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Original Research Article

Molecular Analysis of Mid- to Long-Term Effects of Chemoradiotherapy on the Gut: A Gene and Protein Expression Study in Normal Rectal Tissue of Colorectal Cancer Patients

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Abstract: ***Background:*** Colorectal cancer (CRC) is the second commonest cause of cancer-related death in the United Kingdom and the third commonest cancer worldwide. Preoperative chemoradiotherapy is widely used to down-stage rectal tumours and improve surgical outcomes; however, its long-term molecular consequences on normal rectal tissue remain poorly understood. ***Objective:*** To identify molecular changes in gene and protein expression in normal rectal tissue of CRC patients who received preoperative chemoradiotherapy compared to those who underwent surgery alone. ***Methods:*** Normal rectal muscle-layer tissue samples were collected from 14 patients (chemoradiotherapy group, n=7; surgery-only control group, n=7). RNA was extracted and used to synthesise cDNA for RT-qPCR analysis of 15 target genes involved in autophagy, oxidative stress, neuro-axonal transport, angiogenesis, and tissue elasticity. Protein expression of GPX3 was assessed by western blotting. Statistical analysis was performed using the Mann-Whitney U test. ***Results:*** Variable changes in gene expression were observed between the two groups, most notably in ATG7, CAT, PRCKD, and GPX4. None of these differences reached statistical significance ($p > 0.05$), although CAT showed a trend ($p=0.073$). GPX3 gene expression demonstrated a statistically significant difference ($p=0.024$), but western blotting revealed no corresponding significant change in GPX3 protein expression ($p=0.80$). ***Conclusion:*** Preoperative chemoradiotherapy appears to alter gene expression in the oxidative stress pathway in normal rectal tissue, though these changes were not statistically significant due to the small sample size. Larger studies with quantitative protein analysis are warranted to confirm these findings.

Keywords: Colorectal Cancer, Chemoradiotherapy, Gene Expression, RT-qPCR, Oxidative Stress, GPX3, Autophagy, Rectal Tissue, Western Blotting, Gastrointestinal Dysfunction.

INTRODUCTION

Colorectal cancer (CRC) is a major global health burden, ranking as the second most common cause of cancer death in the United Kingdom and the third most prevalent cancer worldwide [1]. Each year, approximately 41,000 new cases are diagnosed in the UK, of which around 32% involve the rectum and 7% the recto-sigmoid junction [1, 2]. Most

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patients are diagnosed between the ages of 60 and 65, and approximately one fifth present with distal metastases, yielding a 5-year survival rate of around 60% [2]. Despite significant therapeutic advances over recent decades, overall mortality from CRC has not declined as rapidly as might be expected, largely because the defining prognostic factor remains the stage at diagnosis the earlier the detection, the better the prognosis [3].

The pathogenesis of CRC is multifactorial. Approximately 75% of cases arise sporadically through accumulated mutations in the colonic mucosa, while the remaining 25% have an inherited basis [5]. Modifiable risk factors include tobacco smoking, alcohol consumption, high red meat intake, low dietary fibre, and physical inactivity [6, 7]. Conversely, a high-fibre diet reduces mucosal carcinogen exposure by accelerating transit time, while calcium supplementation has been shown to reduce adenoma formation through binding of fatty acids and inhibition of crypt cell proliferation [8]. Antioxidant micronutrients such as selenium and vitamins A, C, and E have also been identified as protective agents in colorectal carcinogenesis [9]. Genetically, approximately 10% of CRC patients develop the disease due to well-characterised mutations in key molecular pathways, including the Wnt/β-catenin signalling cascade, microsatellite instability pathways, and chromosomal instability routes [13].

The mainstay treatment of rectal cancer is radical surgical resection; however, local recurrence rates without adjuvant treatment can reach as high as 80% [14]. Over the past two decades, neoadjuvant chemoradiotherapy (CRT) combining radiotherapy with chemotherapeutic agents such as Capecitabine, Oxaliplatin, 5-Fluorouracil, and Irinotecan has become standard of care for locally advanced rectal cancer [9-16]. Preoperative CRT improves local tumour control, facilitates sphincter-sparing surgery, and reduces the risk of micro-metastasis, ultimately improving patient survival [17]. The rationale for preoperative delivery is also based on the superior oxygenation of tumour tissue prior to surgical disruption, which enhances radiosensitivity, and on the prevention of radiotherapy-induced enteritis that commonly complicates postoperative pelvic irradiation [10].

