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Molecular screening for hereditary nonpolyposis colorectal cancer in Bulgaria

Tanya K. Kadiyska*[1], Teodora E. Goranova[1], Ganka K. Dineva[1], Dimitar G. Nedin[2], Alexandrina B. Alexandrova[2], Antonina T. Gegova[2], Radka P. Kaneva[1], Damyan N. Damyanov[2], Ivo M. Kremensky[1], Vanio Iv. Mitev[3]

[1] *Laboratory of Molecular Pathology, University Hospital of Obstetrics and Gynaecology "Maichin Dom", Sofia-1431, Bulgaria*

[2] *Clinic of Abdominal Surgery, Queen Giovanna Hospital, Sofia-1526, Bulgaria*

[3] *Department of Chemistry and Biochemistry, Medical University, Sofia-1431, Bulgaria*

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Abstract: Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant disease, caused by germline mutations in DNA mismatch-repair genes (MMR). These mutations lead to microsatellite instability (MSI). It has been found that the MSI is not confined to the setting of hereditary disease and may be seen in approximately 12-17% of the sporadic CRCs. In 1998 a National Registry for CRC was instituted in Queen Giovanna Hospital, Sofia. A total of 150 patients have been selected for MSI analysis and 25 tumors showed to be unstable, 14 with loss of heterozygosity (LOH). These tumors were further analyzed for MLH1 promoter hypermethylation and a significant association between this epigenetic change and MSI/LOH sporadic cases. We proposed this method as a step that follows the analysis for MSI and prior to the screening for MMR mutations. The mutation screening detected four known and two novel mutations, one unpublished and four known intronic polymorphisms in both hMLH1 and hMSH2 genes. The use of IHC analysis has been found effective in the investigation of some unclear molecular variations.

We developed an efficient diagnostic strategy for HNPCC testing and the mutation status of 80% MSI HNPCC cases could be detected.

Keywords: HNPCC, immunohistochemistry, hMLH1, hMSH2, MSI

* E-mail: alextanya@excite.com

1 Introduction

Colorectal cancer (CRC) is the most common gastro- intestinal neoplasia. In terms of incidence, colorectal cancers rank fourth in frequency in men and third in women [1]. Approximately 20-25% are determined as familial CRC. Hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, is the most common type of familial CRC and accounts for approximately 5-8% of all colorectal cancers [2]. HNPCC is an autosomal dominant disease with a high risk for colorectal and HNPCC-related cancers, caused by germline mutations in DNA MMR genes [3]. These mutations lead to genomic instability, manifested by MSI. It has been found that the phenomenonn of DNA instability is not confined to the setting of hereditary disease and may be seen in approximately 12-17% of CRC in those with no family history of disease [4]. In the case of these so-called sporadic tumours, mutation of MMR genes is relatively infrequent whereas biallelic hypermethylation of the promoter of MLH1 appears to be the most important mechanism for inactivation of MMR genes [5]. The development of efficient DNA testing of the disease is important for the patient management and in some populations may need an individual approach. This is the first report for molecular screening in CRC for Bulgaria. We aimed to investigate the molecular changes found in our patient group and to describe the nature and frequency of mutations in our region.

2 Materials and methods

2.1 Establishment of a National Registry for Colorectal cancer

In 1998 a National Registry for Colorectal cancer was instituted in Queen Giovanna Hospital, Sofia, Bulgaria. The medical records, blood samples and tissue specimens were obtained from 294 incident CRC cases diagnosed in the period 1998-2005. They were collected regardless of age or the presence or absence of a family history of cancer. Family information was obtained and analysed, and an accurate genealogical tree was prepared, in which the main causes of morbidity and mortality of first and second-degree relatives were recorded. All patients received genetic counseling and informed consent was obtained. Patients were subgrouped into four main categories: 1. HNPCC families, according to the Amsterdam criteria; 2. Families according to the Bethesda criteria; 3. “Family” cases group- represented by families with one or more first-degree relatives of the proband affected by tumours at any site; 4. ”Sporadic” cases whose families did not show any other cancer cases among relatives or without sufficient family information.

The following clinical and histological information data were recorded- patient’s age at diagnosis, tumor localization, tumor stage and differentiation, presence of synchronous or metachronous tumours.

2.2 Microsatellite instability analysis

A total of 150 patients with colorectal cancer have been included in the current study. DNA from fresh blood and/or frozen normal and tumor tissue was isolated using standard protocols. A set of five polymorphic markers—BAT26, D2S123, D5S346, D18S35, and FGA—were selected for analysis of MSI. The repeat markers were amplified from both normal and tumour DNA samples and electrophoresis was performed on an automated fluorescence sequencer (ALF Express, Pharmacia).

2.3 Analysis for MLH1 promoter hypermethylation

In a previous study, a total of 25 MSI positive tumours (10 sporadic cases and 15 with family history), 13 with loss of heterozygosity (eight sporadic cases and five with family history) and 15 random selected sporadic MSI-negative cases, the methylation status of the MLH1 promoter has been analyzed using polymerase chain reaction (PCR)-based HpaII restriction assay [6].

