The role of Double Strand Break Repair, Translesion Synthesis and Interstrand Crosslinks in Colorectal Cancer progression – clinicopathological data and survival.

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Abstract

Abstract Aim: To evaluate the prognostic value of molecular modulation of double strand break (DSBR) - XRCC2 and XRCC5 - core components; DNA damage tolerance/translesion synthesis (DDT/TLS) - POLH, POLK and POLQ – and, finally, of interstrand crosslink repair (ICLR) - DCLRE1A – pathways in sporadic colorectal cancer (CRC) progression. Method: Tumour specimens and matched health mucosal tissues from 47 patients with CRC who underwent surgery were assessed for gene expression of XRCC2, XRCC5, POLH, POLK, POLQ and DCLRE1A by qRT-PCR; protein expression of Polk, Ku80, p53, Ki67 and mismatch repair MLH1 and MSH2 components were assessed by immunohistochemistry (IHC); CpG island promoter methylation of XRCC5, POLH, POLK, POLQ and DCLRE1A was performed. Uni and multivariate analyses were employed to determine associations with clinicopathological features and prognostic value of molecular data. Results: Neoplastic tissues exhibited induction of POLK (p<0.001) and DCLRE1A (p<0.001) expression and low expression of POLH (p<0.001) and POLQ (p<0.001) in comparison to healthy paired mucosa. Low expression of POLH was associated to mucinous histology and T1-T2 tumors (p=0.038); low tumour expression of POLK was associated to distant metastases (p=0.042). POLK promoter methylation was associated to early stages CRC (p=0.011) and POLH promoter methylation to high grade tumors (p=0.023). CRC harbouring POLK promoter methylation exhibited better disease free survival (DFS) (p=0.005). Conclusion: This study mainly demonstrated that low expression or unmethylated POLH and POLK were related to worse biological behavior tumors. However, POLK methylated was associated with better DFS. POLK and POLH are potential prognostic biomarkers in CRC.

1. Background

Colorectal cancer (CRC) is considered the third major cause of cancer-related death worldwide (1-3). Survival rates and therapeutic decisions for CRC patients essentially depend on pathology-related staging following the tumour-node-metastasis (TNM) classification (4). However, despite modifications to improve prognostic staging, this algorithm still fails to predict recurrence and survival after resection for stage II and III CRC patients, resulting in heterogeneous and controversial oncological outcomes (5).

In the pursuit for eliminating TNM inconsistencies, CRC molecular complexity and its heterogeneous clinical presentations have been leading to the research of novel prognostic and predictive biomarkers, including DNA repair components. For example, 15% of sporadic CRC patients which harbour DNA mismatch repair (MMR) system defects and, consequently, microsatellite instability (MSI) (6), have better stage-adjusted survival and reduced likelihood of metastasis when compared to microsatellite stable (MSS) tumors (7,8). Regrettably, MSI has several limitations that restrict its use as a practical prognostic factor across all stages of CRC, as its clinical value is restricted to stage II CRC, where adjuvant chemotherapy is not recommended (9).

Nevertheless, associations of DNA damage and imbalances in other pathways engaged in their repair with CRC risk, progression, response to therapy and prognosis have been widely reported. We and others recently reviewed that disturbances in gene and/or protein expression of DNA damage response sensors and effectors - including double strand break repair (DSBR), DNA damage tolerance/translesion synthesis (DDT/TLS) and interstrand crosslink repair (ICLR) pathways - have minimal association with clinicopathological features and response to therapy in CRC (10,11). Despite the lack of a definitive evidence so far, a plethora of reports have been suggesting an intersection between CRC and DNA repair systems, which may be mediated by MMR defects (by inducing other somatic mutations that disrupt DNA repair mechanisms) or not (12).

Double strand breaks (DSBR) are the most critical type of genotoxic stress and its repair is a central cellular mechanism to preserve genomic stability (13). DSBs are processed by homologous recombination (HR) or classical nonhomologous end joining (NHEJ) DNA repair pathways, and disruptions of these pathways favours the accumulation of damage in rapidly dividing cells, leading to mutagenesis or apoptosis (14). Since DSBs result in the loss of integrity of both complementary strands, proficiency of error-prone repair are required. However, loss of genetic information and genomic instability arise are immediate consequences in order to guarantee cell survival. DNA damage tolerance (DDT) mechanisms are mediated by Y-family translesion DNA polymerases (such as pol κ, pol η and pol θ), which bypass DNA adducts, imbalanced dNTP pools and unusual template structures. As a consequence, in order to impede fork collapse and apoptosis due to unrepaired DSB,
translesion DNA polymerases induce mutation (15,16). So far, although a number of investigations have focused on the role of MMR, NER and BER genes in CRC, much less studies have evaluated DSBR (Double strand break repair), DDT/TLS and ICLR roles from the perspective of expression characteristics and prognostic roles in CRC (10,17-20).

