Identification of novel neuroendocrine-specific tumour genes

E Hofsli*1,2, TE Wheeler1, M Langaa2, A Lægreid1 and L Thommesen1,4
1Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway; 2Oncology Unit, St Olavs Hospital HF, Trondheim, Norway; 3Department of Mathematical Sciences, Norwegian University of Science and Technology, Trondheim, Norway; 4Department of Food and Medical technology, Sør-Trøndelag University College, Trondheim, Norway

Neuroendocrine tumours (NETs) comprise a heterogenous group of malignancies with an often unpredictable course, and with limited treatment options. Thus, new diagnostic, prognostic, and therapeutic markers are needed. To shed new lights into the biology of NETs, we have by cDNA transcript profiling, sought to identify genes that are either up- or downregulated in NE as compared with non-NF tumour cells. A panel of six NET and four non-NET cell lines were examined, and out of 12 743 genes examined, we studied in detail the 200 most significantly differentially expressed genes in the comparison. In addition to potential new diagnostic markers (NEFM, CLDN4, PEROX2), the results point to genes that may be involved in the tumorigenesis (BEX1, TMEPAI, FOSSL, RAB32), and in the processes of invasion, progression and metastasis (MME, STAT3, DCCBLD2) of NETs. Verification by real time qRT–PCR showed a high degree of consistency to the microarray results. Furthermore, the protein expression of some of the genes were examined. The results of our study has opened a window to new areas of research, by uncovering new candidate genes and proteins to be further investigated in the search for new prognostic, predictive, and therapeutic markers in NETs.

**Keywords:** neuroendocrine tumours; gene expression; microarray; neuroendocrine markers; cell lines

Neuroendocrine (NE) tumours (NETs) belong to a heterogenous group of neoplasms arising from malignant transformation of various types of NE cells (Falkmer, 1993; Wick, 2000; DeLellis, 2001; Hofsli, 2006). Although the majority of NETs are rather slow growing, their biology is often unpredictable, making their management a great challenge (Stephenson, 2006; Vilar et al, 2007). Thus, new insight into the biology of these fascinating tumours could not only make prognostication easier, but also guide in the selection for the right treatment strategy, and contribute in the search for new drug targets. This last issue is of vital importance, as up till now, only surgery has the potential to cure patients with NET disease.

Prediction of the biological behaviour of NETs may be difficult based upon histological criteria alone (Wick, 2000; Stephenson, 2006). Well-differentiated NETs are easily recognised by routine tissue staining and conventional light microscopic (LM) examination, combined with immunohistochemical (IHC) detection of NE markers such as chromogranin A (CHGA) and synaptophysin (SYP). However, dealing with poorly differentiated tumours, it may be difficult to decide whether a tumour exhibits an NE character. Thus, new diagnostic markers are warranted.

In addition to classical NETs, it has been increasingly recognised that both mixed endocrine–exocrine malignant tumours, as well as NE differentiation in common epithelial cancers, may occur (Capella et al, 2000; Sørhaug et al, 2007). The picture is even more complex, as recent research has indicated that use of more sensitive methods such as the tyramide signal amplification technique, will identify more NE tumour cells than today's routine diagnostic procedures manage to do (Sørhaug et al, 2007). With respect to prognosis and treatment, the impact of such NE differentiation in epithelial cancers is mostly unknown.

To shed new lights into the biology of NETs, we have compared the gene expression pattern of a selection of NE tumour cells, with that of a group of non-NF tumour cells. By this approach, we have identified genes that are differentially expressed in NE vs non-NF tumour cells. We propose that some of the genes and their gene products may represent interesting new molecular factors with regard to tumorigenesis, prediction of prognosis and treatment response, as well as may represent novel therapeutic targets.

**MATERIALS AND METHODS**

**Cell culture**

Six NE and four non-NF cell lines were used in the gene expression analysis. All cell lines, except the BON cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). BON cells (Evers et al, 1991) were a generous gift from Professor Kjell Öberg, Department of Medical Science, Uppsala University Hospital, Uppsala, Sweden, and cultured as described in Hofsli et al (2005). The six NE cell lines represent various NETs: neuroblastomas (SK-N-AS, SK-N-FI), bronchial carcinoids (NCH727, UMC-11), gastrointestinal carcinoid (BON), and medullary thyroid carcinoma (TT). The non-NF cell lines were colorectal adenocarcinomas (WiDr, SW480), lung adenocarcinoma (A-427) and glioblastoma (A-172). All these cell lines were cultured according to the requirements given by ATCC.
Isolation of RNA

Cells were cultured in 75 cm² culture flasks until 80% confluence, harvested and directly subjected to RNA isolation. Total RNA was isolated using RNeasy midi kit (Qiagen, Germantown, MD, USA), according to the manufacturer’s instruction. Two independent biological experiments were performed with each cell line. The quality of the RNA was examined by use of Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The samples were kept frozen at –80°C until further processing.

