High Interleukin-6 mRNA Expression Is a Predictor of Relapse in Colon Cancer

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Abstract. *Aim: To investigate the expression of interleukin-6 (IL6) in colon cancer tissue, and to examine if the risk of relapse is influenced by IL6 expression. Materials and Methods: Fresh-frozen biopsies from tumor and normal adjacent tissues were taken from patients with colon cancer during surgery and stored at −80˚C. mRNA expression for interleukin-6 was evaluated with reverse transcription real time quantitative polymerase chain reaction. Survival analyses were carried-out using a Cox competing risk regression model. Results: IL6 mRNA was significantly more highly expressed in tumor tissue compared to normal adjacent tissue (p<0.001). We found no significant association with regard to IL6 expression and histological differentiation or cancer stage. We found a significant association between high IL6 expression and risk of relapse (Hazard Ratio=2.23, 95% CI=1.10-4.53; p<0.05), also when adjusted for clinicopathological characteristics (Hazard Ratio=2.16, 95% CI=1.07-4.40; p<0.05). Conclusion: Interleukin-6 is up-regulated in colon cancer tissue at the transcriptional level and is significantly associated with increased risk of relapse.*

Colorectal cancer is one of the most common malignancies in the western world and the second most common cause of cancer-related death (1). Colon cancer is curable by surgical resection of the tumor-bearing segment. The risk of recurrence is reduced in colonic cancer stage II and III if treated with adjuvant chemotherapy but overall survival is not improved for low risk stage II cancer (2-4). It has been a disappointment however, that despite considerable research, it has proved surprisingly difficult to find new reproducible predictive biomarkers to stratify these patients (5). Up to date, the only widely implemented predictive biomarker in colonic cancer is Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation for therapy guidance of response to epidermal growth factor receptor-targeted therapy (6).

The development of cancer is a progressive transformation of normal cells into their malignant counterparts. This involves known critical mutations in oncogenes and tumor-suppressor genes (7-9). This enables tumor cells to evade apoptosis, have limitless self-renewal potential and self-sufficiency in growth signaling, as well as the capability to invade and metastasize into adjacent tissue and organs (8).

Inflammatory cytokines, expressed by tumor stromal cells and cancer cells in the tumor milieu, might promote cancer progression through enhancing proliferation and migration of tumor cells (10, 11). The binding of pro-inflammatory cytokines to their receptor on the epithelial cells activates oncogenic transcription factors and induces epithelial to mesenchymal transition (12). Interleukin-6 (IL6) is proposed to play a key role in chronic inflammation and carcinogenesis (13). Through its downstream transcription factors, *e.g.* signal transducer and transcription 3 (STAT3), it stimulates proliferation and migration in cancer cells and mouse models (11, 12). A raised level of circulating IL6 in plasma has also been linked to increased risk of colorectal adenoma in human patients (14, 15).

We aimed to investigate the expression of the inflammatory cytokine IL6 in colonic cancer tissues. Secondarily, we wanted to examine if the risk of tumor relapse was influenced by IL6 expression.

Patients and Methods

Patients. Tumor samples were obtained from patients diagnosed with colonic cancer who underwent colonic resection at the Department of Surgery, Roskilde University Hospital, Denmark, between September 2006 and May 2012. The study was approved by the Danish National Committee on Biomedical Research Ethics (protocol no.: Ø-2006-1-11G and SJ-373). The inclusion criteria were: signed informed

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Table I. *Baseline characteristics of the study population.*

Characteristic	Patients (n=189)
Median age (range), years	68.0 (43-90)
Gender (%)	
Males	114 (60)
Females	75 (40)
Tumor grade of differentiation (%)	
Poor	49 (26)
Moderate	96 (51)
Well	44 (23)
Cancer stage (%)	
I	18 (10)
II	94 (50)
III	67 (35)
IV	10 (5)
Tumor location (%)	
Right colon	96 (51)
Left colon	93 (49)
Relapse during follow-up (%)	
Yes	31 (16)
No	158 (84)
Death from other causes during follow-up (%)	19 (10)
Median follow-up time (range), months	40.1 (0.17-62)

n: Number of patients.

