Amyloid precursor-like protein 1 is differentially upregulated in neuroendocrine tumours of the gastrointestinal tract

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Abstract

We have examined the global gene expression profile of small intestinal carcinoids by microarray analysis. High expression of a number of genes was found including amyloid precursor-like protein 1 (APLP1). Quantitative real-time PCR and western blot analysis demonstrated higher expression of APLP1 in carcinoid metastases relative to primary tumours indicating a role of APLP1 in tumour dissemination. Tissue microarray analysis of gastroentero-pancreatic tumours demonstrated a high frequency of APLP1 expression and a low frequency of APLP2 expression in neuroendocrine (NE) tumours when compared with non-NE tumours at the same sites. Meta-analysis of gene expression data from a large number of tumours outside the gastrointestinal tract confirmed a correlation between APLP1 expression and NE phenotype where high expression of APLP1 was accompanied by downregulation of APLP2 in NE tumours. Cellular localization of APLP1, APLP2 and amyloid precursor protein (APP) in carcinoid cells (GOT1) by confocal microscopy demonstrated partial co-localization with synaptophysin. This suggests that the APP family of proteins is transported to the cell membrane by synaptic microvesicles and that they may influence tumour cell adhesion and invasiveness. We conclude that APLP1 is differentially upregulated in gastrointestinal NE tumours and that APLP1 may be important for the dissemination of small intestinal carcinoids. Identification of APLP1 in NE tumours offers a novel target for treatment and may also serve as a tumour-specific marker.

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Introduction

Little is known about the causes and genetic changes involved in the formation of gastrointestinal neuroendocrine (NE) tumours. The majority of these tumours are sporadic with no family history and no known causative agents. Differentiated NE tumours generally grow slowly but frequently metastasize to regional lymph nodes and liver. Metastatic tumours may give rise to symptoms due to hormone overproduction (carcinoid syndrome). The classification of NE gastrointestinal tumours is based on the identification of characteristic growth patterns, expression of NE markers and specific hormone products. However, the prognosis of these tumours is difficult to predict. A better understanding of the genes involved in NE tumour formation and progression is needed. In an attempt to characterize the gene expression in gastrointestinal NE tumours, we performed global gene expression analysis of metastatic lesions from malignant ileal carcinoids. One of the differentially upregulated genes was amyloid precursor-like protein 1 (APLP1) which we choose to investigate further regarding its role in NE tumour formation and progression.

The highly conserved family of amyloid precursor proteins (APP) contains the three homologous proteins, APP, APLP1 and APLP2 (Wasco et al. 1992, 1993, Sprecher et al. 1993, Paliga et al. 1997). APP and APLP2 are expressed in virtually all tissues and cell
types, while APLP1 is mainly expressed in neural tissue (Tanzi et al. 1988, Slunt et al. 1994, Lorent et al. 1995).

The functions of the APP-family proteins in non-neuronal tissues are poorly understood. APP, the best characterized member of the family, has been associated with cell adhesion, cell motility, cell proliferation, neuroprotection, neurite outgrowth, synapse formation, vesicular transport and gene regulation (Saitoh et al. 1989, Schubert et al. 1989, Milward et al. 1992, Mattson et al. 1993, Perez et al. 1997, De Strooper & Annaert 2000, Sabo et al. 2001). In order to perform such diverse functions, APP is proteolytically processed to generate several peptide fragments, each with specific functions. APP is digested by α-, β- and γ-secretase to generate the extracellular peptides, β-amyloid peptide and soluble N-terminal domain (sAPP), as well as the APP intracellular C-terminal domain fragment (AID). APLP1 and APLP2 are processed in a similar way to APP, leading to the secretion of the large N-terminal domain and release of the intracellular peptides ALID1 and ALID2 respectively (Slunt et al. 1994, Paliga et al. 1997, Scheinfeld et al. 2002, Eggert et al. 2004). However, the β-amyloid domain is not present in the APLP1 and APLP2 proteins. Similarities in structure, proteolytic processing and tissue distribution of the APP family members suggest that the proteins share common functions. To elucidate the individual functions of the APP proteins, knockout mice have been generated (Zheng et al. 1995, von Koch et al. 1997, Heber et al. 2000). Double knockouts of the combinations APLP2/APP and APLP2/APLP1 were found to be lethal; the mice died shortly after birth demonstrating that APP proteins have a key function in cell growth and survival. Mice with single knockouts of APP, APLP1 and APLP2 and the double knockout APLP1/APP were found to be viable, suggesting that the functions of the proteins are partially redundant. However, knockdown of APLP1 expression in cell culture reduced cell viability and neurite outgrowth (Sakai & Hohjoh 2006).

