Supplementary Materials and methods

Clinical details

There were 15 patients with SBNE Ts included in the 1st profiling. Patients were diagnosed between 2007 and 2012. The median age was 69 years (range 38-84 years; Supplementary Table 1). Most patients were alive at follow-up (mean 52 months; range 25 to 124 months), except for three (patients 2, 9 and 13). None had MEN1 mutations. Four patients had carcinoid syndrome, whilst the remainder had non-functioning tumors.

Nine patients had distant metastases (liver, n=8 and peritoneum, n=1). Patient number 7 had liver metastases and also metastases to the uterus, omentum, ovaries and adnexal structures. Thirteen of the 15 patients (86.7%) had LN metastases. All tumors were either grade 1 (n=12) or grade 2 (n=3) based on the Ki-67 proliferative index. Details of this cohort have been previously documented (Miller, et al. 2014).

Tissue for the 2nd profiling study came from the Zentralklinik Bad Berka, Germany.

Ethical approval was obtained from the Imperial College Healthcare Tissue Management committee and a National Research Ethics Committee (REC reference 07/MRE09/54).

RNA extraction from tissues

Formalin fixed paraffin embedded (FFPE) tissues were used in the 1st profiling study and fresh-frozen tissues for the 2nd profiling study. In the 1st profiling, 47 tissue samples were included. Matching primary SBNETs (n=15), adjacent normal small bowel (NSB, n=12), matched LN metastases (n=9), normal LNs (n=7), adjacent normal liver (n=2) and liver metastases (n=2) were included from each patient where available. For two patients, tissue was available from liver metastases and adjacent normal liver tissue. A 2nd “validation” profiling experiment was performed using 43 fresh-frozen patient samples and included more liver metastases (SBNET, n=13; NSB, n=2; LN metastases, n=15 and liver metastases, n=13. Combining the 2 cohorts, a total of 90 patient samples were profiled for miRNA expression.

A representative section from each FFPE block was stained with hematoxylin & eosin (H&E) and marked by an experienced histopathologist for the area of interest. H&E slides for all tissues were marked up in the same way. Five 10µm sections from each FFPE block were then deparaffinised and stained with haematoxylin only prior to dissection with a scalpel.

Total RNA was extracted from the tissue using the miRNeasy FFPE kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions. RNA quality was checked and RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Oxfordshire, UK). Enough RNA was available for the global miRNA profiling and the qRT-PCR validation experiments.

The RNA extraction from the frozen tissue was done using a cryostat to section the tissue into 20µm sections. The RNA was extracted from these using TRIzol method according to the manufacturer's instructions. The resulting RNA was cleaned up using QIAGEN columns from the miRNeasy FFPE kit (see above) and the RNA quality and quantity validated as before.

NanoString miRNA profiling

The Nanostring nCounter Human miRNA Expression Array (http://www.nanostring.com/) (NanoString Technologies, Seattle, USA) was used to obtain
global miRNA expression profiles (Geiss, et al. 2008). The NanoString miRNA panel contains 800 probes for human miRNAs based on miRBase version 18. Total RNA (100 ng) was used as input for nCounter miRNA sample preparation reactions and these were performed according to manufacturer's instructions (NanoString Technologies). Small RNA sample preparation involves the ligation of a specific DNA tag onto the 3’ end of each mature miRNA. These tags normalize the melting temperatures (Tms) of the miRNAs and provide a unique identification for each miRNA species in the sample. Excess tags were then removed, and the resulting material was hybridized with a panel of miRNA: tag-specific nCounter capture and barcoded reporter probes. Hybridized probes were then purified and immobilized on a streptavidin-coated cartridge using the nCounter Prep Station (NanoString Technologies). Data collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following the manufacturer’s instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high density scan (600 fields of view) was performed.

MiRNA expression data processing

Raw data from the NanoString nCounter Human miRNA Expression Arrays was normalized and analyzed using the DESeq2 package from R/Bioconductor. Poorly expressed miRNAs were filtered out before the analysis. Mean miRNA expression was then calculated for each sample group. Fold change and Log2 fold change (log2 FC) was calculated for each miRNA between the sample groups. Two-sample t tests were applied to find significant differentially expressed miRNA between the sample groups. The False Discovery Rate (FDR) technique was used to adjust the P value for multiple-testing and was calculated using R/Bioconductor. An adjusted P value of <0.05 was considered significant.

Validation of candidate miRNAs by qRT-PCR

The “top hits” from the global miRNA profiling were then validated. MiRNAs chosen from the NanoString data were the top up-regulated (n=3) in SBNET vs. NSB tissues; and those miRNAs significantly down-regulated in LN metastases vs. SBNETs (n=2).

For the validation experiments, candidate miRNAs were quantified by qRT-PCR using specific TaqMan primers (Life Technologies). Samples were run on the qPCR plates in duplicate. RNU44 and U6 were used as endogenous control genes. As similar results were found for both these controls, the data for U6 are presented. For the qRT-PCR validation, the mean delta CT was calculated and miRNA expression was normalized against RNU44 and U6 expression for each sample. Mean delta CT for each miRNA was compared between sample types and an unpaired, one-tailed t test applied using a P value of <0.05 as significant.

