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Chemoprevention with cyclooxygenase and epidermal growth factor receptor inhibitors in familial adenomatous polyposis patients: mRNA signatures of duodenal neoplasia

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Abstract

To identify gene expression biomarkers and pathways targeted by sulindac and erlotinib given in a chemoprevention trial with a significant decrease in duodenal polyp burden at 6 months ($p < 0.001$) in familial adenomatous polyposis (FAP) patients, we biopsied normal and polyp duodenal tissues from patients on drug versus placebo and analyzed the RNA expression.

RNA sequencing was performed on biopsies from the duodenum of FAP patients obtained at baseline and 6-month endpoint endoscopy. Ten FAP patients on placebo and ten on sulindac and erlotinib were selected for analysis. Purity of biopsied polyp tissue was calculated from RNA expression data. RNAs differentially expressed between endpoint polyp and paired baseline normal were determined for each group and mapped to biological pathways. Key genes in candidate pathways were further validated by quantitative RT-PCR.

RNA expression analyses of endpoint polyp compared to paired baseline normal for patients on placebo and drug show that pathways activated in polyp growth and proliferation are blocked by this drug combination. Directly comparing polyp gene expression between patients on drug and placebo also identified innate immune response genes (IL12 and IFN γ) preferentially expressed in patients on drug.

Gene expression analyses from tissue obtained at endpoint of the trial demonstrated inhibition of the cancer pathways COX2/PGE2, EGFR and WNT. These findings provide molecular evidence that the drug combination of sulindac and erlotinib reached the intended tissue and was on target for the predicted pathways. Furthermore, activation of innate immune pathways from patients on drug may have contributed to polyp regression.

Keywords

duodenal polyps; gene expression; chemoprevention; familial adenomatous polyposis; EGFR; cyclooxygenase

INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disorder due to germline mutations in the *APC* (adenomatous polyposis coli) gene (1, 2). FAP is characterized by the formation of hundreds to thousands of adenomatous polyps in the colorectum and a nearly 100% lifetime risk of colorectal cancer (CRC) if left untreated (3). Prophylactic colectomy has become the standard of care once the extent of colorectal polyposis is beyond endoscopic control. FAP patients are also at greatly increased risk for duodenal neoplasia, with duodenal adenomas eventually forming in >50% of FAP patients and duodenal adenocarcinoma occurring in up to 12% (3–8). Since mutations in the *APC* gene are central to the initiation and development of CRC with 80% of sporadic CRCs having APC loss or inactivation, FAP is an excellent model to study the molecular events leading to development of CRC and other intestinal cancers.

Chemoprevention studies in FAP patients can provide clues as to how drugs modify tumor progression. Multiple studies have shown that sulindac, a cyclooxygenase (COX) inhibitor and nonsteroidal anti-inflammatory drug (NSAID), significantly inhibits colorectal adenomatous polyps in FAP patients (9, 10). However, sulindac has failed to show a significant reduction in duodenal adenomas in FAP patients (11, 12). This is thought to be due to increased *COX2* expression in the duodenal tissue compared with colonic tissue of FAP patients (13). Studies have suggested that APC inactivation and epidermal growth factor receptor (EGFR) signaling promote *COX2* expression, leading to the development of intestinal neoplasms (14, 15). The convergence between the WNT and EGFR signaling pathways and COX2 activity was demonstrated in a mouse model of FAP in which a combination of a COX and an EGFR inhibitor diminished small intestinal adenoma development by 87% (16). These results led us to test the hypothesis that a combination of COX and EGFR inhibition would impede adenoma formation in the duodenum of subjects with FAP. We recently reported on a positive clinical study where patients with FAP were treated with either placebo or sulindac and erlotinib (sulindac-erlotinib). At 6 months, the median total duodenal polyp burden had increased by 6mm from baseline in the placebo arm and decreased by 9mm in the sulindac-erlotinib arm ($P<0.001$) (17).

Here, we report the gene expression analyses from duodenal tissue at endpoint compared to baseline for those subjects enrolled in the trial. We show that the EGFR and COX2 pathways are activated in duodenal polyps and that the drug combination of sulindac-erlotinib blocks this activation. In addition, we found evidence for activation of interferon gamma ($IFN\gamma$) and interleukin 12 (IL12) signaling pathways; suggesting that the recruitment of both Th1 and natural killer T cells may have contributed to the polyp regression (size and number) observed in the drug-treated arm (18), (19).

