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DNA Sequencing of Small Bowel Adenocarcinomas Identifies Targetable Recurrent Mutations in the ERBB2 Signaling Pathway

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Abstract

Purpose: Little is known about the genetic alterations characteristic of small bowel adenocarcinoma (SBA). Our purpose was to identify targetable alterations and develop experimental models of this disease.

Experimental Design: Whole-exome sequencing (WES) was completed on 17 SBA patient samples and targeted-exome sequencing (TES) on 27 samples to confirm relevant driver mutations. Two SBA models with *ERBB2* kinase activating mutations were tested for sensitivity to anti-ERBB2 agents *in vivo* and *in vitro*. Biochemical changes were measured by reverse-phase protein arrays.

Results: WES identified somatic mutations in 4 canonical pathways (WNT, ERBB2, STAT3, and chromatin remodeling), which were validated in the TES cohort. While *APC* mutations were

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present in only 23% of samples, additional WNT-related alterations were seen in 12%. *ERBB2* mutations and amplifications were present in 23% of samples. Patients with alterations in the *ERBB2* signaling cascade (64%) demonstrated worse clinical outcomes (median survival 70.3 months vs. 109 months; log-rank hazard ratio 2.4, $p=0.03$). Two *ERBB2*-mutated (V842I and Y803H) cell lines were generated from SBA patient samples. Both demonstrated high sensitivity to *ERBB2* inhibitor dacomitinib ($IC_{50}<2.5nM$). In xenografts derived from these samples, treatment with dacomitinib reduced tumor growth by 39% and 59%, respectively, while it had no effect in an SBA wild-type *ERBB2* model.

Conclusions: The *in vitro* and *in vivo* models of SBA developed here provide a valuable resource for understanding targetable mutations in this disease. Our findings support clinical efforts to target activating *ERBB2* mutations in patients with SBA that harbors these alterations.

Keywords

small intestine; duodenum; survival; targeted therapy

Introduction

The incidence of small bowel adenocarcinoma (SBA) is 10- to 15-fold lower than that of colorectal adenocarcinoma despite their anatomic proximity. The reason for this discrepancy is not known currently, and our ability to explain it is limited by our lack of both molecular characterization and model systems available for studying this relatively rare cancer (1). Although both SBA and colorectal cancer (CRC) are observed in the intestinal cancer syndromes familial adenomatous polyposis and hereditary non-polyposis colon cancer, the majority of SBAs are sporadic, and little is known about the risk factors for these cases (2).

So, little is known about SBA that, at present, its clinical management is primarily extrapolated from that for CRC. The number of clinical trials exploring novel therapies for SBA is limited. In fact, a recent clinical trial of anti-EGFR therapy in *RAS* wild-type SBA demonstrated no responses, in sharp contrast with findings from CRC, reinforcing the critical need for improved understanding of the molecular mechanisms underlying SBA(3).

The one known major genetic difference between SBA and CRC is the frequency of *APC* gene rearrangements, which are far less prominent in SBA than in CRC. Although some reports of DNA sequencing for SBA have been published in the last 5 years (4–6), the patient cohorts in these studies were heterogeneous and small, and most of the studies targeted a limited number of genes. A 2017 study that compared 317 SBA cases with 6353 CRC cases found that SBA tumors are characterized by a higher mutation rate, greater numbers of atypical *BRAF* mutations and *ERBB2* point mutations, and lower rates of *APC* and *SMAD4* mutations than CRC (7).

In the present study, our purpose was to identify targetable alterations and develop experimental models of SBA. We examined exomic mutations in samples from a cohort of patients with SBA at various clinical stages, both sporadic and familial forms; we also expanded our findings to a validation cohort in which we used targeted deep sequencing of archived paraffin SBA samples. Our results identified mutations and amplifications of

growth factor *ERBB2* in 23% of the tumors tested. We further demonstrate *in vitro* and *in vivo* that mutated *ERBB2* is a relevant target in SBA.

Methods

Patient characteristics

Our cohorts comprised patients with SBA treated at The University of Texas MD Anderson Cancer Center between 1998 and 2016. The whole-exome sequencing (WES) cohort consisted of 16 matched samples of tumor and tissues with normal germline DNA (peripheral blood in 4 and adjacent histologically normal tissue in 12) plus one unpaired tumor sample selected because of availability of frozen tissue and 50% cellularity noted on gastrointestinal pathology review (H. W.). For the targeted DNA sequencing (TES) cohort (n=27), cases with paraffin-embedded tumor tissue with a cellularity threshold 30% were selected. Clinicopathological characteristics and survival were collected from retrospective chart review.

