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POLYMORPHIC VARIATION OF MONONUCLEOTIDE MICROSATELLITES IN HEALTHY HUMANS AND ITS IMPLICATION FOR MICROSATELLITE INSTABILITY SCREENING

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ABSTRACT – ***Background*** - Colorectal cancer is the sixth most common tumor and the fifth in mortality in Brazil. Molecular markers have been associated with disease prognosis, especially in relation to therapeutic response and overall survival rates. Among these, microsatellite instability has been extensively studied. Microsatellite stability status is usually determined by comparison of normal and tumoral tissues from the same patient and instability is characterized by the difference in the PCR-amplification profile of these tissues at a given *locus*. Usually, a panel of five markers is used for this purpose. Two of them (BAT-25 and BAT-26) are considered monomorphic in populations of European origin. ***Aim*** - To analyse the frequency of constitutive polymorphic variation at BAT-25 and BAT-26 *loci* in a sample of individuals from Southern Brazil. ***Methods*** - Two-hundred and sixteen healthy and unrelated individuals were analised to assess the frequency of allelic variation at the BAT-25 and BAT-26 *loci* in DNA extracted from peripheral blood. Analysis was done by polymerase chain reaction – single strand conformation polymorphism (PCR-SSCP). ***Results*** - From the sample of patients studied, 7% and 6% of the patients had possible constitutive allelic variation at the BAT-25 and BAT-26 *loci*, respectively. ***Conclusions*** - These results indicate that significant constitutive allelic variation of these *loci* does occur in heterogeneous populations such as ours, and reinforce the importance of comparative studies between tumoral and corresponding normal tissue to determine microsatellite stability status and correctly identify microsatellite instability in selected populations.

HEADINGS – Colorectal neoplasms. Genomic instability. Polymorphism, genetic. Microsatellite repeats. Tumor markers, biological.

INTRODUCTION

Among tumors, colorectal cancer (CRC) is the third in frequency and the second in mortality in developed countries. In Brazil, it is the sixth most common type of cancer and the fifth in mortality[11]. Currently, the determination of disease prognosis is mainly based on clinical, pathological and morphological parameters. Molecular markers have also been associated with prognosis, especially in relation to therapeutic response and overall survival rates. Among them, microsatellite instability (MSI) has been extensively studied. It is a common finding in tissues prone to replication errors caused by a deficiency in the DNA mismatch repair system (MMR), which leads to the progressive accumulation of mutations, especially in mono- and dinucleotide microsatellites. Tumors that show microsatellite instability (MSI+) tend to be associated with better prognosis[3, 12, 14, 18]. In addition, MSI is present in more than 90% of colorectal tumors from hereditary nonpolyposis colorectal cancer syndrome (HNPCC) patients and in only 15% of sporadic