MATERIALS AND METHODS

Patient Cohort

This study was approved by the University of Utah Institutional Review Board (IRB#39278), conducted in accordance with recognized ethical guidelines, and informed consent was obtained from each subject. A randomized, two-arm chemoprevention trial was conducted between July 2010 and June 2014 in which FAP patients were treated either with placebo or with sulindac (150 mg twice daily) plus erlotinib (75 mg per day) for six months (registered with [ClinicaTrials.gov](https://clinicaltrials.gov) as NCT 01187901). Seventy-three individuals with FAP completed the study. For patients with significant discomfort or evidence of toxicity, the dose was lowered during the course of the study as described previously (17). Endoscopic duodenal biopsies were taken for grossly uninvolved tissue at baseline and endpoint, while polyps were obtained at endpoint only. Tissues were placed in RNA *later* Stabilization Solution (ThermoFisher).

Sample Selection and RNA Isolation

A subset of tissue from 10 research participants who responded to the drug combination (sulindac-erlotinib) and 10 research participants who progressed on placebo were selected for molecular analyses (Table 1). Total RNA was prepared from biopsies using a Qiagen RNeasy Mini Kit (Qiagen #74106) following manufacturer's instructions and including the on-column RNase-free DNase treatment. RNA quantity and quality was determined using a Thermo Scientific NanoDrop Spectrophotometer and Agilent Bioanalyzer.

RNA Sequencing

Fifty-two endpoint (20 uninvolved, 32 adenomas) and 17 baseline (all uninvolved) total RNA samples were treated with RiboZero Gold (Illumina) to remove ribosomal RNA prior to cDNA library preparation using the Illumina TruSeq Stranded Total RNA Sample Prep Protocol. PCR-amplified libraries were sequenced on Illumina HiSeq 2500 instrument using 50 cycle single-read chemistry. Sequencing data sets are deposited to NCBI GEO submission #GSE94919. Sequencing reads were aligned to the RefSeq Hg38 human genome using the Novoalign application (Novocraft). Differentially expressed genes were calculated using the USeq application 'DefinedRegionsDifferentialSeq' (DRDS) as previously described (20, 21). The DRDS application uses DESeq2 negative binomial statistics together with a Benjamini and Hochberg false discovery rate to identify differentially expressed genes (22). For paired sample comparisons the DESeq2 paired analysis application in R was used.

Total RNA from twelve endpoint polyps (6 from placebo and 6 from drug endpoint) of similar 3 to 4 mm size, noted in Table 1, was further evaluated by targeted gene expression using the Human Inflammation and Immunity Transcriptome 475 gene panel (Qiagen # RHS-005Z). This targeted approach improves sequencing depth and reduces biases in amplification. The panel includes a diverse set of cytokines, growth factors and transcription factors important in mediating general and specialized immune responses. RNA is converted to cDNA and amplified with a multiplex primer panel that labels each cDNA molecule with a unique molecular tag. The quantified indexed library DNA was pooled to an equimolar

concentration alongside other samples. The pooled library DNA was amplified by emulsion PCR and enriched for positive Ion Sphere Particles (ISPs) using the Ion Torrent One Touch System II (Life Technologies) and the Ion PI Hi-Q OT2 Kit (Life Technologies). Templated ISPs were sequenced on a Piv3 micro-chip using the Ion Torrent Proton Machine (Life Technologies) and the Ion PI Hi-Q Sequencing 200 kit (Life Technologies) for 130 cycles (520 flows). Sequence reads were aligned to the RefSeq Hg38 human genome using the STAR RNA read mapper. Reads that aligned to more than 3 sites in the human genome or did not have at least 60 bp aligned were excluded. Differentially expressed genes between treatment groups was determined using the QIASeq secondary data analysis tool (Qiagen). Student's t-test was applied after data normalization using total molecular tag counts.

RNA Quantification by reverse transcription quantitative PCR (RT-qPCR)

The expression of *MMP7*, *CD44*, *FOS*, *TM4SF5*, *EGFR* and *PTGS2* was confirmed by RT-qPCR. cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen #11754250) following the manufacturer's instructions. Predesigned IDT primer-probe sets were used together with PrimeTime Gene Expression Master Mix (IDT #1055771) to measure expression of the following genes: *CD44* (IDT #Hs.PT.58.2004193), *EGFR* (IDT #Hs.PT.58.20590781), *FOS* (IDT #Hs.PT.58.15540029), *PTGS2* (#Hs.PT.58.77266), *MMP7* (IDT #Hs.PT.58.40068681) and *TM4SF5* (IDT #Hs.PT.58.39684117). Relative gene expression was determined after normalization to the geometric mean expression of the internal control genes *UBC* (IDT #Hs.PT.39a.22214853), *GAPDH* (IDT #Hs.PT.58.40035104) and *KRR1* (IDT #Hs.PT.58.4223891). Each gene was run in triplicate on a Bio-Rad CFX96 Real-Time PCR System. Data analysis was performed with the Bio-Rad CFX Manager software. GraphPad Prism 7 was used for plotting RT-qPCR results and for statistical analysis.