Thus, since tumour heterogeneity and genomic instability are hallmarks of CRC, to pinpoint a role for DSBR, DDT/TLS and ICLR may offer a better understanding of these features. Finally, alterations in gene/protein expression within DSBR, DDT/TLS and ICL components could affect the response to chemotherapy and, ultimately, the overall survival of these patients. Thus, we aimed to evaluate the prognostic role of molecular modulation of key DSBR, DDT/TLS and ICL repair components in sporadic CRC patients.

2. Methods

2.1. Patients

A total of 47 CRC patients who underwent surgical treatment between 2013 and 2015 at Irmandade Santa Casa de Misericórdia de Porto Alegre Hospital were included in this study. Patients who had received neoadjuvant treatment and with a family history of hereditary CRC were excluded. Clinical data for each patient comprised age, sex, preoperative carcinoembryonic antigen (CEA) levels and chemotherapy regimen completed. Pathological data comprised: tumor site, histology, tumor grade, presence of lymph vascular and perineural invasion and staging (according to 8th edition of AJCC/UICC) (21).

2.2. Tumor samples

Fresh tissue specimens comprising tumour tissues (with at least 70% of neoplastic cells) and adjacent normal tumour-free regions (>10 cm distance from tumour) of primary sporadic CRC were collected and assessed for gene expression, gene promoter methylation and BRAFV600 mutation status. Formalin-fixed paraffin-embedded CRC samples were assed for protein expression.

2.3. Quantitative reverse transcription PCR (RT-qPCR)

Gene expression of XRCC2 and XRCC5 (DSBR), POLH, POLK and POLQ (DDT/TLS), DCLRE1A (ICL repair) and MLH1 and MSH2 (MMR) was carried out in colorectal tumors and healthy paired tissues by RT2 Profiler™ PCR Array (SABiosciences/Qiagen). RNA extraction and cDNA synthesis were performed using RNeasy Mini Kit and RT2 PCR Array First Strand Kit (SABiosciences/Qiagen), respectively. Catalogued PCR primers were used. Reaction was prepared using RT2 SYBR-Green/Rox PCR Master Mix (SABiosciences/Qiagen) under standard amplification conditions. Data analysis was based on the 2−ΔΔCq method (Livak et al, 2001) with normalization of raw data to two housekeeping genes (EIF2B and PPIA). Median fold change (Log2(neoplastic tissue/normal tissue)) for each gene was used to categorize tumors into high or low expressors.

2.4. Methylation PCR Analysis

Methylation status of CpG islands of five genes (XRCC5, POLH, POLK, DCLRE1A) was performed by methylation-sensitive restriction qPCR analysis using EpiTect Methyl II PCR assay (Sa Biosciences/Qiagen). Digested DNA was obtained with EpiTect Methyl II DNA restriction kit (Sa Biosciences/Qiagen, #335452) and used as template for qPCR Assay using RT2 SYBR® Green qPCR Mastermix (Sa Biosciences/Qiagen) under standard amplification conditions. Catalogued Epitect II Methyl PCR primers used were as follows: POLH (EPHS5112501-1A); POLK (EPHS511608-1A); XRCC5 (EPHS108851-1A) and
DCLRE1A (EPHS101928-1A) which were all purchased from Qiagen. Gene promoter methylation status was classified into unmethylated (<5%) and methylated (>5%).

2.5. Immunohistochemistry (IHC)

IHC for MLH1, MSH2, XRCC5 (Ku80), Polk, p53 and ki67 was carried out according to MacDonald et al. (22). The sections were incubated with the following primary antibodies, all purchased from Abcam: anti-MLH1 (1:100), anti-MSH2 (1:200), anti-XRCC5 (1:200), anti-DNA Polymerase Kappa (1:300), anti-p53 (1:250) and anti-Ki67 (1:100) and then incubated with appropriate secondary antibodies (Spring). Diaminobenzidine (DAB) was used as chromogen and the sections were counterstained with haematoxylin. Five hot spot fields containing at least 200 cells were captured and the positive cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD). Protein expression was evaluated using QuickScore (QS) and two observers scored all samples independently and blinded (22).