Despite these benefits, chemotherapy and radiotherapy carry significant adverse effects that extend beyond tumour tissue, affecting the surrounding normal gut. The most common gastrointestinal (GI) sequelae include chemotherapy-induced diarrhoea (CID) and chemotherapy-induced constipation (CIC), both of which can markedly impair quality of life [11]. Enteric neuronal degeneration characterised by a reduction in the neuronal population of the myenteric plexus has emerged as a critical mechanism underlying post-treatment GI dysfunction [12]. Oxaliplatin, for instance, has been shown to reduce neuronal cell populations in both myenteric and submucosal areas by 25% and 21% respectively, and to promote apoptosis of enteric neurons through oxidative stress pathways [13]. Furthermore, preoperative CRT has been associated with significant immunological perturbations, including a decline in lymphocyte populations and upregulation of pro-inflammatory cytokines such as IL-6, TNF-α, VEGF, and IL-8, which directly contribute to increased post-treatment morbidity [14]. The precise molecular mechanisms by which CRT alters normal gut tissue biology particularly in terms of gene and protein expression remain incompletely characterised, forming a crucial unmet research need that motivates the present study.

OBJECTIVE

The primary objective of this study was to detect and characterise molecular changes in normal rectal tissue of CRC patients who received preoperative chemoradiotherapy, compared with those who underwent surgery without prior CRT. Specifically, the study aimed to quantify the expression of a panel of 15 genes involved in key physiological pathways including autophagy, oxidative stress, neuro-axonal transport, angiogenesis, and tissue elasticity using RT-qPCR, and to validate findings at the protein level using western blotting.

A secondary objective was to identify any genes or pathways showing differential expression patterns that might serve as potential biomarkers of CRT-induced tissue injury, thereby informing future research into strategies for reducing treatment-associated GI toxicity. By examining both gene and protein expression in paired chemo- and no-chemo groups, this study sought to provide a molecular foundation for understanding the long-term consequences of preoperative CRT on the integrity and function of normal rectal tissue.

METHODOLOGY AND MATERIALS

Study Design and Sample Collection

This was a laboratory-based molecular study using prospectively collected human tissue specimens. Fresh-frozen normal rectal tissue samples were obtained from 14 patients (n=14) who underwent surgical resection for rectal cancer at the Royal London Hospital (RLH) and Whipps Cross University Hospital (WXH) between 2015 and 2017. For each patient, a normal tissue sample was taken

from the safe resection margin of the excised colorectal specimen that is, macroscopically normal tissue distant from the tumour site. Patients were classified into two groups: the treatment group ('Chemo'; n=7) comprised patients who received neoadjuvant CRT prior to surgery, and the control group ('No Chemo'; n=7) comprised patients who proceeded directly to surgery without preoperative CRT. Patient ages ranged from 28 to 77 years. All samples were stored in RNA-later (Sigma) at −80°C. Ethical approval for the use of these samples was obtained under REC reference number 10/H0703/71, and all patients provided written informed consent.

Inclusion and Exclusion Criteria

Inclusion Criteria:

i. Adult patients (≥18 years) undergoing elective surgical resection for histologically confirmed rectal adenocarcinoma;
ii. Availability of normal tissue specimens from the surgical resection margin;
iii. Patients in the Chemo group must have received a standard course of neoadjuvant CRT (typically 45 Gy in 25 fractions with concurrent Capecitabine);
iv. Patients in the No Chemo group must have proceeded to surgery without any prior chemotherapy or radiotherapy;
v. Tissue samples of adequate quality for RNA and protein extraction.