Bioinformatic Analyses

Tumor purity was assessed using the R application Estimation of Stromal and Immune cells in Malignant Tumors using Expression data (ESTIMATE) as previously described. (23, 24). Normalized RPKM values from all expressed genes (≥ 10 reads) were used to determine RNA expression from immune and stromal cells in each of the 32 duodenal polyp RNA-Seq datasets. Percent tumor purity was then calculated by combining immune and stromal scores.

Principal component analysis (PCA) and hierarchical clustering was used to identify sets of differentially expressed genes that separate samples based on placebo versus drug treatment (25, 26). Ingenuity Pathway Analysis (IPA) was used to predict signaling pathways changing between the groups. Signaling pathways regulated by known transcription factors, cytokines, growth factors and kinases (upstream regulators) were identified using a Fisher exact test.

We determined a z-score using IPA that infers the activation state of an upstream regulator based on the direction of fold change of its target genes (27). A z-score ≥ 2 was considered statistically significant.

Immunohistochemistry

Formalin fixed paraffin embedded (FFPE) polyp tissue from 10 patients on placebo (19 duodenum, 3 colon) and 10 patients on drug (7 duodenum and 3 colon) resected for clinical pathologic evaluation were stained for CD56 (a marker for natural killer cells) according to ARUP clinical laboratory test number 2003589 (Salt Lake City, UT). Briefly, sections were cut at 4 microns, melted at 60°C for 30 minutes, stained on the BenchMark™ Ultra (Ventana Medical Systems, Tucson, AZ.) using the CD56 mouse monoclonal antibody, clone 123C3.D5 (Abgent, San Diego, CA, catalog # AH10009) at a dilution of 1:40, and detected using the UltraView Universal DAB Detection Kit (Roche). The sections were counterstained with hematoxylin. CD56 positive cells were counted in up to 10 high power fields (HPF, 40×). Some polyp tissue had less than 10 HPF of dysplastic tissue. Associations of CD56 counts with receiving drug treatment were estimated through Poisson regression, offset by the log of high power fields present. An alpha level of 0.05 was used to determine statistical significance. SAS 9.4 was used for statistical analysis. Using a likelihood ratio test comparing Poisson and negative binomial models, we found that our data are over-dispersed using the Poisson model, so a negative binomial model was used.

RESULTS

The primary objective of this study was to determine if the sulindac-erlotinib drug combination was affecting the intended molecular targets/pathways in duodenal polyps. Duodenal tissue from twenty individuals, ten from each arm of the trial, was selected based on change in duodenal polyp burden over the 6 month investigation period. Some patients underwent dose reduction, so the average consumed daily dose is reported (Table 1). Additionally, we report the reported adverse events, the individual polyps used from the patients, reported as size in mm, and the corresponding calculated tumor purity.

Gene Expression Comparisons from RNA Sequencing (RNA-Seq)

The average number of unique aligned reads per sample was 14.8 million. Approximately 74% of human RefSeq genes (18698 of 25199) had a minimum of 10 exonic reads in one or more samples and were therefore considered expressed in the human duodenum. Figure 1 is a schematic showing the number of subjects and samples used to identify differences in gene expression between: 1) baseline uninvolved and endpoint uninvolved; 2) baseline uninvolved and endpoint polyp; and 3) endpoint uninvolved and endpoint polyp. Overall, the dynamic range of gene expression observed across these relatively small adenomas (< 10mm) was less than typically seen in invasive cancers (28). Thus, a 2.0-fold change cut-off was used to select genes that distinguish between subjects treated with placebo versus drug. A 1.5-fold change was used for analyses involving pathway discovery.

Endpoint vs Baseline Comparisons—Principal component analysis of differentially expressed genes from endpoint vs baseline comparisons showed clear separation of polyps from patients on drug vs polyps from patients on placebo (Figure 2A). Principal component 1 (PC1) accounted for 20% of the variability observed in the data and separated polyps from patients on drug from patients on placebo. Principal component 2 (PC2) accounted for 14% of the variation in the data and separated 2 polyps (6mmA_017 and 6mmB_017), bottom

center, from Patient 2 on drug, from the other eleven polyps from patients on drug. These two polyps may represent acquired resistance to the drug. Principal component3 accounted for 8% of the variation in the gene expression data.

