamali-Moghaddam M, Landegren U: ProteinSeq: High-performance proteomic analyses by proximity ligation and next generation sequencing. *PLoS One* 2011;6:e25583.
- 45 Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, Landegren U: Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* 2006;3:995–1000.
- 46 Clauson CM, Allalou A, Weibrecht I, Mahmoudi S, Farnebo M, Landegren U, Wahlby C, Soderberg O: Increasing the dynamic range of in situ PLA. *Nat Methods* 2011;8:892–893.
- 47 Pu Y, Zhu Z, Liu H, Zhang J, Liu J, Tan W: Using aptamers to visualize and capture cancer cells. *Anal Bioanal Chem* 2010;397:3225–3233.
- 48 Barbas AS, Mi J, Clary BM, White RR: Aptamer applications for targeted cancer therapy. *Future Oncol* 2010;6:1117–1126.
- 49 Zhou J, Rossi JJ: Cell-specific aptamer-mediated targeted drug delivery. *Oligonucleotides* 2011;21:1–10.
- 50 Berezovski MV, Lechmann M, Musheev MU, Mak TW, Krylov SN: Aptamer-facilitated biomarker discovery (AptaBiD). *J Am Chem Soc* 2008;130:9137–9143.
- 51 Hanrieder J, Wicher G, Bergquist J, Andersson M, Fex-Svenningsen A: MALDI mass spectrometry based molecular phenotyping of CNS glial cells for prediction in mammalian brain tissue. *Anal Bioanal Chem* 2011;401:135–147.
- 52 Ouyang Z, Cooks RG: Miniature mass spectrometers. *Annu Rev Anal Chem (Palo Alto Calif)* 2009;2:187–214.
- 53 Bergstrom M, Muhr C, Lundberg PO, Bergstrom K, Gee AD, Fasth KJ, Langstrom B: Rapid decrease in amino acid metabolism in prolactin-secreting pituitary adenomas after bromocriptine treatment: a PET study. *J Comput Assist Tomogr* 1987;11:815–819.
- 54 Bergstrom M, Muhr C, Lundberg PO, Langstrom B: PET as a tool in the clinical evaluation of pituitary adenomas. *J Nucl Med* 1991;32:610–615.

- 55 Yorimitsu H, Murakami Y, Takamatsu H, Nishimura S, Nakamura E: Synthesis and bio-imaging of positron-emitting ^{15}O -labeled 2-deoxy-D-glucose of two-minute half-life. *Chem Asian J* 2007;2:57–65.
- 56 Van der Lely AJ, de Herder WW, Krenning EP, Kwekkeboom DJ: Octreoscan radioreceptor imaging. *Endocrine* 2003;20:307–311.
- 57 Velikyan I, Sundin A, Eriksson B, Lundqvist H, Sorensen J, Bergstrom M, Langstrom B: In vivo binding of [^{68}Ga]-DOTATOC to somatostatin receptors in neuroendocrine tumours – impact of peptide mass. *Nucl Med Biol* 2010;37:265–275.
- 58 Orlefors H, Sundin A, Garske U, Juhlin C, Oberg K, Skogseid B, Langstrom B, Bergstrom M, Eriksson B: Whole-body (^{11}C)-5-hydroxytryptophan positron emission tomography as a universal imaging technique for neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and computed tomography. *J Clin Endocrinol Metab* 2005;90:3392–3400.
- 59 Koopmans KP, Neels OC, Kema IP, Elsinga PH, Sluiter WJ, Vanghillewe K, Brouwers AH, Jager PL, de Vries EG: Improved staging of patients with carcinoid and islet cell tumors with ^{18}F -dihydroxy-phenyl-alanine and ^{11}C -5-hydroxy-tryptophan positron emission tomography. *J Clin Oncol* 2008;26:1489–1495.
- 60 Kauhanen S, Seppanen M, Ovaska J, Minn H, Bergman J, Korsoff P, Salmela P, Saltevo J, Sane T, Valimaki M, Nuutila P: The clinical value of [^{18}F] fluoro-dihydroxyphenylalanine positron emission tomography in primary diagnosis, staging, and restaging of neuroendocrine tumors. *Endocr Relat Cancer* 2009;16:255–265.
