High Resolution Integrative Analysis Reveals Widespread Genetic and Epigenetic Changes After Chronic *In-Vitro* Acid and Bile Exposure in Barrett's Epithelium Cells

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Barrett's epithelium (BE) is a premalignant condition resulting from chronic gastroesophageal reflux that may progress to esophageal adenocarcinoma (EAC). Early intervention holds promise in preventing BE progression. However, identification of high-risk BE patients remains challenging due to inadequate biomarkers for early diagnosis. We investigated the effect of prolonged chronic acid and bile exposure on transcriptome, methylome, and mutatome of cells in an *in-vitro* BE carcinogenesis (BEC) model. Twenty weeks acid and bile exposed cells from the BEC model (BEC20w) were compared with their naïve predecessors HiSeq Illumina based RNA sequencing was performed on RNA from both the cells for gene expression and mutational analysis. HELP Tagging Assay was performed for DNA methylation analysis. Ingenuity pathway, Gene Ontology, and KEGG PATHWAY analyses were then performed on datasets. Widespread aberrant genetic and epigenetic changes were observed in the BEC20w cells. Combinatorial analyses revealed 433 from a total of 863 downregulated genes had accompanying hypermethylation of promoters. Simultaneously, 690 genes from a total of 1,492 were upregulated with accompanying promoter hypomethylation. In addition, 763 mutations were identified on 637 genes. Ingenuity pathway analysis, Gene Ontology, and KEGG PATHWAY analyses associated the genetic and epigenetic changes in BEC20w cells with cellular and biological functions. Integration of high resolution comparative analyses of naïve BAR-T and BEC20w cells revealed striking genetic and epigenetic changes induced by chronic acid and bile exposure that may disrupt normal cellular functions and promote carcinogenesis. This novel study reveals several potential targets for future biomarkers and therapeutic development.

INTRODUCTION

Barrett's epithelium (BE) is a premalignant condition predisposing to esophageal adenocarcinoma (EAC). BE is a columnar epithelium that replaces the normal squamous tissue at the junction of distal esophagus and stomach in response to chronic gastroesophageal reflux (Lagergren et al., 1999; Nehra et al., 1999; Wild and Hardie, 2003). EAC is the fastest growing malignancy amongst all cancers (almost 6-fold over the past few decades) in the United States and Western Europe (Devesa et al., 1998). EAC occurrence is 30-to 125-fold higher in gastro-esophageal reflux disease (GERD) patients complicated with BE (Haggitt, 1994). Early detection is vital in the management of BE progression to adenocarcinoma. Thus it is critical to identify molecular changes that occur during neoplastic transformation so that novel prognostic and therapeutic strategies can be developed for the management of this disease.

Carcinogenesis is accompanied by accumulation of (Hanahan and Weinberg, 2011) genetic changes that provide selective advantage to the cells and enable their transformation (Nowell, 1976; Michor et al., 2004; Merlo et al., 2006). Epigenetic alterations are common during neoplastic transformation and may precede major genetic changes that lead to cancer (Eden et al., 2003; Gaudet et al., 2003; Feinberg and Tycko, 2004). Global hypomethylation (Feinberg and Vogelstein, 1983) and regional hypermethylation (Jones and Laird, 1999) were

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Received 13 February 2013; Revised 31 July 2013; Accepted 1 August 2013
DOI 10.1002/gcc.22106
Published online 3 October 2013 in Wiley Online Library (wileyonlinelibrary.com).

reported in the pioneering epigenetic studies in cancer and can contribute to genomic instability (Chen et al., 1998) and altered gene expression (Baylin et al., 1991; Bird, 1992). Progression from BE to EAC is a multistage carcinogenesis process (McManus et al., 2004; Paulson and Reid, 2004) pathologically observed as metaplasia → dysplasia → carcinoma. Barrett's esophagus therefore has been a uniquely suited model to investigate *in vivo* clonal expansion and genetic instability prospectively as predictors of progression to cancer in humans (Neshat et al., 1994; Rabinovitch et al., 2001; Reid et al., 2001; Wong et al., 2001). The development of genome-wide genetic and epigenetic assays can allow us to study stepwise molecular alterations that occur during these various stages before the development of frank adenocarcinoma.(Xu et al., 2002; Selaru et al., 2002; Wang et al., 2006; Razvi et al., 2007).

In-vitro models of BE are helpful since only 0.5–1% of BE patients may progress to EAC per year (Haggitt, 1994; Jankowski et al., 2002; Shaheen and Ransohoff, 2002) therefore large cohort must be followed for many years before progression to EAC can be observed. Also, it may be difficult to observe in BE patients complicated with GERD the repeated injury and repopulation cycles possibly leading to selection of mutant clones with survival advantages (Dvorakova et al., 2005). Mostly because, such injury may be modified in BE patients who are adequately treated with acid-suppressive proton pump inhibitors (PPIs) (Triadafilopoulos, 2000). Therefore, longitudinal sampling during disease progression and inability to distinguish between progressive and non-progressive BE are considerable limitations in clinical studies.