The fundamental role of APP proteins in survival and proliferation of normal cells suggests that these proteins may also play a role in tumour development. Increased expression of amyloid protein family members can influence cancer development in several different tumour types (Nakagawa et al. 1999, Meng et al. 2001, Hansel et al. 2003, Quast et al. 2003, Baldus et al. 2004, Ko et al. 2004, Mauri et al. 2005, Li et al. 2006). Neuroblastoma, a malignant tumour of neural crest origin, is the only tumour type that has been reported to express high levels of APLP1 (Wasco et al. 1992). Here, we demonstrate for the first time that APLP1 is highly expressed in NE tumours of gastrointestinal tract. The aim of this study was to describe the expression profile and cellular localization of APLP1, APLP2 and APP in these NE tumours and to relate these findings to tumour site, proliferative activity and metastasis.

Material and methods

Cell culture

Two different NE cell lines were used in expression microarray, quantitative real-time PCR (qRT-PCR) and western blot (WB). The GOT1 cell line was established from a liver metastasis of a small intestinal carcinoid (Kölby et al. 2001) and the BON cell line was derived from a metastasis of a malignant pancreatic carcinoid (Evers et al. 1991). The GOT1 cell line was cultured in RPMI-1640 medium with 5 μg/ml transferrin and 5 μg/ml insulin, and the BON cell line was grown in Dulbecco’s Modified Eagle’s Medium (DMEM)/F-12 medium, both supplemented with 10% bovine serum, 200 IU/ml penicillin and 200 μg/ml streptomycin, and incubated in a 5% CO2 humidified atmosphere at 37 °C.

RNA extraction

Fresh biopsies of small intestinal (ileal) carcinoids, gastrointestinal stromal tumours (GIST), colorectal cancers (adenocarcinoma), as well as normal tissues from intestinal mucosa and brain were collected at the time of operation and stored at −135 °C. Tissue used for gene expression microarray analysis was stabilized with RNAlater (Qiagen). To confirm the diagnosis and estimate the percentage of tumour cells in the biopsies, formalin-fixed and paraffin-embedded tissues were taken in direct proximity to the biopsies, stained with hematoxylin and eosin, and examined microscopically. Biopsies with a density of at least 90% tumour cells were used. Total RNA was prepared by homogenizing tumour biopsies in TRIZol Reagent (Invitrogen) using a Tissuelyser (Qiagen), followed by RNA purification using RNeasy Mini spin columns (Qiagen). Cultured cells were lysed in TRIZol and further purified using the RNeasy Mini spin columns. RNA was quantified by reading the absorbance at 260 nm (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and the ratio of absorbance at 260/280 nm was > 1.9 in all samples. The quality of extracted RNA was visually examined by gel electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA, USA).
Expression microarray

Expression microarray analysis was performed on biopsies from the liver metastasis of intestinal (ileal) carcinoids (five patients) and triplicate samples of GOT1 and BON cell lines. cDNA was synthesized from total RNA (5 μg per sample) and labelled with Cy-3 (Amersham Biosciences), using the Pronto! Plus version 1.2 direct labelling and hybridization kit (Promega). cDNA of the universal human reference RNA (URR; Stratagene, La Jolla, CA, USA) was labelled with Cy-5. The reference RNA containing pooled total RNA from 10 different cell lines was used to obtain broad gene expression coverage. Microarray glass slides were printed at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden (http://swegene.onk.lu.se). The arrays consisted of 27,000 unique probes of the Human Array-Ready oligonucleotide libraries version 2.1 and version 2.1.1 (Operon Biotechnologies, Cologne, Germany) printed in duplicate. The microarray slides were hybridized with 50 pmol Cy-3-labelled sample cDNA and 50 pmol Cy-5-labelled reference cDNA at 62 °C for 18 h, then washed to remove excess labelled cDNA and finally scanned using an Agilent G2505B microarray scanner (Agilent Technologies). Fluorescence intensities were converted to removal of labelled cDNA and finally scanned using an Agilent G2505B microarray scanner (Agilent Technologies). Fluorescence intensities were converted to numeric data using Agilent G2567AA Feature Extraction 7.5 software (Agilent Technologies). Statistical analyses were performed using Genespring software (Agilent Technologies). For each hybridization, fluorescence ratios (Cy-3/Cy-5) were normalized with the LOWESS algorithm. Differentially expressed genes related to the URR reference RNA passed a Student’s t-test with P value of <0.02 and Benjamin and Hochberg false discovery rate.

Quantitative real-time PCR (qRT-PCR)