This study was approved by the Institutional Review Board and Institutional Animal Care and Use Committee of MD Anderson and conducted in accordance with U.S. Common Rule. Prospective informed consent was obtained for SBA model generation.

Tumor xenograft and cell-line models

To establish *in vitro* and *in vivo* patient-derived xenograft (PDX) models from freshly excised SBA specimens, we implanted one 4-mm³ piece of tumor subcutaneously into one NOD SCID gamma mouse. After each transplanted tumor was established, pieces of that tumor were in turn transplanted into nude mice and allowed to grow for 3 weeks in preparation for anti-*ERBB2* drug response testing. In parallel, pieces of each transplanted tumor were also cultured *in vitro* for development of cell lines; the cells were mechanically dissociated, subjected to centrifugation in a phosphate-buffered saline solution (PBS) containing antibiotics, and incubated at 37°C in Advanced MEM culture medium supplemented with collagenase IV (1 mg/mL) for 30 min. After the collagenase was removed, cells were fed with 1% Advanced MEM containing 1% fetal calf serum (FCS) and plated into collagen-coated 6-well plates for cell growth.

For *in vivo* testing of sensitivity to *ERBB2* inhibition, mice implanted with tumor as described in the previous paragraph were randomly assigned to receive chow containing dacomitinib (SelleckChem, Boston, MA) or control (5 mice/group). The concentration of dacomitinib in the chow was calculated according to the following formula: $DD = (SD \times BW) FI$, where DD is diet dose, SD is single daily dose, FI is daily food intake (3.5 grams), and BW estimated mean body weight (24g). Chow containing dacomitinib was prepared by Research Diets, Inc. (New Brunswick, NJ) to achieve an approximate daily drug dose of 10mg/kg/day. The largest diameter (D) and the smallest diameter (d) of each mouse's tumor were measured every week, and the volume of each tumor was calculated according to the following formula: $Vol = d^2 \times D / 2$.

For testing sensitivity of the newly established SBA cell lines to anti-*ERBB2* agents, the cells were seeded (5.0×10^3 cells per well) in flat-bottomed 96-well microplates coated with

rat collagen I (Corning, Radnor, PA). After 48 hours, the cells were treated with various concentrations of lapatinib (SelleckChem) or dacomitinib. Cell proliferation was estimated by using the MTS colorimetric assay (Promega Corp., Madison, WI). The significance of differences between treatment groups was defined using a standard *t*-test on triplicate values.

Sequencing analyses

WES was performed by the sequencing core facility at MD Anderson under contract by Illumina (San Diego, CA) to meet the typical depth of at least 50×, with 94% of the known genome being sequenced to at least 8× coverage while achieving a Phred base calling quality score of at least 30 over at least 80% of mapping bases. Quality control metrics were computed on a per lane basis using FastQC (<http://www.bioinformatics.mdacc/projects/fastqc>). Exome capture was performed using the manufacturer's protocol (Illumina Multiplexing_SamplePrep_Guide_1005361_D) as described on the MD Anderson–Illumina core website. Sequence reads were aligned to the hg19 human genome build. Aligned reads were then sorted into genome coordinate order and duplicate reads marked using <http://broadinstitute.github.io/picard>. Somatic single nucleotide variants (SNVs) and INDELs were detected using Indelocator. To increase accuracy, additional filters were applied to high-confidence calls. Quality control modules within the Firehose pipeline (<http://www.broadinstitute.org/cancer/cga/Firehose>) were applied to all sequencing data for assessment of genotype concordance between tumor and normal paired samples. Contamination of samples was estimated using ContEst (8). All samples were required to have <0.5% contamination. MuTect 2.0 was applied to identify somatic SNVs for 16 of the samples. For the one tumor sample without a paired normal (I-755T), the GATK HaplotypeCaller (GATK version 3.7) was applied to identify the mutations. Identified variants were annotated by using Oncotator (version 1.2.7.0) (9). To improve the specificity of mutation calls, only mutations with an allelic fraction >5% were considered. The remaining variants were removed if they were present at greater than 0.5% in the 1000 Genomes (phase 1, version 3) or ExAC (version 0.3) (10) databases and not present in COSMIC (version 74). Oncoprint and mutation mapping were done using c-bio portal tools (11,12).

