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Original Article

MICROSATELLITE INSTABILITY – MSI MARKERS (BAT26, BAT25, D2S123, D5S346, D17S250) IN RECTAL CANCER

Instabilidade de microssatélite – msi nos marcadores (BAT26, BAT25, D2S123, D5S346, D17S250) no câncer de reto

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ABSTRAT – ***Background*** - Colorectal cancer has an important genetic component. Microsatellites are considered phenotypic markers of prognosis, therapeutic response and identify patients with mutations in DNA repair genes. ***Aim*** – To evaluate the molecular profile of tumors underwent to transanal endoscopic microsurgery – TEM in surgical treatment of rectal cancer. ***Method*** – Thirty eight surgical specimens were evaluated according to pathological staging and the region of the tumor were dissected and submitted to DNA extraction. The colorectal tumors were tested for microsatellite instability – MSI using a panel of five markers (BAT25, BAT26, D2S123, D5S346, and D17S2720) technique of Polymerase Chain Reaction (PCR). ***Result*** – From total 63% were male and 47% female, with mean age of 58.4 years. In relation to tumor type adenomas were 58%, 24% low-grade adenomas and 76% high grade; 42% were carcinomas. The depth of resection 80% included the rectal perirenal fat and 20% the muscularis propria. The most frequent microsatellite amplification was BAT26 (100%) and lowest D17S2720 (85.4%). Sixteen patients (42%) were MSI, ten were carcinomas, two low grade adenomas and four high grade. Twenty-two cases (68%) showed microsatellite stable – MSS. The allelic loss of microsatellite markers was statistically significant in cases of carcinoma in relation to adenomas. The most frequent microsatellite amplification was BAT26 (100%) and lower D17S2720 (85.4%), 16 patients (42%) had microsatellite instability – MSI thereof ten were carcinomas, two low grade adenomas, four high-grade adenomas and 22 cases (58%) were microsatellite stable – MSS. ***Conclusion*** - Microsatellite instability (MSI-H) was significantly associated with rectal carcinomas, confirming its use as a prognostic marker in colorectal carcinogenesis.

HEADINGS – Microsatellite instability. Tumor markers, biological. Trasanal endoscopic microsurgery.

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RESUMO – ***Racional*** - O câncer colorretal tem importante componente genético. Os microssatélites são considerados marcadores fenotípicos de prognóstico, resposta terapêutica e de identificação de pacientes com mutação nos genes de reparo do DNA. ***Objetivos*** – Avaliar o perfil molecular dos tumores submetidos à microcirurgia endoscópica transanal (TEM) para tratamento do câncer de reto. ***Método*** – Foram selecionados 38 espécimes avaliados segundo o estadiamento patológico. Foram escolhidas amostras da região tumoral e realizada dissecação e extração do DNA. Os tumores colorretais foram testados para instabilidade de microssatélite – MSI utilizando um painel composto de cinco marcadores (BAT25, BAT26, D2S123, D5S346 e D17S2720), técnica da reação em cadeia da polimerase (PCR). ***Resultados*** – Nos 38 casos observou-se que 63% eram do sexo masculino e 47% feminino com média de idade de 58,4 anos. Em relação ao tipo tumoral 58% eram adenomas, sendo 24% adenomas de baixo grau e 76% de alto grau; 42% eram carcinomas. Quanto à profundidade de ressecção, verificou-se que 80% dos casos incluíam a gordura perirretal e 20% até a muscular própria. O microssatélite com maior frequência de amplificação foi o BAT26 (100%) e o menor D17S2720 (85,4%). Dezesseis casos (42%) apresentaram MSI; eram dez carcinomas, dois adenomas de baixo grau e quatro de alto grau. Vinte e dois casos (68%) tinham microssatélite estáveis – MSS. A perda alélica dos marcadores de microssatélites foi estatisticamente significante nos casos de carcinoma em relação a adenomas. O microssatélite com maior frequência de amplificação foi o BAT26 (100%) e o menor D17S2720 (85,4%); 16 casos (42%) apresentaram instabilidade de microssatélite – MSI. Desses, dez eram carcinomas, dois adenomas de baixo grau e quatro de alto grau; 22 casos (58%) apresentaram microssatélite estáveis – MSS. ***Conclusão*** - A instabilidade de microssatélite (MSI-H) foi significantemente associada com carcinomas retais, confirmando sua utilização como marcador prognóstico na carcinogênese retal.

DESCRITORES - Instabilidade de microssatélites. Marcadores biológicos de tumor. Microcirurgia endoscópica transanal.