consent, histologically verified adenocarcinoma of the colon, no prior chemo- or radiotherapy, tumor samples with RNA quality adequate for reverse transcription real time quantitative polymerase chain reaction (RT-qPCR) (RNA integrity number of >5, median in included samples=7.9) (16, 17) and paired tissue from tumor and adjacent normal tissue. All the patients were preoperatively assessed with a computed tomographic (CT) scan of the abdomen and a CT or X-ray of the thorax. The tumors were classified according to the fifth edition of the Union for International Cancer Control, TNM classification (18). Postoperative surveillance was performed in accordance with the Danish Colorectal Cancer Groups recommendations (19) and patients were followed until relapse, death from other causes, or for a maximum of five years. The end of follow up was 4. June 2014.

Tissue samples, RNA extraction and cDNA synthesis. Tumor tissue samples were obtained from the luminal side of the intestine, close to the transition zone, and snap frozen in liquid nitrogen immediately after surgical removal of the primary tumor then stored at −80˚C. Samples of adjacent healthy tissue were taken for comparison. From the same biopsies, a paraffin-embedded tissue section was made, and evaluated for content of invasive cancer cells by a specialist in gastropathology. Before RNA isolation, tumor samples were homogenized with an Ultra-Turrax (IKA, Staufen, Germany). RNA was extracted using mirVana RNA isolation kit (Ambion, Life Technologies, Naerum, Denmark) according to the manufacturer's protocol. The quality of RNA was assessed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with a RNA integrity number (RIN) greater than 5 were included. Complementary DNA (cDNA) was synthesized with qScript cDNA SuperMix (Quanta Biosciences Inc., Gaithersburg, MD, USA) with 1 μg of total RNA, according to the manufacturer's protocol.

Table II. *Analysis of variance for IL6 with regard to cancer stage and differentiation grade.*

	IL6	
	F-value	*p*-Value
One-way ANOVA		
Cancer stage	1.05	0.37
Differentiation grade	0.21	0.82
Two-way ANOVA		
Cancer stage	1.07	0.36
Differentiation grade	0.32	0.73
Stage × grade interaction	1.75	0.11

Table III. *Association of different variables on risk of relapse using competing risk regression models.*

Variable	HR (95% CI)	*p*-Value
Unadjusted Cox regression		
High IL6 expression	2.23 (1.10-4.53)	0.025*
Adjusted Cox regression		
High IL6 expression	2.16 (1.07-4.40)	0.03*
Male gender	0.99 (0.48-2.03)	0.98
Age (years)	1.04 (0.99-1.10)	0.06
Low cancer stage (I+II)	0.32 (0.14-0.70)	0.005*
Well-differentiated tumor	0.77 (0.44-1.34)	0.36

HR: Hazard ratio on logit scale; CI: confidence interval. *Significant at the 0.05 level.

mRNA quantification. Copy numbers of mRNA for *IL6* were determined with real-time quantitative RT-PCR using the primers specified below. Intron-spanning primers were designed using PRIMER3 software (20). For validation, the primers were initially used in a PCR reaction with *Taq* polymerase (Thermo Fisher scientific, Slangerup, Denmark) using human intestinal cDNA as template, and the PCR products were run in a 3% agarose gel to confirm the expected size. The products were gel purified with NucleoSpin Extract II gel extraction kit (Macherey-Nagel, Düren, Germany) and used as standards. The PCR products were verified by DNA sequencing (Eurofins Genomics, Ebersberg, Germany). RT-qPCR was carried out using a Lightcycler LC480 from Roche using SYBR-Green Master Mix (Roche Lifesciences, Hvidovre, Denmark) mixed with cDNA. For quantification of mRNA copies, a serial of 10-fold dilutions of gel-purified PCR products were used to calculate standard curves. Beta-2-microglobulin (*B2M*) mRNA, a widely accepted standard, suitable for use in colonic cancer tissue, was used as reference gene (21). Primer sequences for *B2M* were: forward: GTGCTCGCG CTACTCTCTC and reverse: GTCAACTT CAATGTCGGAT (Accession number: NM_004048.2) and for IL6: forward: AGACAGCCACTCA CCTCTTC and reverse: ACCAGGC AAGTCTCCTCATT (Accession number: NM_000600.3). Melting curves were inspected after each run to rule out the occurrence of unwanted amplified PCR fragments.