Gene target bioinformatic analyses

TargetScan (Human version 6.2) was used to predict the gene targets of the validated differentially expressed miRNAs (Friedman, et al. 2009; Lewis, et al. 2005). These were compared to publically available SBNET gene expression datasets. Gene Expression Omnibus (GEO) dataset accession number GSE27162 was used for the SBNET vs. LN metastases comparison (Edfeldt, et al. 2011). Datasets GSE9576, GSE6272 and E-TABM-389 (EBI’s Array-Express) were used for the SBNET vs. normal small bowel comparison (Kidd, et al. 2014; Leja, et al. 2009). GEO2R was used to identify up- and down-regulated genes between the specific tissue types of interest for GSE27162 and GSE9576, and genes were considered significant if the P value was <0.05 (Davis and Meltzer 2007). For the GSE6272 and E-TABM-389 datasets, data on differentially expressed genes in SBNET vs. normal small bowel was available and those with a Log fold-change cut-off of +/- 1.4 and P value of 0.05 were considered significant (Kidd et al. 2014). As gene symbols often varied between datasets, all genes were checked against the HUGO Gene Nomenclature
Committee (HGNC) database to make sure that each gene had the same official symbol in the gene lists used for the enrichment analysis. Interesting miRNA-mRNA interactions were also investigated using miRanda-mirSVR (http://www.microrna.org/microrna/home.do).

**Gene ontology and pathway analyses of mRNA expression**

We used DAVID (http://david.abcc.ncifcrf.gov/) to perform enrichment analyses of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa, et al. 2010) and GO terms (biological process) (Ashburner, et al. 2000) for the list of protein-coding genes deregulated by differentially expressed miRNAs (Benjamini-Hochberg adjusted t-test $P$-value $P<0.05$). A selection of the top enriched GO biological processes and KEGG pathways for genes can be seen in Supplementary Tables 5.

**Cell culture**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin/glutamine. Human small intestinal NET cell line KRJ-I was kindly provided by our co-author Dr Roswitha Pfargner (University of Graz, Graz, Austria) (Pfragner, et al. 1996). KRJ-I cells were cultured in Ham’s F12 medium (Gibco) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin/glutamine.

**Luciferase Reporter Assay**

HEK293T cells were seeded onto 24 well plates at a density of 50,000 cells/well in antibiotic-free medium. Twenty-four hours later, cells were co-transfected with 100nM of precursor (pre-miRs) or inhibitor (anti-miRs) miRNAs (Applied Biosystems) together with FOSB or NUAK2 3’UTR reporter constructs (pLightSwitch_3UTR GoClone vectors, SwitchGear Genomics) at 100ng/well using Lipofectamine2000 (Invitrogen). After 24 hours, cells were lysed with passive lysis buffer (Promega) and luciferase activity was measured using the LightSwitch Assay System (SwitchGear Genomics) following the manufacturer’s instructions. The mean luciferase activity for each precursor miRNA is shown relative to the mean for the negative control (Figure 5).

**Transfection of KRJ-I cells**

KRJ-I cells were seeded on 6 well-plates at a density of 400,000 cells/well in antibiotic-free medium. Twenty-four hours later, cells were transfected with 5nM of precursor (pre-miRs) or inhibitor (anti-miRs) miRNAs (Applied Biosystems) using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. Forty-eight hours post-transfection, cells were lysed for RNA or protein extraction.

**Measuring gene and miRNA expression by qRT-PCR**

Total RNA was extracted using TRI Reagent® (Sigma) and Direct-zol™ RNA MiniPrep Kit (Zymo Research) following the manufacturer’s protocols, including DNase I treatment. qRT-PCR of mature miRNAs was then performed using TaqMan miRNA assay kit (Applied Biosystems). We used small nuclear RNA U47 as an endogenous control. For gene expression, cDNA was synthesized from 1 μg of purified DNase-treated RNA using RevertAid reverse transcriptase and random hexamer primers (Thermo Scientific), according to the manufacturer’s protocols. We performed qRT-PCR on a StepOne™ Real-Time PCR System using Fast SYBR® Green Master Mix (both from Applied Biosystems). The following primers were used: NUAK2-F TTGCCATCCCATGAAGTGGTG, NUAK2-R CATACTCCATGACGATCCACGA; FOSB-F GGACGAAATAAACATAAGCA, FOSB-R ATCTCCAGTCAAGTCCTC; GAPDH-F TGAAGGTCGGAGTCAACGGATTT, GAPDH-R GCCATGGAATTTGCCCATGGGTGG.
Western blotting

Whole cell lysates were prepared in RIPA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche). Protein concentration was determined using the Bradford Protein Assay (Bio-Rad). Twenty micrograms of lysates were separated on Bolt® 4–12% Bis-Tris Plus gels using Bolt™ MOPS SDS Running buffer (Life Technologies) following manufacturer’s instructions and transferred onto Hybond C super nitrocellulose membrane (GE Healthcare). The following primary antibodies were used: FosB (Santa Cruz Biotechnology, sc-48, 1:200), NUAK2 (Proteintech, 11592-1-AP, 1:400) and GAPDH (Abcam, ab9482, 1:1000). Following incubation with the appropriate HRP-conjugated secondary antibodies (Dako), the signal was detected using the Amersham ECL Western Blotting detection system (GE Healthcare).

REFERENCES FOR SUPPLEMENTARY MATERIAL