RNA sequencing was performed by the sequencing core facility at MD Anderson using the Applied Biosystems (Foster City, CA) SOLiD system. Total RNA for whole-transcriptome sequencing was prepared according to vendor's protocols and individual prepared "barcode" libraries were quantified and pooled equally together for multiplexing. The sequencing runs were performed on SOLiD v 3.0. To identify SBA-related differentially expressed genes, the "reads per kilobase of exon per million mapped sequence reads" (RPKM) values of the human RefSeq genes were calculated using the RNA-seq flow in the Partek® Genomics Suite™ (version 6.5 beta, Partek Inc., St. Louis, MO) and then log transformed. Single-factor analysis of variance (ANOVA) was used to detect differentially expressed genes among 18,890 protein-coding genes: $P < 9.5 \times 10^{-4}$ (false discovery rate [*FDR*] < 0.05) was used as the cut-off in the two-group comparison tumor versus adjacent normal tissue.

TES was performed by Genewiz, Inc. (South Plainfield, NJ) from formalin-fixed, paraffin embedded (FFPE) samples. Targeted whole-exome capture libraries were constructed from

tumor DNA after sample shearing, end repair, phosphorylation, and ligation to barcoded sequencing adaptors. DNA was then subjected to hybrid capture using Agilent SureSelect Gene Enrichment workflow. The minimum coverage was set as 20×, although most of the samples had 300×–400× coverage and a variant probability of 90%; required variant count was 2. The resulting single nucleotide polymorphisms (SNPs)/INDELs were further filtered to limit the potential of false positives (min. base Q=30; min. forward/reverse balance=0.05; min. frequency=1.0%). The marginally filtered SNPs/INDELs generated above were annotated using the dbSNP138.txt (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/>) and common_no_known_medical_impact_20140430.vcf from the NCBI. A list of genes included in this panel is shown in Table S1.

Mutation rates and nucleotide transition signatures

The mutation rate for each sample was calculated by dividing the number of final mutation calls after filtering by the total genomic territory sequenced (in megabases). To identify mutation signatures that match known signature patterns found in other types of solid tumors from The Cancer Genome Atlas (TCGA), we applied the R package DeconstructSigs to estimate underlying source mutation signatures for our SBA tri-nucleotide mutation profiles (13). We obtained a measure of tumor heterogeneity for each tumor sample based on a technique that clusters mutation allele frequencies (14). We then estimated tumor cellularity and ploidy for each sample by applying Sequenza, which jointly analyzes mutation allele frequencies and copy number profiles (15).

Immunohistochemical (IHC) staining and ERBB2 copy number aberrations

Immunohistochemical testing for ERBB2 (14 of 27 TES samples) and for microsatellite instability-high by MLH1, MSH2, MSH6 and PMS2 (42 of 44 TES and WES samples) was performed on FFPE tissue sections as previously reported (16). To detect *HER2* overexpression, *HER2* fluorescent in situ hybridization (FISH) was performed using the HER2IQ FISH PharmDx kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Appropriate positive and negative control tissues were assayed in parallel. The carcinoma component of the specimen was scored based on evaluation of hematoxylin and eosin (H&E)-stained slides. *HER2* and *CEN-17* signals were counted for a total of 20 cells using the manufacturer's recommended interpretation of scoring. Specimens with a *HER2/CEN-17* ratio >2.2 were considered *HER2* gene-amplified (17). *ERBB2* absolute copy-number alterations for each of the 17 WES samples was estimated using the BAM multiscale reference and NGS-quadratic correction (Nexus 9.0_biodiscovery, 2017). Segments were analyzed using SNP-FAST2 Segmentation, with high gain defined as higher than 0.6 and high loss as at least -1.2.

For PDX IHC analysis slides were stained for Ki67 (Cell Signaling, #9027, 1:50, 30min) and for phosphorylated-ERK1/2 (Cell Signaling, pERK clone D13.14.4E; 1:150) as previously described (18).

Immunofluorescence, confocal analysis, and in vitro kinase assay

For immunofluorescence analysis, cells were seeded into 8-chamber collagen-coated chamber slides (Thermo Fisher Scientific, Rochester, NY). The cells were fixed with 3.7%

formaldehyde (Tsoumis, Rockville, MD), extracted with chilled ethanol/methanol (1:1 volume) and blocked with 10% normal goat serum. Primary antibodies against E-cadherin and auto-phosphorylated EGFR (Cell Signaling Technology, Danvers, MA) were added, and the cells were incubated overnight and Z-sectioning was used for 3D virtual reconstruction, as described elsewhere (19).