Microarray hybridisation

Human cDNA arrays with 15,000 probes in duplicate were obtained from Norwegian Microarray Consortium, Oslo, Norway (http://www.microarray.no). These arrays were prepared using sequence-verified human genes (Research Genetics, Huntsville, AL, USA). Additional information of cDNA clone preparation and printing is described in detail within the platform GPL3313, of the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL3313). Two negative controls and ten different cDNA spike-in controls from Arabidopsis thaliana (Stratagene SpotReporter, La Jolla, CA, USA) were included in all arrays. Total RNA (2 μg) from the cell lines and from Universal Human Reference RNA (Stratagene, La Jolla, CA, USA), was reverse transcribed and labelled with Cy3- and Cy5-attached dendrimer, respectively, using the Genisphere 3DNA Array 350 Expression Array Detection kit (Genisphere, Montvale, NJ, USA), as described in the manufacturer’s protocol and previously by us (Yadetie et al, 2003; Norsett et al, 2004; Hofsi et al, 2005). To reduce the artefacts because of different sensitivity to photo-bleaching, the biologic replicates of each of the 10 cell lines were randomised by dye-swaps. The arrays were scanned separately by two wavelengths (532 and 633 nm) using ScanArray Express HT scanner (Packard BioScience, Billerica, MA, USA).

Microarray data analysis

The microarray data were prepared according to the MIAME recommendations (Brazma et al, 2001). Image analysis was carried out using the GenePix Pro 4.1 software (Axon Instruments, Union City, CA, USA). All subsequent statistical analysis was performed using the statistical package R (R Development Core Team, 2004), dendrimer, respectively, using the Genisphere 3DNA Array 350 Expression Array Detection kit (Genisphere, Montvale, NJ, USA), as described in the manufacturer’s protocol and previously by us (Yadetie et al, 2003; Norsett et al, 2004; Hofsi et al, 2005). To reduce the artefacts because of different sensitivity to photo-bleaching, the biologic replicates of each of the 10 cell lines were randomised by dye-swaps. The arrays were scanned separately by two wavelengths (532 and 633 nm) using ScanArray Express HT scanner (Packard BioScience, Billerica, MA, USA).

Table 1 Primers and probes

| Symbol/gene Bank Accession No. | Sequence (5’→3’) | Forward reverse probe | Product length |
|-------------------------------|-------------------|-----------------------|---------------|
| BAALC NM_024812 | actgcccatggcatgtctct | S | 66 |
| FOSL1 NM_005438 | tccagccatggagagcgc | AS | |
| GSTP1 NM_000852 | tggagggtgcctggaagcttc | Probe | |
| SCG2 NM_003469 | acctcgccgacagcccccc | F | 81 |
| M160 NM_174941 | aaggtctgtaagcccc | AS | |
| GAPD NM_002046 | aatgcaacggctctcctcacttt | Probe | |

Genes, primers, and probe sequences of selected genes for confirmation studies. The length, product length, and orientation are given here.
Belgium, and had an optimal annealing temperature of 56 and 68°C, respectively. TaqMan real-time PCR was performed with 1 x Quantitect Probe PCR Master Mix (Qiagen, Germantown, MD, USA), 400 nM of each primer, 200 nM TaqMan Probe (Eurogentech) or sybergreen and cDNA equivalent to 62.5 ng total RNA in a total reaction volume of 25 µl. The Real-Time PCR was performed in Stratagene’s Mx3000P Real Time PCR system; 15 min at 95°C followed by 40 cycles of 15 s at 94°C, 40 thermal cycles of 15 s at 94°C, 30 min at 56°C and 30 s at 72°C. Each sample was measured in triplicate. A negative control without the cDNA template was included, and contamination by genomic DNA was ruled out by performing PCR analysis on template where reverse transcriptase had been omitted in the RT reactions. GAPDH was run in parallel as controls to monitor RNA integrity reverse transcriptase had been omitted in the RT reactions. Fold induction of gene expression level was estimated by the 2-DDCt method, where: Fold change = 2-DDCt and ΔΔCt = (CtGEN−CtGAPDH)treated − (CtGEN−CtGAPDH)treated (Livak and Schmittgen, 2001). This was accomplished by using the same universal human reference RNA in both the microarray and the real-time RT–PCR analysis;

Western blot

Whole cell lysates were prepared from 5–7 × 10⁶ cells which were washed two times in PBS, scraped and harvested directly in 2000 µl SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8; 8.7% glycerol; 2% w/v SDS; 5% v/v 2-β mercaptoethanol; 0.09% w/v bromophenol blue). Viscosity was reduced by drawing the suspension through a 21-G needle, cell debris were removed by centrifugation (15 000 g, 15 min), and the supernatant was stored at −80°C. Each extract (15 µl) was boiled and separated on an SDS 10% polyacrylamide gel (running buffer: 25 mM Tris-HCl, pH 8.3; 190 mM glycine, 0.1% w/v SDS) before electrophoresis onto Hybond-P membranes (Amersham Pharmacia Biotech, Pittsburgh, PA, USA). The transfer was performed in 25 mM Tris-HCl, 190 mM glycine and 20% methanol, pH 8.3, for 1 h at 175 mA. The membranes were treated with 5% nonfat dry milk (Nestlé, Vevey, Switzerland) in TBS (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl) for 1 h at room temperature and incubated with primary antibodies diluted (1:500:1:1000) in TBS with 1% BSA and 0.05% Tween 20 for 2 h, 20°C. The blots were then incubated with peroxidase-conjugated secondary antibodies (1:1000) in TBS with 1% BSA and 0.05% Tween 20 for 1 h at room temperature. After washing (4 × 15 min in TBS with 0.05% Tween 20), binding of secondary antibodies was visualised by the ECL-detection system (Amersham) before they were digitally exposed with the KODAK Image Station 2000R (Kodak, Rochester, NY, USA) for 5 min. GAPDH levels were used to verify protein loading.

