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Original article

Prognostic impact of changes in base excision repair machinery in sporadic colorectal cancer

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ABSTRACT

Objective: to evaluate the prognostic value of base excision repair proteins in sporadic colorectal cancer.
Methods: Pre-treatment tumor samples from 72 patients with sporadic colorectal adenocarcinoma were assessed for APC, MPG, Polβ, XRCC1 and Fen1 expression by immunohistochemistry. The associations of molecular data were analyzed in relation to clinical features and TNM staging as a prognosis predictor and disease-free survival.
Results: Higher levels of MPG, Polβ and XRCC1, but not Fen1, were associated with unfavorable pathological outcomes, such as poor cellular differentiation, advanced TNM stages, presence of lymphatic and perineural invasions and metastatic lymph nodes. MPG and Polβ overexpression were associated with right-sided CRC. However, only MPG high expression is associated with shorter disease-free survival in CRC patients.
Conclusions: Our results suggest that increased expression of MPG, Polβ and XRCC1 are more likely to evolve to poor pathological outcomes, but only the elevated expression of MPG protein predicts recurrence. The BER proteins appear to be suitable candidates to refine the TNM current staging of colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is one of the most frequently neoplasia in Western countries (10–15% of all forms of cancer) and ranks second in cancer related deaths [1,2]. While only 6% of all cases present a hereditary genetic etiology, the sporadic CRC (~80% of all cases), which is the most prevalent form, still has a lack of knowledge about the etiological factors that triggers this disease [1,2]. Despite survival rates have increased in the past few years, at least a third of patients who undergo curative resection experience local tumor recurrence or metastasis [3,4]. Pathological staging is the only prognostic classification used in clinical practice to select patients for adjuvant chemotherapy. Furthermore, drug resistance is also a critical problem in CRC patients with comprehensive treatment, which is directly associated to the absence of predictive markers [5,6].

Among the earliest events leading to the development of sporadic CRC are the mutations in the central area of the *adenomatous polyposis coli* (*APC*) gene, which are strongly associated with familial predisposition to CRC and with the sporadic CRC [7,8]. Appropriate levels of functional APC are essential to many cellular and tissue integrities [9,10]. The major role of APC is to regulate β-catenin and Wnt signaling, interfering in processes such as apoptosis, cell adhesion, chromosomal instability, cell cycle and DNA repair [11].

The DNA repair system has evolved to deal with the modification or loss of DNA bases, as a sophisticated manner to fight against the mutations. However, changes in its normal functions respond as a major cause of human diseases, including cancer. Among these mechanisms, base excision repair (BER) is the most prevalent pathway for the

Abbreviations: CRC, colorectal cancer; TNM, tumor-node-metastasis; APC, *adenomatous polyposis coli*; BER, base excision repair; dRP, deoxyrbose phosphate; AP site, apurinic/abasic site; SN-BER, single-nucleotide base excision repair; LP-BER, long-patch base excision repair; MPG, N-methylpurine DNA glycosylase; Fen1, flap endonuclease; Polβ, DNA polymerase beta; XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1; QS, multiplicative quick score method; MMR, Mismatch Repair; LCRC, Left-sided colorectal cancer; RCRC, right-sided colorectal cancer

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removal of damaged bases generated by alkylation, oxidation or reduction [12] and proceeds through a sequence of reactions requiring several different enzymes. The first step involves excision of the damaged base by a DNA glycosylase enzyme, which leads to the formation of a potentially cytotoxic abasic site intermediate (AP site). Subsequently, the AP site is processed by an AP endonuclease (APE1), generating a strand break and a flap. At this point, DNA polymerase fills the gap, and DNA ligase seals the remaining nick, thus completing the BER process [13].

It has been reported that APC protein has a DNA repair inhibitory domain located towards the N-terminus, which interacts with the BER proteins Polβ and Fen1 [14]. APC has the ability to block Polβ −directed strand displacement synthesis in long patch-BER (LP-BER) or it can inhibit its lyase activity, thus blocking single nucleotide-patch BER (SN-BER) [15,16]. The consequence of the blocked LP- and SN-BER on cellular fate is not clear, but specially because both Fen1 and APC are considered tumor suppressors [16,17] and their levels are critical for the repair of the BER pathways in colon cancer [18] this combination of factors can differently drive the tumor cells in terms of aggressiveness.

DNA repair imbalance is related to malignant transformation by allowing greater vulnerability to the accumulation of DNA damage [19]. The elaboration of an expression profile that could produce reliable biomarkers is a priority need to guide colorectal cancer treatment and monitor therapeutic response, as well as for surveillance to detect recurrence. Thus, bearing in mind the importance of DNA repair in the disease development and therapy response, it seems quite reasonable to consider a categorization of colorectal tumors based on DNA repair characteristics.