Endogenous ERBB2 was pulled down using an anti-ERBB2 antibody (cat. 2165S, Cell Signaling Technology) from CRC cell line KM12L4 (wild-type; Korean Cell Line Bank), and SBA cell lines SBA-6 (V841I) and SBA-16 (Y803H) and incubated with its substrate (Glu, Tyr; cat. CS0730; Sigma-Aldrich, St Louis, MO) at a ratio of 4:1 per manufacturer's instructions. Substrate phosphorylation was detected by dot blot assay using anti-phosphotyrosine PY69 (Cell Signaling Technology). Recombinant EGFR pretreated with EGF was used as positive control. IgG pull-down was used as negative control.

RPPA analysis of ERBB2-targeted therapy

To identify changes in ERBB2-mutant and wild-type tumors in response to ERBB2 inhibition, we used reverse-protein phase array (RPPA) analysis. Cellular proteins derived from the tumors were arrayed on nitrocellulose coated slides (Grace BioLabs, Bend, OR) by an Aushon 2470 Arrayer (Aushon BioSystems, Billerica, MA). Each slide was probed with a validated primary antibody with a Pearson correlation coefficient between RPPA and western blotting of greater than 0.7. Each dilution curve was fitted with a logistic model ("Supercurve Fitting" developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, <http://bioinformatics.mdanderson.org/OOMPA>).

Statistical analyses

Gehan-Breslow-Wilcoxon test and Fisher exact test were used to test associations between mutations and continuous and nominal variables. Pearson's correlation was used to assess the linear relationship between continuous variables. Log-rank test and Cox proportional hazards models were applied for the time-to-event outcome analysis. A P-value 0.05 was considered statistically significant. Factors in multivariate modeling included age, microsatellite instability status, small bowel location, TNM stage, histological grade, perioperative chemotherapy use, and ERBB2 signaling cascade (ESC) mutation status.

Results

Mutational landscape by whole-exome sequencing

In the 16 paired WES samples, the overall mutation rate was 44.1 mutations/Mb in microsatellite instability high (MSI-high) samples and 12.3 mutations/Mb in microsatellite stable (MSS) samples. From these mutations, we identified 3 underlying mutational processes (Figure S1) (13). The most frequent mutation signature was Signature 1, a pattern associated with spontaneous deamination of 5-methylcytosine (5-mC) that accounts for a significant percentage of the critical somatic driver mutations observed in most cancers, including CRCs. For example, 60% of the *APC* nonsense mutations in SBA derived from 5-mC>T deamination. The second most common signature observed was a defective DNA mismatch repair signature (Signature 6), which is most frequently found in colorectal and

uterine cancers and is highly prevalent in MSI-high tumors. As anticipated, the contribution of this signature to the mutation profiles was associated with MSI status, where percent contributions for MSI and MSS SBA tumors were 33.8% and 7.7%, respectively ($p=0.001$; Wilcoxon rank-sum test). The third most frequent signature was Signature 5, whose etiology is currently of unknown but is represented in all cancer types. Two cases (I.135 and I.1755) demonstrated high mutational rates but were MSS by intact expression of mismatch repair proteins and did not demonstrate evidence of the defective DNA mismatch repair mutational signature 6. The etiology of the high mutational rate in these two cases is not known, as neither case demonstrated POLE/POLD mutations, which has been associated with high mutational rates in CRC.

A frequency-based analysis of the somatic mutations in the WES cohort identified several tumor drivers with relatively higher recurrent mutation rates, such as *APC* (36%), *NFI* (36%), *ERBB2* (36%), *KRAS* (32%), or *KMT2D* (23%) (Figure 1). Pathway enrichment analysis using the Ingenuity platform (IPA; Qiagen Bioinformatics, Redwood City, CA) of the top 200 exomic mutations revealed several signaling pathways that were frequently mutated in the WES cohort, including WNT, ERBB2, STAT3, and chromatin remodeling (Table S2).

Mutation validation by TES

To further validate the frequently mutated genes found in the WES cohort, we used TES on an additional 27 samples. The targeted gene panel included over 250 genes that have been described to be associated with CRC or other solid tumors as reported in the COSMIC database (Table S1). The most frequently mutated genes in the TES cohort included genes that code chromatin modifiers/epigenetic signaling, STAT3 and WNT signaling, and the ERBB2 signaling pathway (Figure 2A).