Hierarchical clustering analysis of the top genes that compose principal component 1 and 2 and are in the WNT-signaling pathway (14 genes), PGE2 pathway (10 genes), or EGFR pathway (10 genes) are shown in Figure 2B. Endpoint vs baseline comparisons show a separation of polyps from patients on drug versus polyps from patients on placebo. The gene expression patterns of these genes suggest that the drug combination blocks adenoma progression through WNT-signaling and specifically blocks the targeted PGE2- and EGFR-signaling processes. Two outlier polyps identified from Patient 2 (D6mmB_017 and D6mmA_017) in principal component analysis showed high expression of a subset of genes (*MMP7*, *AXIN2*, *CXCL5*, *EGR1*, *FOS*, Figure 2B) suggesting a lack of drug effect, and possible drug resistance in these polyps.

When comparing endpoint samples to paired baseline uninvolved duodenal tissue, three patients (one on drug and two on placebo) were excluded due to a lack of baseline tissue (Table 1). Five polyps were also excluded from the final analysis because tumor purity was estimated at <50% (Table 1, Supplemental Figure S1). Differential gene expression with the <50% tumor purity included and excluded is presented in Supplemental Table 1. When comparing endpoint polyp samples with paired baseline uninvolved duodenum from patients randomized to placebo, we identified 977 differentially expressed genes (fold change ≥ 2.0 ; false discovery rate (FDR) < 0.05) by DESeq2 analysis (Supplemental Table 1). The same comparison from patients on drug yielded only 51 differentially expressed genes, suggesting that the drug combination restores the normal duodenal biology (Supplemental Table 1). Comparing endpoint uninvolved duodenum with paired baseline uninvolved duodenum, we identified 1 differentially expressed gene in patients on sulindac-erlotinib and 11 differentially expressed genes in patients on placebo (Supplemental Table 1).

Endpoint Only Comparison—In order to evaluate if there were drug-specific effects on normal tissue, we compared endpoint uninvolved duodenum between patients on sulindac-erlotinib and patients on placebo (Supplemental Table 2). No differentially expressed genes (fold change ≥ 2.0 FDR < 0.05) were found. Only one differentially expressed gene, *NANOS3*, was found comparing endpoint adenomas to paired endpoint uninvolved duodenum from drug treated patients. In contrast, 493 differentially expressed genes were found comparing endpoint adenomas to paired endpoint uninvolved duodenum from patients on placebo (Supplemental Table 2). Again, this set of differentially expressed genes includes multiple known genes involved in adenoma polyp progression including *CD44*, *MMP7* and *CEMIP* (also known as *KIAA1199*) (29).

RT-qPCR Validation—*CD44*, *MMP7*, *FOS*, *TM4SF5*, *EGFR* and *PTGS2* gene expression, representing the three target signaling pathways WNT, EGFR and COX2, were evaluated by RT-qPCR using PrimeTime gene expression assays (IDT, Figure 3). Relative gene expression was determined by the 2^{-CT} method using *GAPDH*, *KRR1*, and *UBC* as the internal control genes (30). *CD44* and *MMP7* show a significant increase in gene expression in polyp as compared to normal from the placebo group ($p < 0.05$), consistent with

previous reports (29, 31) (Figure 3). In polyps from subjects on placebo, we observe upregulation of *EGFR* mRNA, a major effector after APC transformation (32, 33). We also observe downregulation of *TM4SF5* which has been reported to be associated with elevated TM4SF5 protein expression suggesting activation of a previously observed feedback mechanism involving proteasome inhibition in response to elevated TM4SF5 protein levels (34). Notably for all six genes, there is no significant change in gene expression of normal versus polyp from the drug group. When comparing gene expression between polyps from subjects on placebo versus subjects on drug the *FOS* gene is the only one to show a significant difference ($p < 0.05$). The two polyps from Patient 2 may represent acquired resistance to the drug since both had higher FOS expression and were outliers (Figure 2). Certain genes, such as *PTGS2*, had wide variation in gene expression; thus additional confirmation was performed using RT-qPCR. The different platforms had high correlation in measurement suggesting biologic (and not experimental) variability (Supplemental Figure S2). Previous studies evaluating the levels of *PTGS2* mRNA in intestinal polyps have been mixed, with some studies showing elevated RNA levels (35, 36) and others showing no change compared to uninvolved tissue (37), which is consistent with the major regulation of COX2 being post-translational (38). Even in studies showing statistical differences in the level of *PTGS2*, induction is significantly varied across subjects, which is similar to our findings in duodenal polyps from FAP patients.