2.6. **BRAF**\(^{V600E}\) mutation analysis

The exon 15 of the **BRAF** gene was amplified by polymerase chain reaction through Platinum Taq DNA Polymerase Kit\(^ {\text{®}}\) (Invitrogen by Life technologies) and appropriate primer pair: forward 5′-CTTCATAATGCTTGCTCTGATAGGA-3′ and reverse 5′-CAGGGCCAAAAATTTAATCAGTGGA-3′. Sanger sequencing reaction was performed with the BigDye Terminator V3.1 Cycle Sequencing Kit (Life Technologies).

2.7. Statistical Analysis

Gene expression means between normal and neoplastic tissue were compared using independent sample *t*-Student or Mann-Whitney tests after Kolmogorov-Smirnov tests. For correlation and survival analyses, continuous variables were dichotomized as previously stated. Association between molecular and clinical features were assessed by Chi-square (\(\chi^2\)) test and Fisher’s exact test. Kaplan–Meier analysis, with log-rank test was used to determine the overall survival (OS) and disease-free survival (DFS). Cox regression analysis for independent correlation of individual parameters with patients’ OS and DFS. Statistical analysis was performed using SPSS software version 22.0.0. A two-sided test with \(p<0.05\) was considered statistically significant.

2.8. Availability of data and materials

Any supplementary supporting data relating details of clinical and pathological analysis are available upon request from the corresponding author and can be found in the electronic medical record system of Irmandade of Santa Casa of Misericórdia of Porto Alegre.

3. Results

3.1. Characteristics of CRC patients

The main patient characteristics are shown in Table 1. A total of 47 patients were included in the final statistical analysis.

3.2. Molecular changes in DSBR, ICLR and DDT/TLS in CRC tumors
**3.3. Associations of DSBR, ICL repair and DDT/TLS key components with clinicopathological and molecular features of CRC patients**

Tumors with low expression of \( \text{POLH} \) exhibited mucinous histology (\( p=0.05 \)), but smaller invasive depth (\( p=0.038 \)). Low tumour expression of \( \text{POLK} \) was associated with presence of distant metastases (\( p=0.042 \)). Promoter methylation of \( \text{POLK} \) was associated to smaller invasive depth (\( p=0.011 \)) and methylation of \( \text{POLH} \) to well differentiated tumors (0.023). In addition, \( \text{POLK} \) promoter methylation was associated with tumors with high Ki67 contents (\( p=0.036 \)) and low expression of DCLRE1A was associated with tumors with low Ki67 contents (\( p=0.042 \)) (Table 3). Overexpression of \( \text{POLK} \) was associated with tumors expressing MLH1 (\( p=0.042 \)) (Supplementary Tables S2, S3, S4). High tumor protein expression of MSH2 was associated with absence of distant metastases (\( p=0.035 \)), while overexpression of Ki67 with lower preoperative CEA levels (\( p=0.042 \)) (Table 4). More detailed associations between clinicopathological features and molecular data are provided in supplementary tables S5, S6 and S7.

**3.4. Prognostic value of DNA repair component modulation in patients with CRC**

Kaplan-Meier's survival analyses indicated that patients whose tumors harboured \( \text{POLK} \) promotermethylation presented better DFS (\( p=0.005 \)). Statistical tendencies were found for \( \text{POLK} \) promoter methylation and better OS (\( p=0.053 \)); overexpression of \( \text{POLQ} \) and better OS (\( p=0.076 \)) and DFS (\( p=0.068 \)); overexpression of XRCC5 expression and better survival (\( p=0.057 \)) (Figure 2). Other survival analyses are provided in supplementary figures S1, S2, S3, S4, S5, S6.

Univariate Cox regression analysis showed prognostic significance of N+, M+, lymph vascular invasion, perineural invasion, stages III and IV, low tumour \( \text{POLQ} \) gene expression, tumour unmethylated \( \text{POLK} \) gene promoter, and high XRCC5/Ku80 protein expression on overall survival. Unfortunately, these associations were not confirmed in our multivariate analysis (Table 5). For DFS, univariate analysis showed that male, preoperative CEA >5ng/mL, N+, lymph vascular invasion, perineural invasion, chemotherapy realized stages III, low expression of \( \text{POLQ} \), unmethylated \( \text{POLK} \) promoter and low or absent MSH2 protein expression were predictors of poor DFS, but not confirmed in multivariate analysis (Table 6).