Pathways of interest across TES and WES cohorts

Twenty-three percent of the samples harbored *APC* mutations, 70% of which were truncating mutations (Figure 2A). An additional 12% of samples displayed genetic alterations in other WNT-signaling inhibitors, including *RNF43* and *ZNRF3* (Figure 2B), although the latter was not tested in the TES cohort. In total, 27 (61%) of the 44 samples had an alteration in a WNT signaling-related gene.

We also assessed WNT activation by assessing the differential expression of known WNT-related genes between normal and tumor samples using RNAseq analysis of 15 WES samples (11 tumor and 4 normal). Several known secreted WNT inhibitors, including *WIF1* and *SFRP1*, were significantly downregulated in >90% of the tumor samples (Figure S2A). Furthermore, a consistent upregulation of WNT-regulated stem cell markers, such as *LGR5*, *HOPX*, and *BMII*, was evident across all tumor samples. Finally, CTNNB1 direct targets, such as *AXIN2* and *WISPI*, were consistently upregulated in tumor samples, including those devoid of WNT pathway gene mutations (Figure S2A). Altogether, these results suggest that WNT activation is widely represented across SBA.

Another striking difference from CRC in the SBA samples was the relatively large number of samples with truncation mutations upstream of the SET domains of two histone-lysine *N*-

methyltransferases, *KMT2C* and *KMT2D* (Table S3). Twenty-nine percent of samples displayed nonsense mutations in either *KMT2D* or *KMT2C*, and 2 samples displayed nonsense mutations for both methyltransferases (Figure 2A). Suppressor of zeste 12 homolog (*SUZ12*) is a zinc finger gene and a polycomb family member which also cooperates with *KMT2C* and *KMT2D* in the histone methylation process. *SUZ12* was mutated in one third of the WES samples (Figure 2A), underscoring the important interplay between various epigenetic regulatory mechanisms in SBA.

Of the greatest possible clinical relevance was our finding of *ERBB2* alterations in 23% (10/44) of samples, with somatic point mutations in the *ERBB2* gene in 7 samples and *ERBB2* amplification in 3 samples. Of the 7 *ERBB2* mutations, 4 were in the kinase domain (V777L, V842I, D769Y, Y803H), one in the extracellular domain (S310Y), and 2 samples were non-activating mutations (R678Q) (Figure 2B).

As previously mentioned, IPA of the top 200 WES mutations demonstrated that the STAT3 canonical signaling pathway was significantly altered in our SBA cohorts (Table S2). STAT3 can serve as a network hub, where various receptor tyrosine kinase activators, including *ERBB2*, *EGFR*, *TGFBR2*, or *IGFR2*, can converge and induce its activation. In addition, 14% of samples demonstrated alterations in the tumor suppressor tyrosine phosphatase receptor type T (*PTPRT*), which has been shown to directly regulate STAT3 (20,21). Further confirmation of *ERBB2* and STAT3 signaling activity was provided by a transcriptome analysis of the 11 tumor samples from the WES cohort. IPA confirmed that STAT3 and *ERBB2* were significantly deregulated (Table S4).

Clinical relevance of ERBB family cascade mutations in SBA

For the entire cohort of 44 SBA cases, the median follow-up interval was 84 months, and 53% were alive at the time of last follow-up. We evaluated the clinical impact of activating mutations in 8 members of the ESC (Figure S2B), using a grouping methodology similar to that described previously (22), and generated an ESC-mutated group (n=27) and an ESC-non-mutated group (n=17) (Figure S2C). In univariate analysis, the ESC-mutated group demonstrated a poorer survival duration than the ESC-non-mutated group (Figure 2C), with a median survival of 70.4 months compared to 109 months (log-rank test $p=0.03$ and log rank hazard ratio [HR]=2.4). In multivariate modeling, ESC mutation was the only significant variable (HR 3.6, 95% confidence interval 1.2 to 10.7, $p=0.02$).

SBA cell lines and in vitro targeting of ERBB2 mutations

To further validate *ERBB2* as a target, we developed two SBA patient-derived cell lines: SBA-6 from human sample I-797, which harbors the *ERBB2* mutation V842I, and SBA-16 from human sample I-577, which harbors the mutation Y803H (Figure 2B). These lines, when injected into nude mice, developed tumors with typical adenocarcinoma features. In addition, both cell lines formed spheres when grown in low-attachment plates (Figure 3A and B, middle panels) or formed 3-dimensional (3D) structures when attached on collagen- or vitronectin-coated plates (Figure 3A and B, right panels). A typical 3D-reconstructed structure is shown in Figure 3C, where red represents EGFR-Y1068, green represents E-cadherin, and blue is nuclear stain Topro-3. Altogether, these results suggest that tumor-

derived small intestine cell lines are highly differentiated and maintain an organized cellular architecture.