Pathway Discovery

The differentially expressed list of 2637 genes representing 1.5-fold differential expression and $FDR < 0.05$ (Supplemental Table 1) were uploaded into Ingenuity Pathway Analysis (IPA) software (2591 of the genes annotated in IPA) to identify the pathways affected in duodenal tissue when normal epithelia becomes a polyp. The software also identified which of these pathway changes were repressed by sulindac-erlotinib treatment. Multiple known signaling pathways important in adenoma development were predicted to be activated in adenomas from patients on placebo, including CTNNB1 (WNT), EGFR, TNF and PGE2 pathways (Table 2, Supplemental Table 3). In contrast, adenomas from patients on drug showed almost complete loss of cancer pathway signaling in agreement with the reduction in polyp burden observed in these patients. The z-score predicts the activation state of the upstream regulator based on the direction of fold change of its gene targets. A z-score ≥ 2 suggests the upstream regulator is significantly activated. The Fisher exact p-value is based on the overlap of differentially expressed genes and known regulator targets with no information regarding activation state. We also observed inhibition of multiple signaling pathways in polyps from patients on placebo that include pathways related to intestinal development and tumor suppression (CDX2) (39, 40), heterochromatin assembly (CBX5), cell adhesion (CTNNA) and interferon signaling (IFN α and IFN γ).

IPA analysis also revealed immune signaling pathways that are downregulated in placebo polyps and not downregulated in drug polyps; most notably IFN α and IFN γ . Because PGE2, a product of COX2 enzyme, is potent inducer of interleukin 10 (IL10), which in turn suppresses IFN γ signaling (41), this observation would be consistent with increased COX2 expression in placebo polyps. Similarly, polyps from patients treated with sulindac-erlotinib would not have COX2 overexpression and would not have suppression of IFN γ . In order to

further explore this finding, an inflammation and immunity transcriptome panel was run on 6 placebo polyps and 6 drug polyps to enhance coverage. These results confirmed that IFN α , IFN γ and IL12 are more active in polyps from patients on drug, whereas PGE2 is less active in polyps from patients on drug (Table 3, Supplemental Tables 4 and 5).

The RNA expression data suggest activation of the specific T-cell and natural killer mediated immune response through IL12 and IFN γ . We thus stained FFPE tissues from the clinical trial for the presence of natural killer cells using immunohistochemistry for CD56 (neural cell adhesion molecule; NCAM). We observe an increase in natural killer cells in polyps from patients on drug versus placebo, but the observation is not statistically significant. Polyps from the group treated with drug had an average CD56 count of 1.18 per HPF ranging from 0 to 3.57 per HPF. The placebo group had an average CD56 count of 0.846 per HPF, ranging from 0 to 1.83. Upon fitting the data to a negative binomial model, we find a 1.43 increase in count per HPF in the polyps from patients on drug (95% CI: 0.77, 2.66; P=0.2641). Although there is a slight increase in natural killer cells, which may represent activation of T-cell mediated immune response, it is not statistically different from the polyps from patients on placebo.

DISCUSSION

We recently completed and reported the clinical findings of a chemoprevention trial aimed at preventing progression of duodenal adenomas in patients with FAP (17). Combined treatment with sulindac-erlotinib resulted in a 56% reduction in duodenal polyp burden after 6 months whereas the placebo arm had a 31% increase in duodenal polyp burden. The goal of this present study was to characterize the molecular changes associated with adenomatous polyp regression in FAP patients treated with sulindac-erlotinib. RNA-Seq technology was used to define the human duodenum transcriptome in FAP patients treated with sulindac-erlotinib or placebo.

One of the challenges resulting from the success of the clinical trial was that there was very limited polyp tissue available from patients on drug. Consequently, the power to discover molecular changes was limited, ranging from 51% to 80% depending on the number of paired comparisons (Supplemental data). Even with this limitation, the robustness of these gene expression-based signatures provided convincing evidence that the sulindac-erlotinib drug combination was inhibiting the intended WNT, EGFR and PGE2 pathways. This fits with our current biologic and genetic understanding of the progression of colon cancer, expands our understanding of how these drugs drive the regression of duodenal polyps in FAP patients and identifies new biomarkers for diagnostics and therapeutics.

Adenomas from patients on placebo displayed a wide range of gene expression changes including changes in RNA transcripts associated with increased WNT, EGFR and PGE2 signaling. Increased expression of many WNT signaling targets including *AXIN2*, *LGR5*, *MMP7* and *MYC* observed in our study are in agreement with previously published gene expression studies from sporadic and/or FAP patient cohorts (29, 31, 42). These genes play an important role in regulating cell proliferation and tumor progression in colon adenomas. *AXIN2* and *LGR5* are both considered negative regulators of WNT signaling while *MMP7*

and *MYC* are positive regulators. AXIN2 or conductin protein is part of the multiprotein APC complex that regulates the stability of β -catenin, and LGR5 is an established cell surface protein marker of intestinal stem cells (43, 44). MMP7 protein plays a role in the breakdown of extracellular matrix, T-cell migration and tumor metastasis (45). *MYC* protein plays multiple roles in the development of cancer including the regulation of cell proliferation, apoptosis and epithelial to mesenchymal transition (46). The upregulation of these genes provides strong support of WNT activation in FAP duodenal adenomas in our study similar to previous gene expression studies.