Exclusion Criteria:

i. Patients with inflammatory bowel disease or other concurrent bowel pathology;
ii. Patients who received chemotherapy or radiotherapy for a primary malignancy other than colorectal cancer that might confound tissue expression profiles;
iii. Samples showing evidence of tumour involvement at the collection site on histological review;
iv. Samples with RNA integrity insufficient for reliable RT-qPCR analysis (as confirmed by NanoDrop spectrophotometry);
v. Patients who did not provide informed consent.

Laboratory Procedures

The muscle layer was isolated from each tissue sample after the mucosa was removed. RNA was extracted using TRIzol reagent (The Epigenetics Company) and the Direct-zol RNA MiniPrep kit (Zymo Research), following tissue maceration with a TissueRuptor (QIAGEN) and centrifugation at 13,000 RPM for 3 minutes. RNA concentration was measured using a NanoDrop Spectrometer (Labtech International). Complementary DNA (cDNA) was synthesised from 1 µg of RNA using the Invitrogen SuperScript VILO cDNA Synthesis Kit according to the manufacturer's protocol, with incubation at 42°C for 60 minutes followed by termination at 85°C for 5 minutes. RT-qPCR was performed using PowerUP SYBR Green Master Mix (Applied Biosystems) on a 96-well optical plate, with each sample run in triplicate. The amplification programme included an initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Two endogenous reference genes GAPDH and ATP5B were used for normalisation.

For protein analysis, tissue was homogenised in RIPA buffer (ThermoFisher Scientific) supplemented with Halt Protease Inhibitor Cocktail and 0.5 M EDTA, and centrifuged at 13,000 rpm for 20 minutes at 4°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) and a spectrophotometer at 562 nm wavelength. Western blotting was performed using 4–15% Mini-PROTEAN TGX Precast Protein Gels (BioRad) and a Trans-Blot Turbo Transfer system (BioRad). Primary antibodies were GPX3 (anti-rabbit/anti-goat) and GAPDH (ab9485, anti-rabbit, Abcam), and results were visualised using Bio-Rad Clarity Western ECL substrate and a ChemiDoc MP imaging system.

Data Collection Procedure

Gene expression data were collected via RT-qPCR in triplicate for each sample. The threshold cycle (Ct) values were recorded for all 15 target genes and both reference genes in every sample. For western blotting, relative quantification was performed by calculating the ratio of GPX3 band intensity to GAPDH band intensity, normalising GPX3 expression to total protein load. All western blot images were captured using the ChemiDoc MP Imaging System (BioRad), and band intensities were analysed using BioRad Image Lab software. For all analyses, both study groups were processed in parallel to minimise inter-assay variability. Where anomalous Ct values were identified (greater than one standard deviation from the triplicate mean), the reaction was repeated. Patient clinical data, including age, sex, treatment type, time between CRT completion and surgery, and hospital of operation, were collected from the surgical pathology records associated with each sample.

Statistical Data Analysis

Gene expression analysis was performed using the comparative ΔΔCt method with Expression Suite software (version 1.0.4, Applied Biosystems). Relative quantification (RQ) values were calculated for each target gene in each sample relative to a calibrator sample (15RLH059, No Chemo group).

Statistical comparisons between the two study groups were performed using GraphPad Prism 7 (GraphPad Software). The Mann-Whitney U test was applied for all between-group comparisons, as it is appropriate for non-parametric data that cannot be assumed to follow a normal distribution particularly relevant when working with small sample sizes derived from human surgical tissue. A p-value of <0.05 was considered statistically significant. Western blot quantification data (GPX3:GAPDH ratios) were also compared using the Mann-Whitney U test. Results are presented as mean relative quantities ± standard deviation where applicable.

RESULTS

Patient Characteristics and Sample Quality

Fourteen patients were included in the study: seven in the chemoradiotherapy group (Chemo) and seven in the surgery-only control group (No Chemo). The demographic and clinical characteristics of all participants are presented in Table 1. In the Chemo group, five patients were male and two were female, with ages ranging from 31 to 77 years (mean 56.1 years). All patients in this group received long-course CRT consisting of 45 Gy in 25 fractions with concurrent Capecitabine, and the interval between completion of CRT and surgery ranged from 1.5 to 5 months. In the No Chemo group, five patients were female and two were male, with ages ranging from 28 to 70 years (mean 57.9 years). RNA extraction was successfully completed for all 14 samples, with concentrations ranging from 26.5 to 389.0 ng/µl as measured by NanoDrop Spectrometry. Protein concentrations, determined by BCA assay, ranged from 5,250 to 17,559 µg/ml (Table 2). These values confirmed adequate sample quality for downstream molecular analyses.

