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The Inflammatory Microenvironment in Colorectal Neoplasia

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Abstract

Colorectal cancer (CRC) is a major cause of mortality and morbidity worldwide. Inflammatory activity within the stroma of invasive colorectal tumours is known to be a key predictor of disease activity with type, density and location of immune cells impacting on patient prognosis. To date, there has been no report of inflammatory phenotype within pre-malignant human colonic adenomas. Assessing the stromal microenvironment and particularly, inflammatory activity within colorectal neoplastic lesions is central to understanding early colorectal carcinogenesis. Inflammatory cell infiltrate was assessed by immunohistochemistry in paired colonic adenoma and adjacent normal colonic mucosa samples, and adenomas exhibiting increasing degrees of epithelial cell dysplasia. Macrophage phenotype was assessed using double stain immunohistochemistry incorporating expression of an intracellular enzyme of function. A targeted array of inflammatory cytokine and receptor genes, validated by RT-PCR, was used to assess inflammatory gene expression. Inflammatory cell infiltrates are a key feature of sporadic adenomatous colonic polyps with increased macrophage, neutrophil and T cell (specifically helper and activated subsets) infiltration in adenomatous colonic polyps, that increases in association with characteristics of high malignant potential, namely, increasing degree of cell dysplasia and adenoma size. Macrophages within adenomas express iNOS, suggestive of a pro-inflammatory phenotype. Several inflammatory cytokine genes (CCL2, CCL3, CCL4, IL1β, CCL23, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5) are dysregulated in adenomas. This study has provided evidence of increased inflammation within pre-malignant colonic adenomas. This may allow potential mechanistic pathways in the initiation and promotion of early colorectal carcinogenesis to be identified.

Introduction

Colorectal cancer (CRC) is a major health burden causing significant morbidity and mortality, with over a million cases diagnosed each year worldwide [1]. The majority of CRC is sporadic, that is, associated with no identifiable heritable genetic mutation. The accepted pathogenetic framework for this malignancy is the adenoma-carcinoma sequence [2]. In understanding pathogenesis of malignancy, the emphasis has previously rested on epithelial cell behaviour, but over the last decade, the focus has moved to the microenvironment as a whole and the importance of stromal cell and epithelial cell interaction is now widely recognised [3,4]. Inflammatory activity within the stroma of invasive colorectal tumours is known to be a key predictor of disease activity with type, density and location of immune cells impacting on patient prognosis [5]. The point at which this inflammatory infiltrate exerts influence on colorectal carcinogenesis is unknown. In breast cancer models, non-invasive tumour cells have been shown to recruit macrophages which induce angiogenesis and promote malignant transformation [6]. To date, there has been no report of inflammatory phenotype within pre-malignant human colonic adenomas. Assessing the stromal microenvironment and particularly, inflammatory activity within colorectal neoplastic lesions is central to understanding early colorectal carcinogenesis, and in particular, understanding the mechanisms involved in the transition of pre-invasive to invasive disease. The aim of this paper therefore was to define the inflammatory microenvironment within pre-malignant human adenomatous colonic polyps, and to investigate how this changes along with adenoma characteristics linked to high malignant potential.