2. Patients and methods

2.1. Study patients and collection of samples

In this study (case-series design), we retrospectively selected resection specimens of 72 individuals diagnosed with adenocarcinoma of the colon and rectum and who were admitted to colorectal surgery with curative intent by the same surgical team between 2010 and 2012 in South Brazil. Patients were excluded if at least one of the follow criteria was identified: suspicion of hereditary colorectal cancer (familial adenomatous polyposis and hereditary non-polypoid colorectal cancer); presence of colorectal cancer associated with inflammatory bowel disease; realization of neoadjuvant chemoradiation therapy. This study was approved by the Ethics Committee in Human Research of the participating institutions (No. 321.069). Written informed consent was obtained from all patients before their enrolment in the study.

Epidemiological, clinical and pathological data were obtained from the hospital medical records. Histopathological data (such as tumor subtype, depth of invasion, lymph node and/or metastasis distance and staging) were also extracted from the pathological reports. TNM system was used as the staging scale for prognosis. Colon tumors was classified into left-sided colorectal cancer (LCRC) and right-sided colorectal cancer (RCRC)

2.2. Immunohistochemistry

Formalin-fixed and paraffin-embedded samples were cut into 4 μm sections. After deparaffinization and rehydration, the sections were quenched with 3% H_2O_2 in methanol to block endogenous peroxidase. 5% bovine serum albumin was then applied to prevent non-specific binding. The sections were incubated with APC (dilution 1:100, AB15270, Abcam), Polβ (dilution 1:500, AB26343, Abcam), XRCC1 (dilution 1:50, AB1838, Abcam), Fen1 (dilution 1:800, AB17993, Abcam), MPG (dilution 1:100, EPR10959 (B), Abcam), then treated with the rabbit conjugated to horseradish peroxidase (DAKO) antibodies. Diaminobenzidine was used as chromogen and the sections were counterstained with haematoxylin. Tissues used as positive controls were recommended by the antibodies manufacturer as it follows: human testis tissue (anti-MPG, anti-Fen, anti-XRCC1); human small cell lung cancer tissue (anti-Polβ); and normal human colon tissue (anti-APC). Omission of the primary antibody was used as a negative control. Each immunohistochemical stain was performed in a group to prevent potentially staining irregularities encountered with separate immunohistochemistry runs.

Histological sections used for diagnostic and experimental purposes were obtained from the same tumoral area to minimize intratumoral heterogeneity bias. The quality (number, intensity, and pattern) of every staining procedure has been comparatively evaluated using consecutive control sections an independent experienced pathologist blinded to the objectives of this study. After the immunostaining, two observers assessed all cases independently. The few cases with discrepant scoring were re-evaluated jointly on a second occasion, and agreement was reached in all cases. Non-representative samples or samples with only a few tumor cells (< 100) were excluded from the data analysis.

2.3. Immunohistochemistry results evaluation

Positive staining for APC and BER proteins was defined as the observation of shades of brown nuclear staining the microscope ($\times 400$). Five hot spot fields containing at least 200 cells were captured and the positive cells were manually counted using the NIH-ImageJ software. To assess the immunohistochemical expression we used the multiplicative quick score method (QS) [20]. In order to minimize intratumoral heterogeneity bias, based on the distribution and intensity of staining, we used a semiquantitatively score (corresponding to staining intensity and percentage of reactive nuclei). According to the number of positive staining cell, the staining density was expressed semi-quantitatively as follows: 0, less than 5%; 1, 5–25%; 2, 25–50%; 3, 50% to 75%; or 4, more than 75%. We also evaluated the staining intensity was scored as follows: 0-negative staining; 1-weak staining; 2-moderate staining and 3-strong staining. Both values were multiplied together, and the staining score was stratified into two groups of immune reactivity: weak (score range, 0–4) or strong (score range, 5–12) (Supplementary Fig. 1).

2.4. Statistical analysis

Statistical analysis was performed using SPSS software version 22.0. Immunohistochemistry and clinical features correlations were analyzed through contingency tables, chi-square ($\chi 2$) test and Fisher's exact test. For tumor protein expression and associations with the disease-free interval, the Kaplan-Meier survival table method was used. To test the significance of the differences between the curves of the disease-free interval and protein expression levels, the Log-rank (Mantel-Cox) test was used. All statistical tests were two sided and $P \leq 0.05$ was considered significant.

2.5. 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 the Irmandade Santa casa de Misericórdia de Porto Alegre hospital.

3. Results

3.1. Clinicopathological findings

Table 1 summarizes the clinicopathological characteristics of the 72 included patients. The patients' ages varied from 29 to 88 years. Considering the anatomic location, 59 (80%) of the tumors were located in