The RNA levels of many target genes of both EGFR and PGE2 signaling were also significantly increased in duodenal adenomas from patients on placebo but not on drug. The increased expression of immediate early genes *FOS* and *EGR1* in duodenal adenomas are consistent with increased EGFR signaling and cell proliferation. Increased PGE2 signaling was associated with increased mRNA levels of the stem cell marker *CD44*. PGE2 increases the number and survival of CD44 positive stem cells in human colorectal cancer and animal models of colon tumor metastasis (47). The increased activation of EGFR and PGE2 pathways in adenomatous polyps from FAP patients in our study further supports the use of the combined sulindac-erlotinib therapy in our Phase II clinical trial for the treatment of duodenal adenomas in FAP patients.

Pathways selectively downregulated in placebo polyps but not drug polyps compared to paired uninvolved duodenal tissue included α -catenin (CTNNA) and chromobox 5 (CBX5). α -catenin is an actin binding protein whose cellular distribution is regulated by β -catenin (48). Reciprocally α -catenin inhibits Wnt/ β -catenin mediated transcription through dual binding of β -catenin and actin. The observed decrease in α -catenin signaling in duodenal polyps observed in our study may be the result of altered α -catenin localization and/or reduced transcriptional regulation through α -catenin. Chromobox 5, also known as heterochromatin protein 1 alpha (HP1 α), is a nuclear protein that mediates transcriptional silencing through interactions with H3K9 methyltransferase and DNA methyltransferases 1 and 3 (49). HP1 α is also important in accurate chromosomal segregation during mitosis (50). Decreased HP1 α signaling observed in placebo polyps may reflect an increase in transcriptional activity and/or decrease in chromosomal stability necessary for duodenal polyp development and progression. Together, the reduced activity of α -catenin and HP1 α are both suggestive of increased transcriptional activity associated with tumor development and growth.

Following sulindac-erlotinib therapy, a pronounced reduction in the number of genes differentially expressed in adenomas was observed from the patients on drug. The average size of adenomas collected from patients on drug was smaller than those collected from patients on placebo, yet retained their adenomatous appearance upon pathological examination. The pronounced regression of duodenal adenomas in our Phase II clinical trial was surprising based on previous trials using sulindac therapy alone (11, 12). At best, we anticipated an inhibition of further duodenal polyp growth in our FAP patient cohort. Although this is clearly speculative, and further work will be required, our results may suggest that the combination therapy of sulindac-erlotinib is restoring some level of innate immune surveillance and adenoma cell killing in the duodenum of FAP patients on drug.

The derepression of IFN α signaling and presence of CD56 positive NK cells support this idea. It has been shown that increased levels of PGE2 cause a decrease IL12 signaling, which is important in the recruitment of natural killer (NK) cells and cytotoxic T lymphocytes (51). Both mouse and human cancer cells deficient in cyclooxygenases (COX) or PGE2 show increased immune signaling and T-cell dependent growth control compared to cancer cells expressing COX (52). These findings are consistent with our studies that show sulindac-erlotinib restores the expression of genes important in the innate immune response and NK cell surveillance and function.

Based on the RNA analysis, we observed potential resistance to the drug combination in multiple polyps from Patient 2. This chemoprevention trial, however, was limited in its ability to evaluate resistance by the minimal polyp tissue that was from patients on drug and available at the end of the trial. It will be important for future work to examine the molecular changes in polyps that are persistent, do not regress, and likely represent resistance to EGFR and COX inhibition. Future chemoprevention trials with this drug combination will benefit by extending the treatment period beyond 6 months by which time resistant polyps would be more established and evident. In addition sampling for future studies should consider use of highly sensitive new technologies such as single-cell RNA and DNA sequencing, liquid biopsy for circulating tumor DNA and cytokines in blood, single-cell mass cytometry for protein expression, and metabolomics. These other endpoints would enable one to overcome limitations of sample number as well as detecting distinct responsive and resistant cells within a single polyp. In a follow-up clinical trial (NCT02961374) efficacy and tolerability of erlotinib alone, given once weekly, in FAP patients with Spigelman stage 2 to 3 duodenal polyposis is being examined. Secondary aims will similarly evaluate the gene expression profiles of duodenal tissues. These analyses will be important to examine if inhibition of the COX2 pathway is required to derepress IFN α signaling.