We have developed an *in vitro* model of BE carcinogenesis (BEC) (Das et al., 2011) from exposing-hTERT immortalized benign Barrett's epithelial cells BAR-T (Jaiswal et al., 2007) to 5 min pulses of acidified bile (200 μM glycochenodeoxycholic acid at pH4, B4) daily over a period of 65 weeks. This model highlights primary events of progression of BE such as increased columnar/colonic phenotype (Bajpai et al., 2008) and neoplastic properties like changes in morphology, clumping loss of contact inhibition, and loss of adherence dependence (Das et al., 2011). We hypothesize that elucidation of the basic mechanisms underlying early stages of carcinogenesis in this model may lead to the identification of potentially useful predictors of carcinogenesis progression or candidate clinical biomarkers and therapeutic targets.

In this study, we used a comprehensive approach integrating large-scale genomic, and epigenomic datasets to identify molecular alterations (Alvarez et al., 2011) in the BEC model acquired during the 20 weeks of acid and bile exposure. We observed widespread novel changes in transcriptome, methylome, and mutatome upon comparing the naïve BAR-T cells with those exposed to 20 weeks of acid and bile (BEC20w). Finally, integrated bioinformatics analyses revealed a core set of genes that were dysregulated by a combination of epigenetic and genetic changes, and the pathways modulated by these genes that can be studied in the future.

MATERIALS AND METHODS

Cell Line, Medium, and Cell Culture

BAR-T cells and BEC20w cells were grown in special supplemented keratinocyte medium (KBM2) from Cambrex Bioscience (East Rutherford, NJ), as described elsewhere (Jaiswal et al., 2007). Six independent replicates of the BEC20w cells were developed after exposing six different flasks of naïve BAR-T cells to B4 for 5 min every day for up to 20 weeks per our protocol for the BEC model (Das et al., 2011). Hydrochloric acid (A) was used to adjust the culture medium to pH4. The bile acid, glycochenodeoxycholic acid, GCDA (Sigma, St. Louis, MI) was diluted to optimum working concentration of 200 μM (B) with the culture medium already adjusted to pH4 (B4). The cells were rinsed with phosphate buffered saline (PBS) before and after incubation with B4. The control untreated cells were grown in parallel in the KBM2 medium mentioned above.

Massively Parallel RNA Sequencing (RNAseq), for Identification of Mutations, and Changes in Gene Expression

The transcriptome of untreated/naïve BAR-T cells and BEC20w cells was examined by RNA sequencing (RNA-seq) for differentially expressed mRNA and SNPs/mutations. RNA was isolated by phenol chloroform extraction and checked for quality by bioanalyzer before and after depletion of ribosomal RNA using RiboMinus kit (Invitrogen, Grand Island, NY). Purified mRNA was phosphatase-treated, followed by polynucleotide kinase-treatment to add a 5′ phosphate to the RNA molecule. The 3′ Illumina adapter with a 5′ preadenylated nucleotide was ligated to the 3′ end of the RNA molecule and a 5′ adapter (containing a

5nt bar code for indexing) was ligated to the 5′ end using T4 RNA ligase. Reverse transcription was then performed to create a RNA-cDNA hybrid molecule. This product was used as the template for 15 cycles of PCR with Phusion polymerase to generate the library for Illumina sequencing. Single-end 100-bp sequencing was done on Illumina HiSeq 2000. Initial data processing was performed using Illumina Sequence Control Software (SCS) and Pipeline 1.n software packages. These software components perform image analysis (Firecrest), base-calling (Bustard) and alignment of sequence tags to the appropriate reference genome (using Efficient Large-Scale Alignment of Nucleotide Databases, ELAND). For transcriptional profiling, to determine the relative counts of sequences from each gene relative to each other, we obtained counts normalized by the total number of reads to allow inter-sample comparisons.

Muti-sample variant analysis was performed on genome-wide HISEQ data obtained from paired untreated BAR-T and BEC20w cells. Mutations were analyzed by GeneSifter software (Geospiza, Seattle, WA). Fastq files were analyzed by Burrows-Wheeler alignment tool and SAMtools software package. GeneSifter aligned the reads to the HG19 and multi-sample variant analysis determined the nucleotide differences/SNPs/ mutations in these samples.