The Y803H mutation found in sample SBA-16 has not been previously reported in the COSMIC database, whereas mutation V842I has previously been reported to increase the kinase activity of ERBB2 and improve response to lapatinib (23). An *in vitro* kinase assay using immunoprecipitated endogenous ERBB2 confirmed that both mutations are kinase activating (Figure 4A). Exposure to an ERBB2 inhibitor caused significant decreases in cell proliferation, with IC₅₀ at 25nM for lapatinib (SBA-6 and SBA-16) and at 1nM (SBA-6) or 2.5nM (SBA-16) for dacomitinib (Figure 4B). A reduction in downstream ERBB2 signaling was seen after exposure to lapatinib (100nM) for 72 hours (Figure 4C). In particular, phospho-AKT, phospho-ERK1, and phospho-MEK1 demonstrated reductions, as did total STAT3.

ERBB2 targeting in SBA PDX models

To determine whether the 2 ERBB2 kinase activating mutations are responsive to ERBB2 inhibitors *in vivo*, we implanted nude mice with one of 3 different SBA tumors, one ERBB2 wild-type and 2 ERBB2 mutant. Because both SBA-6 and SBA-16 cell lines displayed slightly higher *in vitro* sensitivity to dacomitinib than to lapatinib, we decided to treat the mice with dacomitinib.

All 3 of the PDX models, the wild-type *ERBB2* mouse model PDX20 (derived from human sample T20) and the 2 *ERBB2*-mutant models PDX6 (derived from SBA-6 cells) and PDX16 (derived from SBA-16), displayed a *KRAS* or *NRAS* mutation in either codon 12 or 13. Dacomitinib significantly reduced tumor growth in both ERBB2 mutant models, whereas it had no effect in the wild-type model (Figure 5A). Microscopic analysis of H&E-stained sections representative of control and treated tumors in each model showed similar adenocarcinoma features with a somewhat richer mucinous component in the wild-type PDX20 model than in the mutant PDX6 and PDX16 models (Figure 5B, left panels and Figure S3). The uniform glandular morphology was drastically disrupted in PDX6 and PDX16 by dacomitinib, with evident areas of necrosis, whereas no significant changes were evident in the PDX20 tumors (Figure 5B and Figure S3). Both Ki-67 and phospho-ERK stains were reduced in ERBB2 mutant models ($p=0.033$ and $p=0.048$, respectively) with dacomitinib treatment in contrast to control, whereas no difference was seen in the ERBB2 wild-type model (PDX20), (Figure 5C and D). Unsupervised clustering of significantly differentially expressed proteins ($p<0.05$) from RPPA analysis of tumors after 3 weeks of treatment revealed 2 types of differences among the mouse models used. Differences in protein expression between ERBB2-mutant and wild-type models may reflect differences in pro-survival mechanisms and autophagy-related responses, as suggested by differences in HIAP/BIRC3 and ATG7 expression (Figure 5E, yellow vertical bar). Differences related to dacomitinib response in ERBB2-mutant and wild-type models included reductions in the mutant models of expression of proteins directly related to tumor-specific glycolysis: FASN, MCT4, PKM2, LDHA, and SDHA (Figure 5E, green vertical bar).

Discussion

This large-scale analysis demonstrates alterations in previously reported genes of interest in SBA, such as *APC*, *KRAS*, and *ERBB2*, but also genes less recognized as implicated in SBA biology, such as the tumor suppressor *PTPR*, the epigenetic regulators of *H3K27me3* (*SUZ12*, *KMT2C*, and *KMT2D*), and the WNT activators *RNF43* and *ZNRF3*. This work demonstrated in novel SBA models that harbor activating mutations in *ERBB2*, furthermore, that small-molecule inhibitors of ERBB2 have anticancer activity both *in vitro* and *in vivo*. Given the rarity of SBA, in which only limited clinical trials have been conducted, laboratory efforts are critical to identifying and informing novel clinical approaches for patients with this cancer.

An important finding from this study is that 23% of samples harbor either *ERBB2* missense activating mutations or gene amplifications. Our results accord with previous reports identifying ERBB2 as a targetable molecule in SBA (5–7). We found not only that small-molecule ERBB2 inhibitors were effective against tumor growth both *in vitro* and *in vivo*, but also that downstream changes resulting from ERBB2 inhibition included inhibition of glycolysis and lactic acid transport, suggesting a metabolic phenotype in ERBB2-mutant tumors (24). In addition, we found that SBA with genomic alterations in the ERBB2 signaling cascade demonstrated worse survival, though given the limited sample size, further replication of this finding is needed.