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colorectal tumors. Therefore, MSI analysis is also an important screening tool in the differential diagnosis of hereditary CRCs. In general, microsatellite stability (MS) status is determined by the comparison between normal and tumoral tissues from the same patient and instability is characterized by the difference in the amplification profile of specific markers between these tissues. The International Workshop on Microsatellite Instability and Replication Error Repair (RER) Phenotypes in Cancer Detection and Familial Predisposition has recommended that MS status be studied through a panel of five markers: two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D2S123, D5S346 and D17S250) markers[2, 6]. The presence of instability in two or more markers defines a tissue as MSI high (MSI-H), presence of instability in only one marker classifies the tissue as MSI low (MSI-L) and absence of instability in all five markers defines a tissue as microsatellite stable (MSS)[6]. The BAT-26 locus contains a 26-repeat adenine tract and is located within the fifth intron of the *hMSH2* gene, whereas the BAT-25 locus contains a 25-repeat thymine tract located within intron 16 of the *c-kit* oncogene. These mononucleotide markers are considered quasi-monomorphic in RER-negative tumors or normal tissue of European individuals, exhibiting little repeat size variation (~2bp) or no variation at all[5, 9]. Thus, analysis of only tumoral tissue without comparison with the corresponding normal tissue has been proposed and considered sufficient for instability detection in patients at risk for HNPCC by many authors[8, 9, 20]. In addition, since small unstable alleles can be easily distinguished from normal ones by SSCP (single strand conformation polymorphism) or sequencing, and BAT-26 is highly sensitive to detect MSI-H colorectal tumors, some investigators have proposed that analysis of BAT-26 alone can determine MS status in CRCs with greater than 99% accuracy[2, 4, 6, 7, 19]. Therefore, many published reports have determined MS status using only tumoral tissue and the two markers BAT-25 and BAT-26 instead of the panel of 5 markers originally recommended by the National Cancer Institute (NCI)[9, 10, 20]. Recently, PYATT et al.[13] showed a high frequency of allelic variation at the BAT-25 and BAT-26 *loci* in a sample of African-American individuals, suggesting that similar variations may be encountered in other populations. Most importantly, this observation reinforced the concern with the accuracy of the analysis when only tumoral tissue is used to determine MS status. Finally, a study of MSI in endometrial adenocarcinomas suggested a polymorphic profile of BAT-25 and BAT-26[19]. Therefore, allelic variation of these markers in specific populations should be determined before a decision is made on the most reliable clinical protocol for MSI screening. Little has been published on MS status in sporadic and hereditary CRC in Brazilian patients and healthy individuals[7].

The present study reports the frequency of individuals showing constitutive polymorphic variation at the BAT-25 and BAT-26 *loci* in a sample of healthy individuals from the southernmost state of Brazil, Rio Grande do Sul.

METHODS

Two-hundred and sixteen healthy and unrelated individuals from Rio Grande do Sul, Brazil, were studied anonymously. The study was approved by the institutional review board and associated ethics committee. DNA was extracted from peripheral blood by a standard salting-out procedure. Screening for constitutive polymorphic variation at the BAT-25 and BAT-26 *loci* was performed by PCR-SSCP as described previously[6, 10]. All samples were compared to a normal control (WT size) and all those exhibiting a different size were considered allelic variants.

RESULTS

All 216 samples were successfully amplified for BAT 26 and 215, were amplified for BAT 25. Fifteen (7%) and 13 (6%) individuals showed variant alleles at the BAT-25 and BAT-26 *loci*, respectively. Only one individual (0.5%) showed simultaneous allelic variation at both *loci.* Figure 1 depicts a typical SSCP mini-gel, where samples homozygous for the usual sized-alleles and allelic variants of BAT-25 and BAT-26 can be seen.

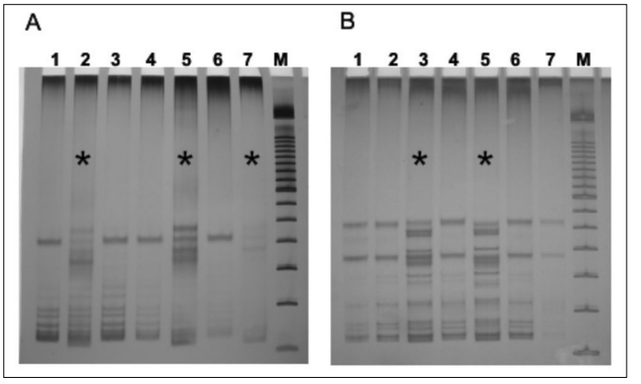

FIGURE 1. SSCP results of PCR-amplified sequences from lymphocyte DNA. A) BAT-26 amplification products: Lanes 1, 3, 4, and 6 depict the allelic pattern of fragments containing the usual 26 adenine repeats (so called common or 'large' allele); lanes 2, 5 and 7 show a variant pattern (asterisks). B) BAT-25 amplification products: Lanes 1, 2, 4, 6 and 7 depict the pattern of o fragments containing the usual 2o fragments containing the usual 25 thymine repeats; lanes 3 and 5 show a variant pattern (asterisks). M = 100 bp ladder.