TABLE 1. Primers Used for Real Time RT-PCR

Genes	Primers
AGR2	F 5′-ATG AGT GCC CAC ACA GTC AA-3′
	R 5′-GGA CAT ACT GGC CAT CAG GA-3′
BRCA1	F 5′-CTT AGA GTG TCC CAT CTG TCT GG-3′
	R 5′-GCC CTT TCT TCT GGT TGA GA-3′
BRCA2	F 5′-GCG CGG TTT TTG TCA GCT TA-3′
	R 5′-TGG TCC TAA ATC TGC TTT GTT GC-3′
CDC25A	F 5′-CCTCCGAGTCAACAGATTCA-3′
	R 5′-GGGTCGATGAGCTGAAAGAT-3′
CHEK2	F 5′-CCC AAG GCT CCT CCT CAC A-3′
	R 5′-AGT GAG AGG ACT GGC TGG AGT T-3′
GJA1	F 5′-TCT GAG TGC CTG AAC TTG C-3′
	R 5′-ACT GAC AGC CAC ACC TTC C-3′
MCAM	F 5′-TGG TTT GTA CAC CTT GCA GAG TAT TC-3′
	R 5′-TGG GCA GCC GGT AGT TGA-3′
S100A4	F 5′-CAT GGC GTG CCC TCT G-3′
	R 5′-TGC CCG AGT ACT TGT GGA AG-3′
SYK	F 5′-CATGTCAAGGATAAGAACATCATAGA-3′
	R 5′-AGTTCACCACGTCATAGTAGTAATT-3′
FOS	F 5′-AAA AGG AGA ATC CGA AGG GAA A-3′
	R 5′-GTC TGT CTC CGC TTG GAG TGT AT-3′
ANGPT1	F 5′-GATGTCAATGGGGGAGGTT-3′
	R 5′-CTCTGACTGGTAATGGCAAAAATA-3′
ETS2	F 5′-TCAGCTCTGAGCAGGAGTTTCAGA-3′
	R 5′-GGTTGGCTTATTGAGGCAGAGAGA-3′
ITGB3	F 5′-GTG ACC TGA AGG AGA ATC TGC-3′
	R 5′-TTC TTC GAA TCA TCT GGC C-3′
JUN	F 5′-CCC CCA GCG TAT CTA TAT GGA A-3′
	R 5′-GCT GTC CCT CTC CAC TGC AA-3′

Real Time RT-PCR

Validation of randomly selected differentially expressed genes was performed by qRT-PCR using the Lightcycler (Roche Applied Bioscience, Indianapolis, IN) and SYBR green PCR kit (Qiagen, Valencia, CA). Total RNA was extracted from paired samples (untreated BAR-T and BEC20w cells) one pair duplicate of set for genome-wide analyses and six separate BEC20w replicates, using the RNeasy Mini Kit from Qiagen. Samples were run in duplicate with test primers sets and actin as the internal control. All samples underwent denaturing at 95°C for 45 sec followed by 30 cycles of amplification at 62°C for 10 sec each and 72°C for 12 sec. each. Fold changes were calculated by normalizing the test crossing thresholds (C_t) with the beta actin and G6PDH (internal control) C_t. using the $\Delta\Delta C_t$ method. Deviation in fold changes between replicates is expressed as ±SEM. The genes and primers used for qRT-PCR are listed in Table 1. Cancer specific genes (associated with cell-cycle regulation, adhesion, and known oncogenes) with consideration for gene families mentioned by Nancarrow et al. (2011) to be common in most GWAS studies on EAC, were selected for validation.

HELP-Tagging for Genome-wide Methylation Analysis

The HELP-tagging assay uses massively parallel sequencing to analyze the status of 1.8 CpGs across the entire genome (Suzuki et al., 2010). For HELP-tagging assays, DNA samples were digested with HpaII and ligated to customized Illumina adapters with a complementary cohesive end. These adapters also contain an EcoP15I site that cuts into the adjacent sequence 27 bp away, allowing ligation with another Illumina adapter for library generation by PCR. The presence of "CCGG" and EcoP15I sequences at the ends of the reads allow removal of spurious sequences. Before sequencing, qRT-PCR was performed with primers that measure the proportion of adapter dimer complexes in the library, usually a very small proportion (<5%) of the total library. Following sequencing, low quality or unmapped reads and piled up reads on each locus were removed. An output for each locus in terms of read frequency was created. The HpaII signal was

normalized with that of the deeply sequenced MspI profiles, as performed previously (Suzuki et al., 2010). Results were generated using the WASP system and linked the UCSC Genome Browser for visualization.

Methylation Analysis

HELP-tagging data were analyzed using an automated pipeline, as described (Suzuki et al., 2010). Loci were defined in a continuous variable model, given the quantitative nature of this and comparable published assays (Suzuki et al., 2010). Methylation values were depicted from a range of 0 to 100, with 0 representing fully methylated to 100 representing fully hypo methylated loci.

Pathways Analysis Tools

Ingenuity Pathway Analysis IPA (Ingenuity Systems®, Redwood City, CA www.ingenuity.com) was used to identify biological and molecular networks. This web-based entry tool allows for the mapping of gene expression data into relevant pathways based on their functional annotation and known molecular interactions. This literature from published, peer-reviewed scientific publications, is stored in the Ingenuity Pathways Knowledge Base (IPKB), and is continuously updated. The Gene Ontology database, a database curate by GO Ontology consortium, was used to identify relevant gene products in terms of their associated biological processes, cellular components, and molecular functions. The KEGG PATHWAY maps are a collection of manually drawn graphical diagrams, representing molecular pathways for metabolism, genetic information processing, environmental information processing, and other cellular processes.