The following antibodies were used: mouse anti-human GAPDH (1:1000) (Abcam, Cambridge, UK); mouse anti-human PRDX2 (1:1000) (Abcam); rabbit anti-human HPN (1:1000) (Cayman, Michigan, USA); rabbit anti-human SCG2 (1:500) (Abcam); and a secondary antibody conjugated to horseradish peroxidase (Fierce, Rockford, IL, USA).

Immunohistochemical and ultrastructural examinations

For IHC investigations, cell pellet was conventionally fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Sections, about 4–5 micron thick, were employed for the IHC examinations, using the Vectastain ABC kit (Vector Lab., Burlingame, CA, USA), and/or Tyramide signal amplification technique (NEN LifeScience Products, Boston, MA, USA), as previously described (Ovrigstad et al, 1999). Chromogranin A antiserum (1:500) was provided by Incstar (Stillwater, MN, USA), monoclonal mouse antisyntaptophysin antiserum (1:20) by Dako (Glostrup, Denmark), anti rat neuron-specific enolase (1:500) by Polysciences (Warrington, PA, USA), and antineurofilament M (1:4000) by Fitzgerald (MA, USA).

For the electron microscopic (EM) investigations, the pellet was fixed in 2% neutral glutaraldehyde, post-fixed in 2% osmium tetroxide, contrasted with 1% lead citrate and 4% uranyl acetate, and conventionally embedded in Epon. Finally, conventional ultra thin sections were cut and analysed by means of our transmission EMs (JEOL 100CX and Phillips SEI Tecnai 12).

RESULTS

Confirmation of the NE character

To confirm the NE and non-NE character of the cell lines, respectively, IHC and EM investigations were performed in addition to conventional LM examination. The employed NE cell lines (NCI-H727, UMC-11, SK-N-AS, SK-N-FI, TT, BON) encompass NE features with the expression of CHGA and SYP as the confined NE marker. The four cell lines known to be of non-NE character (WiDr, A-172, A-427, SW480), showed no staining with CHGA and SYP (data not shown). In addition, the cells were examined for the expression of ENO2 (enolase 2/neuron-specific enolase), an NE marker thought to be less specific than the conventional NE markers CHGA and SYP. All the presumed NE cell lines showed positive immunoreactivity to enolase 2, and this was also the case for the non-NE cell lines A-427 and SW480 (data not shown). EM investigations demonstrated occurrence of typical NE secretion granules in all the NE tumour cells, but not in any of the non-NE tumour cells, thus confirming the predefined NE/non-NE characteristics of the cell lines used.

Genes differentially expressed in NE vs non-NE tumour cells

Having confirmed the NE and non-NE character of the cell lines, respectively, we performed transcript profiling by cDNA micro-array analysis in an effort to identify new NE-specific genes, and by this, get more insight into the biology of NETs. By using the convex decreasing density estimator for the proportion of true null hypotheses as presented in Langgaas (2005), we expect 5.5% of the genes studied to be differentially expressed in NE vs non-NE cells. The 200 most significant genes (P-value 0.008/FDR 0.49) in the comparison of the NE vs non-NE tumour cell groups are sequence verified, and 153 genes are given as Supplementary Information in the gene expression omnibus (GEO) GSE4328.

Based on information from the GO annotation database and literature search, these genes are displayed with the log ratio and biological processes in which they are likely to be involved. The up- and downregulated genes range from log2 5.87 to −2.92, respectively. The 70 most highly up- and downregulated genes, are shown in Table 2. A hierarchical cluster analysis of the 48 most significantly differentially expressed genes (P-value 0.0014/FDR 0.2823) is shown in Figure 1.

The three most highly overexpressed genes: SCG3 (26.6 fold), SCG2 (15.3 fold) and DDC (9.6 fold) (Table 2), have previously been shown to be linked to NE tumour biology, thus confirming the reliability of our study design. SCG3 and SCG2 are both members of the chromogranin–secretogranin family of NE secretory, acidic glycoproteins (Taupenot et al, 2003), and DDC has more recently been shown to be expressed in various NETs (Uccella et al, 2006). Furthermore, the high expression of MAOA in our study, support previous findings of high expression of monoaminooxidase A in various NETs (Orlefors et al, 2003).