In summary, our analysis of gene expression in duodenal tissue from patients on drug compared to patients on placebo describes key changes in cancer, inflammation and innate immunity signaling pathways. Many of the genes and pathways described in our study support previous findings of molecular changes observed in colon adenomas from FAP patients. The observed reduction in duodenal polyp number and size together with the inhibition of WNT, EGFR and PGE2 signaling and increase in IFN γ signaling provide important insights into the mechanisms of duodenal polyp regression in FAP patients treated with sulindac-erlotinib.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations list

FAP	familial adenomatous polyposis
CRC	colorectal cancer
COX	cyclooxygenase
NSAID	nonsteroidal anti-inflammatory drug
sulindac-erlotinib	sulindac and erlotinib
RT-qPCR	reverse transcription quantitative PCR
PCA	principal component analysis
FFPE	formalin fixed paraffin embedded
HPF	high power fields
FDR	false discovery rate

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TRANSLATIONAL RELEVANCE

Familial adenomatous polyposis (FAP) patients have a 100% lifetime risk of colorectal cancer and are also at increased risk for duodenal neoplasia, with duodenal adenomas eventually forming in >50% of FAP patients. In most cases, prophylactic colectomy and frequent endoscopy is the standard of care for these patients. We recently completed a phase II clinical trial of sulindac and erlotinib in a FAP patient cohort and found a significant decrease in duodenal polyp burden at six months for patients on drug versus placebo. Here, we present the duodenal polyp RNA expression data from this cohort, which shows almost complete inhibition of the tumor signaling pathways WNT, EGFR and COX2/PGE2 and activation of innate immunity signaling pathways IL12 and IFN γ in adenomas from patients on drug. The drug combination of sulindac and erlotinib provides a promising alternative treatment of duodenal polyps in FAP patients.

Figure 1. Flow diagram of patient samples used for gene expression analysis
Duodenal polyp and uninvolved tissue samples from 10 patients on sulindac-erlotinib (drug)
and 10 patients on placebo were used for gene expression analysis.

Figure 2. Differential gene expression in duodenal polyps from subjects treated with sulindac-erlotinib versus placebo and focused on intended targeted pathways of WNT, EGFR and PGE2

A. Principal component analysis using the 977 genes differentially regulated at 2-fold or higher in placebo polyps as compared to baseline uninvolved. Figure includes endpoint polyps with > 50% purity by ESTIMATE from subjects on drug (red) or on placebo (blue).
 B. Hierarchical clustering values are represented as log₂ ratios of endpoint polyps with > 50% purity compared to paired baseline uninvolved duodenum from each patient. Samples are labeled as drug (D) or placebo (P), the size of polyp in mm, and the sample number,

corresponding to Table 1. Red denotes increased expression, blue decreased expression and black no change compared to baseline uninvolved duodenum.

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Figure 3. Quantitative reverse transcription PCR confirmation of differentially regulated genes representing WNT, EGFR and PGE2 signaling pathways

Panel A and B represent genes in WNT signaling pathway. Panel C and D are representative of EGFR signaling pathway. Panel E and F represent PGE2 signaling pathway. Each individual samples is graphed along with the median and 95% confidence interval. P-values are reported for Mann-Whitney tests. Non-significant p-values are noted as (ns). The open circles are the five < 50% tumor purity polyps. RT-qPCR failed for the endpoint uninvolved duodenum sample from patient 176, so this data point is absent from all graphs.

Table 1

Characteristics of participants and biopsies studied.

