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Changes in gene expression in small bowel neuroendocrine tumors associated with progression to metastases

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Background. Small bowel neuroendocrine tumors (SBNETs) present frequently with metastases, yet little is known about the molecular basis of this progression. This study sought to identify the serial differential expression of genes between normal small bowel, primary small bowel neuroendocrine tumors, and liver metastases.

Methods. RNA isolated from matched normal small bowel tissue, primary small bowel neuroendocrine tumors, and liver metastases in 12 patients was analyzed with whole transcriptome expression microarrays and RNA-Seq. Changes in gene expression between primary small bowel neuroendocrine tumors and normal small bowels, and liver metastases versus primary small bowel neuroendocrine tumors were calculated. Common genes that were differentially expressed serially (increasing or decreasing from normal small bowel to primary small bowel neuroendocrine tumors to liver metastases) were identified, and 10 were validated using qPCR.

Results. Use of 2 transcriptome platforms allowed for a robust discrimination of genes important in small bowel neuroendocrine tumors progression. Serial differential expression was validated in 7/10 genes, all of which had been described previously in abdominal cancers, and with several interacting with members of the AKT, MYC, or MAPK3 pathways. Liver metastases had consistent underexpression of PMP22, while high expression of SERPINA10 and SYT13 was characteristic of both pSBTs and liver metastases.

Conclusion. Identification of the serial differential expression of genes from normal tissues to primary tumors to metastases lends insight into important pathways for SBNETs progression. Differential expression of various genes, including PMP22, SYT13 and SERPINA10, are associated with the progression of SBNETs and warrant further investigation. (Surgery 2017;160:XXX-XXX.)

Arising from the enterochromaffin (EC) cells of the small bowel, small bowel neuroendocrine tumors (SBNETs) have become the most common neoplasm of the small intestine,[1] and, although they generally grow slowly, a substantial number of patients will progress to metastatic disease by the time of presentation. Despite the increased incidence of these neoplasms, little is known regarding the genetic steps accompanying the transformation of primary neoplasms and their progression to metastases. Improved understanding of these changes would aid in the identification of genes and pathways important to the evolution of SBNETs and assist potentially in the development of new diagnostic and therapeutic strategies.

Exome sequencing of SBNETs has revealed nonrecurring mutations in a variety of genes as well as frequent sites of deletion or amplification involving genes in the AKT and SMAD pathways.[2] Francis et al also reported a low frequency of somatic mutations in the cell cycle checkpoint gene *CDKN1B*,[2,3] which was confirmed by others, with an incidence of 3 – 8.5%.[4,5] Studies at the RNA level in neuroendocrine tumors (NETs) have shown utility for diagnosis,[6]

RNA-Seq data presented herein were obtained at the Genomics Division of the Iowa Institute of Human Genetics, which is supported, in part, by the University of Iowa Carver College of Medicine and the Holden Comprehensive Cancer Center (National Cancer Institute of the National Institutes of Health under Award Number P30CA086862). T32: T32CA148062 (K.K.) and SPORE: P50CA174521 (J.H., J.D., P.B., G.L., T.O., A.B.).

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identification of the sites of unknown primaries,[7] and discrimination of SBNETs from pancreatic NETs in primaries and metastases.[8,9]

Transcriptome analysis also has the potential to improve our understanding of the pathways central to progression of primary neoplasms to metastases. Recognition of genes serially over or underexpressed beginning with normal tissue and primary neoplasms, followed by even greater differential expression in metastases, could contribute to this understanding. In this study, we set out to compare changes in whole transcriptome expression between normal small bowel, primary SBNETs, and synchronous SBNET liver metastases using 2 different but complimentary platforms to identify genes associated with this progression.

Methods

RNA isolation

Patients presenting to the University of Iowa with SBNETs were consented for genetic studies and entered into a tumor registry approverd by the institutional review board. Tissues collected during operative procedures performed on patients with SBNETs were placed in RNAlater solution (Thermo Fisher Scientific, Waltham, MA). Twelve patients who had histologic confirmation of SBNETs and tissue samples from normal small bowel (Nl), a primary SBNET (pSBT) and a SBNET liver metastasis (lMet) were selected for transcriptome analyses. RNA was isolated from tissues using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) with DNA digestion and resuspension in H_2O per the protocol recommended by the manufacturer. RNA quality was then assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with a requirement that samples have RNA integrity numbers (RIN) > 6.