NETs in general are relatively slow growing tumours with a less invasive character than many epithelial cancers. Several genes thought to play a role in the processes of invasion, tumour
## Table 2  Differentially expressed genes in NE vs non-NE tumour cells

| Gene symbol | Gene name                          | UGCluster | Ratio |
|-------------|-----------------------------------|-----------|-------|
| **Upregulated** |                                   |           |       |
| SCG3        | Secretogranin III                | Hs.232618 | 26.56 |
| SCG2        | Secretogranin II (chromogranin C) | Hs.516726 | 15.29 |
| DDC         | Dopa decarboxylase (aromatic L-amino acid decarboxylase) | Hs.359698 | 9.65  |
| BAALC       | Brain and acute leukemia, cytoplasmic | Hs.533446 | 7.78  |
| NEF3        | Neurofilament 3                   | Hs.58657 | 7.66  |
| C8orf13     | Chromosome 8 open reading frame 13 | Hs.24299 | 7.27  |
| BEK1        | Brain expressed, X-linked         | Hs.33470 | 6.76  |
| RAPGEF5     | Rap guanine nucleotide exchange factor (GEF) | Hs.174768 | 6.01  |
| PLXNA2      | Plecin A2                         | Hs.497626 | 5.88  |
| MGC17299    | Hypothetical protein MGC17299     | Hs.104476 | 5.11  |
| PRDX2       | Peroxiredoxin 2                   |           | 5.04  |
| M160        | Scavenger receptor cysteine-rich type I protein M160 | Hs.49636 | 5.01  |
| DNAJC12     | DnaJ (Hsp40) homologue, subfamily C, member 12 | Hs.260720 | 4.50  |
| MAOA        | Monoamine oxidase A               | Hs.183109 | 4.48  |
| FAM6A       | Family with sequence similarity 46, member A | Hs.10784 | 4.29  |
| NEF3        | Neurofilament 3                   |           | 3.70  |
| ITGA10      | Integrin, α 10                    | Hs.158237 | 3.88  |
| HLA-DOA     | Major histocompatibility complex, class II, DO-α | Hs.351874 | 3.15  |
| CNTN1       | Contactin 1                       | Hs.14536 | 3.12  |
| NR0B2       | Nuclear receptor subfamily 0, group B, member 2 | Hs.27055 | 3.57  |
| TAGLN3      | Transgelin 3                      | Hs.169330 | 3.45  |
| FEG10       | Paternally expressed 10           | Hs.147492 | 3.37  |
| EGLN3       | Egfl nine homologue 3 (C. elegans) | Hs.41599 | 3.36  |
| MBP         | Myelin basic protein              | Hs.551713 | 3.36  |
| ABC6C       | ATP-binding cassette, subfamily C (CFTR/MRP), member 6 | Hs.13188 | 3.26  |
| SPHBT2      | Smc-like with four mbt domains 2   | Hs.407983 | 3.17  |
| C9orf150    | Chromosome 9 open reading frame 150 | Hs.12319 | 3.15  |
| HIPK2       | Homeodomain interacting protein kinase 2 | Hs.39746 | 3.10  |
| C1orf28     | Chromosome 17 open reading frame 28 | Hs.11067 | 3.05  |
| C3orf14     | Chromosome 3 open reading frame 14 | Hs.49716 | 2.94  |
| UMDQ1       | UIM domains containing 1          | Hs.193370 | 2.92  |
| HPN         | Hepsin (transmembrane protease, serine 1) | Hs.182385 | 2.82  |
| MDS010      | x 010 protein                     | Hs.231750 | 2.77  |
| MSH1        | Membrane-spanning 4-domains, subfamily A, member 1 | Hs.438040 | 2.75  |
| NAPB        | N-ethylmaleimide-sensitive factor attachment protein, β | Hs.92671 | 2.69  |
| PBX1        | Pre-B-cell leukemia transcription factor 1 | Hs.493096 | 2.63  |
| APG4A       | APG4 autophagy 4 homologue A       | Hs.8763 | 2.60  |
| ARHGAP26    | Rho GTPase-activating protein 26   | Hs.293593 | 2.56  |
| GAB2        | GRB2-associated binding protein 2  | Hs.429434 | 2.53  |
| AQP3        | Aquaporin 3                        | Hs.234642 | 2.45  |
| MGC4645     | Hypothetical protein MGC4645       | Hs.395306 | 2.44  |
| PTP4A3      | Protein tyrosine phosphatase type IVA, member 3 | Hs.43666 | 2.40  |
| TP53I1I     | Tumour protein p53-inducible protein 11 | Hs.554791 | 2.36  |
| C14orf132   | Chromosome 14 open reading frame 132 | Hs.64934 | 2.33  |
| SCSOD1      | Sterol C5-desaturase               | Hs.287749 | 2.29  |
| CENTB5      | Centaurin, β 5                    | Hs.535257 | 2.15  |
| ARHGAP5     | Rho GTPase-activating protein 5    | Hs.25287 | 2.13  |
| KIAA0924    | KIAA0924 protein                   | Hs.50656 | 2.10  |
| C6orf1      | Chromosome 6 open reading frame 1  | Hs.381300 | 2.10  |
| SCFTB       | Small glutamine-rich tetratricopeptide repeat (TPR) | Hs.87871 | 2.05  |
| NNT         | Nicotinamide nucleotide transhydrogenase | Hs.482043 | 2.05  |
| BRPF3       | Bromodomain and PHD finger containing, 3 | Hs.520096 | 1.99  |
| TBC1D16     | TBC1 domain family, member 16      | Hs.36989 | 1.98  |
| IF2BP2      | Interferon regulatory factor 2-binding protein 2 | Hs.350268 | 1.94  |
| DIFCPZpH34H226 | Limb1 domain containing 2 (DIFCPZpH34H226) | Hs.294103 | 1.93  |
| CALM1       | Calmodulin 1 (phosphorylase kinase, delta) | Hs.282410 | 1.82  |
| C6orf209    | Chromosome 6 open reading frame 209 | Hs.271643 | 1.79  |
| ZCCHC3      | Zinc finger, CCHC domain containing 3 | Hs.28608 | 1.78  |
| IRS2        | Insulin receptor substrate 2       | Hs.442344 | 1.76  |
| RGS18       | Regulator of G-protein signalling 18 | Hs.440890 | 1.70  |
| SCFD1       | Sec1 family domain containing 1    | Hs.369168 | 1.69  |
| TCEAL8      | Transcription elongation factor A (SII)-like 8 | Hs.389734 | 1.67  |
| SEC23B      | Sec23 homologue B (S. cerevisiae)  | Hs.369373 | 1.59  |
| MECP2       | Methyl CpG-binding protein 2        | Hs.200716 | 1.59  |