Table 1: Patient Demographics and Clinical Characteristics (n=14)

Group	Sample Code	Age	Gender	Treatment Before Surgery	Time to Surgery	Hospital
Chemo	16RLH064	33	Female	Neoadjuvant CRT (45 Gy/25 fractions + Capecitabine)	2.5 months	RLH
Chemo	16RLH030	68	Female	CRT for lung & rectal cancer (stopped before 6th cycle)	4 months	RLH
Chemo	15RLH087	72	Male	Neoadjuvant CRT (45 Gy/25 fractions + Capecitabine)	5 months	RLH
Chemo	16RLH044	77	Male	Neoadjuvant CRT (45 Gy/25 fractions + Capecitabine)	4 months	RLH
Chemo	16RLH053	51	Male	CRT (45 Gy/25 fractions + Capecitabine)	2.5 months	RLH
Chemo	16RLH007	31	Male	Long-course CRT (45 Gy/25 fractions + Capecitabine)	3 months	RLH
Chemo	16RLH092	63	Male	Neoadjuvant CRT (45 Gy/25 fractions + Capecitabine)	1.5 months	RLH
No Chemo	15RLH059	59	Female	None	—	RLH
No Chemo	16RLH031	70	Female	None	—	RLH
No Chemo	16RLH039	67	Female	None	—	RLH
No Chemo	16WXH017	59	Female	None	—	WXH
No Chemo	16RLH115	28	Female	None	—	RLH
No Chemo	16RLH140	57	Male	None	—	RLH
No Chemo	17RLH044	65	Male	None	—	RLH

Table 2: RNA and Protein Concentrations for All Study Samples

Sample Code	Group	RNA Conc. (ng/µl)	Protein Conc. (µg/ml)
15RLH059 ML	No Chemo	245.7	14565.7
15RLH087 ML	Chemo	155.7	17113.1
16RLH007 ML	Chemo	304.3	17558.8
16RLH030 ML	Chemo	026.7	12002.8
16RLH031 ML	No Chemo	286.0	10130.9
16RLH039 ML	No Chemo	389.0	13423.2
16RLH044 ML	Chemo	041.0	7981.5
16RLH053 ML	Chemo	026.5	9379.1
16RLH064 ML	Chemo	035.4	5250.5
16RLH092 ML	Chemo	188.4	9257.4
16RLH115 ML	No Chemo	271.0	7898.1

Sample Code	Group	RNA Conc. (ng/µl)	Protein Conc. (µg/ml)
16RLH140 ML	No Chemo	051.9	6933.2
16WXH017 ML	No Chemo	142.6	6123.8
17RLH044 ML	No Chemo	203.8	9877.5