RESULTS

Chronic Acid and Bile Exposure Altered Gene Expression in BEC20W Cells

Whole genome sequencing on total RNA derived from BEC20w cells and untreated BAR-T controls was performed using Illumina HISEQ 2000 was performed. Satisfactory coverage of the genome was achieved and gene expression changes were evaluated by pair-wise analyses. A total of 1,492 genes were upregulated and 863 genes downregulated in BEC20w cells when compared with corresponding untreated BAR-T controls. Analysis of the dataset by the KEGG PATHWAY analysis (www.genome.jp/kegg/pathway.html) tool showed that the upregulated genes were significantly associated with 22 pathways, (with positive z scores >2, indicating enrichment

TABLE 2. KEGG Pathway Analysis of Genes Up-regulated in BAR20w Cells

KEGG pathway	No of genes	z-score (Up)
Metabolic pathways	140	6.91
Protein processing in endoplasmic reticulum	27	4.26
RNA transport	26	4.35
Huntington's disease	25	3.31
Systemic lupus erythematosus	25	4.83
Purine metabolism	22	2.93
Spliceosome	22	4.17
Alzheimer's disease	20	2.33
Oxidative phosphorylation	17	2.77
Parkinson's disease	17	2.85
Pyrimidine metabolism	17	3.61
Cytokine–cytokine receptor interaction	12	−2.08
Pathways in cancer	12	−2.76
Amino sugar and nucleotide sugar metabolism	10	3.46
Proteasome	10	3.79
Glutathione metabolism	9	2.78
RNA polymerase	9	4.76
Valine, leucine and isoleucine degradation	8	2.65
Cysteine and methionine metabolism	6	2.05
DNA replication	6	2.05
Ether lipid metabolism	6	2.13
Propanoate metabolism	6	2.38

TABLE 3. KEGG Pathway Analysis of Genes Down-regulated in BAR20w Cells

KEGG pathway	No of genes	z-score (Down)
Pathways in cancer	24	3.72
Focal adhesion	17	3.76
ECM-receptor interaction	14	6.39
Regulation of actin cytoskeleton	14	2.35
Lysosome	11	3.26
Wnt signaling pathway	10	2
Amoebiasis	9	2.74
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	9	3.96
Axon guidance	9	2.06
Osteoclast differentiation	9	2.09
Hypertrophic cardiomyopathy (HCM)	8	2.8
Melanogenesis	8	2.33
Dilated cardiomyopathy	7	2.13
Protein digestion and absorption	7	2.47
Basal cell carcinoma	6	2.91
Inositol phosphate metabolism	6	2.81
p53 signaling pathway	6	2.31