| **Downregulated** |                                   |           |       |
| MME          | Membrane metallo-endopeptidase     | Hs.307734 | 0.12  |
| STAT3        | Signal transducer and activator of transcription 3 (acute-phase response factor) | Hs.463059 | 0.13  |
| Gene symbol | Gene name | UGCluster | Ratio |
|-------------|-----------|-----------|-------|
| MEOX1       | Homeobox protein MOX-1 | Hs.438     | 0.14  |
| TMEM41      | Transmembrane, prostate androgen induced RNA | Hs.517155  | 0.16  |
| RAB3B       | RAB3B, member RAS oncogene family | Hs.2886714 | 0.17  |
| KL2         | Kruppel-like factor 2 (lung) | Hs.107740  | 0.17  |
| ZNF354A     | Zinc finger protein 354A | Hs.486254  | 0.17  |
| LOC255104   | Hypothetical protein LOC255104 | Hs.517155  | 0.17  |
| S100A10     | S100 calcium-binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11)) | Hs.148783  | 0.18  |
| DCBLD2      | Discoidin, CUB and LCCL domain containing 2 | Hs.203691  | 0.18  |

**Table 2 (Continued)**

| Gene symbol | Gene name | UGCluster | Ratio |
|-------------|-----------|-----------|-------|
| ZF          | HCF-binding transcription factor Zhangfei | Hs.535319  | 0.25  |
| AMOTL2      | Angiomotin like 2 | Hs.426312  | 0.25  |
| LMO2        | LIM domain only 2 | Hs.34560   | 0.25  |
| CETN2       | Cenitin, EF-hand protein, 2 | Hs.82799   | 0.27  |
| RUNX1       | Runt-related transcription factor 1 (acute myeloid leukaemia 1; amll oncogene) | Hs.149261  | 0.27  |
| HRASL3      | HRAS-like suppressor 3 | Hs.502775  | 0.28  |
| APEH        | N-acetylaspartyl-aspartate dipeptidase | Hs.715969  | 0.28  |
| ERBB2       | V-erb-b2 erythroblastoma viral oncogene homologue 2, neuro/glioblastoma derived oncogene homologue | Hs.446352  | 0.28  |
| YAP1        | Yes-associated protein 1, 65kDa | Hs.503692  | 0.31  |
| CD9         | CD9 antigen (p24) | Hs.114286  | 0.31  |
| TNNT2       | Troponin I, skeletal, fast | Hs.523403  | 0.32  |
| FBN1        | Fibulin 1 | Hs.24601   | 0.32  |
| S100A8      | S100 calcium binding protein A8 (calgranulin A) | Hs.416073  | 0.33  |
| LOC388990   | Novel 58.3 KDA protein | Hs.29090   | 0.34  |
| CTSL        | Cathepsin L | Hs.418123  | 0.34  |
| PRPS2       | Phosphoribosyl pyrophosphate synthetase 2 | Hs.104123  | 0.35  |
| TM8B10      | Thymosin, β 10 | Hs.446574  | 0.37  |
| TSPM2       | Trophoblast motility 2 (β) | Hs.30077    | 0.37  |
| SHKBP1      | SH3-domain kinase-binding protein 1 | Hs.447770  | 0.38  |
| FOSL1       | FOS-like antigen 1 | Hs.283565  | 0.39  |
| ODCL1       | Omidine decarboxylase I | Hs.467701  | 0.39  |
| MRLC2       | Myosin regulatory light chain MRLC2 | Hs.464472  | 0.39  |
| LOC527228   | Hypothetical protein from clone 643 | Hs.558253  | 0.39  |
| CD164       | CD164 antigen, salomucin | Hs.520313   | 0.41  |
| CAMK1       | Calcium/calmodulin-dependent protein kinase I | Hs.438475  | 0.41  |
| RPA3        | Replication protein A3, 14kDa | Hs.487540  | 0.41  |
| VIL2        | Villin 2 (ezrin) | Hs.487027  | 0.42  |
| IFRD2       | Interferon-related developmental regulator 2 | Hs.315177  | 0.42  |
| NLGN2       | Neurexin 2 | Hs.26229    | 0.43  |
| CD59        | CD59 antigen p18-20 | Hs.278573   | 0.43  |
| ZBTB4       | Zinc finger and BTB domain containing 4 | Hs.35096   | 0.45  |
| TXNRD1      | Thioredoxin reductase I | Hs.337766   | 0.46  |