To analyse expression levels of APLP1, APLP2 and APP in small intestinal carcinoids during tumour dissemination qRT-PCR analysis was performed on biopsies from normal small intestinal mucosa (n = 3), primary small intestinal (ileal) carcinoids (n = 6) and liver metastases of small intestinal carcinoids (n = 5) (the same biopsies as for expression microarray). As controls served normal brain tissue (n = 1), and the GOT1 (n = 3) and BON (n = 3) cell lines. The primary tumours and metastases did not originate from the same patients. To quantify the expression levels of APLP1, APLP2 and APP in different gastrointestinal tumour types, we analysed primary colorectal adenocarcinoma (n = 10), GIST (n = 10) and small intestinal carcinoids (n = 12). Total RNA was treated with DNase1 (DNA-free kit; Ambion, Austin, TX, USA) and reverse transcribed using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster city, CA, USA). A master mix was prepared for the qRT-PCRs by mixing water, 2 × TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan gene expression assays (Applied Biosystems) containing a 20 × solution of primer and probe specific for APLP1 (Hs00193069_m1), APLP2 (Hs00155778_m1), APP (Hs00169098_ml) and the glyceraldehyde-3-phosphate dehydrogenase gene ((GAPDH) (Hs99999905_ml)). The comparison of primary carcinoid tumours with metastases was performed in a 7700 Real-Time PCR System (Applied Biosystems). The comparison of different gastrointestinal tumour types was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems). The samples were subjected to PCR cycling conditions recommended by Applied Biosystems. The cycle threshold (Ct) of target genes and housekeeping gene GAPDH was determined manually within the exponential phase of amplification (7700 Real-Time PCR System) or automatically (7500 Fast Real-Time PCR System) by the software. The expression levels of target genes were related to endogenous expression of GAPDH which was chosen as housekeeping gene since it showed low variability in expression levels (Ct value) between the different tumour samples. Statistical significance was calculated by the two-tailed unpaired t-test using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) and a P value of <0.05 was considered significant.

Antibodies

Antibodies raised against the C-terminal part of APLP1 (aa643–653), APLP2 (aa752–763) and APP (aa751–770) (Cat no: 171615, 171616, 171610; Calbiochem, San Diego, CA, USA) were used for immunohistochemistry (IHC), immunofluorescence (IF) and WB. The dilutions used for these C-terminal antibodies were as follows: APLP1 1:1000 (IHC), 1:500 (IF) and 1:5000 (WB); APLP2 1:800 (IHC), 1:500 (IF) and 1:1000 (WB); and APP 1:400 (IHC), 1:2000 (IF) and 1:5000 (WB). Antibodies to the N-terminal part of APLP1 (cat no: NE1009; Oncogene, Sandiego, CA, USA), APLP2 (cat no: 171617; Calbiochem) and APP (aa44-62) (Cat no: ab15272; Abcam, Cambridge, UK) were used for IHC. The dilutions used for these C-terminal antibodies were as follows: APLP1 1:100, APLP2 1:2000 and APP 1:200. Antibodies to chromogranin A (CHGA) at 1:500 (Cat no: MAB319; Chemicon, Temecula, CA, USA), synaptophysin at 1:20 (Cat no: M0776; DakoCytomation, Glostrup,
Differentiated and 11 poorly differentiated. Two core adenocarcinomas (1 well differentiated, 9 moderately and 1 poorly differentiated) and pancreatic (ductal) colorectal adenocarcinomas (24 moderately differentiated adenocarcinomas (9 diffuse type and 16 intestinal type), 12 low risk, 2 intermediate risk and 12 high risk), gastric functioning tumour (malignant)), GIST (2 very low risk, Von Hippel-Lindau disease (VHL)-associated non-MEN1-associated non-functioning tumour (malignant); 1 type 1 (MEN1)-associated insulinoma (malignant); 2 sporadic glucagonoma (malignant); 1 sporadic insulinoma, 6 benign and 3 malignant; 2 (12 non-functioning, 1 benign and 11 malignant; 9 sporadic insulinoma, 6 benign and 3 malignant; 2 sporadic glucagonoma (malignant); 1 sporadic VIPoma (malignant); 1 multiple endocrine neoplasia type 1 (MEN1)-associated insulinoma (malignant); 2 MEN1-associated non-functioning tumour (malignant); 1 Von Hippel-Lindau disease (VHL)-associated non-functioning tumour (malignant)), GIST (2 very low risk, 12 low risk, 2 intermediate risk and 12 high risk), gastric adenocarcinomas (9 diffuse type and 16 intestinal type), colorectal adenocarcinomas (24 moderately differentiated and 1 poorly differentiated) and pancreatic (ductal) adenocarcinomas (1 well differentiated, 9 moderately differentiated and 11 poorly differentiated). Two core biopsies, 0.6 mm in diameter, were taken from each donor block. Each biopsy was mounted in a recipient paraffin block (NALLCRSET-1) using a robotized tissue puncher/arrayer (ATA-27; Beecher Instruments, Silver Spring, MD, USA). Normal tissue samples from colorectal mucosa/submucosa, gastric mucosa/submucosa, exocrine and endocrine pancreas, small intestinal mucosa/submucosa and cerebral cortex were obtained and placed at the start, the centre and at the end of the recipient block. These tissues served as negative and positive controls for the immunocytochemical staining. Tissue microarray was constructed in collaboration with the Swegene National Tissue Microarray Center, Malmö, Sweden. The tissue array was validated by examining sections stained with hematoxylin and eosin, and immunostained with antibodies against the NE markers chromogranin A and synaptophysin.

Western blot

WB analysis was performed on primary small intestinal (ileal) carcinoids (n = 4), liver metastases (n = 4), normal tissues from intestinal mucosa (n = 1), liver (n = 1) and the GOT1 and BON cell lines. Brain tissue (n = 1) served as a positive control. The primary tumours and metastases did not originate from the same patients. Whole-cell lysates were prepared as described previously and analyzed by western blotting (Jakobsen et al. 2002). The chemiluminescent signals were detected by an image reader (LAS 3000; Fujifilm, Tokyo, Japan) and quantified using MultiGauge version 3.0 software (Fujifilm). GAPDH was analyzed to estimate the amount of protein transferred to membranes.