**4. Discussion**
A growing body of evidence has been strengthening the need for more accurate tools to minimize the inconsistencies of the TNM staging system as a prognostic and therapeutic guidance for CRC patients. Contribution of aberrant DNA repair and DNA damage response in carcinogenesis and its response to treatments is notoriously established. Furthermore, the study of DNA repair components as oncological molecular markers have already reached clinical practice, including MGMT promoter methylation status (glioblastoma) (23), BRCA1/2 mutation (breast and ovarian cancer) (24-26) and MMR deficiency (colorectal, endometrial, ovarian and other cancer types) (27-32).

POLK and POLH encode members of DNA polymerase type-Y family of proteins, Pol κ and Pol η, respectively. Variations in expression or activity of Y-family DNA polymerases could possibly produce TLS pathway imbalance and, therefore, mutagenesis (33). However, the magnitude to which these alterations are oncogenic drivers or whether it impacts clinical outcomes is still unknown.

In our study, we found upregulation of POLK and downregulation of POLH in neoplastic tissues in comparison to paired normal tissues. The oncological relevance of pol κ and pol η in cancer is most firmly established concerning response to treatment. Upregulation of pol κ confers resistance to temozolomide in glioblastoma (34,35), and upregulation of pol η to platinum drugs in HNSCC, lung, gastric adenocarcinomas and ovarian cancers (36-38). Contrary to our results, low levels of POLK were previously observed in CRC (39,40). Conversely, others reported increase of pol κ expression in brain and lung cancers (41,42).

Low expression of POLH and POLK were found in tumors with mucinous histology and vascular metastasis, although in early stages of development. POLK promoter methylation was strongly associated with better DFS. Conversely, unmethylated POLH and POLK promoters were associated with more advanced and poorly differentiated tumors.

Despite finding more aggressive colorectal tumors harbouring high POLK levels, this fact was not a predictor of DFS and OS. In counterpart, POLK promoter methylation was associated with better DFS, but we could not confirm it as an independent prognostic factor. Surprisingly, despite POLK gene and protein expression were associated (p=0.001), such connection was not found between POLK expression and promoter methylation. It may indicate that promoter methylation is not the main mechanism regulating POLK transcription.

On its turn, POLQ (A-family) encodes pol θ DNA polymerase and is a component of an end-joining pathway for DSB. Defects in POLQ lead to double-strand break-mediated genomic instability (43). Differently from previous reports (44,45), our patients presented downregulation of POLQ, but no association with clinicopathological parameters was detected. Overexpression of pol θ has been implicated as an indicator of poor prognosis and decreased survival in breast, colorectal and NSCLC (45-47). Nevertheless, to date, POLQ overexpression presented a weak association for better OS (p=0.076) and DFS (p=0.068).

DSBR (represented in our study by XRCC2 and XRCC5) did not present alterations in gene expression between neoplastic and normal tissues nor associations with clinicopathological variables in CRC patients. To date, low XRCC5/Ku80 expression suggested poor OS in CRC patients included in our study (p=0.057). XRCC5/Ku80 is associated with risk of development of several tumors (48,49) and its activity may inhibit or promote the carcinogenic process, depending on the tumor type (50). In CRC, downregulation of XRCC5 and/or its protein product (Ku80) was associated with poor prognosis and better response to radiotherapy (10,51-53). Regarding ICLR, despite DCLRE1A being upregulated in neoplastic tissues, it did not present associations with clinical features or survival in this study. DCLRE1A encodes SNM1A nuclease, and it has been linked to an important function in human ICL repair (54).

Finally, despite its sample size limitation, to the best of our knowledge, our study is one the few to report associations between POLK, POLH modulation and clinical features and prognosis of CRC patients. Furthermore, we believe that this is the first study to evaluate DCLRE1A gene expression and promoter methylation in colorectal tumors.

5. Conclusion
Components of the pathways involved in DSBR, DDT/TLS and ICLR are a new horizon in the DNA repair pathway discussion. There are few reports about these and the influence on clinico-pathological features and survival is still a big question. This study mainly revealed that low expression or unmethylated \textit{POLH} and \textit{POLK} were related do worse tumors. In this context, \textit{POLK} methylated was strongly associated with better DFS with a propensity for a better OS. On the other hand, another interesting finding is the high score of XRCC5/Ku80 in IHQ suggests a better survival. Finally, even with little information about these pathways in relation to their clinicopathological influence and survival, this knowledge may help to clarify the utility of specific adjuvant treatments based on the individual's genotype in the future.