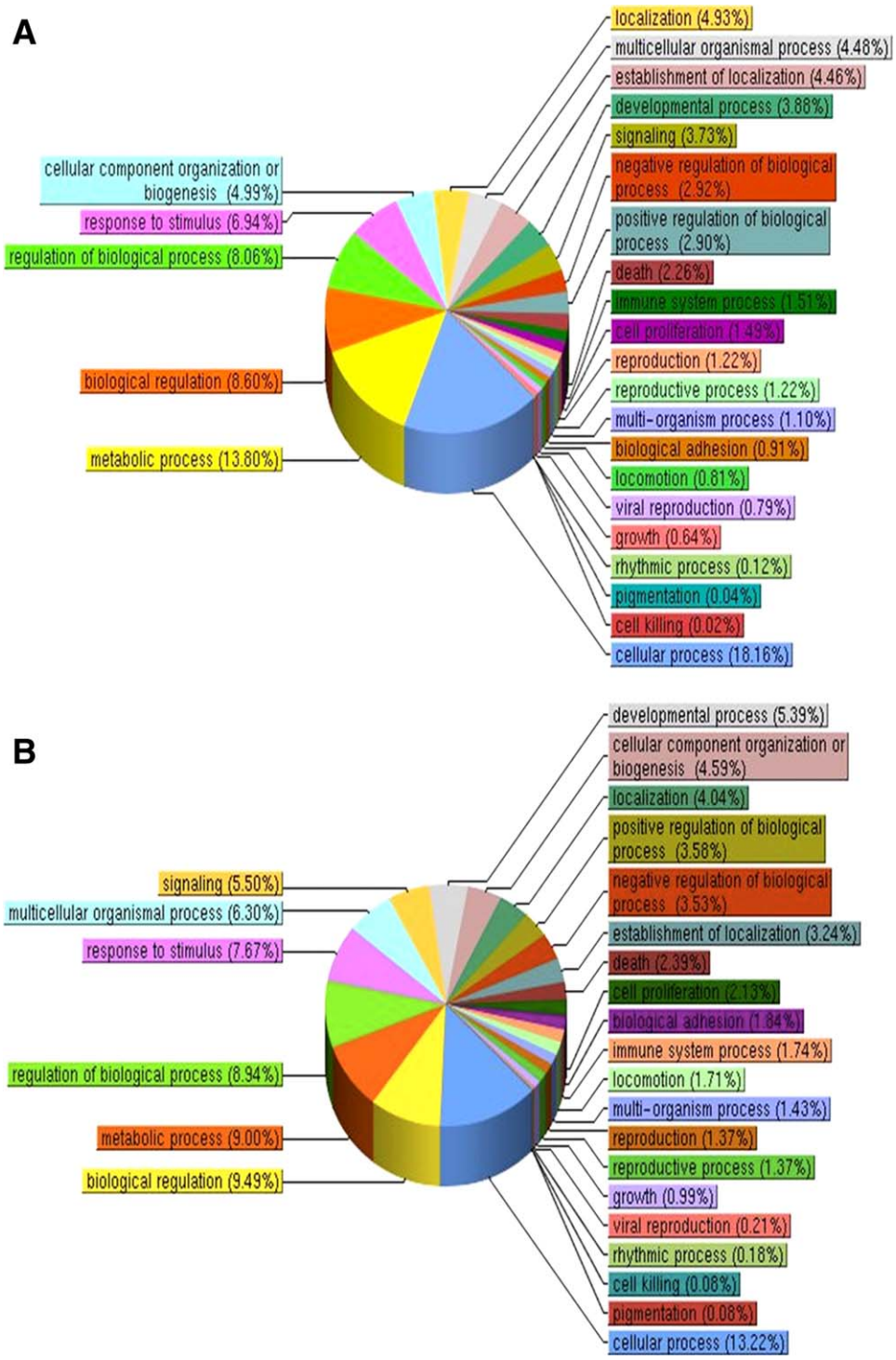
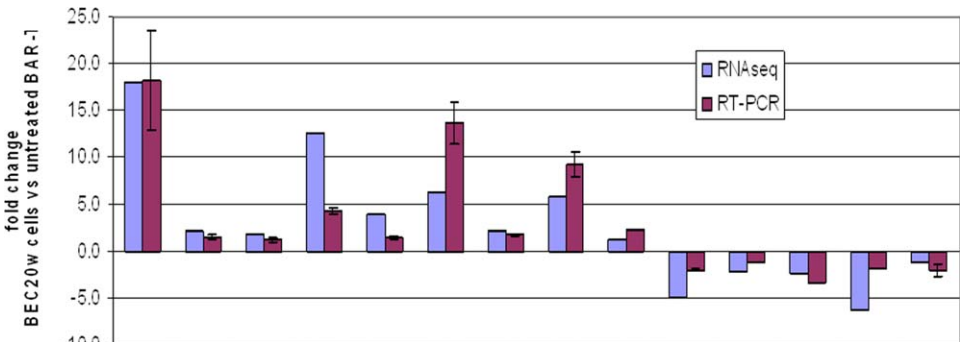


Figure 1. Ontology pathways upregulated (A) and downregulated (B) in BEC20w cells. Gene Ontology pathway analysis of HiSeq datasets reveals widespread changes in cellular and metabolic processes, biological regulation and cell signaling associated gene expression in BAR20w cells due to chronic acid and bile exposure. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

beyond random chance) that regulated metabolic process ($z = 6.91$) and various other cellular process. The downregulated genes were significantly associated with 15 pathways (with positive z scores >2) regulating cell adhesion, developmental processes and cell proliferation (Tables 2 and 3). When the same dataset (>2-fold difference, Likelihood Ratio, Benjamini, and Hochberg correction) was



	AGR2	BRCA1	BRCA2	CDC25A	CHEK2	GJA1	MCAM	S100A4	SYK	FOS	ANGPT1	ETS2	ITGB3	JUN
RNA seq	18.0	2.1	1.7	12.6	3.9	6.2	2.1	5.7	1.3	-5.1	-2.3	-2.4	-6.4	-1.2
RT-PCR	18.2	1.5	1.2	4.2	1.4	13.7	1.7	9.2	2.3	-2.0	-1.2	-3.3	-1.8	-2.1

Figure 2. QRT-PCR validation of alterations in the transcriptome observed by RNAseq in BEC20w cells. Fold change in gene transcript levels as detected by HiSeq analysis was validated in six independent replicates of the BEC20w cell line. Quantitative real time PCR analysis showed similar dysregulation of genes selected for validation. The error bars denote variation between replicates when compared with the paired untreated BAR-T cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

grouped using the Gene Ontology database (http://www.geneontology.org/) dysregulation of functionally similar biological pathways was observed (Figs. 1A and 1B).

Validation of Illumina HiSeq RNA Sequencing by qRT-PCR Showed Consistent Changes in the Transcriptome

Quantitative RT-PCR analysis was performed on selected genes that were dysregulated in the RNA-sequencing dataset. Quantitative PCR validated the findings of RNA sequencing and confirmed the significant changes in expression of these genes between naive BAR-T and BEC20w cells (Fig. 2). We found these changes to be consistent in six independent replicates of the BEC20w cells, obtained after exposing six different flasks of naïve BAR-T cells to B4 for 20 weeks. This confirmed that the genetic events were real and reproducible.