Materials and Methods

Assessment of inflammatory cell phenotype

Inflammatory cell phenotype was assessed by immunohistochemistry on 65 colonic adenomatous polyps and 36 adjacent normal mucosal biopsies, obtained from 36 patients at CRC screening colonoscopy, as described previously [7]. The polyp
sample set was expanded to include 40 colonic adenomas exhibiting low grade dysplasia (LGD), 40 with high grade dysplasia (HGD) and 40 adenomatous polyps with supervening invasive adenocarcinoma (cancer polyps (CaP)) [7]. Each specimen was evaluated to confirm histological diagnosis by an experienced consultant gastrointestinal pathologist (GIM). The macroscopic size of each polyp was measured post fixation, providing a consistent measurement of size distribution between the 40 LGD polyps, 40 HGD polyps or the 40 CaP polyps. Tissue was stained using the Envision™ doublestain kit or CSA II biotin-free tyramide signal amplification (Dako, K1497), dependant on antibody requirements (Table 1). Primary antibody was applied for 60 minutes following antigen retrieval (Table 1), identified by peroxidase linked immunoreactivity. Sequential staining with CD68 detected macrophage infiltrate using an alkaline phosphatase linked detection signal. Staining was performed using either the G2 doublestain Envision™ kit or Envision™ doublestain kit, dependant on supplier availability (Dako, K5361 & K1395, respectively). Heat induced antigen retrieval was performed. Levamisole was added to the liquid permanent red chromagen to suppress endogenous alkaline phosphatase activity. Optimisation ensured no cross-reactivity or quenching of signal. The area of most positive macrophage infiltration within one HPF (×40 magnification), distinct from lymphoid aggregation, was identified under fluorescent light at 580 nm using a Texas red filter set and digitally imaged, and also captured under standard optical light. In relation to degree of cell dysplasia, the area of defined histological abnormality was marked and digitally imaged. The 2 images were imported into Corel Paint X3, version 13 and macrophage infiltrate assessed for expression of either iNOS or arginase I.

Assessment of macrophage phenotype

Macrophage phenotype was assessed in 42 adenomatous polyps and 25 adjacent normal mucosal biopsies (randomly selected from the larger prospectively collected sample set) and polyps of increasing epithelial cell dysplasia, as described above. A double stain immunohistochemical technique was used, incorporating detection of an intracellular enzymatic marker of macrophage function, namely iNOS (pro-inflammatory classically activated macrophage) or arginase I (alternatively activated macrophage) (9,10)(Table 1), identified by peroxidase linked immunoreactivity. Sequential staining with CD68 detected macrophage infiltrate using an alkaline phosphatase linked detection signal. Staining was performed using either the G2 doublestain Envision™ kit or Envision™ doublestain kit, dependant on supplier availability (Dako, K5361 & K1395, respectively). Heat induced antigen retrieval was performed. Levamisole was added to the liquid permanent red chromagen to suppress endogenous alkaline phosphatase activity. Optimisation ensured no cross-reactivity or quenching of signal. The area of most positive macrophage infiltration within one HPF (×40 magnification), distinct from lymphoid aggregation, was identified under fluorescent light at 580 nm using a Texas red filter set and digitally imaged, and also captured under standard optical light. In relation to degree of cell dysplasia, the area of defined histological abnormality was marked and digitally imaged. The 2 images were imported into Corel Paint X3, version 13 and macrophage infiltrate assessed for expression of either iNOS or arginase I.

Profiling inflammatory gene expression

Tissue representative of each stage of the adenoma-carcinoma sequence (normal mucosa, adenoma and adenocarcinoma) was obtained from 7 colectomy specimens as part of an ongoing CRC tissue specimen bank, as previously published [11]. Histological analysis confirmed pathological diagnosis. RNA was extracted

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**Table 1. Characteristics of the antibodies used for the immunohistochemical analysis of inflammatory cell infiltrate.**