**Abbreviations**

- CEA: carcinoembryonic antigen
- CRC: colorectal cancer
- DDT/TLS: DNA damage tolerance/translesion synthesis
- DFS: disease free survival
- DSBR: double strand break
- HR: homologous recombination
- ICLR: interstrand crosslink
- IHC: immunohistochemistry
- MSI: microsatellite instability
- MSS: microsatellite stable
- OS: overall survival
- NHEJ: nonhomologous end joining
- TNM: tumour-node-metastasis

**Declarations**

Ethics and consent to participate: This study was approved by the Institutional Review Board of Irmandade Santa Casa de Misericórdia de Porto Alegre and Universidade Federal de Ciências da Saúde de Porto Alegre (09761613.4.0000.5345, 34145614.9.0000.5335, 58299916.3.00005335, 58299916.3.3001.5345). The informed written consent was obtained from all participants according national regulations.

Competing Interests: No commercial interest involved in this study.

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Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors' contributions: GAL and NML conceived of the presented idea. GAL and NML developed the theory. GAL, HCG, DBA performed the experiment. GAL verified the analytical methods. JS and ANK supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.
Consent for publication: the authors agree with the publication

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Table

Table 1: Clinicopathological features of patients with CRC included in this study (n=47)

| Variable                      | n(%) |
|-------------------------------|------|
| **Total cases**               | 47   |
| **Age (mean±SD)**             | 67.77 ± 11.49 |
| **Age (year)**                |      |
| ≤ 65                          | 19 (40.4) |
| > 65                          | 28 (59.6) |
| **Gender**                    |      |
| Female                        | 28 (59.6) |
| Male                          | 19 (40.4) |
| **Preoperative CEA ng/mL**    |      |
| ≤ 5                           | 25 (53.2) |
| > 5                           | 22 (46.8) |
| **Tumour location**           |      |
| Right side                    | 17 (36.1) |
| Left side                     | 30 (63.9) |
| **Histology**                 |      |
| Well or moderately differentiated| 19 (40.4) |
| Poorly differentiated         | 28 (59.6) |
| **Mucinous**                  |      |
| No                            | 43 (91.5) |
| Yes                           | 4 (8.5) |
| **Tumour invasive depth**     |      |
| 1-2                           | 12 (25.5) |
| 3-4                           | 35 (74.5) |
| **Lymph node status**         |      |
| N-                            | 24 (51.1) |
| N+                            | 23 (48.9) |
| **Vascular metastasis**       |      |
| No                            | 40 (85.1) |
| Yes                           | 7 (14.9) |
| **Lymph vascular invasion**   |      |
| No                            | 23 (48.9) |
| Yes                           | 24 (51.1) |
| **Perineural invasion**       |      |
| No                            | 20 (42.6) |
| Yes                           | 27 (54.4) |
| **Chemotherapy**              |      |
| No                            | 21 (45.7) |
| Yes                           | 25 (54.3) |
| **TNM stage**                 |      |
| I-II                          | 23 (48.9) |
| III-IV                        | 24 (51.1) |
| **Relapse**                   |      |
| No                            | 32 (80) |
| Yes                           | 6 (20) |
Table 2: Protein levels (Polk, Ku80, Mlh1, Msh2, Ki67 and p53), methylation (POLH, POLK, XRCC5 and DCLRE1A) and BRAF mutation in neoplastic tissue.

| Variable | n (%) | n (%) |
|----------|-------|-------|
| **Methylation** | Unmethylated | Methylated |
| **POLH** | 20 (57.1) | 15 (42.9) |
| **POLK** | 19 (52.8) | 17 (47.2) |
| **XRCC5** | 25 (67.5) | 12 (32.5) |
| **DCLRE1A** | 17 (58.6) | 12 (41.4) |
| **IHC** | | |
| XRCC5/Ku80 | 22 (46.8) | 25 (53.2) |
| Pol k | 21 (44.6) | 26 (55.4) |
| MLH1 | 7 (14.9) | 40 (85.1) |
| MSH2 | 6 (12.7) | 41 (87.3) |
| p53 | 32 (68.1) | 15 (31.9) |
| Ki67 | 7 (14.9) | 40 (85.1) |

Table 3: Correlations between DNA gene repair expression of POLH, POLK, POLQ, XRCC and XRCC5, methylation of POLH, POLK, XRCC5 and DCLRE1A, BRAF, IHC of XRCC5, Pol k, MLH1, MHS2, p53 and Ki67 scores with clinical parameters.