- 61 Orlefors H, Sundin A, Lu L, Oberg K, Langstrom B, Eriksson B, Bergstrom M: Carbidopa pretreatment improves image interpretation and visualisation of carcinoid tumours with ^{11}C -5-hydroxytryptophan positron emission tomography. *Eur J Nucl Med Mol Imaging* 2006;33:60–65.
- 62 Hofmann M, Maecke H, Borner R, Weckesser E, Schoffski P, Oei L, Schumacher J, Henze M, Heppeler A, Meyer J, Knapp H: Biokinetics and imaging with the somatostatin receptor PET radioligand (^{68}Ga)-DOTATOC: preliminary data. *Eur J Nucl Med* 2001;28:1751–1757.
- 63 Kwekkeboom DJ, de Herder WW, van Eijck CH, Kam BL, van Essen M, Teunissen JJ, Krenning EP: Peptide receptor radionuclide therapy in patients with gastroenteropancreatic neuroendocrine tumors. *Semin Nucl Med* 2010;40:78–88.
- 64 Ruf J, Heuck F, Schiefer J, Denecke T, Elgeti F, Pascher A, Pavel M, Stelter L, Kropf S, Wiedenmann B, Amthauer H: Impact of multi-phase (^{68}Ga)-DOTATOC-PET/CT on therapy management in patients with neuroendocrine tumors. *Neuroendocrinology* 2010;91:101–109.
- 65 Li S, Sun F, Jin ZY, Xue HD, Li ML: Whole-body diffusion-weighted imaging: technical improvement and preliminary results. *J Magn Reson Imaging* 2007;26:1139–1144.
- 66 Takahara T, Imai Y, Yamashita T, Yasuda S, Nasu S, Van Cauteren M: Diffusion weighted whole body imaging with background body signal suppression (DWIBS): technical improvement using free breathing, STIR and high resolution 3D display. *Radiat Med* 2004;22:275–282.
- 67 Poeppel TD, Krause BJ, Heusner TA, Boy C, Bockisch A, Antoch G: PET/CT for the staging and follow-up of patients with malignancies. *Eur J Radiol* 2009;70:382–392.
- 68 Pichler BJ, Judenhofer MS, Pfannenberger C: Multimodal imaging approaches: PET/CT and PET/MRI. *Handb Exp Pharmacol* 2008;185:109–132.
- 69 Gaa J, Rummeny EJ, Seemann MD: Whole-body imaging with PET/MRI. *Eur J Med Res* 2004;9:309–312.
- 70 Liapi E, Geschwind JF, Vossen JA, Buijs M, Georgiades CS, Bluemke DA, Kamel IR: Functional MRI evaluation of tumor response in patients with neuroendocrine hepatic metastasis treated with transcatheter arterial chemoembolization. *AJR Am J Roentgenol* 2008;190:67–73.
- 71 Leach MO, Brindle KM, Evelhoch JL, Griffiths JR, Horsman MR, Jackson A, Jayson GC, Judson IR, Knopp MV, Maxwell RJ, McIntyre D, Padhani AR, Price P, Rathbone R, Rustin GJ, Tofts PS, Tozer GM, Vennart W, Waterton JC, Williams SR, Workman P: The assessment of antiangiogenic and antivascular therapies in early-stage clinical trials using magnetic resonance imaging: issues and recommendations. *Br J Cancer* 2005;92:1599–1610.
- 72 Mross K, Dreves J, Muller M, Medinger M, Marme D, Hennig J, Morgan B, Lebowitz D, Masson E, Ho YY, Gunther C, Laurent D, Unger C: Phase I clinical and pharmacokinetic study of PTK/ZK, a multiple VEGF receptor inhibitor, in patients with liver metastases from solid tumours. *Eur J Cancer* 2005;41:1291–1299.
- 73 O'Connor JP, Naish JH, Parker GJ, Waterton JC, Watson Y, Jayson GC, Buonaccorsi GA, Cheung S, Buckley DL, McGrath DM, West CM, Davidson SE, Roberts C, Mills SJ, Mitchell CL, Hope L, Ton NC, Jackson A: Preliminary study of oxygen-enhanced longitudinal relaxation in MRI: a potential novel biomarker of oxygenation changes in solid tumors. *Int J Radiat Oncol Biol Phys* 2009;75:1209–1215.