| Inflammatory cell type | Inflammatory cell marker | Type | Antigen retrieval method* | Positive tissue control | Dilution | IHC detection protocol | Supplier | Code | Isotype/ Clone |
|------------------------|--------------------------|------|--------------------------|------------------------|---------|-----------------------|----------|------|----------------|
| T helper cell          | CD4                      | mouse monoclonal | M20-high pH | Tonsil | 1:100 | Envision | Novocastra | NCL-CD4-If6 | IgGσ, If6 |
| cytotoxic T cell       | CD8                      | mouse monoclonal | M20          | Tonsil | 1:160 | Envision | Dako | M7103 | IgG1, CB/144B |
| activated T cell       | CD25                     | mouse monoclonal | M20          | Tonsil | 1:600 | Tyramide signal amplification | Novocastra | NCL-CD25-305 | IgG2a, 4C9 |
| B cell                 | CD20                     | mouse monoclonal | M20          | Tonsil | 1:400 | Envision | Dako | M0755 | IgG1a, L26 |
| Plasma cell            | CD138                    | mouse monoclonal | M20          | Tonsil | 1:1000 | Envision | Dako | M7077 | IgG1, VS38C |
| NK cell                | CD56                     | mouse monoclonal | M20          | Appendix | 1:150 | Envision | Monosan | MON9006-1 | IgG1 |
| Macrophage             | CD68                     | mouse monoclonal | T16/M20     | Tonsil | 1:300 | Envision | Dako | M0814 | IgG1a, KP[14] |
| Classically activated  | iNOS                     | mouse monoclonal | M20          | Appendix/colonorectal carcinoma | 1:400 | Tyramide signal amplification | BD biosciences | 610328 | 6, IgG2a |
| Alternatively activated | Arginase I              | rabbit polyclonal | M20          | Liver | 1:40 | Envision | Santa Cruz | sc-20150 | H-52 |
| Neutrophil             | Neutrophil elastase      | mouse monoclonal | nil | Tonsil | 1:100 | Envision | Dako | M0752 | IgG1a, NP57 |
| Mast cell              | Mast cell tryptase       | mouse monoclonal | M20          | Tonsil/Appendix | 1:30,000 | Envision | Dako | M7052 | IgG1a, AA1 |

*M = heat induced by microwaving in 10mM citrate buffer, pH 6.0, T = enzymatic digestion performed at 37° C, in 0.2% trypsin, 0.1% calcium chloride solution, pH 7.8, numerical values = time for antigen retrieval.

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using the RNaseasy mini kit (Qiagen, Crawley, UK, 74104), incorporating Qiashredder column tissue homogenisation and on-column DNase digestion. Agilent BioAnalyzer® profiling assessed yield and quality of the extracted total RNA. 500ng of total RNA was used to synthesise biotinylated cRNA using the Oligo-GEArray Reagent kit (Superarray Biosciences, USA, GA-054). Following quality assessment by Nanodrop spectrophotometry® this was hybridised to a commercially available targetted Oligo-GEArray gene filter, Human Inflammatory Cytokines and Receptors (OHS-011, Superarray Bioscience, USA), representing 112 inflammatory cytokine and receptor genes. Array images were captured using a Fuji LAS1000 cooled CCD camera. Hybridised arrays from each patient were imaged together. Signal intensity was analysed using AIDA Image Analyser programme v3.21 (Raytest Isotopenmessgeräte GmBH, Straubenhardt, Germany).

Microarray data was validated by quantitative reverse transcription real-time PCR. Reverse transcription was performed using 500ng total RNA and random primers, catalyzed with SuperScript™ II reverse transcriptase (concentration 200units/500ng total RNA and random primers, catalyzed with SuperScript II reverse transcriptase (concentration 200units/500ng total RNA and random primers, catalyzed with SuperScript II reverse transcriptase (concentration 200units/500ng total RNA and random primers, catalyzed with Taq DNA polymerase). A 10 fold serial dilution standard curve was included for each gene of interest and both normalisers on each plate. Data was included if the PCR efficiency fell between 80% to 105%. A no template control was included. The PCR cycling programme (15 minutes, 95°C to activate the Taq polymerase, followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 2°C for 30 seconds) was followed immediately by a default melting curve program to 96°C. Sequence verification of PCR amplicons was performed by either cloning into pGEM®-T easy vector system (Promega, Southhampton, UK, A1360) and the universal M13 primer, or purification of RT-PCR product and sequencing using custom designed gene specific primers based on reference positions indicated from RefSeq accession number supplied with Superarray primer assays.