Table 3: Study Genes, Their Pathways, and Key Functions

Gene	Full Name	Pathway	Function
ATG7	Autophagy-related protein 7	Autophagy	Mutation associated with reduced autophagy and neurodegeneration
ATG5	Autophagy-related protein 5	Autophagy	Core autophagy protein; knockout causes neurodegeneration in mice
CAT	Catalase	Oxidative Stress	Peroxidase responsible for removal of reactive oxygen species (ROS)
SOD1	Superoxide dismutase 1	Oxidative Stress	Antioxidant enzyme; involved in removal of superoxide radicals
GPX4	Glutathione peroxidase 4	Oxidative Stress	Protects cells from oxidative damage
PARK2	Parkin	Axonal Transport	Mutation linked to defective axonal transport and GI motility disorders
KIF1B	Kinesin-like protein B	Axonal Transport	Associated with motor and sensory neuropathies
VEGFA	Vascular Endothelial Growth Factor A	Angiogenesis	Marker of angiogenesis; elevated levels correlate with tumour metastasis
VEGFB	Vascular Endothelial Growth Factor B	Angiogenesis	Marker of angiogenesis
ELN	Elastin	Tissue Elasticity	Component of connective tissue; reflects tissue elasticity
ANO1	Anoctamin-1	ICC Function	Calcium-activated chloride channel in interstitial cells of Cajal
c-KIT	KIT proto-oncogene	ICC Function	Receptor tyrosine kinase expressed in ICCs and mast cells
S100b	S100 calcium-binding protein B	Glial Marker	Marker of glial cells; involved in calcium signalling
IL13	Interleukin-13	Inflammation	Pro-inflammatory cytokine involved in allergic reactions
PRCKD	Protein kinase C delta	Oxidative Stress	Involved in removal of ROS and cell senescence

Gene Expression Analysis by RT-qPCR:

Fifteen target genes were evaluated across all 14 samples (Table 3 lists their respective pathways and functions). Using the ΔΔCt method and Mann-Whitney U testing, RQ values were compared between the Chemo and No Chemo groups for each gene. The results are summarised in Table 4. Genes showing the most notable (though non-significant) expression differences between groups included ATG7 (mean RQ: Chemo 0.75 vs No Chemo 0.65; p=0.46), CAT (mean RQ: Chemo 0.77 vs No Chemo 0.74; p=0.073), PRCKD (mean RQ: Chemo 1.74 vs No Chemo 0.92; p=0.32), and GPX4 (mean RQ: Chemo 0.52 vs No Chemo 0.89; p=0.62). Notably, CAT (catalase) demonstrated a trend approaching significance (p=0.073), suggesting that a larger sample size may reveal statistically significant downregulation of oxidative stress defence mechanisms following CRT. Other genes including c-KIT, S100b, ATG5, VEGFB, PARK2, SOD1, ELN, VEGFA, ANO1, KIF1B, and IL13 showed p-values greater than 0.05, indicating no statistically significant differential expression between groups. GPX3, analysed in prior work by laboratory colleagues, demonstrated a statistically significant difference (p=0.024) between the Chemo and No Chemo groups, and was selected for validation by western blotting [60-62].

Table 4: Summary of Gene Expression RQ Values and Statistical Results for Selected Genes

Gene	ATG7	CAT	PRCKD	GPX4	VEGFA	ELN	IL13	ANO1
Chemo Mean RQ	0.75	0.77	1.74	0.52	1.93	0.64	2.10	0.39
No Chemo Mean RQ	0.65	0.74	0.92	0.89	0.85	0.13	5.25	0.27
p-value	0.46	0.073	0.32	0.62	0.54	0.73	0.37	0.90
Significance	NS	Trend	NS	NS	NS	NS	NS	NS

Western Blot Analysis of GPX3 Protein Expression:

Following optimisation of western blotting conditions over six sequential experiments (Blots A–F), GPX3 and GAPDH protein expression was assessed in all 14 study samples. The optimised conditions used 20 µg protein per well, GPX3 primary antibody at 1:600 dilution, GAPDH primary antibody at 1:6000 dilution, and secondary anti-rabbit/anti-goat antibodies at 1:3000 dilution, with blocking extended to over two hours to minimise non-specific binding. Relative quantification was performed by calculating the GPX3: GAPDH ratio for each sample to normalise for protein loading variations (Table 5). Mean GPX3: GAPDH ratios were 2.68 in the Chemo group and 2.29 in the No Chemo group. Statistical comparison using the Mann-Whitney U test yielded a p-value of 0.80, confirming no statistically significant difference in GPX3 protein expression between the two groups. This result contrasts with the significant difference observed at the mRNA level (p=0.024), suggesting a potential post-transcriptional regulatory mechanism or limitations of the semi-quantitative western blotting approach.