INTRODUCTION

The mapping of the human genome, together with the development of new molecular techniques, has allowed not only the gene discovery of genetic predisposition to cancer, but also the possibility of large scale of genomic analysis. The development of cancer is the result of a series of steps involving gene mutations, chromosomal breaks and losses, gene amplifications and genomic instability, and epigenetic mechanisms. Among the multiple genes involved in this process, is highlighted the proto-oncogenes, tumor suppressor genes, related to DNA repair[18,25].

The DNA is a molecule that frequently suffers alterations through changes of segments and variations in the process of cell division. To correct such changes exist some proteins with the function of repairing and maintaining the integrity of DNA. These proteins are produced from repair genes (mismatch repair genes - MMR), and its function is exerted continuously, preserving cellular tissues[12,13]. The microsatellite instability - MSI results from inactivation of the DNA repair proteins and constitutes the molecular basis of non polipoid hereditary colorectal cancer - HNPCC. Six genes related to DNA repair is involved in MMR (mismatch repair genes), such as: hMLH1 (Gene Human mut-L Homologue 1), hMSH2 (Gene Human mut-S Homologue 2) and hMSH6 (Gene Human mut-S Homologue 6)[19]. The hMLH1 gene is located on chromosome 3p21-23[14,15] and the hMSH2 on chromosome 2p21, in an area originally identified as important region for genes involved in HNPCC[22,24]. Observation of large changes in the number of microsatellite in certain tumor tissue shows no normal function on DNA repair. It represents an indirect action on the deficiency of proteins repair caused by mutations in genes previously mentioned. This error is called replication error (replications errors - RER +)[11,30]. The MSI represent a marked phenotypic characteristic of HNPCC being present in 80-90% of tumors, and in sporadic cases reaches up to 15%[2,4].

While genes are susceptible to a random number of chemical changes (mutations), the majority of them are eliminated by DNA repair system. There are a variety of repair mechanisms, catalysed by different sets of enzymes. Almost all mechanisms depend on the existence of two copies of genetic information, one on each tape of DNA double helix. If the sequence on a tape is damaged accidentally, the information is not lost because a "backup" version remains in a complementary nucleotide sequence on the other tape[18]. Besides the damage in repair system, is required the occurrence of more than one mutations to transform normal cells into tumor. The mutations must occur at least five or six times independently within a single cell to establish the conditions necessary for cancer development. Such mutations can be dominant or recessive. Dominant mutations lead to a gain of function and may cause the development of oncogenes, that are mutated forms of proto-oncogenes. They act promoting growth and division of normal cells. But, in mutated form, they promote cancer development. Recessive mutations lead to loss of function and may alter or inactivate tumor suppressor genes (TSG). Its products are required to inhibit cell growth, division cycle and prevent the development of cancer. Such mutations, however, do not destroy mutated cells and provide competitive advantage over normal neighboring cells[3,16]. The repair genes act ensuring that each genetic information is copied correctly during DNA replication. Mutations in these genes lead to increase other mutations[8].

From the point of view of genetic, colorectal cancer (CRC) can be divided into two groups: 1) sporadic, occurring without family liaison and correspond about 85% of cases, and 2) hereditary, based on genetic defect in the family, generation after generation. Hereditary cases occurs at earlier ages - from age 20 - and may be present on several people within the same family[6,9]. The results of treatment of CRC is directly related to early diagnosis, ie, the sooner you make the diagnosis, the higher the cure rate, reaching over 90% in early cases. In recent years, with the rapid and growing development of molecular techniques, are identified specific genetic defects in at least two major hereditary syndromes on large intestine, the familiar adenomatous polyposis (FAP) and hereditary colorectal cancer without polyposis (HNPCC). Both are vertically transmitted from one generation to another, through Mendelian inheritance pattern, in general type autosomal dominant[28]. The proto - oncogene (K-ras), tumor suppressor genes (APC, DCC and TP53) and DNA repair genes or MMR (English, mismatch repair - genes called MSH2, MLH1, PMS1, PMS2 and MSH6) have central participation in the development of CRC. Approximately 25% to 40% of DNA molecule is formed by repetitive nucleotide sequences, which might be seen several times throughout the genome, and subdivided into repetitive dispersion and repetitive tandem (or satellite)[10]. Such repetitions can be classified according to the extent of repetitive sequences: satellite, minisatellite and microsatellite depending on the number of nucleotides. The number of minisatellite and microsatellite varies among

individuals, that´s why they are considered DNA fingerprinting and used in paternity tests[1]. The microsatellite instability - MSI is the observation that the DNA extracted from cells of certain tumors show changes in the number of repeating units in one or more microsatellites compared to the same existing microsatellite DNA samples from normal tissue of the same individual - blood cells, for example. Therefore, tumor cells have "fingerprints" defective in their DNA compared to other body tissues.