Tissue microarray

Formalin-fixed and paraffin-embedded tumour tissues were retrieved from the archives at the Department of Clinical Pathology and Cytology, Sahlgrenska University Hospital, Göteborg, Sweden. Tissue blocks from the following primary tumours were used: gastric carcinoids (7 enterochromaffin-like (ECL) cell tumours (benign) in chronic atrophic gastritis, 4 sporadic ECL cell tumours (malignant)), small intestinal (ileal) carcinoids (32 enterochromaffin cell tumours (malignant)), appendiceal carcinoids (18 classical type, 15 benign and 3 malignant; 3 mixed endocrine and exocrine type, 1 benign and 2 malignant), rectal carcinoids (23 L cell type, 19 benign and 4 malignant), endocrine pancreatic tumours (12 non-functioning, 1 benign and 11 malignant; 9 sporadic insulinoma, 6 benign and 3 malignant; 2 sporadic glucagonoma (malignant); 1 sporadic VIPoma (malignant); 1 multiple endocrine neoplasia type 1 (MEN1)-associated insulinoma (malignant); 2 MEN1-associated non-functioning tumour (malignant); 1 Von Hippel-Lindau disease (VHL)-associated non-functioning tumour (malignant)), GIST (2 very low risk, 12 low risk, 2 intermediate risk and 12 high risk), gastric adenocarcinomas (9 diffuse type and 16 intestinal type), colorectal adenocarcinomas (24 moderately differentiated and 1 poorly differentiated) and pancreatic (ductal) adenocarcinomas (1 well differentiated, 9 moderately differentiated and 11 poorly differentiated). Two core biopsies, 0.6 mm in diameter, were taken from each donor block. Each biopsy was mounted in a recipient paraffin block (NALLCRSET-1) using a robotized tissue puncher/arrayer (ATA-27; Beecher Instruments, Silver Spring, MD, USA). Normal tissue samples from colorectal mucosa/submucosa, gastric mucosa/submucosa, exocrine and endocrine pancreas, small intestinal mucosa/submucosa and cerebral cortex were obtained and placed at the start, the centre and at the end of the recipient block. These tissues served as negative and positive controls for the immunocytochemical staining. Tissue microarray was constructed in collaboration with the Swegene National Tissue Microarray Center, Malmö, Sweden. The tissue array was validated by examining sections stained with hematoxylin and eosin, and immunostained with antibodies against the NE markers chromogranin A and synaptophysin.

Immunohistochemistry

Sections of paraffin-embedded tissue were subjected to antigen retrieval by boiling in Tris–EDTA (pH 9) for 15 min, followed by blocking with 0.5% non-fat milk and incubation with primary antibodies to APLP1, APLP2 and APP. Bound antibodies were visualized by the peroxidase-based EnVision+ Dual Link System (Dako-Cytomation). Immunohistochemical staining of tumour biopsies in tissue microarrays was judged as being either positive or negative. A biopsy was considered to be positive when more than 25% of tumour cells were labelled in the cytoplasm, the nucleus or the cell membrane. Similarly, nuclear labelling was judged to be positive when more than 25% of tumour cell nuclei were labelled. Tissue microarrays were evaluated by two pathologists (A Bergström and O Nilsson).

Confocal laser scanning microscopy

GOT1 cells were grown on chamber slides for 3 days and fixed in 4% paraformaldehyde in PBS. The slides were incubated with primary antibodies for 1 h at room temperature in 1% BSA, 0.2% Triton X-100 and 0.1% sodium azide in PBS. The cells were incubated for 1 h with a mixture of 0.006% Hoechst 33258 for nuclear staining and a 1:2000 dilution of secondary antibodies conjugated with Alexa Fluor 594 (goat anti-rabbit, cat no: A11037; Invitrogen) or Alexa Fluor 488 (goat anti-mouse, cat no: A11029; Invitrogen). The primary antibody was omitted in negative controls. Coverslips were mounted using ProLong Gold anti-fade reagent (Molecular Probes, Eugene, OR, USA). The fluorescent cells were analyzed by confocal microscopy using a Zeiss LSM 510 META system with the LSM-5 software supplied by the manufacturer. Emission spectra were...
recorded with the META multichannel detector using the following settings: Hoechst 33258, diode laser 405 nm excitation, 422–476 nm emission detection; Alexa Fluor 488, argon laser 488 nm excitation, 497–550 nm emission detection; and Alexa Fluor 594, DPSS-laser 561 nm excitation, 583–668 nm emission detection. Images were recorded with an optical section of 0.8 μm using an αPlan-FLUAR 100X/1.45 N.A. oil immersion objective.

**Ethics**

These studies were approved by the Regional Ethical Review Board in Go¨teborg, Sweden.