**Statistical methods**

Differences in inflammatory cell infiltrate between tissue types and in relation to adenoma size was assessed using paired t-tests. One way ANOVA assessed the relationship between inflammatory infiltrate and degree of dysplasia. One paired t test assessed macrophage phenotype. Both absolute and relative differences in function were tested in a one way ANOVA. SAS 9.1.3 for Windows XP (SAS Institute, Cary, NC, USA) was used for statistical analyses.

Gene array signal intensities were normalised to background signal, log-transformed and rescaled to ensure each data point lay between lowest and highest signal intensity. Data from short and long exposure images were analysed in combination and a weighted average generated. ANOVA was conducted with Patient as blocking variable. Gene expression signals between normal, adenoma and CRC were compared and greater than 2 fold difference in expression pattern identified. RT-PCR data was analysed using a paired t-test, incorporating the $2^{-\Delta\Delta C_T}$(Livak) method as previously published [12].

Statistical significance was set at $p<0.05$ throughout.

**Ethics**

Ethical approval for this study was obtained from Grampian Ethics Committee. Written informed consent was obtained from all participants.

**Results**

**Assessment of inflammatory cell phenotype**

Patient and adenoma characteristics are shown in Table 2. Macrophage ($p = 0.0002$), neutrophil ($p = 0.0001$), helper T cells

| Table 2. Adenoma and patient characteristics. |
|------------------------------------------------|
| Adenoma and patient characteristics          | Adenomas collected within paired polypos-normal sample set (n = 64) | LGD (n = 40) | HGD (n = 40) | CaP (n = 40) |
|-----------------------------------------------|---------------------------------------------------------------|--------------|-------------|--------------|
| Colonic site                                  | Left sided*                                                   | 54 (85%)     | 35 (88%)    | 37 (92%)     | 37 (92%)     |
|                                              | Right sided                                                  | 10 (15%)     | 5 (12%)     | 3 (8%)       | 3 (8%)       |
| Polyp size                                    | Small (1cm)                                                  | 35 (55%)     | 11 (27%)    | 8 (20%)      | 5 (12%)      |
|                                              | Large (>1cm)                                                 | 29 (45%)     | 29 (73%)    | 32 (80%)     | 35 (88%)     |
| Histological classification                  | Tubular adenoma                                              | 50 (78%)     | 32 (80%)    | 15 (38%)     | 13 (32%)     |
| Degree of dysplasia                          | Tubulo-villous adenoma                                       | 14 (22%)     | 8 (20%)     | 25 (62%)     | 27 (68%)     |
|                                              | Low grade                                                    | 56 (88%)     | 0 (0%)      | 0 (0%)       | 0 (0%)       |
|                                              | High grade                                                   | 2 (3%)       | 40 (100%)   | 0 (0%)       | 0 (0%)       |
|                                              | Cancer polyp                                                  | 6 (9%)       | 0 (0%)      | 40 (100%)    | 40 (100%)    |
| Source                                       | CRC screening                                                | 64 (100%)    | 32 (80%)    | 2 (5%)       | 5 (12%)      |
|                                              | Diagnostic database                                          | 0 (0%)       | 8 (20%)     | 38 (95%)     | 35 (88%)     |
| Patient age (years)                          | 63 (53-69)                                                   | 63 (55-79)   | 71 (45-92)  | 67 (38-89)   |
| Gender                                       | Male                                                         | 58%          | 60%         | 55%          | 53%          |
|                                              | Female                                                       | 42%          | 40%         | 45%          | 47%          |

*distal to splenic flexure.