| Variable | GENE EXPRESSION | METHYLATION | IHC |
|----------|----------------|-------------|-----|
| **POLH** | POLK POLQ XRCC2 XRCC5 DCLRE1A | | |
| Age, Y | 0.548 0.095 0.452 0.452 0.143 0.318 | 0.347 0.419 0.176 0.261 | 0.408 0.115 0.285 0.169 0.363 0.6 |
| Gender | 0.318 0.143 0.452 0.095 0.452 0.238 | 0.224 0.603 0.228 0.314 | 0.169 0.117 0.6 0.535 0.16 0.285 |
| CEA, ng/mL | 0.075 0.340 0.340 0.580 0.580 0.207 | 0.127 0.175 0.165 0.602 | 0.503 0.413 0.447 0.092 0.393 0.042 |
| Tumour location | 0.544 0.092 0.310 0.237 0.310 0.544 | 0.205 0.627 0.102 0.398 | 0.391 0.123 0.499 0.372 0.241 0.499 |
| Histology | 0.452 0.548 0.548 0.318 0.548 0.548 | 0.023 0.341 0.051 0.630 | 0.169 0.117 0.133 0.209 0.363 0.4 |
| Mucinous | 0.050 0.288 0.679 0.288 0.679 0.679 | 0.457 0.562 0.704 0.452 | 0.257 0.61 0.512 0.568 0.381 0.488 |
| T | 0.038 0.402 0.402 0.337 0.598 0.337 | 0.168 0.011 0.311 0.579 | 0.228 0.104 0.417 0.151 0.415 0.243 |
| N | 0.557 0.443 0.234 0.095 0.443 0.557 | 0.500 0.209 0.243 0.149 | 0.562 0.448 0.525 0.646 0.46 0.525 |
| M | 0.525 **0.042** 0.190 0.190 0.475 0.525 | 0.251 0.072 0.350 0.650 | 0.426 0.377 0.057 **0.035** 0.054 0.296 |
| Lymph vascular invasion | 0.557 0.443 0.443 0.095 0.443 0.557 | 0.253 0.324 0.121 0.252 | 0.438 0.237 0.525 0.646 0.234 0.525 |
| Perineural invasion | 0.433 0.337 0.567 0.337 0.337 0.433 | 0.485 0.286 0.401 0.615 | 0.467 0.064 0.648 0.201 0.532 0.352 |
| Chemotherapy | 0.194 0.18 0.374 0.607 0.374 0.5 | 0.163 0.132 0.502 0.37 | 0.165 0.48 0.601 0.422 0.198 0.399 |
| TNM stage | 0.230 0.562 0.155 0.334 0.155 0.562 | 0.363 0.121 0.407 0.252 | 0.241 0.241 0.574 0.397 0.381 0.574 |

Table 4: Correlations between DNA repair gene expression, methylation and IHC with BRAF mutation and IHC for MLH1, MSH2, p53 and Ki67
| Variable | GENE EXPRESSION | METHYLATION | IHC |
|----------|----------------|-------------|-----|
|          | POLH POLK POLQ XRCC2 XRCC5 DCLRE1A | PolH PolK XRCC5 DCLRE1A | XRCC5 Pol k |
| BRAF     | 0.484 0.19 0.484 0.109 0.125 0.125 | 0.271 0.438 0.704 0.726 | 0.082 0.549 |
| MLH1     | 0.226 0.042 0.475 0.226 0.525 0.226 | 0.386 0.13 0.47 0.65 | 0.129 0.265 |
| MSH2     | 0.646 0.085 0.312 0.646 0.646 0.354 | 0.543 0.58 0.609 0.212 | 0.235 0.603 |
| p53      | 0.124 0.3 0.54 0.54 0.234 0.46 | 0.564 0.627 0.609 0.267 | 0.451 0.177 |
| Ki67     | 0.19 0.525 0.226 0.475 0.19 0.042 | 0.655 0.036 0.609 0.452 | 0.623 0.574 |