Patient number	Germline APC mutation	Sample number	Sex	Age	Duodenal Polyp Burden			Drug Exposure		adverse events	Endoscopic Tissues for mRNA Analysis			
					Arm	Base sum diam	Endpoint sum diam	Change	Avg daily dose erlotinib (mg)		Baseline uninvolved	Endpoint uninvolved	Endpoint polyp(s) size (in mm)	Tumor purity
1	c.3927_3931del5	008	Female	41	Drug	95	32	−66%	47	mucositis(1)	yes	yes	4 [#]	0.67
2	c.1660C>T	017	Male	45	Drug	56	26	−54%	58	rash(1)	yes	yes	4 [#] ; 5; 6; 6	0.52; 0.79; 0.80; 0.85
3	c.426_427delAT	028	Male	55	Drug	22	5	−77%	70	rash(1)	yes	yes	2	0.56
4	c.1690C>T	027	Female	40	Drug	85	31	−64%	46	rash(1)	yes	yes	3 ^{##}	0.46
5	c.426_427delAT	055	Male	53	Drug	13	4	−69%	27	mucositis (1); rash (1)	yes	yes	2 [*]	0.47
6	clinical dx	073	Female	47	Drug	42	23	−45%	45	rash(1)	yes	yes	3 [#]	0.51
7	c.426_427delAT	155	Female	58	Drug	63	23	−63%	49	mucositis (1); rash (1)	no	yes	3 [#]	0.7
8	del promoter 1B	173	Female	44	Drug	49	28	−43%	21	rash(1)	yes	yes	3 [#]	0.54
9	del promoter 1B	176	Male	28	Drug	13	4	−69%	21	mucositis (2); rash (2)	yes	yes	2	0.57
10	c.4612_4613delGA	005	Male	56	Drug	700	700	0% [^]	75	mucositis(2); rash(1)	yes	yes	6; 6; 6; 6	0.76; 0.78; 0.67; 0.70
11	c.531+2_531+3insT	021	Male	52	Placebo	61	163	167%	NA		yes	yes	8; 6	0.68; 0.71
12	c.2093T>G	024	Male	46	Placebo	12	25	108%	NA	rash(1)	yes	yes	2	0.67
13	c.2093T>G	031	Female	56	Placebo	22	51	132%	NA	mucositis (1)	yes	yes	4 [*] ; 4 [#]	0.49; 0.65
14	del exon 11–18	072	Female	50	Placebo	55	83	51%	NA		yes	yes	3 [#]	0.54
15	c.694C>T	080	Female	37	Placebo	20	61	205%	NA		yes	yes	2 [*]	0.44
16	c.904C>T	095	Male	59	Placebo	107	185	73%	NA		yes	yes	4 [*] ; 4 [#]	0.49; 0.64
17	del promoter 1B	150	Male	54	Placebo	112	181	62%	NA		no	yes	4 [#] ; 4	0.63; 0.58
18	c.4612_4613delGA	151	Female	38	Placebo	38	98	158%	NA		no	yes	3 [#]	0.55
19	c.531+2_531+3insT	175	Male	19	Placebo	12	21	75%	NA		yes	yes	4 [#]	0.8
20	c.3927_3931del5	013	Female	58	Placebo	98	135	38%	NA		yes	yes	8, 6, 4	0.66; 0.72; 0.75

The “sum diam” refers to the sum of the diameter of all polyps endoscopically identified as described previously (17).

^{*} Indicates polyp excluded for differential expression analysis (<50% tumor purity) (2 drug; 3 placebo)

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Indicates 3 to 4 mm polyp used in Human Inflammation and Immunity Transcriptome panel (6 drug; 6 placebo).
^ Patient 10 was on drug and had no change in sum diameter but a reduction in volume due to a change in polyp height.

Table 2

Ingenuity (IPA) upstream regulator analysis of differentially expressed genes in endpoint polyp compared to uninvolved baseline

Upstream Regulator	Symbol	Molecule Type	Placebo Polyp (n=10, 2591 genes)		Drug Polyp (n=13, 236 genes)	
			z-score	p-value	z-score	p-value
Tumor necrosis factor	TNF	Cytokine	2.06	4.95E-16	1.62	3.08E-02
Beta catenin	CTNNB1	Transcription factor	3.04	2.29E-11	1.89	4.61E-05
Epidermal growth factor	EGF	Growth factor	2.38	3.31E-07	NA	NS
Epidermal growth factor receptor	EGFR	Kinase	3.38	3.66E-05	1.56	1.80E-02
Prostaglandin E2	PGE2	Endogenous chemical	1.95	1.83E-03	1.10	3.52E-02
Chromobox 5	CBX5	Transcription factor	-3.09	1.90E-11	-1.89	1.28E-04
Interferon gamma	IFNG	Cytokine	-1.17	3.18E-10	0.393	1.76E-03
Caudal type homeobox 2	CDX2	Transcription factor	-2.29	1.23E-09	0.00	3.85E-02
Interferon alpha	IFNA	Group	-3.74	3.78E-04	-1.27	3.33E-02
Alpha catenin	CTNNA	Group	-2.14	2.86E-03	NA	NS

NA = not applicable because upstream regulator was not significantly represented in differentially expressed gene list

NS = not significant

Table 3

Top signaling pathways in Inflammation and Immunity Transcriptome panel which are activated or inhibited in polyps from patients treated with sulindac-erlotinib compared to patients on placebo

Upstream Regulator	Symbol	Molecule Type	z-score	p-value
Interferon alpha	IFNA	Group	3.063	4.52E-22
Interferon gamma	IFNG	Cytokine	2.965	5.29E-21
Interleukin 12	IL12	Complex	2.675	1.55E-15
Signal transducer and activator of transcription 4	STAT4	Transcription factor	2.403	3.00E-06
Prostaglandin E2	PGE2	Chemical	-2.225	1.52E-05

(+) positive z-score or (–) negative z-score indicates that these pathways are more or less active in patients treated with sulindac-erlotinib versus placebo.