RNA sequencing

RNA-Seq was performed at the University of Iowa Institute of Human Genetics (Iowa City, IA) using the Illumina TruSeq protocol (Illumina, Inc., San Diego, CA). Total RNA (500 ng) was fragmented, converted to cDNA, and ligated to sequencing adaptors. The molar concentrations of the indexed libraries were measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and combined equally into pools for sequencing. The concentration of each pool was determined using the Illumina Library Quantification Kit and sequenced on the Illumina HiSeq 4000 genome sequencer using a 75 bp paired-end sequencing-by-synthesis chemistry. The resulting FASTQ data were then aligned using the human hg19 genome assembly for mapping and annotation. TopHat (version 2.1.0, Center for Computational Biology at Johns Hopkins University, Baltimore, MD) was used to perform mapping, Cuffquant for quantitation, and Cuffnorm and Cuffdiff for normalization and differential expression analysis.[10] The 10th percentile of the level of expression was added to the kilobase of transcript per million mapped reads values reported by Cuffdiff to regularize the expression values in order to diminish artifacts of large- or small-fold change values as a result of a measured value for expression being close to zero. Statistically significant expression change was determined by the false discovery rate (FDR) adjusted *P* value (*Q* value).

Whole transcriptome microarrays

A total of 10 ng of total RNA was extracted and converted to cRNA utilizing the GeneChip WT Pico Reagent Kit (Affymetrix, Inc., Santa Clara, CA); then cRNA was hybridized to the GeneChip Human Transcriptome Array 2.0 (HTA; Affymetrix), and fluorescence was measured using the GeneChip Scanner 3000 (Affymetrix). Data were processed using the Affymetrix Expression and Transcriptome Analysis consoles, and comparisons were tested using analysis of variance (ANOVA) with significant differential expression defined as ANOVA *P* value and FDR *P* value < .05.

Expression data analysis

Genes with significant differential expression between pSBTs and Nls, lMets and Nls, and lMets and pSBTs by RNA-Seq were identified using a regularized, log-fold change >1 or < –1 (approximately 2-fold and –2-fold, respectively). Common genes expressed differentially in pSBT versus Nl, lMet vs. Nl, and lMet versus pSBT analyses were identified, and genes with either significant serially increased expression from normal tissue to liver mets (lMet > pSBT > Nl) or serially decreased expression (Nl > pSBT > lMet) were selected. The data from the HTA microarrays were analyzed in a similar fashion, and we complied a list of genes satisfying the criteria of significant differential expression of greater than 2-fold increase or decrease in serial expression (from Nls to pSBTs then lMets). The lists obtained from RNA-Seq and HTA expression studies were analyzed, and genes common to both lists were identified.

PCR validation

Genes were selected for qPCR (quantitative polymerase chain reaction) validation based on a combination of the magnitude of the differences in expression observed and involvement in cancer formation or progression as identified by Ingenuity Pathway Analysis (IPA; Ingenuity System Inc., Qiagen). Total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), then used as a template for qPCR reactions with Taqman primers from 10 genes meeting the criteria outlined above, as well as the control genes *POL2RA* and *HPRT1* using a 7900HT Fast Real-Time PCR System (Applied Biosystems). These validation assays were carried out using RNA from all 3 tissue sites in 40 additional patients. Assays were performed in quadruplicate, dCt calculated for each gene, and ddCt calculated for each tissue comparison. The concordance of qPCR results with HTA and RNA-Seq results was assessed by confirming statistically significant differential expression between tissue sites (pSBT versus Nl, lMet versus Nl, and lMet versus pSBT) using paired *t* tests. Gene expression levels were also assessed for the ability to discriminate between pSBTs and lMets using classification trees.

Results

RNA-Seq analysis revealed 1,270 genes in the pSBT versus Nl list that met criteria for presumed clinically relevant upregulation ($P < .05$ and regularized log-fold change >1), with 1,136/1,270 of these genes also meeting criteria in the lMet versus Nl analysis (Table I). There were 727 genes in the pSBT versus Nl groups that met criteria for clinically relevant downregulation ($P < 0.05$ and regularized log-fold change < –1), and 598/727 also were downregulated in the lMet versus Nl results. When the same selection criteria of log-fold changes were applied to the lMet versus pSBT list, there were 157 upregulated genes and 565 downregulated genes. A total of 34 of the 157 genes were serially upregulated and also were seen in the 1,136 genes common to the upregulation of pSBT versus Nl and lMet versus Nl (and thus were not highly expressed specifically in the liver or small bowel). Serial downregulation (expression 2-fold lower in lMet than pSBT, and 2-fold lower in pSBT than Nl) was seen in 143 of the 565 genes differentially expressed between pSBT and lMet, from the 598 common downregulated genes identified in the SBT and lMet versus Nl comparisons. Thus, the final numbers for further consideration were 34 serially upregulated genes (expression 2-fold greater in lMet than pSBT, and 2-fold greater in pSBT than Nl) and 143 serially downregulated genes.

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