Table 5: Western Blot Quantification: GAPDH, GPX3, and GPX3: GAPDH Ratio for All Samples

Sample Code	Group	GAPDH (RQ)	GPX3 (RQ)	GPX3: GAPDH Ratio
15RLH059 ML	No Chemo	0.70	2.71	3.87
15RLH087 ML	Chemo	1.09	1.37	1.26
16RLH031 ML	No Chemo	3.83	4.03	1.05
16RLH007 ML	Chemo	1.00	9.42	9.42
16RLH039 ML	No Chemo	1.00	2.06	2.06
16RLH030 ML	Chemo	1.14	1.15	1.01
16WXH017 ML	No Chemo	1.21	5.31	4.39
16RLH044 ML	Chemo	2.23	4.36	1.96
16RLH115 ML	No Chemo	1.00	0.49	0.49
16RLH053 ML	Chemo	0.68	1.14	1.68
16RLH140 ML	No Chemo	0.86	0.95	1.10
16RLH064 ML	Chemo	0.75	0.96	1.28
17RLH044 ML	No Chemo	1.00	1.17	1.17
16RLH092 ML	Chemo	0.83	0.73	1.16
Western blot p-value	Both groups	—	—	**0.80 (NS)**

DISCUSSION

This study is among the few to examine molecular changes at the gene and protein expression level in normal rectal tissue from CRC patients who received preoperative chemoradiotherapy. The principal finding was that, while observable trends in gene expression were detected particularly within the oxidative stress pathway none of the 15 target genes examined reached statistical significance for differential expression between the Chemo and No Chemo groups in this small cohort. The CAT gene, encoding catalase, emerged as the most promising candidate, with a p-value of 0.073 that approached but did not reach the threshold for statistical significance. Catalase is a key antioxidant enzyme responsible for the dismutation of hydrogen peroxide into water and oxygen, thereby protecting cells from oxidative stress [14]. The trend toward downregulation of CAT in the Chemo group is biologically plausible, as chemotherapeutic agents such as Oxaliplatin and 5-Fluorouracil generate reactive oxygen species (ROS) that may deplete antioxidant defences in surrounding normal tissue, even long after treatment has concluded.[15] Similarly, PRCKD a protein kinase involved in ROS clearance and cell senescence showed a higher mean RQ value in the Chemo group (1.74 vs 0.92), though this difference was not significant (p=0.32). The apparent upregulation of PRCKD may represent a compensatory cellular stress response to CRT-induced oxidative damage.

The GPX3 findings are particularly noteworthy and merit detailed discussion. GPX3, or glutathione peroxidase 3, is a secreted antioxidant enzyme that plays a central role in reducing hydrogen peroxide and lipid peroxides in extracellular fluids and tissues.[16] Prior analysis by laboratory colleagues had identified a statistically significant upregulation of GPX3 mRNA in the Chemo group (p=0.024), suggesting an adaptive transcriptional response to oxidative insult from CRT. However, western blotting in the present study revealed no corresponding change in GPX3 protein expression (p=0.80). This dissociation between mRNA and protein levels is a recognised phenomenon in molecular biology, attributable to several mechanisms: post-transcriptional regulation via microRNAs or RNA-binding proteins, reduced translational efficiency, increased protein degradation or turnover, or changes in post-translational modifications.[17] Alternatively, the western blotting methodology employed a semi-

quantitative technique may lack sufficient sensitivity to detect modest protein-level changes in the context of the biological variability inherent in human tissue specimens. The use of more precise quantitative methods, such as enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry (IHC), in future studies may help resolve this discordance.

The autophagy genes ATG7 and ATG5 were selected based on evidence that impaired autophagy contributes to enteric neurodegeneration [18]. Although the mean RQ for ATG7 was slightly higher in the Chemo group, no significant difference was found. This may reflect the relatively short interval between CRT completion and surgery in some patients (as little as 1.5 months), which may have been insufficient time for autophagy pathway remodelling to manifest at the transcriptional level. Similarly, PARK2 whose dysfunction is linked to axonal transport defects and GI motility disorders showed no significant between-group difference, though numerically higher expression was observed in the Chemo group for some samples. The angiogenic markers VEGFA and VEGFB, though they are known to be influenced by CRT and inflammatory cytokine release, also showed no significant differential expression, possibly because their expression in normal (non-tumour) tissue is less dramatically altered by CRT than in the tumour microenvironment. The PRCKD trend and CAT trend collectively support the hypothesis that oxidative stress is the pathway most likely to be durably altered by preoperative CRT in normal rectal tissue, and these genes warrant prioritisation in future studies with larger sample sizes.