Table 5: Overall survival calculated with univariate and multivariate cox regression tests.
| Variable                        | Univariate analysis | Multivariate analysis |
|--------------------------------|---------------------|-----------------------|
|                                | HR                  | CI (95%)              | p value   | HR                  | CI (95%)              | p value   |
| Age > 65 years                 | 1.708               | (0.526-5.547)         | 0.373     |                     |                       |           |
| Male sex                       | 1.917               | (0.644-5.71)          | 0.242     |                     |                       |           |
| CEA > 5                        | 1.234               | (0.404-3.774)         | 0.712     |                     |                       |           |
| Left side                      | 1.309               | (0.403-4.253)         | 0.654     |                     |                       |           |
| Poor differenciated            | 1.534               | (0.472-4.982)         | 0.477     |                     |                       |           |
| Mucinous                       | 2.081               | (0.461-9.394)         | 0.341     |                     |                       |           |
| T3-T4                          | 5.034               | (0.654-38.764)        | 0.121     |                     |                       |           |
| N+                              | 4.021               | (1.103-14.654)        | 0.035     | 1.983               | (0.176-22.359)        | 0.58      |
| M+                              | 3.059               | (0.938-9.976)         | 0.064     | 1.63                | (0.443-6.004)         | 0.462     |
| Lymph vascular invasion        | 4.021               | (1.103-14.654)        | 0.035     | 1.394               | (0.194-10.006)        | 0.741     |
| Perineural invasion            | 3.582               | (1.099-11.673)        | 0.034     | 2.54                | (0.643-10.038)        | 0.184     |
| Chemotherapy                   | 2.911               | (0.799-10.598)        | 0.105     |                     |                       |           |
| Stage III-IV                   | 4.14                | (1.136-15.087)        | 0.031     | 1.096               | (0.144-8.353)         | 0.929     |
| Low Exp POLH                   | 1.839               | (0.602-5.624)         | 0.285     |                     |                       |           |
| Low Exp POLK                   | 1.213               | (0.407-3.609)         | 0.729     |                     |                       |           |
| Low Exp POLQ                   | 2.782               | (0.855-9.055)         | 0.089     | 1.254               | (0.215-7.33)          | 0.801     |
| High Exp XRCC2                 | 1.131               | (0.38-3.368)          | 0.825     |                     |                       |           |
| Low Exp XRCC5                  | 1.738               | (0.568-5.32)          | 0.332     |                     |                       |           |
| Low Exp DCRLE1A                | 1.616               | (0.528-4.944)         | 0.401     |                     |                       |           |
| Unmetilated POLH              | 1.134               | (0.346-3.718)         | 0.835     |                     |                       |           |
| Unmetilated POLK              | 3.363               | (0.908-12.46)         | 0.07      | 1.756               | (0.306-10.062)        | 0.451     |
| Unmetilated XRCC5              | 1.533               | (0.406-5.786)         | 0.529     |                     |                       |           |
| Unmetilated DCLRE1A            | 2.778               | (0.717-10.766)        | 0.139     |                     |                       |           |
| Pol k IHC Low                  | 1.54                | (0.517-4.586)         | 0.438     |                     |                       |           |
| XRCC5 IHC Low                  | 2.968               | (0.912-9.654)         | 0.071     | 1.802               | (0.376-8.646)         | 0.461     |
| BRAF mutated                   | 1.363               | (0.177-10.497)        | 0.766     |                     |                       |           |
| MLH1 IHC Low                   | 2.06                | (0.566-7.491)         | 0.273     |                     |                       |           |
| MSH2 IHC Low                   | 1.266               | (0.281-5.715)         | 0.759     |                     |                       |           |
| p53 IHC High                   | 1.352               | (0.442-4.135)         | 0.597     |                     |                       |           |
| Ki67 IHC Low                   | 2.312               | (0.301-17.785)        | 0.421     |                     |                       |           |