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Assessment of macrophage phenotype

There were no iNOS positive pro-inflammatory macrophages in the normal colonic mucosal biopsies. In comparison, within paired adenomas, these cells were a key feature of the stroma with 84% (61%–93%) of the macrophage population expressing iNOS (p = 0.001) (Table 5). Arginase I expression within the macrophage population was not a prominent feature of either the normal mucosal biopsies or the adenomas. Overall, there appeared to be more arginase I expressing macrophages within the stroma of the polyps, when analysed as both the absolute number of arginase I positive cells and the percentage of arginase I positive cells within the total macrophage population, and this was statistically significant with p = 0.001 and p = 0.023, respectively, but the overall number of positive cells were low (Table 5). 43%, 67% and 34% of the total macrophage population expressed iNOS in the low grade dysplastic, high grade dysplastic and cancer polyp groups, respectively. This is compared to 12%, 5% and 15% of macrophage expressing arginase I. The difference in this pattern of enzymatic expression favours a pro-inflammatory phenotype. The relative proportion of iNOS expressing pro-inflammatory macrophages to arginase I expressing regulatory macrophages was greatest in the low grade dysplastic (p = 0.001) and high grade dysplastic groups (p = 0.001). The relative proportion of regulatory to pro-inflammatory macrophage was higher in the cancer polyp group suggesting that regulatory macrophage are more abundant within areas of invasive disease.

Profiling of inflammatory gene expression

The average age of the patients was 71 years (range 55–79). Five of the patients were male. Five of the cancers originated on the left side of colon. On staging, one was Duke’s A, 4 Dukes B and 2 Dukes C. All of the 9 differentially expressed genes are linked to inflammation and key cell functions important in tumour biology. IL-8 is a known potent chemoattractant of neutrophils and macrophages to areas of inflammatory activity. IL-8 is also mitogenic [13], angiogenic [14] and, through linked expression of MMPs, influences tumour cell motility such that invasion is enhanced [15]. CCL20 is a chemokine involved in directing lymphoid cell migration through binding to its receptor CCR6. CCR6 is significantly up-regulated in both colorectal cancer and associated liver metastases and data suggests that it plays a role in the recruitment of CCR6+ tumour cells to the site of distant metastases [16]. CXCL1 expression is up-regulated in colorectal adenomas and adenocarcinoma [17], inhibiting apoptosis and inversely

| Inflammatory cell | Normal Colon | Adenomatous Polyp | Mean Difference (95% CI) | P-Value |
|-------------------|--------------|--------------------|--------------------------|---------|
| T helper cell     | 13.19 (11.84)| 21.80 (14.67)      | 8.61 (2.95, 14.27)       | 0.004   |
| Cytotoxic T cell  | 6.25 (7.97)  | 6.00 (8.31)        | −0.25 (−2.66, 2.16)      | 0.83    |
| B cell            | 2.78 (6.56)  | 4.11 (7.31)        | 1.33 (−1.60, 4.27)       | 0.36    |
| Activated T cell  | 2.32 (2.67)  | 7.69 (5.71)        | 5.33 (3.34, 7.72)        | <0.0001 |
| NK cell           | 0.19 (1.01)  | 2.11 (3.39)        | 1.92 (0.11, 3.72)        | 0.04    |
| Macrophage        | 10.83 (10.20)| 19.63 (10.69)      | 8.89 (4.50, 13.32)       | 0.0002  |
| Mast cell         | 10.00 (5.35) | 10.56 (9.63)       | 0.56 (−1.97, 3.40)       | 0.59    |
| Neutrophil        | 1.56 (3.22)  | 14.83 (14.76)      | 13.28 (8.37, 18.19)      | <0.0001 |
| Plasma cell       | 5.50 (7.19)  | 6.00 (8.18)        | 0.50 (−2.61, 3.61)       | 0.75    |