**Results**

**Global gene expression analysis of small intestinal carcinoids demonstrated high expression of APLP1**

In order to identify highly expressed genes in liver metastases of small intestinal carcinoids, we established a gene expression profile by microarray analysis. For comparison, the expression profile of the two carcinoid cell lines, GOT1 and BON, was also determined. One thousand four hundred three significantly differentially expressed genes were analyzed by hierarchical clustering using Pearson correlation (Fig. 1A). The clustering of these samples showed two main branches, one branch with the BON cell line alone and the other branch containing a group with the five intestinal carcinoids and a sub-branch with the GOT1 cell line. The expression profiles of the intestinal carcinoids were highly correlated, indicating that these tumours as a group have great phenotypic similarities. When comparing the gene expression profiles of the intestinal carcinoids with the two carcinoid cell lines GOT1 and BON, cluster analysis demonstrated the closest relationship between intestinal carcinoids and GOT1. This finding suggests that GOT1 cell line has an expression profile more similar to tissue samples of intestinal carcinoid than BON cells have. GOT1 cells may therefore be more representative for small intestinal carcinoids. This is in agreement with the fact that GOT1 cell line was established from an ileal carcinoid, while BON cells were derived from a pancreatic carcinoid. Analysis of differentially expressed genes in intestinal carcinoids revealed genes related to MAPK, Wnt, Hedgehog and Notch signalling, as well as a number of genes related to NE functions. NE-related genes were found to be highly expressed in carcinoid tumours, e.g. those for CHGA, secretagogin (SCGN), synaptotagminXIII (SYT13), DOPA decarboxylase (DDC), synapsin1 (SYN1), secretogranin III (SCG3) and vesicle-associated membrane protein 2 (VAMP2) (Table 1). Unexpectedly, we found high expression of APLP1, which has not been investigated previously in small intestinal carcinoids. We further explored the expression pattern and possible function of the APP gene family in NE tumours.

**Meta-analysis revealed differential upregulation of the APLP1 gene in NE tumours of the lung**

To explore the APLP1 expression in a wider range of tumours, we performed meta-analysis of published data sets using the ONCOMINE database (www.oncomine.org). The differential expression of the APLP1 gene in normal and cancer tissues was analyzed in available data sets (April 2006) containing 35 different microarray studies covering 14 different tumour types including tumours of the blood, brain, head and neck, salivary gland, thyroid, lung, adrenal gland, pancreas, liver, prostate, ovary, uterus, kidney and urinary bladder (Fig. 1B). The sets contained no gastrointestinal tumours. There were significant changes in expression of APLP1 (P value <0.02) in 8 out of the 35 available comparisons. Three out of the eight studies showed significant upregulation of APLP1. These were all NE tumours of the lung, representing the only NE tumour types within the 35 data sets. These lung tumours also showed significant down-regulation of APLP2 (data not shown). Tumours of the brain, exocrine pancreas and multiple myeloma displayed downregulation of APLP1.

**Tissue microarray demonstrated differential upregulation of APLP1 expression in NE tumours of the gastrointestinal tract**

To explore the expression of APLP1, APLP2 and APP proteins in NE and non-NE tumours of the gastrointestinal tract, we performed IHC on tissue microarrays comprising 214 primary tumours. NE tumours were more frequently positive for APLP1 than non-NE tumours; 84% of NE tumours (gastric, small intestinal, appendiceal and rectal carcinoids, and endocrine pancreatic tumours) were positive, in contrast to 26% of non-NE tumours (GIST, gastric, colorectal and pancreatic adenocarcinoma) (Table 2). The frequency of positive APLP1 staining in NE tumours was similar in benign and malignant cases. APLP2 was frequently expressed in all types of tumours investigated except for small intestinal carcinoid and endocrine pancreatic tumour; therefore, NE tumours showed a significant lower frequency of APLP2 expression (77%) than non-NE tumours (97%).
APP was also frequently expressed in gastrointestinal tumours, but with no significant difference between NE tumours (85%) and non-NE tumours (92%). APLP1, APLP2 and APP labelling of tumours were confined to tumour cells, while tumour stroma was consistently negative. The tumour cells showed cytoplasmic labelling using antibodies recognizing the C-terminal domain (Fig. 2A). Positive labelling of tumour cell nuclei was detected in some NE and non-NE tumours using the antibody to the C-terminal domain of APLP2. Nuclear labelling was most prominent in pancreatic adenocarcinomas (80%), but was also seen in gastric adenocarcinomas (43%) and gastric carcinoids (14%). N-terminal

Figure 1 Global gene expression analysis. (A) Hierarchical clustering of carcinoid tumours reveals homogenous expression patterns. Gene expression data from five small intestinal carcinoid tumours and the carcinoid cell lines GOT1 and BON using 1403 significantly expressed genes. Each column in the diagram represents a tumour sample and each row a gene. Gene expression values are represented as colours; red represents relatively higher expression and blue relatively lower expression than the reference URR. (B) Data search of the database ONCOMINE reveals upregulation of

Table 1 Significantly upregulated neuroendocrine and neuronal genes in small intestinal carcinoid tumours in comparison to Universal human reference RNA (URR)