As expected, *APC* mutations were found in less than 30% of the SBA samples tested, confirming the known difference between SBA and sporadic large intestine tumors, in which *APC* mutations represent the dominant somatic mutation (>70%). Furthermore, we identified loss-of-function alterations in the upstream WNT repressors *RNF43* and *ZNRF3* in samples without *APC* truncations. In total, 27 (61%) of the 44 samples had an alteration in a WNT signaling-related gene. Transcriptome analysis revealed that a WNT-driven phenotype is not the exception in SBA but is probably the norm since all tumors displayed downregulation of secreted WNT suppressors such as *WIF1* and *SFRP1* and upregulation of *LGR5* and *LGR6*, which mark actively dividing stem cell populations (25), or of *HOPX* and *BIMI*, which mark WNT-driven dormant stem cell populations. We found that WNT ligand activation by *ZNRF3* and *RNF43* loss of function could be responsible for WNT activation in at least 12% (5/42) of SBAs. This pathway of WNT activation is vulnerable to Porcupine, an enzyme catalyzing the acylation of WNT proteins (25). Trials investigating the anticancer effects of Porcupine inhibition are currently ongoing, though primarily focused upon CRC (NCT01351103). Prior work in SBA have noted abnormal nuclear accumulation of β -catenin as a marker of WNT activation in 20–41% of cases, and in one study correlated with worse overall survival.(26,27) In CRC, despite near universal alterations in the APC gene, abnormal β -catenin expression is observed in a limited number of cases, often heterogenous within a tumor, and of unclear prognostic significance.(28,29) Thus, as in CRC, additional factors in addition to single alterations in the WNT pathway genes are likely involved with downstream WNT-related protein expression.

One of the most intriguing findings of our study is that 70% of the SBA samples (WES and TES combined) have somatic mutation in *KMT2C* and/or *KMT2D* methyltransferases,

while only approximately 10% of CRC samples have such a mutation (30–32). Methylation of histone H3 lysine 4 is one method that cells use to mark promoters, enhancers, and super-enhancers for further recruitment of transcription co-activators, such as p300 (33). Several studies have underscored the roles of *KMT2C* and *KMT2D* as important suppressors of various cancers (34,35). Nonsense mutations constitute 37% and 60%, respectively, of the total *KMT2D* and *KMT2C* mutations in many malignancies, including those of the esophagus and prostate (36,37) (38,39). Importantly, recent studies comparing intensive mapping of enhancer modulation by *KMT2C* and *KMT2D* between normal crypts and tumor areas in CRC revealed differential enrichment that translates into modified expression of genes of various signaling pathways, including the WNT pathway (40). Moreover, *KMT2D* deficiency alone can induce tumor formation by transcriptional stress, which triggers abnormalities in early replicating fragile sites within the chromosome (41,42). Recently the N-terminal domain of *KMT2C* was demonstrated to interact with the histone H2A deubiquitinase and tumor suppressor BAP1 and may reflect the mechanism seen in SBA as, the majority of *KMT2C* mutations, 72%, occurred in the N-terminal domain.(43)

A final finding of potential clinical relevance was the consistent finding of STAT3 pathway activation in SBA. A known suppressor of STAT3 activation, *PTPRT*, was mutated in 14% of SBA samples. These mutations were all identified within the catalytic domains of *PTPRT* and occurred in samples without upstream alterations in receptor tyrosine kinases such as *ERBB*, *EGFR*, or *TGFBR2*.

Although this study represents one of the largest known molecular analyses of SBA tumor samples, our analyses were limited by sample size, and validation in additional datasets is needed. In particular, as frozen tumor samples were collected from a referral hospital, there is likely a selection bias for more indolent cases, as the higher than expected MSI-high rate may reflect. However, the use of *in vivo* and *in vitro* models for the identified *ERBB2* mutations lends strong support to the importance of *ERBB2* alterations in this tumor type. Given the overall low frequency of *ERBB2* alterations in this rare cancer, however, clinical trials investigating *ERBB2* targeting are unlikely to occur. Thus, the development of novel model systems, as reported here, represents a critical step forward in our efforts to both understand this cancer and guide development of potential novel therapies for patients with metastatic SBA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