In this analysis, the maximum infiltrate value was used for the polyp when more than 1 polyp existed. Numerical data represents mean cell counts (with mean of standard deviation).
linked to expression of fibulin-1, an extra-cellular matrix protein implicated in control of tumour cell migration. Wang et al. (2006) [18] reported that expression of CXCL1 induced by PGE2 was linked to angiogenesis in colorectal cancer in vitro and in vivo and thus provided a link between COX-2 up-regulation and chemokine induced tumour associated endothelial cell migration. Of interest, is the fact that expression of several chemokines was down-regulated in adenomas. CCL23 is an immune mediator involved in the chemotaxis of monocytes but not neutrophils [19]. CCL23 inhibits the release of neutrophils and monocytes from bone marrow, suggesting that it may be an important mediator in regulating bone marrow response during immune stimulation [20]. In addition, CCL23 confers angiogenic properties [21], mediated through up-regulation of MMP2 gene expression in endothelial cells [22]. CCL5 drives T cell and monocyte migration and activation, with increased expression linked to a number of malignancies [23,24]. CCL5 was not found to be up-regulated in colorectal cancer compared to adjacent normal mucosa by Baier and colleagues [25] and this would be in keeping with the present study. CCL19 and CCL21 are structurally related chemokines that share the common receptor, CCR7. They play a pivotal role in the development of secondary lymphoid tissue as seen in animal models of deficiency [26,27] and are involved in T cell activation through interaction with dendritic cells within secondary lymphoid organs. It is difficult to speculate on the biological ramifications of differential expression of each of these chemokines individually. However, the dysregulation of these inflammatory cytokine and chemokine genes in adenomatous polyps compared to adjacent

Table 4. Assessment of inflammatory cell infiltrate in colonic adenomas in relation to increasing degree of epithelial cell dysplasia (one way ANOVA).

| Inflammatory cell | Low grade dysplasia | High grade dysplasia | Cancer polyps | P value |
|------------------|---------------------|----------------------|---------------|---------|
| T helper cell    | 14.20 (40)          | 13.80 (40)           | 21.95 (38)    | **0.009** |
| Activated T cell | 4.95 (40)           | 5.43 (40)            | 8.26 (39)     | 0.06    |
| Macrophage       | 40.37 (39)          | 55.89 (40)           | 82.33 (39)    | **<0.0001** |
| Neutrophil       | 8.13 (40)           | 17.80 (40)           | 31.44 (39)    | **<0.0001** |

Numerical data denotes mean cell counts. () = n.
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Figure 1. Inflammatory cell infiltration in colorectal adenomas compared to normal colonic mucosa. Macrophage infiltrate was increased in polyps (B) compared to adjacent normal mucosa (A). Neutrophil infiltrate was increased in polyps (D) compared to adjacent normal mucosa (C). CD25+ activated T cells were increased in polyps (F) compared to adjacent normal mucosa (E). doi:10.1371/journal.pone.0015366.g001
normal mucosa complements the cellular findings of an active inflammatory stromal microenvironment and is worthy of further investigation. Several gene expression studies have previously been performed on normal and diseased colonic tissue. In general, a genome wide approach has been applied to identify genes dysregulated in malignant tissue that can be targeted in treatment strategies and for staging [28–30]. In contrast, there are fewer reports of gene expression studies in pre-malignant colorectal adenomas [31–34]. Each stage of the adenoma-carcinoma sequence may have an identifying gene signature primarily involving genes related to cell cycle, cell growth, RNA and protein processing and cell signalling. Sabates-Bellver et al. [35] assessed 32 pedunculated colorectal adenomas compared to normal mucosa and identified differential expression of several inflammatory genes in adenomas, corresponding to those identified from our data analysis. Specifically, CCL19 and CCL5 had reduced expression in polyp lesions. In addition, IL-8, CXCL1, CXCL2, CXCL3, and CCL20 had higher levels of expression in the polyps compared to normal, which is in keeping with the current study.

The gut exerts a continuous low grade ‘physiological’ mucosal inflammatory activity, in response to continual challenges from luminal contents, with T helper cell, macrophage, CD8+ T cells, and plasma cell infiltrates [36,37]. The normal mucosal biopsies in this study revealed mucosal immunoreactivity as expected. The demonstration that polyps harbour an increased cellular inflammatory infiltrate over and above what is expected in “normal” colonic tissue is an important finding. This study has shown that adenomatous polyps are rich with pro-inflammatory macrophage, neutrophil and T helper cell infiltration and these are likely to exert a significant influence on their surrounding microenvironment within the polyp lesion. NK cells were also found to be increased in polyp compared to normal mucosa. However, the majority of polyps did not have any evidence of CD56+ NK cell infiltrate and those that did had an average of only 5 positive cells/HPF. This only marginally achieved statistical significance (p = 0.04) and the relevance of this in the biological microenvironment is debatable and requires further evaluation.