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APP was also frequently expressed in gastrointestinal tumours, but with no significant difference between NE tumours (85%) and non-NE tumours (92%). APLP1, APLP2 and APP labelling of tumours were confined to tumour cells, while tumour stroma was consistently negative. The tumour cells showed cytoplasmic labelling using antibodies recognizing the C-terminal domain (Fig. 2A). Positive labelling of tumour cell nuclei was detected in some NE and non-NE tumours using the antibody to the C-terminal domain of APLP2. Nuclear labelling was most prominent in pancreatic adenocarcinomas (80%), but was also seen in gastric adenocarcinomas (43%) and gastric carcinoids (14%). N-terminal

APL1 in NE tumours of the lung. The differential expression of APLP1 in normal tissue compared with cancer tissue is depicted using box plots. Normalized expression units are given on the x-axis (log transformed, array median set to zero and array standard deviation set to one). Eight analyses with significant differences (i.e. with P value <0.02) were found. For every class, there is a box with three vertical lines corresponding to the median, lower quartile and upper quartile of the expression values. The ends of the whiskers correspond to the tenth and ninetieth percentiles. Dots correspond to minimum and maximum values. The number of samples in each class compared (normal versus cancer) is given in parentheses.
domain-specific antibodies gave similar results regarding the expression of APLP1, APLP2 and APP as antibodies directed to the C-terminal domain (data not shown). However, N-terminal domain-specific antibodies clearly labelled tumour cell membrane in ileal (94%) and appendiceal (81%) carcinoids in contrast to gastric (0%) and rectal (0%) carcinoids.

To quantify the differential expression of APLP1, APLP2 and APP in gastrointestinal tumours, we performed qRT-PCR analysis of small intestinal carcinoids,

### Table 2 Tissue microarray analysis of amyloid precursor-like protein 1 (APLP1), APLP2 and amyloid precursor protein (APP) expression in gastrointestinal tumours

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<th>APP</th>
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GIST, gastrointestinal stromal tumour. Data for tissue microarray were obtained by incubation with C-terminal directed antibodies against APLP1, APLP2 and APP.

aTumours were judged to be positive when ≥25% of tumour cells were stained.

bSignificant difference in immuno-positive staining between NE and non-NE tumours determined by Binomial two sample test.

Figure 2 Expression of APLP1, APLP2 and APP in gastrointestinal tumours. (A) APLP1, APLP2 and APP are expressed in small intestinal carcinoids. Immunohistochemical analysis of a primary small intestinal carcinoid demonstrating strong cytoplasmic expression of APLP1, APLP2 and APP in tumour cells with no expression in stroma cells. (B) APLP1 is highly expressed in small intestinal carcinoids. The expression levels of RNA encoded by the APLP1, APLP2 and APP genes in primary tumours of colorectal adenocarcinoma (n=10), GIST (n=10) and small intestinal carcinoid (n=12) were determined by qRT-PCR. The values given are relative to the housekeeping gene GAPDH. Asterisks indicate statistically significant changes (unpaired t-test): *P<0.05 and **P<0.01.
GIST and colorectal adenocarcinoma (Fig. 2B). APLP1 showed the greatest difference in expression between the three tumour types, with the highest expression in small intestinal carcinoids and lowest expression in colorectal adenocarcinoma and GIST. APLP2 also showed differences in expression levels between the three tumour types, with the highest expression in GIST followed by carcinoids and colorectal adenocarcinoma. The level of APP expression was found to be high in all three tumour types with least expression in colorectal cancer.

APLP1 is upregulated in disseminated small intestinal carcinoids

In order to explore APLP1, APLP2 and APP expression in small intestinal carcinoids during tumour dissemination, we performed qRT-PCR and WB analysis on biopsies from normal intestinal mucosa, primary carcinoid tumours and liver metastases. qRT-PCR analysis revealed elevated expression of APLP1 and APP in both primary and metastatic carcinoid tumours relative to the expression in normal intestinal mucosa (Fig. 3A). On the other hand, APLP2 gene expression was reduced in primary and metastatic tumours compared with intestinal mucosa. Comparison of primary carcinoids with metastatic tumours revealed a significant upregulation of APLP1 expression in metastatic lesions, while APLP2 and APP expression was downregulated, clearly demonstrating a differential upregulation of APLP1 gene expression during carcinoid tumour dissemination. GOT1 cells showed an expression pattern that was similar to that observed in metastatic carcinoid tumours, i.e. high expression of APLP1 and APP but low expression of APLP2. BON cells showed an expression pattern that was different from those of GOT1 cells and intestinal tumour, i.e. they showed low expression of APLP1. WB confirmed high protein levels of APLP1, APLP2 and APP in small intestinal carcinoids and upregulation of APLP1 in metastatic lesions (Fig. 3B). WB also verified the difference in APLP1 protein levels between GOT1 and BON cells and demonstrated intracellular C-terminal fragments (CTFs) in the two cell lines. Faint bands of CTF were also detected by the anti-APLP2 and anti-APP antibodies, suggesting proteolytic processing of APP family proteins in carcinoid tumours and involvement of APP proteins in gene regulation. Proliferation index (percentage of Ki67 positive tumour cell nuclei) was also analysed in biopsies of primary tumours and metastases used for qRT-PCR. In primary tumours, the Ki67 labelling index was 0.4% (mean, range 0.0–1.4%; n = 6) while metastatic lesions had a labelling index of 5.4% (mean, range 0.0–22.2%; n = 5). However, there was no significant correlation between proliferation index and APLP1 expression level in tumour biopsies.