Small bowel adenocarcinoma (SBA) is a rare cancer, with limited data to guide therapeutic decisions. Both the absence of representative cell lines and patient-derived xenograft (PDX) models and the limited knowledge of the molecular alterations within this cancer have hindered design of tumor-specific therapeutic strategies. Currently, the approach to SBA is predicated on extrapolation from colorectal cancer. Here, through exome and targeted next-generation sequencing of over 40 SBA patient samples, and developing relevant cell lines and PDX models, we identified targetable genomic alterations in SBA. Alteration in *ERBB2* was among the most frequent therapeutic targets identified. *ERBB2* targeting resulted in significant growth inhibition in cell lines and tumor xenografts. Moreover, we found that activation of the *ERBB2* signaling cascade predicts a poor outcome in SBA. These findings provide strong support for clinical efforts to identify and target *ERBB2* genomic alterations in patients with SBA.

Figure 1. Whole exome-based somatic mutational landscape in SBA samples.

Color-coded somatic aberrations are shown for by type (missense, nonsense, indels) or location (coding or non-coding) for each whole-exome sequencing (WES) sample (n=17), together with relevant clinicopathologic parameters. Only genes with somatic mutation in at least 3 samples are shown.

Figure 2. Gene set mutations in SBA.

(A) Oncoprint representation of frequently mutated genes in SBA across whole-exome sequencing (WES) and targeted-exome sequencing (TES) cohorts. (B) Non-synonymous mutation mapping in *ERBB2*, *RNF43*, and *ZNRF3* found in WES and TES cohorts. (C) Kaplan-Meier plot representing overall survival (months) in patients with mutations in 8 members of the ERBB2 signaling cascade (ESC) and patients without such mutations (ESC wild-type; hazard ratio 2.4, $p=0.03$).

Figure 3. Morphological similarities of tumors in situ and in tumor-derived cell lines in vitro. (A) Representative images of sample I-797, showing *in situ* tumor staining with H&E (left), tumor-derived cells cultured in low-attachment culture dishes (suspension; middle), and tumor-derived cells cultured in extracellular matrix (ECM)-coated tissue culture dishes (attached; right). (B) Representative images of sample I-577 under the same conditions as in (A). Note the pseudocrypt formation by both cell lines. (C) A 3D-reconstructed pseudocrypt derived from SBA-6 shown from base to top and stained with anti-EGFR-Y1068 (red), anti-E-cadherin (green), and nuclear stain (blue). Note distribution of active EGFR within the pseudocrypt structure.

Figure 4. *In vitro* sensitivity of ERBB2 kinase domain mutant cell lines SBA-6 and SBA-16 to ERBB2 inhibition.

(A) *In vitro* kinase assay using endogenous pulled-down ERBB2 from kinase mutant SBA cell lines (SBA-6 [V842I] and SBA-16 [Y803H]) or kinase wild-type (WT) KM12L4 CRC cells. (B) Log-dose vs response in SBA tumor-derived cell lines SBA-6 (*left*) and SBA-16 (*right*) treated with ERBB2 inhibitor lapatinib (Lapa) or dacomitinib (Daco). (C) Following treatment with Lapa at 100nM for 72hours cells lines were analyzed and fold change of normalized medians for each protein derived from reverse-protein phase array (RPPA) for downstream ERBB2 signaling proteins are shown: phospho-AKT_pT308, phospho-ERK1-pT202_Y204, phospho-MEK1-pS217_S221, and total STAT3.

Figure 5. *In vivo* sensitivity of ERBB2 kinase domain mutant tumors and wild-type control tumors to ERBB2 inhibition.

(A) Sensitivity to daconitinib (Daco) or control (Con) of ERBB2-mutant SBA PDX models PDX6 (derived from SBA-6 cells) and PDX16 (derived from SBA-16 cells) and ERBB2 wild-type (WT) SBA PDX model PDX20. (B) Hematoxylin and eosin-stained representative examples of Daco-treated and control-treated ERBB2-mutant tumors. (C) Ki-67 and phospho-ERK from Daco-treated and control-treated PDX models at 3 weeks. (D) Representative examples of phospho-ERK and Ki-67 stained Daco-treated and control-treated ERBB2-mutant PDX16 tumors. (E) Heatmap representation of normalized median RPPA values for tumors from each of these models at 3 weeks showing cluster classification

of significantly ($p < 0.05$) differentially expressed proteins between WT and ERBB2 mutants, *yellow bar*, or control vs dacomitinib treatment, *green bar*.

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