DISCUSSION

MSI analysis is an important tool in the study of colorectal tumors, and has been extensively used as a screening test to identify patients with the hereditary colorectal syndrome HNPCC. Therefore, a standard panel of five microsatellite markers was established by the International Collaborative Group in HNPCC and by the NCI (USA) for this purpose[5]. The frequency of polymorphic variation at these loci has been established in Europeans, Asians and African-Americans, but little is known about its behavior in the Brazilian population[2, 6, 15]. The International Workshop of Microsatellite Instability and RER phenotypes in Cancer Detection and Familiar Predisposition recommended that all

studies that include BAT-26 in the analysis of MSI should compare its pattern in normal and tumoral tissue, since this marker can show a different allelic profile (i.e. constitutional polymorphism) according to the ethnical background of the population under study[2, 16]. Despite this recommendation, however, the majority of studies that have used BAT-25 and BAT-26 in CRC, including studies with Brazilian individuals, consider these two markers monomorphic and do not compare the amplification profile of tumoral and normal tissue of the same individual to determine MSI. Failure to do so may constitute an important confusion factor if the population studied is heterogeneous and indeed polymorphic at these *loci*, leading to misclassification of the tumor and ultimately to inappropriate management of the patient. There are several evidences in the literature that suggest that BAT-26 has considerable allelic variation[2, 17].

PYATT et al.[13], in a populational study with 103 African American individuals, reported allelic variation of 12.6% and 18.4% in BAT-26 and BAT-25, respectively. Simultaneous allelic variation at both *loci* was observed in 2.9% of individuals. Finally, ALAZZOUZI et al.[1] described that the BAT-26 repetitive region ranges in size from 21 to 27 adenines in healthy individuals.

The present study identified possible BAT-25 and BAT-26 variant alleles in a significant proportion of healthy individuals. If a tumor sample of such individual would be analysed for MSI without analysis of the normal corresponding tissue, this constitutive variant could be misclassified as MSI. Our results reinforce the need for the comparative analysis between normal and tumoral tissue for MSS determination to avoid false-positive results in HNPCC screening in certain populations. They also suggest that allelic variation at these *loci* is common in the heterogeneous Brazilian population.

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RESUMO – ***Racional*** - No Brasil, o câncer colorretal é o sexto tumor em freqüência e o quinto em mortalidade. Marcadores moleculares têm sido associados com o prognóstico da doença, especialmente em relação à resposta terapêutica e taxa de sobrevida. Dentre eles, a instabilidade de microssatélites tem sido amplamente estudada. O estado de instabilidade de microssatélites é usualmente determinado pela comparação entre tecido tumoral e tecido normal correspondente de um mesmo paciente e a instabilidade se caracteriza pela diferença no perfil do produto de amplificação por PCR destes tecidos em um determinado locus. Usualmente, é utilizado um painel de cinco marcadores para este propósito. Dois deles (BAT-25 e BAT-26) são considerados monomórficos em populações de origem européia. ***Objetivo*** - Analisar a freqüência de variação constitutiva nos loci BAT-25 e BAT-26 em amostra de indivíduos do sul do Brasil. ***Métodos*** - Duzentos e dezesseis indivíduos saudáveis e não relacionados foram analisados para determinar a freqüência de variação alélica nestes loci. O rastreamento de variantes alélicas foi feito por "polymerase chain reaction – single strand conformation polymorphism" (PCR-SSCP). ***Resultados*** - Observou-se possível variação alélica constitutiva em 7% e 6% dos pacientes nos loci BAT-25 e BAT-26, respectivamente. ***Conclusão*** - Estes resultados indicam que há significativa variação alélica constitucional nos loci BAT-25 e BAT-26 em grupos selecionados, como nesta amostra de indivíduos brasileiros, e reforça a importância de estudos comparativos entre tecido tumoral e o tecido normal correspondente para identificar instabilidade de microssatélites em populações determinadas.

DESCRITORES – Neoplasias colorretais. Instabilidade genômica. Polimorfismo genético. Repetições de microssatélites. Marcadores biológicos de tumor.

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