APP family proteins are localized to synaptic-like microvesicles and early endosomes, and co-localized with the adaptor protein FE65 in carcinoid tumour cells

In order to determine whether APLP1, APLP2 and APP may function as adhesion molecules, secretory proteins or regulators of gene transcription, we studied the cellular localization of these proteins in carcinoid tumour cells (GOT1) by confocal laser scanning microscopy. C-terminal directed antibodies against APLP1, APLP2 and APP were used in order to visualize the full-length proteins as well as the processed CTFs. Using antibodies directed against the C-terminal part of APLP1, APLP2 and APP, a predominant cytoplasmic distribution was seen, but with minor fractions located at or in the nucleus. The cytoplasmic location of APLP1, APLP2 and APP was further studied in relation to markers for large dense core vesicles (CHGA), synaptic-like microvesicles (synaptophysin) and early endosomes (Rab5) (Fig. 4). All three APP proteins partly co-localized with synaptophysin and Rab-5, indicating localization to synaptic-like microvesicles and early endosomes. There was only weak co-localization of APLP1, APLP2 and APP with CHGA. The adaptor molecule FE65, which interacts with the CTF of APP to regulate transcription (Borg et al. 1996), was found to be highly expressed in GOT1 cells and co-localized with all three amyloid proteins in both the cytoplasm and the nucleus (Fig. 4D).

Discussion

In this paper, the expression pattern of the APP protein family in tumours of the gastrointestinal tract is reported for the first time. We found that APP proteins are highly expressed in gastrointestinal tumours, with differential upregulation of APLP1 in NE tumours. APLP1 was found to be highly expressed in intestinal carcinoids, with enhanced expression in metastatic lesions compared with primary tumours, which suggests that APLP1 may be upregulated during tumour dissemination. Upregulation of APLP1 in intestinal carcinoids was accompanied by downregulation of APL2, indicating differential gene regulation of the APP family in NE tumours. This observation was further supported by a search in
the ONCOMINE database, which demonstrated upregulation of APLP1 and downregulation of APLP2 in NE tumours of the lung. We also studied the cellular co-localization of the APP protein family in a model system with carcinoid cells (GOT1) and found that they co-localized to synaptic-like microvesicles, early endosomes and the adaptor protein FE65, which suggests that APP proteins may have a role in both cell adhesion and gene regulation.

APP has previously been shown to be involved in several fundamental functions related to cancer. The secreted N-terminal fragment of APP (sAPP) has been shown to act as an autocrine growth factor. It promotes cell proliferation in fibroblasts, thyroid epithelial cells

**Figure 3** APLP1 is upregulated in metastases of small intestinal carcinoids. Expression levels of RNA encoding (A) APLP1, (B) APLP2 and (C) APP relative to GAPDH in intestinal mucosa (n=3, circles), primary tumour of carcinoid (n=6, squares), metastases of carcinoid (n=5, up triangles), BON (n=3, down triangles), GOT1 (n=3, crosses) and brain (n=1, diamonds) as determined by qRT-PCR analysis. APLP1 RNA was significantly upregulated in metastases of carcinoid when compared with intestinal mucosa (***P<0.001) and primary tumour (*P<0.02). The average is indicated by a horizontal line. Asterisks indicate statistically significant changes (unpaired t-test). Normal intestinal mucosa, liver, primary tumour of carcinoid (n=4), metastasis of carcinoid (n=4), BON and GOT1 were immunoblotted with antibodies against (D) APLP1, (E) APLP2, (F) APP and (G) GAPDH. Brain tissue was used as positive control. Two C-terminal APLP1 fragments (CTFs) in BON and GOT1 are clearly visible. The relative densities of APLP1, APLP2, APP and GAPDH were quantified by MultiGauge version 3.0 (Fujifilm, Tokyo, Japan).
and pancreatic cancer cells (Saitoh et al. 1989, Pietrzik et al. 1998, Hansel et al. 2003). The activities of the two proteases β- and γ-secretase that are involved in the cleavage of the APP family of proteins can influence tumour growth and angiogenesis (Paris et al. 2005). It was recently shown that homo- and heterocomplexes of the APP family of proteins promote cell adhesion via transcellular interactions. This finding supports the view that the APP proteins may have cell adhesion properties, such as transdimerization similar to cadherins and nectins (Soba et al. 2005). Deregulation of adhesion molecules, and also switching from one set of adhesion molecules to another, e.g. the E-cadherin to N-cadherin switch, have been shown to be important events in tumour dissemination (Hazan et al. 2004). The switch between APLP1 and APLP2 during carcinoid tumour dissemination may be of equal relevance.