Chronic Acid and Bile Exposure Induced Multiple Mutations in BEC20w Cells

To identify SNPs/mutations in BEC-20wk cells, multi-sample variant analysis was performed on the genome-wide data obtained from HiSeq analysis of these and the control untreated cells. Only those SNPs that would lead to a non-synonymous change in amino acid sequence (with prediction score >20) were used for evaluation of mutations. This stringent criterion identified 763 mutations on 637 unique genes in the genome of BEC20wk cells. Ingenuity Pathway Analysis of the dataset revealed the gene networks and important biological functions such as cellular assembly and organization, cell cycle and DNA replication affected by these mutations (Table 4).

Chronic Acid and Bile Exposure Lead to Widespread Epigenetic Changes in the BEC20w Cells

Methylation analysis revealed aberrant hypomethylation was as pervasive as hypermethylation in the genome of BEC20W cells when compared with untreated BAR-T cells using HELP assay (HELP-tagging). In addition to gene promoters, epigenetic changes were also seen in gene bodies, intergenic regions, repetitive elements and CpG islands and shores. Gene promoters, repetitive elements, and CpG shores (regions away from CpG islands) had the most hypomethylation (Fig. 3).

Changes in the Transcriptome Correlate with Epigenetic Changes in the BEC20w Cells

In a combinatorial approach, ingenuity pathway analysis was performed on integrated genetic and epigenetic datasets to identify dysregulated gene networks. Of the total 1,492 genes, 690 genes were upregulated due to accompanying promoter hypomethylation. The gene pathways associated with these genes functionally regulating metabolism, cancer, cell-to-cell signaling and interaction, cellular movement etc. were also upregulated due to aberrant loss of methylation (Table 5).

From a total of 863 downregulated genes, 433 genes had accompanying hypermethylation in their promoters. The gene pathways associated with these genes downregulated by aberrant

TABLE 4. Ingenuity Pathway Analysis Revealed Biological Functions Affected by Non-synonymous Mutations in BEC20w Cells

Genes affected by mutations	Biological Functions
AGFG1, APC,BUB3, BUB1B, CASC5, CENPE, CIB1, CNOT7, COL17A1, DHX15, DST, E4F1, ERBB2IP, EXPH5, GNE, HDAC1, KIF20B, KIFAP3, MACF1, NUP54, PICK1, PNISR, PNN, SACS, SP100, TOB1, TOPORS, UBR4, UBR5, ZWINT	Cellular assembly, cell cycle, DNA replication, recombination and repair
AKAP9, ATG13, BIRC6, CAPZA2, CDC5L, CS, DDX24, MAP4, MYO5B, NRP1, PDK1, POLR1B, POLR2D, POLR3B, PPM1A, PPP1R7, PPP1R11, RAE1, RBBP6, RBL2, RNF41, SCAF8, SEH1L, SEMA3C, SGOL2, TJP2, TRAP1, TRIM33, ZNF451	Cell death, gene expression, cell cycle
AIMP2, APAF1, BIRC2, CASP4, CASP8AP2, CDK12, CHD4, CLTC, DOCK5, DSP, GDI1, GSN, KTN1, MLH1, MLH3, MSH6, N4BP1, NDFIP2, NEDD4L, NLRP1, PKP4, PMS2, RABEP1, SPTAN1, TMPO, UBA1, XRN1, YLPM1	Cancer, gastrointestinal disease, genetic disorder

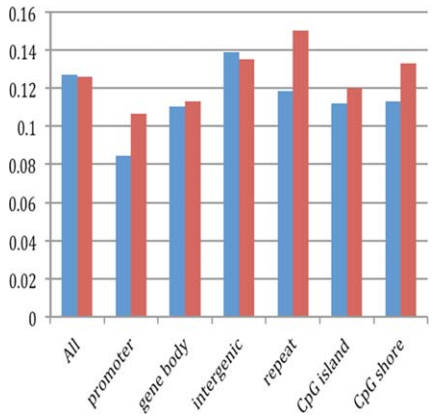


Figure 3. HELP-tagging assay reveals aberrant methylation (both hypo and hyper) throughout the genome of BEC20w cells different from matched controls. Figure shows percentage of changes in the various genomic regions. The paired bars represent hypermethylated (left) and hypomethylated (right) sites within each region. Degree of methylation is represented as a percentage of total loci on the Y-axis. Hypomethylation was more common in promoter region, repetitive elements, CpG islands as well as CpG shores. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hypermethylation regulated protein synthesis, tissue development, cellular development, and similar other biological functions (Table 6).