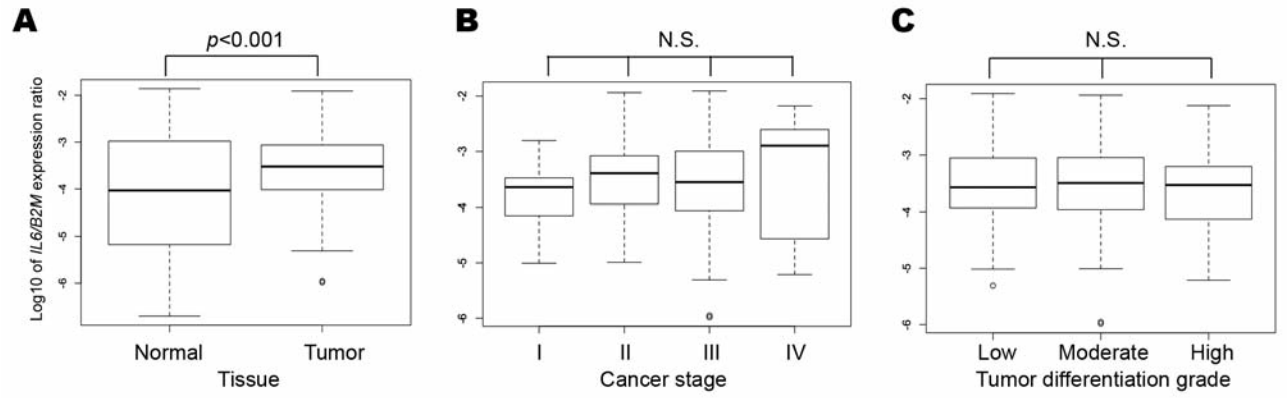

Figure 1. *mRNA expression of interleukin-6 (IL6). A: Paired data from tumor tissue and normal adjacent tissue, showing significantly increased IL6 expression in tumor tissue. B: Expression with regard to cancer stage, showing no significant differences between stages for IL6 expression. C: Expression with regard to tumor differentiation grade, showing no significant differences between low, moderate and high differentiation for IL6 expression. N.S.: Non significant. Box: Median (thick bar) and the inter-quartile range of values. Whiskers: The whole range of values except outliers. Circles: Outliers (outside the 1.5 times length of the interquartile range).*