Table 6: Disease free survival calculated with univariate and multivariate cox regression tests.
| Variable                        | Univariate analysis |                | Multivariate analysis |                |
|--------------------------------|---------------------|----------------|-----------------------|----------------|
|                                | HR                  | CI (95%)       | p value               | HR             | CI (95%)       | p value               |
| Age < 65 years                 | 1.121               | (0.342-3.677)  | 0.851                 | 2.287          | (0.063-82.97)  | 0.652                 |
| Sex. male                      | 2.878               | (0.841-9.851)  | 0.092                 |                |                |                       |
| CEA > 5                        | 5.845               | (1.538-22.068) | 0.009                 | 25.432         | (0.258-2510.47) | 0.258                 |
| Right side                     | 1.425               | (0.435-4.676)  | 0.559                 |                |                |                       |
| Poor differenicated            | 3.414               | (0.736-15.838) | 0.117                 |                |                |                       |
| Mucinous                       | 1.139               | (0.145-8.934)  | 0.901                 |                |                |                       |
| T3                              | 36.142              | (0.157-8313.8) | 0.196                 |                |                |                       |
| N+                              | 6.049               | (1.295-28.245) | 0.022                 | 46.388         | (0.083-25970.4) | 0.235                 |
| Lymph vascular invasion        | 5.587               | (1.201-25.998) | 0.028                 | 15.922         | (0.026-9799.2) | 0.398                 |
| Perineural invasion            | 4.323               | (1.141-16.372) | 0.021                 | 16.76          | (0.467-601.995)| 0.123                 |
| Chemotherapy                    | 4.678               | (1.003-21.807) | 0.049                 | 6.629          | (0.159-276.673)| 0.32                  |
| Stage III                      | 3.687               | (0.971-14.005) | 0.055                 | 42.077         | (0.201-8821.94)| 0.17                  |
| High Exp POLH                  | 1.621               | (0.474-5.546)  | 0.442                 |                |                |                       |
| Low Exp POLK                   | 2.05                | (0.599-7.014)  | 0.253                 |                |                |                       |
| Low Exp POLQ                   | 3.151               | (0.834-11.906) | 0.091                 | 1.63           | (0.244-10.867)| 0.629                 |
| Low Exp XRCC2                  | 1.955               | (0.572-6.682)  | 0.285                 |                |                |                       |
| Low Exp XRCC5                  | 1.936               | (0.566-6.625)  | 0.293                 |                |                |                       |
| High Exp DCLRE1A               | 1.155               | (0.351-3.797)  | 0.812                 |                |                |                       |
| Unmetilated POLH              | 1.23                | (0.33-4.593)   | 0.758                 |                |                |                       |
| Unmetilated POLK              | 10.263              | (1.292-81.531) | 0.028                 | 51.874         | (0.221-12164.7)| 0.156                 |
| Unmetilated XRCC5             | 4.438               | (0.554-35.552) | 0.16                  |                |                |                       |
| Metilated DCLRE1A             | 2.239               | (0.409-12.26)  | 0.353                 |                |                |                       |
| Pol K IHC Low                 | 1.581               | (0.482-5.183)  | 0.45                  |                |                |                       |
| XRCC5 IHC Low                 | 1.532               | (0.467-5.031)  | 0.482                 |                |                |                       |
| BRAF wild                     | 22.171              | (0.1117704.6) | 0.575                 |                |                |                       |
| MLH1 IHC Low                  | 2.966               | (0.776-11.334) | 0.112                 |                |                |                       |
| MSH2 IHC Low                  | 3.253               | (0.857-12.35)  | 0.083                 | 1.837          | (0.343-9.842)  | 0.478                 |
| p53 IHC Low                   | 4.888               | (0.625-38.213) | 0.13                  |                |                |                       |
| Ki67 IHC Low                  | 26.615              | (0.024-29262.2)| 0.358                 |                |                |                       |

**Supplemental File Legend**

Legend supplementary Table S1:

Associations of DNA repair gene expression, methylation and IHC were evaluated using chi-square ($\chi^2$) test and Fisher’s exact test.

Legend supplementary Table S2:

The data were evaluated using chi-square ($\chi^2$) test and Fisher’s exact test. Statistically significant are highlighted (p<0.05).

Legend supplementary Table S3:
The data were evaluated using chi-square ($\chi^2$) test and Fisher’s exact test. Statistically significant are highlighted (p<0.05).

Legend supplementary Table S4:

The data were evaluated using chi-square ($\chi^2$) test and Fisher’s exact test.

Figures

**Figure 1**

Molecular changes in DSBR, ICLR and DDT/TLS compared colonic normal tissue and CRC tumors. A. Gene expression was quantified for a panel of genes by real-time qPCR analysis in neoplastic and normal mucosal tissues from 47 patients with sporadic colorectal cancer. The following genes were examined: MLH1, MSH2, POLK, POLH, POLQ, XRCC2, XRCC2 and DCLRE1A. Gene expression data are shown as scatter diagrams. B. Fold Change between neoplastic and normal tissue quantified real-time qPCR analysis. C. Heat map of individual gene expression changes in sporadic colorectal cancer. Fold changes were calculated for neoplastic tissue vs. adjacent normal tissue. Blue indicates decreased relative gene expression, red indicates increased relative gene expression and white indicates no change in gene expression. Gene expression means between normal and neoplastic tissue were compared using independent sample t-Student or Mann-Whitney tests after Kolmogorov-Smirnov tests.
Figure 2
Overall and disease free-survival for POLQ gene expression, POLK methylation and IHC for XRCC5. The data were evaluated with Kaplan-Meier test.

Supplementary Files
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