The promoter hypermethylation of hMLH1 gene appears to be a major genetic alteration present in CCR. Molecular analysis of mutator via carcinogenesis by detection of MSI has application in clinical practice, since tumors called "mutator" exhibit distinct biological characteristics[3,29]. There is evidence that these tumors are different in several aspects: location (proximal colon), age (less advanced), histological appearance (higher incidence of mucinous tumors) and prognosis (better survival compared to microsatellite stable CCR)[26]. However, MSI tumors are associated with resistance to chemotherapy with 5-fluorouracil (5FU) and shorter survival of patients after treatment with the drug[5,6,15,20]. MSI CCR marker can also be inherited or acquired susceptibility to other cancers such as gastric cancer, uterus and ovary[21]. Several theories have been proposed to explain why MSI-H tumors (High-instability) have a better prognosis in sporadic colorectal carcinomas, despite the unfavorable characteristics.

This study aims to assess the profile of molecular tumors underwent to transanal endoscopic microsurgery (TEM) for the treatment of rectal cancer.

METHODS

Were selected 38 surgical specimens resected by transanal endoscopic microsurgery (TEM) in the period from 2003 to 2006, evaluated according to pathological staging[27].Thereafter, the samples were chosen from region tumor embedded in paraffin, submitted to dissection and extraction of DNA using commercial kit (QIAmp® DNA FFPE Tissue Handbook – Qiagen, Califórnia, USA) Losso, 2010. Colorectal tumors were tested for microsatellite instability - MSI using a panel of five primer pairs (BAT25, BAT26, D2S123, D5S346, D17S2720) (Table 1). The method used for detection of microsatellite was VNTRs (Variable Number of Tandem Repeats)Kashyap et al. 2,004. Were used 100 ng / ul purified DNA in a final volume of 25µl PCR reaction. The PCR conditions were: initial denaturation at 95° C for period of five minutes, followed by 10 continuous cycles of denaturation at 95° C for one minute annealing starting at 60 ° C and lowered 1° C every cycle until reaching the temperature of 51° C in the last cycle extension at 72° C for one minute and at 72° C for one minute. Completing the 30 cycles, the material remained five minutes at 72° C to provide a longer extension for all 40 cycles. Subsequently, the PCR products was analyzed by electrophoresis gel on 10% polyacrylamide (16 hours, 450 V, room temperature) stained with silver nitrate 0.1%.

Statistical analysis

For association analyzes were performed using tests of independence between variables using chi-square test with Yate's correction or Fisher. Were calculated the odds ratio with a confidence interval of 95%. Values of p less than 0.05 were considered significant

RESULTS

From the 38 cases of rectal cancer operated by TEM (Figure 1), 63% were male and 47% female with a mean age of 58.4 years. Regarding tumor type, adenomas were 58% - 24% lower and 76% high grade – and 42% were carcinomas (Table 1). In relationship to the resection depth was found that 80% included the perirectal fat and 20% the muscularis propria.

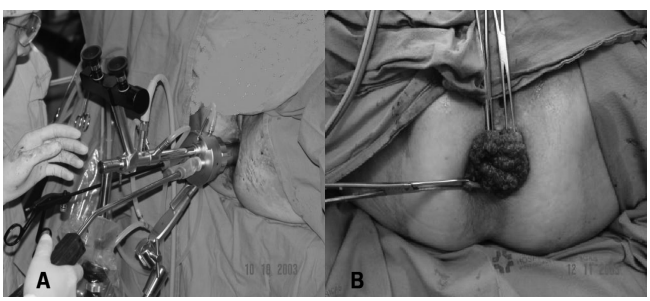

FIGURE 1 – Transanal endoscopic microsurgery (TEM): A) the endoscopic system positioned; B) low rectal adenoma (prolapsed).

TABLE 1 - Panel with five microsatellite markers

Microssatélite	Location	Size	Forward primer	Reverse primer
BAT25	gene c-Kit cr. 4q12	110 - 130 pb	5' -TCG CCT CCA AGA ATG TAA GT - 3'	5' - TCT GCA TTT TAA CTA TGG CTC - 3'
BAT26	gene hMSH2 cr. 2p	100 - 120 pb	5' - TGA CTA CTT TTG ACT TCA GCC - 3'	5' - AAC CAT TCA ACA TTT TTA ACC C - 3'
D2S123	gene hMSH2 cr. 2p	200 - 230 pb	5'- AAA CAG GAT GCC TGC CTT TA - 3'	5' - GGA CTT TCC ACC TAT GGG AC - 3'
D5S346	gene APC cr. 5q21q22	100 - 130 pb	5' - AGC AGA TAA GAC AGT ATT ACT AGT T - 3'	5' - ACT CAC TCT AGT GAT AAA TCG GG - 3'
D17S250(Mfd15CA)	gene BRCA1 cr.17q11.2-q12	140 - 170 pb	5' - GGA AGA ATC AAA TAG ACA AT - 3'	5' - GCT GGC CAT ATA TAT ATT TAA ACC - 3