Neutrophils are a source of N-nitrosamines, especially in the presence of colonic amine producing bacteria, and these are known to be carcinogenic [38,39]. Coussens et al. [40] showed that neutrophil and macrophage derived MMP-9 enhanced progression from dysplasia to overt malignancy through paracrine signalling in a mouse model of squamous skin cancer, with reduced MMP-9 delaying angiogenesis within dysplastic areas and reducing the incidence of invasive disease. In addition, inflammatory cells of haemopoietic origin, introduced by bone marrow transplant, were identified in hyperplastic, dysplastic and neoplastic skin lesions, and produced MMP-9 driven changes associated with early carcinogenesis. Our previous data showed that MMP-9 is expressed within adenomatous polyps [41]. Recently, Jablonska and colleagues [42] have demonstrated that endogenous IFNβ inhibits angiogenesis, by inhibiting pro-angiogenic genes within tumour associated neutrophils, in a transplantable mouse tumour model. Neutrophil elastase, secreted by neutrophils, is a main constituent of phagocytic response, degrading proteins within the local environment such as elastin, collagen and other constituents of extracellular matrix [43]. Elastase deficient mice have significantly reduced skin carcinoma load in response to repeated UV light or chemical exposure [44]. Administration of a specific

| Table 5. Assessment of macrophage phenotype in colonic adenomas (n = 42) and adjacent normal mucosa (n = 25). (l) = 25th and 75th centiles. |
|---------------------------------------------------------------|
| **Gene** | **Normal (N)** | **Adenoma (Ad)** | **CRC** | **P value N vs. Ad** | **P value N vs. CRC** | **P value Ad vs. CRC** |
|---------------------------------------------------------------|
| CCL20 | 2.47 | 3.97 | 3.65 | 0.014 | 0.044 | 0.537 |
| CXCL1 | 1.60 | 3.52 | 3.31 | 0.001 | 0.001 | 0.520 |
| CXCL2 | 1.35 | 3.10 | 3.16 | 0.003 | 0.002 | 0.910 |
| CXCL3 | 0.77 | 1.76 | 1.88 | 0.009 | 0.004 | 0.712 |
| IL-8 | 1.63 | 2.84 | 3.42 | 0.01 | 0.001 | 0.173 |
| CCL5 | 3.61 | 2.40 | 2.59 | 0.007 | 0.017 | 0.660 |
| CCL19 | 2.73 | 1.57 | 1.59 | 0.004 | 0.004 | 0.944 |
| CCL21 | 2.98 | 1.87 | 2.23 | 0.007 | 0.052 | 0.285 |
| CCL23 | 2.02 | 0.96 | 0.91 | 0.001 | 0.001 | 0.831 |

Gene expression in normal colonic mucosa vs. pre-malignant adenomatous polyp (Ad) vs. invasive adenocarcinoma (CRC). Bold denotes genes with increased expression in neoplastic tissue compared to normal mucosa, and italics denotes genes with reduced expression in neoplastic tissue compared to normal mucosa. doi:10.1371/journal.pone.0015366.t006