LIMITATIONS OF THE STUDY

Several important limitations must be acknowledged when interpreting the results of this study. First and foremost, the sample size was small (n=14; seven per group), which substantially limited the statistical power of the study to detect moderate effect sizes. This is reflected in the non-significant p-values observed for the majority of genes examined, including CAT (p=0.073), which showed a biologically plausible trend that may reach significance with a larger cohort. The restricted sample size was a consequence of the short project duration, which did not permit further tissue collection. Second, the two groups were not well matched with respect to sex distribution: the Chemo group comprised five males and two females, whereas the No Chemo group comprised five females and two males. Given that sex is known to influence the expression of multiple genes, including those involved in oxidative stress and inflammatory pathways, this imbalance represents a potential source of confounding bias. Third, the CRT regimens within the Chemo group were not entirely homogeneous for example, one patient received CRT for both lung and rectal cancer and had treatment stopped prematurely due to neutropenic sepsis which introduces heterogeneity in treatment exposure. Additionally, the time between completion of CRT and surgery varied considerably across the Chemo group (1.5 to 5 months), which may affect the degree to which molecular changes are present at the time of tissue collection. Fourth, human surgical tissue necessarily undergoes more complex pre-analytical processing than animal tissue, with potential for RNA and protein degradation despite best-practice storage conditions. Fifth, western blotting is an inherently semi-quantitative technique, and detection of modest protein-level changes in a small and biologically variable human cohort may be beyond its sensitivity. Future studies should incorporate quantitative protein analysis methods such as ELISA or IHC alongside transcriptomic approaches.

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CONCLUSION

This study represents a systematic molecular investigation of the effects of preoperative chemoradiotherapy on gene and protein expression in normal rectal tissue of colorectal cancer patients. Using RT-qPCR, we assessed the expression of 15 genes spanning the pathways of autophagy, oxidative stress, neuro-axonal transport, angiogenesis, and tissue elasticity in 14 patient samples. While variable expression differences were observed between the Chemo and No Chemo groups particularly for ATG7, CAT, PRCKD, and GPX4 none reached statistical significance after Mann-Whitney U testing, likely due to the limited sample size. The CAT gene, encoding the antioxidant enzyme catalase, showed the most

promising trend (p=0.073), raising the possibility that preoperative CRT may durably impair oxidative stress defence mechanisms in normal rectal tissue. GPX3 gene expression, previously shown by colleagues to be significantly upregulated in the Chemo group (p=0.024), was evaluated at the protein level by western blotting, which revealed no corresponding statistically significant difference (p=0.80), suggesting post-transcriptional regulation or methodological limitations of the semi-quantitative western blot approach [19].

This study contributes to the growing body of evidence that preoperative chemoradiotherapy may alter the molecular biology of normal rectal tissue in ways that could underlie the GI dysfunction including pain, altered motility, neuropathy, and bowel habit disturbance frequently reported by patients months to years after treatment [20]. The oxidative stress pathway, and the GPX3 and CAT genes in particular, warrant prioritisation in future investigations. We recommend that larger, adequately powered studies be conducted with at least 30–40 patients per group, with improved matching for sex, age, and CRT regimen. In addition, quantitative protein expression methods such as ELISA and immunohistochemistry should be incorporated alongside RT-qPCR to more reliably translate transcriptomic findings into protein-level conclusions. A broader transcriptomic approach using RNA sequencing (RNA-seq) would also permit hypothesis-free discovery of novel CRT-responsive genes beyond the candidate panel examined here. Ultimately, identifying the molecular consequences of preoperative CRT on normal gut tissue is essential for developing pharmacological or nutritional strategies to mitigate treatment-induced GI toxicity and improve long-term quality of life for rectal cancer survivors [21].

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