The microsatellite with more frequent amplification was BAT26 (100%) and the lowest D17S2720 (85.4%). Sixteen cases (42%) showed MSI. Of these, ten were carcinomas, two adenomas with low-grade and four high. Twenty-two (68%) cases showed stable microsatellite (MSS). The allelic loss of microsatellite markers was statistically significant in the cases of carcinoma in relation to the adenomas (p = 0.0003, OR 16.7; 95% CI: 2.8-98.0) (Table 2 and Figure 2).

TABELA 2 - Loss of allelic microsatellite markers in patients with rectal carcinoma and adenoma

	Allelic loss patients (N = 16)	patients without allelic loss (N=22)	P	OR
Carcinoma	10	2	0,0003	16,7
Adenoma	6	10		IC95% 2,8-97,9

Fisher's exact test

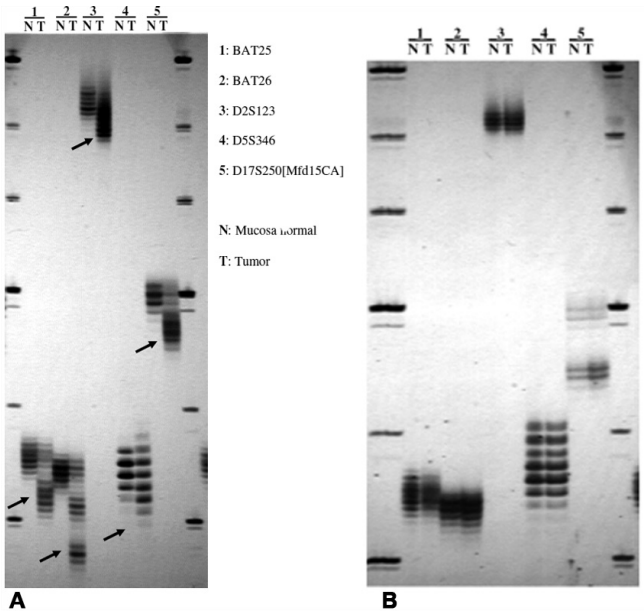

FIGURE 2 – A) Microsatellite instability - MSI-H. The arrow indicates the extra alleles arising from errors of DNA replication in tumor sample as compared to normal DNA sample; B) microsatellite stability (MSS) in a case of colorectal carcinoma and electrophoresis polyacrylamide gel with 6% urea stained with silver nitrate

DISCUSSION

The identification of parameters that reflect the biological behavior of rectal cancer is determining in the prognosis and cancer therapy. The results of this study showed the importance of molecular analysis in the appearance of rectal tumors, and may imply therapeutic design.

The presence of MSI may be significantly corroborated by evaluating the number of concurrent intramural active cytotoxic lymphocytes[27]. This observation supported the hypothesis that MSI-H tumors are associated with the production of new immunogenic epitopes due to defective repair system. This hypothesis attempts to explain why patients with sporadic CRC MSI-H exhibit antitumor immune response more effective than MSS patients with more favorable clinical outcome. In this study it was observed that the presence of MSI-H was significantly higher in carcinomas than in adenomas, confirming the prognostic value of MSI in CRC. It should also be noted that the articles published in CCR about MSI are mainly from Eastern and European populations; few studies were conducted in Brazil. Considering that our country has great genetic heterogeneity - the result of five centuries of miscegenation -, are necessary studies to establish the frequence of MSI in different regions of Brazil.

The survival of patients with metastatic CRC (mCRC) progressively improved over the past decades. Was due primarily to new chemotherapeutic combinations (5-fluorouracil, irinotecan, oxaliplatin), and the introduction of new therapies. Among them are two monoclonal antibodies against the receptor of epidermal growth factor receptor (EGFR) - cetuximab and panitumumab - which have demonstrated efficacy in the treatment of mCRC. However, due to toxicity and cost, it is essential to use tools to select patients most likely to have benefit with the treatment.

Therefore, the identification of cases of rectal cancer with MSI may allow proper neoadjuvant chemotherapy specific to each patient, thus sparing the toxicity of therapy and enabling the implementation of a new therapeutic modality.

CONCLUSIONS

The microsatellite instability (MSI-H) was significantly associated with rectal carcinomas, confirming its use as a prognostic marker in colorectal carcinogenesis.

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