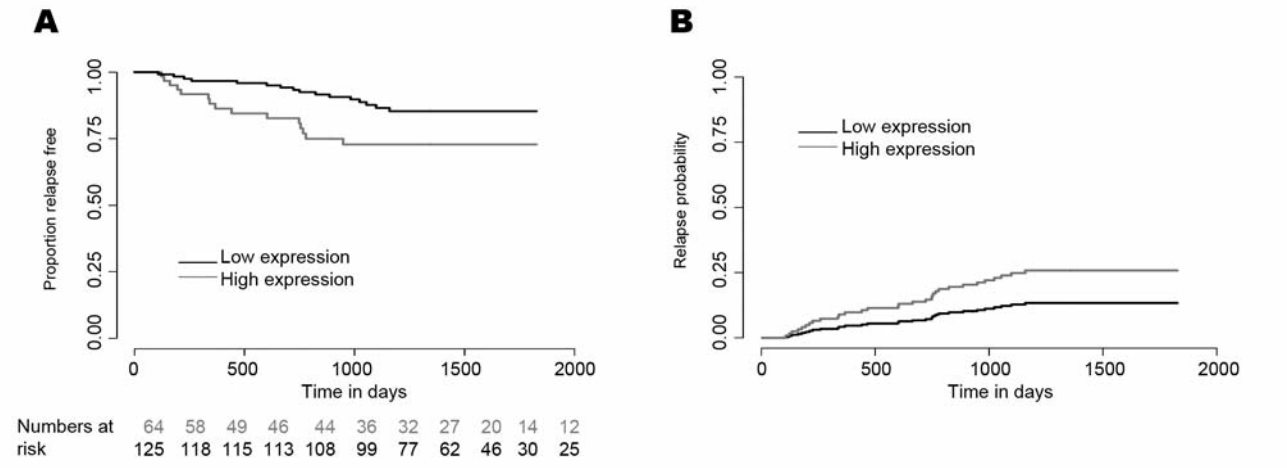

Figure 2. *Risk of relapse with regard interleukin-6 (IL6) mRNA expression. A: Unadjusted Kaplan-Meier curves showing risk of relapse for patients with high and with low expression of IL6. Patient numbers at risk for any given time-point is indicated. B: Cox competing risk regression model showing cumulative incidence curves for risk of relapse for patients with high and with low expression of IL6.*

Statistical analysis. Before analysis, all expression data for the marker gene relative to that for reference gene (*B2M*) were transformed to their common logarithms (log10ratios) (22), and normality was assessed with probability plots. Groups were compared using Student's *t*-test and one or two-way analysis of variance (ANOVA). Two-sided *p*-values were considered significant if less than 0.05.

The patients were divided into two groups with regard to their *IL6* mRNA expression (high and low), using the Youden index for the identification of the optimal cut-off point (23). Cumulative incidences of relapse with regard to mRNA expression were analyzed using a Cox competing risk regression model (24). Both unadjusted and adjusted models were calculated, and results presented as hazard ratios (HR) with 95% confidence intervals (CI). Data were tested for proportional hazards, linearity and interaction using cumulative baseline and residual plots.

All statistical analyses were performed using R (v3.0.3, http://cran.r-project.org/) including packages: polycor, OptimalCut points, timereg, survival, riskRegression and cmprsk.

Results

Patients and clinical characteristics. The study demographics are listed in Table I. In all, 189 patients were included in the analysis. The median age was 68 years and there was a slight predominance of male patients. Most patients were diagnosed with stage II or III colonic cancer, with only a small proportion with stage I and IV. Fifty percent of the tumors were moderately differentiated. The

rest were evenly divided between poorly and well-differentiated. Tumor locations were evenly distributed between the right and left side of the splenic flexure. Thirty-one patients (16%) experienced relapse during the follow-up period. The median follow-up time was 40.1 months.

Expression of IL6 in tumor tissues. Expression data for tumor and normal tissues are presented in Figure 1. We found that *IL6* mRNA was significantly more highly expressed in tumor compared to normal adjacent tissues (paired data) ($p<0.001$), mean difference in log10ratio: 0.52 (95% CI=0.35-0.70).

Using one- and two-way ANOVA, we found no significant association with regard to IL6 expression and histological differentiation or cancer stage. The same was true for anatomical localization of the tumor (data not shown). Results are summarized in Table II and Figure 1.

IL6 expression and risk of relapse. Patients were split into groups of high and low *IL6* mRNA expression, with a cut-off set at log10ratio: -3.19 (identified with Youden index). Figure 2A shows Kaplan-Meier curves for relapse-free follow-up for the IL6 expression groups. An unadjusted risk regression model showed a significant association between high IL6 expression and risk of relapse (HR=2.23, 95% CI=1.10-4.53; $p<0.05$). Figure 2B shows the cumulative incidence curves of risk of relapse in the two IL6 expression groups.