DISCUSSION

The multistage carcinogenesis process in BE is considered an ideal model to study human neoplastic progression. In this report, we have used a dynamic BEC cell culture model that closely imitates the clinical progression of BE to EAC. Under intermittent chronic acid and bile exposure (B4), the immortalized benign BAR-T cells (Jaiswal et al., 2007) undergo gradual neoplastic changes culminating in tumor formation in nude mice (Das et al., 2011). It must be mentioned that the BAR-T cells growing in parallel for the same duration as the BEC model did not develop these changes and remained benign (Bajpai et al., 2008; Das et al., 2011; Bajpai et al., 2012). Progressive genetic instability and clonal selection have been identified as characteristic early events in the molecular pathogenesis of BE (Neshat et al.,1994; Barrett et al., 1996a,b; Galipeau et al., 1996; Barrett et al., 1999; Wong et al., 2001). We identified chromosomal instability in the BEC model, as early as 20w after initiation of B4 exposure (Bajpai et al., 2012). These cells however did not demonstrate the changes in cell morphology or adhesive properties observed in the BEC40w and BEC60w cells (Das et al., 2011). Hence, we examined the BEC20w cells to study global changes early in the BEC process. One of the important objectives of using this model for our study was to compare the status of BE cells growing for the same period of time with and without further exposure to acid and bile. This situation may not be recreated *in vivo* in the same individual, since BE is clinically managed by acid-suppressive drugs.

Clonal selection may be favored by overexpression of oncogenes (Ehrlich, 2002) or transcriptional silencing of tumor suppressor genes (Feinberg and Vogelstein, 1983; Bird, 1996; Herman and Baylin, 2003), which may result from selected gene-specific hypomethylation or hypermethylation of CpG islands. Several investigations on candidate biomarkers for BE have mostly focused on hypermethylation of CpG islands on selected gene promoters (Eads et al., 2001; Schulmann et al., 2005; Prasad et al., 2008; Sato et al., 2008; Smith et al., 2008; Jin et al., 2009). Although very little has been explored regarding hypomethylation in BE, we observed that this phenomenon is a more pervasive epigenetic alteration in BE progression (Alvarez et al., 2011, 2012). Our combinatorial approach elucidated that genetic and epigenetic alterations may occur concurrently and act synergistically in regulation gene expression during BEC process (Alvarez et al., 2011; Agarwal et al., 2012). While several genes associated with metabolic and cellular processes and cancer pathway are modulated, specific upregulation due to hypomethylation is observed in genes associated with cancer development, gastrointestinal diseases, cellular assembly and organization.

TABLE 5. Ingenuity Pathway Analysis of Genes Up-regulated With Accompanying Hypomethylation in BEC20w cells

ID	Top functions	Molecules in network
1	Nucleic acid metabolism, small molecule biochemistry, ami no acid metabolism	ACO2, ADSS, AP1B1, AP1G1, AP1S2, APIP, ARHGAP6, CCDC53, CHRAC1, FARSB, FECH, FXN, GART, GEMIN5, GEMIN7, HAUS1, HAUS6, LNX1, MCM10, NECAP1, NFS1, NME1, NP M3, ORC2, PAICS, PPAT, PTPRZ1, RAB26, SDHB, SORD, SPRY4, UGP2
2	Cancer, gastrointestinal disease, hepatic system disease	CENPA, COPS3, COPS7B, EPCAM, ETFA, FABP5, FKBP4, H2AFZ, HERC5, IBTK, IL15RA, MLF2, NQO2, PDCD5, PPIF, PRMT1, RBCK1, RELT, RGS20, RNF25, RNF166, RRM1, RRM2, TIMM22, TIMM8A, TOMM22, TOMM40, TOMM40L, TRMT112, UBA5, WDR62, WTAP
3	Embryonic development, organismal development, hereditary disorder	ACVR1, ACVR1B, C1QBP, CLN6, CYB5A, MLX, MRPL3, MRPL4, MRPL21, MRPL39, MRPS2 6, MXD1, MYCBP, NDUFA2, NDUFA6, NDUFB2, NDUFV2, ODC1, PEG10, PFDN2, PI NX1, P TX3, RPL35A, RPL36AL, RPL39L, RPLP0, RPLP1, TXN, ZNF512B
4	Cellular assembly and organization, cellular compromise, cellular function and maintenance	ANG, APAF1, CD83, COMMD1, DNAJB9, DNAJC3, DNAJC30, ERN1, FANCC, GCH1, GSAP, HSPA2, HSPA6, HSPA13, HSPA14, HSPA1A/HSPA1B, HSPB11, HSPH1, KCTD12, NQO1, NTN1, RFK, RI PK2, SESN2, TAF5, TRAF5, UNC5B
5	Cell-to-cell signaling and interaction, tissue development, cellular development	ACADM, ADK, BET1, BTC, EREG, ERRFI1, EXOC6, GCDH, GFPT1, HK2, MAL2, NEU3, NRG1, PBK, PDE5A, Pkg, PRDX2, Ptk, SDF2L1, SLC2A4, SRD5A3, SRXN1, STX5, TMEM176B, TP D52, TRAP1, ULBP1, WDR61

TABLE 6. Ingenuity Pathway Analysis of Genes Down-regulated With accompanying Hyper-methylation in BEC20w Cells