The intracellular C-terminal domain of members of the APP protein family participates in the regulation of transcription by a multi-protein complex consisting of AID, ALID1 or ALID2, the histone acetyltransferase Tip60 and also other proteins containing phosphotyrosine-binding domains, the adaptor proteins FE65, X11, Dab1 and the non-receptor tyrosine kinase ABL (Borg et al. 1996, Homayouni et al. 1999, Cao & Sudhof 2001, Zambrano et al. 2001, Scheinfeld et al. 2002). To date, only a few genes have been shown to be regulated by AID, namely tetraspanin-27/KAI-1, which is linked to the regulation of NFκB-related proteins, and the two major acetylcholinesterases, ace-1 and ace-2, of the nematode Caenorhabditis elegans (Baek et al. 2002, Bimonte et al. 2004). It is not presently known whether ALID1 and ALID2 regulate the same genes as AID. We found that in carcinoid tumours all three members of the APP family are processed by γ-secretase to produce the CTFs crucial for signal transduction and gene regulation. We also detected high expression of FE65 in carcinoid tumour cells, co-localized with APP, APLP1 and APLP2 in the cytoplasm and nuclei, although the nuclear staining was faint for APP and APLP1. Together, these findings indicate that APP-dependent gene regulation may be of importance in carcinoid tumours.

One of the characteristics of NE tumours is that they have regulatory secretory pathways by which hormones are released upon stimulation. Two types of secretory organelles have been characterized in NE tumours, large dense core vesicles containing chromogranin A, and synaptic-like microvesicles containing, e.g. synaptophysin, synapsin and synaptobrevin. In this study, we examined the cellular localization of APP proteins by C-terminal-directed
antibodies and found mainly cytoplasmic distribution, localized to synaptic-like microvesicles and early endosomes. Only small amounts of APP proteins were associated with large dense core vesicles. However, N-terminal-directed antibodies clearly demonstrated membrane labelling of tumour cells in ileal and appendiceal carcinoids. The results suggest that members of the APP protein family are transported to the cell membrane by microvesicles and may act as adhesion proteins, influencing tumour migration and invasiveness.

In this study, we found elevated expression of APLP1 in NE tumours of the gastrointestinal tract relative to its expression in non-NE tumours. A search of the ONCOMINE database also showed upregulation of APLP1 and downregulation of APLP2 in NE tumours of the lung. One may speculate that APLP1 in NE tumours controls the degree of NE differentiation by interfering with Notch signalling. Notch has been shown to control differentiation in endocrine cells and in the endocrine pancreatic cell line BON (Nakakura et al. 2005, Bjerknes & Cheng 2006). In the developing endoderm, Notch signalling inhibits NE differentiation by repressing the expression of basic helix-loop-helix transcription factors. Similarities in proteolytic activation by γ-secretase and signal transduction of the three members of the APP family and Notch raise the possibility that signals from these two protein families may interact. In vitro and genetic studies in Drosophila have confirmed that there is cross-talk between the APP family and Notch through interactions between AID and the inhibitors of the Notch receptor Numb and Numb-like proteins (Roncarati et al. 2002, Merdes et al. 2004). A second way of interaction between APP and Notch is by heterodimerization resulting in activation of reciprocal target genes (Fischer et al. 2005).

Recently, a tumour suppressor effect of Notch-1 has been demonstrated in a number of NE-tumours (Kunimalaiyaan & Chen 2007) suggesting that APLP1–Notch1 interaction in carcinoids may interfere with tumour growth. Further studies of APLP1 processing and Notch signalling in carcinoid tumours will help to elucidate the functional significance of APLP1 for NE growth and differentiation in carcinoid tumours.

Current strategies for treatment of carcinoid tumours include surgery, liver embolization, somatostatin receptor-mediated radiation therapy and modulation of the immune system. These regimes improve patient survival, but do not provide cure. Novel-targeted therapies for carcinoid tumours are therefore needed. The expression of APLP1 in NE tumours may offer new therapeutic and diagnostic strategies. One therapeutic option is to administer β- or γ-secretase inhibitors, which would interfere with the proteolytic processing of APP family proteins in tumour cells and block the formation of soluble amyloid peptides and ALID (van Es et al. 2005). This would inhibit APLP1 signalling in tumour cells, which may in turn inhibit tumour dissemination. However, this type of therapy should be targeted to tumour cells to avoid side effects in normal tissue. Another therapeutic option is to utilize APLP1, being a membrane-bound protein, for targeting with radionuclide or antibody therapy. Cleavage of APLP1 generates N-terminal fragments that may be detectable in serum and serve as diagnostic markers.

We conclude that NE tumours of the gastrointestinal tract express all three members of the APP family. APLP1 is highly expressed in NE tumours when compared with non-NE tumours with marked upregulation in metastatic small intestinal carcinoids suggesting a role in tumour dissemination. Identification of APLP1 in NE tumours offers a novel target for treatment and it may also serve as a tumour-specific marker.

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