| MAT2B       | Methionine adenosyltransferase II, 5 | Hs.546462   | 0.46  |
| BMP1        | Bone morphogenetic protein 1 | Hs.12374    | 0.46  |
| HRB2        | HIV-1 rev binding protein 2 | Hs.205558   | 0.47  |
| APPBP1      | Amyloid β precursor protein binding protein 1, 59kDa | Hs.460978   | 0.48  |
| CTNNA1      | Catenin (cadherin-associated protein), α 1, 102kDa | Hs.445981   | 0.49  |
| COMMD6      | COMM domain containing 6 | Hs.508266   | 0.51  |
| MAP4        | Microtubule-associated protein 4 | Hs.501799   | 0.51  |
| PSMD1D2     | Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12 | Hs.4295    | 0.51  |
| PLP2        | Proteolipid protein 2 (colonic epithelium-enriched) | Hs.77422    | 0.52  |
| GPX1        | Glutathione peroxidase 1 | Hs.76686    | 0.52  |
| SYT1        | Synaptophysin-like protein | Hs.202166   | 0.54  |
| PICALM      | Phospholipid-interacting clathrin assembly protein | Hs.69893    | 0.56  |
| PTTP12      | Protein tyrosine phosphatase, non-receptor type 12 | Hs.61812    | 0.56  |
| PSMA5       | Proteasome (prosome, macropain) subunit β type5 | Hs.485246   | 0.58  |
| ST13        | Suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein) | Hs.546303   | 0.58  |
| S140        | U2-associated S140 protein | Hs.25977    | 0.58  |
| PAWR        | PRKC, apoptosis, WT1, regulator | Hs.406074   | 0.59  |
| EGR3        | Early growth response 3 | Hs.534313   | 0.60  |
| HNRP4H1     | Heterogeneous nuclear ribonucleoprotein H1 (H) | Hs.202166   | 0.60  |
| GLTSCR2     | Gloma tumour suppressor candidate region gene 2 | Hs.421907   | 0.61  |
| P38CA       | Proliferating cell nuclear antigen | Hs.47433    | 0.61  |
| PSMB3       | Proteasome (prosome, macropain) subunit β type 3 | Hs.82793    | 0.61  |
| EMR3        | EGF-like module containing, mucin-like, hormone receptor-like 3 | Hs.295626   | 0.62  |

Genes significantly (P<0.007) up- or downregulated in the neuroendocrine cell lines compared with the non-neuroendocrine cell lines. The first half of the table shows downregulated genes whereas the last part of the table shows the upregulated genes. The genes are all given with unigene cluster id’s, official gene name and symbols in addition to their respective ratio (NE vs non-NE).
followed by a similar expression pattern at the protein level, we investigated whether the difference in gene expression level was reflected by the protein level. To this end, we performed protein expression analysis of at least three selected genes using the same RNA samples as those used in the microarray analysis. The three most highly downregulated genes in our study were MME (0.12 fold), STAT3 (0.13) and DCBLD2 (0.14 fold). Our results also point to differences in expression of several genes thought to be involved in the process of tumorigenesis (BEX1, TMEPAI, FOSL1, RAB32, ERBB2) (Table 2 and Supplementary Information). Well-differentiated NETs are in general relatively insensitive to various chemoheurapeutic drugs, and thus it is interesting to note variations between the two groups in the expression of genes known to be involved in the process of drug resistance (STAT3, PRKD2 ABCC6, GSTP1) (Table 2 and Supplementary Information). Nearly 50% of the upregulated, and 16% of the downregulated genes are in the GO database defined as having an unknown function (Supplementary Information).