ID	Top functions	Molecules in network
1	Protein synthesis, gene expression, RNA post-transcriptional modification	ACTB, AGO4, ANG, BACE1, CLTB, CTSD, DBN1, FBXO2, FN1, HIP1R, KANK2, RPL36AL, RPLP0, RPLP1, RPS3, RPS5, RPS8, RPS9, RPS10, RPS13, RPS15, RPS16, RPS21, RPS 23, RPS28, RPS29, RPS3A, RPS4Y1, XRCC1
2	Tissue development, hair and skin development and function, organ morphology	ABCC6, AGRN, AP1 B1, AP1 G1, AP1 M1, APBA1, CDK10, CHRNB1, DLL1, DYSF, ETS2, H19, HOXA5, JAG2, KIF13A, KRT17, LRRN1, MAGI1, NFIB, NFIX, NOTCH1, RAB4B, SIK1, SOX2, VASN, ZEB1
3	Lipid metabolism, molecular transport, small molecule biochemistry	ABCA1, ABCA7, ADAR, AES, ALOX15B, AOX1, CBLC, GAS6, GLUL, HMGB3, IL11, IL32, I RAK3, LIPG, LLGL1, MARK1, MDK, MMAB, MVK, NPC2, PNMA1, USP21, USP35, USP43
4	Connective tissue development and function, skeletal and muscular system development and function, connective tissue disorders	ACAN, AGER, ARHGAP5, ARHGAP6, CD24, CTGF, ERK, FBLN1, IFNLR1, LBH, MMP14, NDRG2, PLXNB1, RPS6, SOX4, SOX9, SPHK1, TCF7L2, TIMP2, WNT10A, WNT5B, WNT 7A, WNT7B, ZNF219
5	Nervous system development and function, cellular development, hematological system development and function	APP, CASZ1, EFCAB7, EGR1, FKBP10, FOXJ2, FRMD8, GEMIN8, KLC4, KRT7, MAGED 2, MEIS1, PCDH7, PHF16, PMP22, RELA, SLC2A4, SNX21, SNX33, TCEA2, TFAP2C, UL K1, ZAP70

A recent study found considerable diversity between observations of 16 GWAS studies comparing histological BE and EAC biopsy samples (Nancarrow et al., 2011). These differences are ascribed to assay technology, extent of normal tissue infiltration in tumor biopsy samples, small sample sizes, and tumor heterogeneity among others (Gu et al., 2010). However, a closer scrutiny revealed that members of keratin, mucin, trefoil, annexin, and S100 calcium binding protein gene families were mostly implicated in BE and EAC (Nancarrow et al., 2011). Our analyses confirms dysregulation of several members of the keratin family (AGR2 upregulated and KRT 7, 13, 14, and 17 downregulated), MUC7 and S100A4; yet there are several other cancer relevant genes aberrations not reported before.

The metaplasia-dysplasia-carcinoma cascade in BE is described as a consequence of genomic instability (Rodriguez et al., 2006) marked by chromosomal rearrangements or aneuploidy (Chen et al., 1998) and elevated mutation rates (Barrett et al., 1999; Reid et al., 2001; Wong et al., 2001; Maley et al., 2006; Paulson et al., 2009). Since EAC is associated with high frequency of mutations (Dulak et al., 2013) it seems likely that numerous mutations were detected in the BEC20w cells. Deletion of the 9p21 locus at the early stages and LOH of TP53 during the later stages of progression of BE are genetic events proposed as specific markers of EAC risk in

BE (Barrett et al., 1996b: Maley et al., 2006). Loss of 9p21 occurred early in the naïve BAR-T cells (Jaiswal et al., 2007) however, no change of TP53 was detected in the BEC20w cells. Perhaps this is because the BEC20w cells represent an earlier premalignant stage of BE and TP53 aberrations are considered to be relatively late events in BEC (Barrett et al., 1999; Maley et al., 2006; Merlo et al., 2006). Some mutations identified in BAR20w cells are supported by observations in EAC tumors, e.g., APC (Choi et al., 2000), MSH6 (Dulak et al., 2013), DOCK5 a DOCK family gene (Jarzynka et al., 2007), PIK3R1 and PIK3C2G from the PIK3 family (Phillips et al., 2006). Several other genes known to be associated with cancer pathway and gastrointestinal disease were also mutated in our study.

In conclusion, this study provides experimental evidence of changes in the transcriptome, mutatome, and methylome as a direct consequence of chronic acid and bile salt exposure. Our novel combinatorial bioinformatics approach integrated the genetic and epigenetic changes and predicted the possible biological outcomes. Further comprehensive investigation involving later stages of the BEC model and pathological samples is necessary to conclude the significance of these aberrations as biomarkers in BEC. Possible unique contribution of the BEC model is foreseeable in identification of biomarkers that discriminate progressive from nonprogressive BE.

ACKNOWLEDGMENTS

Amit Verma and Kiron Das made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; involved in drafting the manuscript; revised manuscript critically for intellectual content; given final approval of the version to be published. Manisha Bajpai made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; involved in drafting the manuscript; revised manuscript critically for intellectual content. Rachel Kessel and Tushar Bhagat made substantial contributions to acquisition of data, analysis and interpretation of data; involved in drafting the manuscript. Sangeeta Nischal and Yiting Yu made substantial contributions to acquisition of data and analyses.

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