In an adjusted competing risk regression model, we grouped cancer stage into low (cancer stage I+II) and high (cancer stage III+IV). Taking into account clinicopathological characteristics, *IL6* expression was still significantly associated with increased risk of relapse (HR=2.16, 95% CI=1.07-4.40, $p<0.05$). Low cancer stage, compared with high, was associated with decreased risk of relapse (HR=0.32, 95% CI=0.14-0.70, $p<0.05$). Table III summarizes the data.

Discussion

The present study was undertaken in order to evaluate the importance and possible implications of *IL6* expression in colonic cancer. Herein we showed, in a cohort of 189 patients, that *IL6* mRNA expression was significantly up-regulated in tumor tissues. We did not find any relationship between *IL6* mRNA expression and tumor differentiation grade or cancer stage. In a competing risk regression model, high *IL6* expression was a significant predictor of relapse, even when adjusted for cancer stage and other clinicopathological characteristics.

It is well-recognized that long-standing chronic inflammation in connection with ulcerative colitis increases the risk of colorectal cancer (25). It is proposed that inflammatory cytokines, like IL6, play a central role in colonic cancer development (12). Circulating IL6 in plasma is increased in patients with colorectal cancer and has also been linked to an increased risk of developing colorectal adenomas and worse outcome of colonic cancer (14, 15, 26). This transformation and progression may be initiated through the ability of IL6 to induce migration and proliferation, which has been shown in several colonic cancer cell line studies (27-30). However, IL6 production in tumor cells is negligible (29), and it is suggested that tumor-associated macrophages and mesenchymal stem cells are the primary origin of IL6 production in colonic cancer (31, 32). Since we did not make any attempt to micro-dissect the cancer cells from the surrounding tumor stroma in our biopsies, the origin of the IL6 expression could not be evaluated in this study. The importance of IL6 in cancer progression is still unclear, especially in sporadic colonic cancer. Our data suggest that IL6 might be of importance in a clinical framework regarding sporadic colonic cancer. Since many studies on inflammatory cytokines have been carried-out in mouse or cell models, the relatively large patient material in this study may suggest a clinical application.

IL6 has been proposed as a possible target in colonic cancer therapy (33, 34). Monoclonal antibodies against IL6 are commercially available and are being tested in various advanced solid cancer forms *e.g.* ovarian and prostate (35-37). These studies are only in phase I/II, and although the treatment is well-tolerated, an effect is yet to be shown (35). High IL6 was significantly associated with risk of relapse in our study in an unadjusted and adjusted competing risk regression model. This might imply a role for treatment with antibodies against to IL6 in colonic cancer.

Although our data point to IL6 as a mediator of tumor progression, many factors have not been addressed in this study. First of all, transcription is only one of many levels at which activity of various proteins can be regulated. The level of translation, phosphorylation of various kinases and receptor status are of course vital for cytokine activity, as well as the regulation of their downstream transcription factors (38). Our tumor samples were taken from the luminal side of the tumor. Given recent studies showing large intra-tumoral heterogeneity, these samples might not reflect the micro-milieu at the invasive front (39-41). Furthermore, it was recently shown that many genes are activated in the tissue adjacent to a colonic tumor which are not activated in mucosa from healthy individuals (42). In particular, inflammatory cytokines may play an active role in this cross-talk between tumor and normal adjacent tissue (42), and this might have led to bias in our results. Furthermore, the implications of known oncogenic mutations, such as of *KRAS* and v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) on the expression profile and competing risk analysis, were not addressed. Finally since no clinically meaningful cut-off point can be made for the normalized mRNA copy number, we chose to use the Youden index for identification of the optimal

cut-point (23), resulting in the conversion of IL6 expression to a dichotomous variable of high *versus* low expression. This has the advantage of being able to find the value that best discriminates between two stages of the disease (*e.g.* relapse *vs.* non-relapse). The technique does, however, carry a risk of over-fitting the data (5, 43).

In conclusion, *IL6* is up-regulated in tumor tissue at the transcriptional level and is significantly associated with an increased risk of relapse. This might suggest IL6 as a possible target in future anticancer therapy.

Conflicts of Interest

The Authors declare there are no conflicts of interests in regarding article.

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