**Validation by real-time qRT–PCR**

To validate the microarray results, we performed real-time quantitative RT–PCR analysis of five selected genes using the same RNA samples as those used in the microarray analysis. The selection of the genes (BAALC, SCG2, GSTP1, FOSL1, M160) were based upon a combination of P-value, differential expression, and biological function. In general, 70% of the genes found to be differentially expressed in the microarray study were confirmed by RT–PCR (Figure 2). This seems to be in accordance with previous studies using cDNA arrays (Kothapalli et al, 2002; Hofsli et al, 2005), and underlines the need to verify microarray data by additional methods.

**Protein expression analysis**

To investigate whether the difference in gene expression level was followed by a similar expression pattern at the protein level, we first performed western blot analysis of cell lysates. The selection of gene products analysed (secretogranin II, peroxiredoxin 2, hepsin) was based upon a combination of the expression level found in the microarray analysis, biological relevance, and availability of antibodies. As seen in Figure 3, the protein expression of the NE marker secretogranin II, correlated well with the gene expression level of SCG2 found in the microarray analysis (15-fold upregulated)(Table 2), and in the real-time RT – PCR analysis (Figure 2). All the NE tumour cell lines express a high level of SCG2, whereas the expression level in the non-NE cell group is almost undetectable. Hepsin (2.8 fold upregulated in the micro-array analysis) was found to be expressed in all cell lines and without any significant difference in NE vs non-NE cells (Figure 3). Thus, hepsin is ruled out as a possible new diagnostic marker of NET disease. On the contrary, the level of peroxiredoxin 2 expression (5 fold upregulated in the microarray analysis) was significantly different in the two groups (Figure 3). Peroxiredoxin 2 was clearly detectable in the NE cell line group, but almost undetectable in the non-NE cell group, thus pointing out peroxiredoxin 2 as an interesting new NE biomarker. The difference in secretogranin II and peroxiredoxin 2 expression was also confirmed by IHC analysis (data not shown).

In addition to secretogranin II and peroxiredoxin 2, our study points to NEFM as another interesting candidate marker of NET disease. NEFM, which was upregulated by a factor of 7.7 in the microarray analysis (Table 2), was by IHC shown to be expressed only in the NE tumour cells group (data not shown).

**DISCUSSION**

Although last year’s genomic and proteomic research have uncovered some genes and gene products thought to have an important function in the context of NET tumour biology (Hofsli, 2006), still much is unknown concerning which factors that are important with regard to the causes and behaviours of NET disease.
diseases. The results of this study contribute to an increased insight into the biology of these tumours, by identifying genes that are differentially expressed in NE tumour cells as compared with non-NE tumour cells. We believe that some of these genes and gene products represent interesting candidates in the search for new prognostic, predictive and therapeutic markers. The study also point to genes that may play a role in the tumorigenesis of NETs.

The three most highly overexpressed genes in the NE vs the non-NE tumour cell group (SCG3, SCG2 and DDC) (Table 2), have all previously been described in the context of NE tumour biology, thus confirming the reliability of our study design. Although secretogranin II and one of its split product (Taupenot et al, 2003; Guillemot et al, 2006) have been shown to be expressed in various types of NETs, investigations of the expression of secretogranin III in NETs have so far not been reported. The enzyme dopa decarboxylase (DDC)(catecholamine biosynthesis) has more recently been shown to be expressed in various NETs, such as bronchial carcinoids and poorly differentiated NE carcinomas of the lung (Uccella et al, 2006). It has also been shown to be a marker of neuroblastoma in children (Bozzi et al, 2004), and of NE differentiation in prostate carcinoma (Wafa et al, 2007). Another gene known to be involved in catecholamine metabolism, MAOA (Toninello et al, 2006), was also identified as highly expressed in the NET group (Table 2), a finding that was confirmed by IHC analysis (not shown). This supports previous findings demonstrating a high expression of MAOA in gastroenteropancreatic (GEP) tumours (Orlefors et al, 2003). To conclude, we believe that SCG3, SCG2 and DDC could represent useful additional biomarkers in NET diseases, and that they perhaps should be implemented in the standard diagnostic panel of NE biomarkers. Furthermore, measurement of MAOA activity may, as recently shown in a baboon model, aid in understanding the pathophysiology of NETs (Murthy et al, 2007).

Figure 2 RT–PCR confirmation of microarray results. A selection of genes (A–E) was also analysed by semi quantitative real-time RT–PCR (grey), and compared to the respective ratios of the microarray analysis (white). The two methods correlated at 9/10 cell lines at best, and the lowest correlated at 6/10 cell lines. Y axis shows the log-transformed ratio of both the microarray and the RT–PCR, based on the fold change ratios and the delta–delta \( \Delta C_t \) calculation, respectively.
expression of this gene in brain, but also in peripheral organs such as liver, pancreas, testis, and ovary (Yang et al, 2002a, b; Alvarez et al, 2005). It has more recently been suggested that BEX1 may play a role as a tumour suppressor in malignant glioma (Foltz et al, 2006). A very low expression was observed for the TMEPAI gene (Table 2), which is involved in androgen receptor signaling, and is proposed to play a role in prostate tumorigenesis (Xu et al, 2003). TMEPAI has been shown to be overexpressed in various solid tumours, probably because of abnormal activation of the EGF pathway (Giannini et al, 2003). Also the oncogenic transcription factor FOSS1, was downregulated in the NE tumour cell group. FOSS1 is upregulated in several solid cancers, and is becoming a new target for cancer intervention (Young and Colburn 2006). The ras family member RAB32, has been proposed to represent a context of the oncogenic pathway of microtubule instability and high gastrointestinal adenocarcinomas (Shibata et al, 2006). In our study, RAB32 was highly downregulated in the NE vs the non-NE group. Also the ERBB2 gene expression level was significantly lower in the NE tumour cell group than in the non-NE group. The expression level of this member of the oncogenic EGF receptor family, has previously been reported as a variable in various NETs (cf. Hofski, 2006). So far, there is no strong evidence that ERBB2 amplification/overexpression could play an important role in NET pathogenesis, or that it could be a potential target for treatment, as is the case in various epithelial cancers (Hsieh and Moasser 2007).