| Table 6. Differential expression of inflammatory genes in colorectal neoplastic progression. |
|-----------------------------------------------|
| **Gene** | **Normal (N)** | **Adenoma (Ad)** | **CRC** | **P value N vs. Ad** | **P value N vs. CRC** | **P value Ad vs. CRC** |
|-----------------------------------------------|
| CCL20 | 2.47 | 3.97 | 3.65 | 0.014 | 0.044 | 0.537 |
| CXCL1 | 1.60 | 3.52 | 3.31 | 0.001 | 0.001 | 0.520 |
| CXCL2 | 1.35 | 3.10 | 3.16 | 0.003 | 0.002 | 0.910 |
| CXCL3 | 0.77 | 1.76 | 1.88 | 0.009 | 0.004 | 0.712 |
| IL-8 | 1.63 | 2.84 | 3.42 | 0.01 | 0.001 | 0.173 |
| CCL5 | 3.61 | 2.40 | 2.59 | 0.007 | 0.017 | 0.660 |
| CCL19 | 2.73 | 1.57 | 1.59 | 0.004 | 0.004 | 0.944 |
| CCL21 | 2.98 | 1.87 | 2.23 | 0.007 | 0.052 | 0.285 |
| CCL23 | 2.02 | 0.96 | 0.91 | 0.001 | 0.001 | 0.831 |

Gene expression in normal colonic mucosa vs. pre-malignant adenomatous polyp (Ad) vs. invasive adenocarcinoma (CRC). Bold denotes genes with increased expression in neoplastic tissue compared to normal mucosa, and italics denotes genes with reduced expression in neoplastic tissue compared to normal mucosa. doi:10.1371/journal.pone.0015366.t006
neutrophil elastase inhibitor suppressed the proliferation and motility of a pancreatic cancer cell line [45], as well as squamous and adenocarcinoma cell lines of lung malignancy [46]. The underlying mechanism is unclear. Degradation of protein transcription factors may disrupt regulatory proteins of gene expression [44]. Reactive oxygen species derived from polymorphs can indirectly affect gene expression by, for example, modulating enzyme activity and promoting changes in transcription factor binding capacity. Neutrophil activation has been implicated in cell cycle G2/M arrest, dependent on expression of p53 and p21, and associated with DNA damage checkpoint mechanisms, in an in vitro co-culture model mimicking colitis [47].

Pro-inflammatory macrophages are a central and potent constituent of innate immunity. Within our cancer polyp group, the macrophage population appears to change with a reduced proportion of iNOS expressing classically activated cells. Macrophages are well recognised at sites of malignancy. Tumour associated macrophage have poor antigen presenting capabilities with a limited anti-tumour response. This reduced tumoricidal capacity has been linked to reduced iNOS expression [48] and would be in keeping with the current data.

There is pharmacological evidence for the role of inflammation in the development of colorectal neoplasia. Several large randomised controlled trials have shown that regular aspirin use can reduce the risk of CRC and adenoma development by up to 50% [49–52]. The use of aspirin or NSAIDs in our study population is unknown.

This study has defined the stromal microenvironment of premalignant colorectal adenomas and identified key inflammatory components involved in adenoma progression to invasive malignancy. It is clear from our data that a phenotypic and genotypic ‘switch’ occurs early in the adenoma-carcinoma sequence, with expression of inflammatory cytokines and chemokines dysregulated in the transition from normal mucosa to adenomatous polyp, rather than at the polyp to invasive disease transition. This data increases the understanding of the environmental influences within the adenoma in relation to disease progression and may have identified potential mechanistic pathways in the initiation and promotion of early colorectal carcinogenesis. Ultimately, this may identify phenotypic markers within adenomas which determine malignant potential.

Supporting Information

Table S1 RT-PCR validation of gene expression in normal colonic mucosa, adenomatous polyp and adenocarcinoma (CRC), normalised to expression of GAPDH and B2M. P value generated from 2 tailed t-test statistical analysis. Bold denotes genes with increased expression in neoplastic tissue compared to normal mucosa, and italics denotes genes with reduced expression in neoplastic tissue compared to normal mucosa.
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Author Contributions

Conceived and designed the experiments: MHM EEO GIM. Performed the experiments: MHM KNS JED NF GLH JT MH. Analyzed the data: GN CM MHM EEO GIM JED JT NAGM. Contributed reagents/materials/analysis tools: MHM GIM KNS NF JED JTG JTG. Wrote the paper: MHM GIM.

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