A hallmark of NETs in general, are that they are relatively slow growing and less invasive in character. Thus, its interesting to note that several genes thought to play a role in the processes of invasion, tumour progression and metastasis (MME, STAT3, DCBLD2, S100A10, CD9, S100A8) were highly downregulated in the NE vs the non-NE tumour group (Table 2). The most highly downregulated gene was MME. A loss or decrease in MME has been reported in a variety of malignancies, and reduced expression results in the accumulation of higher peptide concentrations that could mediate neoplastic progression (Sumitomo et al, 2005). Loss of this endopeptidase also leads to AKT1 (protein kinase B) activation, and contributes to the clinical progression of prostate cancer (Osman et al, 2006). STAT3 (the signal-transducer and activator of transcription 3) is known to play an important role in both tumorigenesis and tumour progression, and is often constitutively activated in tumour cells (Aggarwal et al, 2006). Thus, inhibitors of STAT3 activation have potential for both prevention and therapy of cancer (Huang, 2007). In lung cancer, DCBLD2 has been shown to be highly upregulated in the cell line NCI-H460-LNM35, in association with its acquisition of metastatic phenotype, and also upregulated in high frequency in metastatic lesions from lung cancers (Koshikawa et al, 2002). It is also shown that DCBLD2 may play a role in cell motility (Nagai et al, 2007), and thus it is suggested that this novel gene may become a target of therapy to inhibit metastasis of lung cancers.

The plasminogen receptor S100A10 is found overexpressed in many cancer cells, and seems to play an important role in cancer cell invasiveness and metastasis (Kwon et al, 2005). RNA interference-mediated downregulation of S100A10 gene expression in colorectal cancer cells, has been shown to result in a complete loss in plasminogen-dependent cellular invasiveness (Zhang et al, 2004). More recently it has been shown by IHC analysis that S100A10 expression in thyroid neoplasms contributes to the aggressive characteristic of anaplastic carcinoma (Ito et al, 2007). To conclude, the very low levels of various genes known to be involved in the processes of invasion, tumour progression and metastasis could perhaps reflect the in general more slow growing and less invasive character of NETs.

In addition to the already mentioned STAT3 and PRX2, other genes that have been linked to the phenomenon of drug resistance,

Figure 3  Western blot. Western blot analysis was performed on cell lines (NE and non-NE) with the antibodies against secretogranin II, hepsin, peroxiredoxin 2 and GAPDH. Cells were harvested and prepared as described in Materials and methods.
were identified as differentially expressed (ABCC6, GSTP1). Well-differentiated NETs are in general relatively insensitive to various chemotherapeutic drugs. Thus, it is interesting to note that our study reveals a relatively high expression of ABCC6 (ATP-binding cassette, subfamily C (CFTR/MRP), member 6), one member of the MRP subfamily involved in multi-drug resistance (Beck et al., 2005). Endocrine G-cells in the stomach has been shown to express high level of ABCC6 (Beck et al., 2005). However, our study is the first to report ABCC6 expression in NE tumour cells. The antiapoptosis gene GSTP1 was, however, downregulated in the NET group (Table 2). In prostate cancer, the loss of expression of GSTP1 is the most common genetic alteration reported (Meiers et al., 2007). A comprehensive survey of GSTP1 expression in NETs has so far not been performed, but one study has been undertaken, showing that the expression of this drug-resistant protein is significantly lower in large cell NE carcinoma of the lung than in the other more common histological types of lung cancer (Okada et al., 2003).

In conclusion, the results of our study add new important lights into the understanding of NE tumour biology, by identifying genes differentially expressed in NE as compared with non-NE tumour cells. In addition to potential new diagnostic markers (SCG2, SCG3, DDC, MAOA, NEFM, CLDN4, PEROX2), genes critical in the processes of tumour invasion, progression and metastasis (MME, STAT3, DCBLD2, S100A10, CD9, S100A4B), tumorigenesis (BEX1, TMEPA1, FOSL1, RAB32) and drug-resistance (ABCC6, GSTP1) were identified, as well as several genes with hitherto unknown functions.

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