2.4 MMR mutation screening

We screened for mutations the hMLH1 and hMSH2 genes in 30 patients without detected hMLH1 promoter hypermethylation. PCR amplification of all exons including flanking intronic regions was performed and than analyzed using single-strand conformation polymorphism (SSCP) analysis. Samples showing an altered mobility pattern were then subjected to direct sequencing.

2.5 Immunohistochemistry

The rabbit polyclonal antibody against the C-terminus of the MLH1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution was used, following the manufacturer's protocol, with minor modifications. Two investigators assessed the slides for MLH1 staining independently. Lesions were considered with lack of expression when a complete absence of nuclear staining was evident in tumor cells against the normal epithelium, stromal and blood cells, always used as internal control. Only samples with intact nuclear staining were considered to show positive expression.

3 Results

In order to investigate better the prevalence and the role of genetic instability testing in the molecular diagnostic strategy, we first performed MSI analysis on 150 Bulgarian patients with histologycaly confirmed CRC. Seven patients fulfilled the Amsterdam criteria, 39 were selected according to one or more of the Bethesda criteria, 45 were from the so- called "Family" cases and 59 were sporadic. We detected 25 tumours with MSI

(5 HNPCCs, 9 according to Bethesda criteria, 2 "Family" cases and 9 sporadic) and 14 with LOH (none from the HNPCCs, 4 according to Bethesda criteria and 8 sporadic) in at least two of the loci analyzed.

There is evidence that MSI phenotype is associated with distinct clinicopathological features, notably proximal tumor site, high grade and mucinous production [7]. Our patients were first divided according to the tumor localization. The proximal location was typical for the MSI, but not for the LOH tumors. In the whole group, 59 tumors (39%) were with proximal localization comparing with 21 from 25 (84%) MSI tumors. Only one of the left localized tumors was highly unstable. A mucinous component of more than 50% of the tumor was present in 60% of the MSI and in only 21% of the LOH cases, compared with 19% of the MSS group. The majority of the MSI (72%) and LOH (85%) colorectal tumours were with moderate to high differentiation. No difference was seen in sex, age of onset and staging, or presence of synchronous or metachronous tumours.

Then we tested the MSI and LOH tumors for hMLH1 promoter hypermethylation versus 15 random selected MSS sporadic tumors as controls. A significant association was found between this trend and MSI/LOH ($p<0.04$). The correlation between the MLH1 promoter methylation and the MSI sporadic cases was highly significant ($p<0.01$). In this group there were no patients younger than 50 years ($p<0.02$). The correlation between MLH1 promoter methylation and the LOH sporadic cases was also significant ($p<0.02$).

The IHC showed lack of MLH1 expression in all detected tumours with methylation. Patients whose tumours showed hMLH1 promoter hypermethylation were not included in the screening for MMR mutations. Our search for genetic alterations in hMLH1 and hMSH2 genes found the following variations: four known and two novel mutations, one unpublished and four known intronic polymorphisms in both hMLH1 and hMSH2 genes, presented on Table 1. The frequencies are done for heterozygous variations, excluding one patient who seems to be homozygous for c.655 A>G. Lack of MLH1 protein expression was shown in all tumors with previously described pathogenic mutations as well as in the tumor from the novel c.31 delC. Lack of protein expression was also detected in the c.655 A>G mutation carrier. MLH1 protein was intact in the tumor from the carrier of the novel "silent" mutation and in the two tumors from the c.655 A>G heterozygous.

4 Discussion

In our population- based study, the detected frequency of MSI was about 17%. This percentage is relatively high for an unselected group. In other studies the frequency of MSI was between 12 and 17% [4, 8]. In such a population-based study, Percesepe et al. found MSI in only 8,3% [9]. The clinical characteristics associated with the MSI phenotype in our group were similar to those described by others [10, 11]. An interesting finding was the moderate to high differentiation in our group, which has been previously described as specific for Chinese HNPCC families only [12].

MSI and LOH patients were then investigated for hMLH1 promoter hypermethyla-

Table 1 MLH1 and MSH2 genetic variations, found in 30 MSI or LOH Bulgarian patients.

Gene[1]	Exon/ Intron	Mutation/Polymorphism	Consequence	Frequency
MLH1	**ex.1**	**c.31 del C**	**Leu11fsX6**	**1/30**
MLH1	**ex.1**	**c.6 G>A**	**Ser2Ser**	**1/30**
MLH1	ex.8	c.655 A>G	Ile219Val	3/30
MLH1	IVS 13	c.1558+14 G>A	-	1/30
MLH1	IVS 14	c.1668-19 A>G	-	15/30
MLH1	ex.16	c.1852 AA>GC	Lys618Ala	2/30
MLH1	**IVS 16**	**c.1896-17 C>G**	**-**	**1/30**
MLH1	ex.19	c.2146 G>A	Val716Me	1/30
MSH2	ex.6	c.965 G>A	Gly322Asp	1/30
MSH2	IVS6	c.1077-10 T>C	-	1/30
MSH2	IVS12	c.2006-6 T>G	-	2/30

[1]GenBank reference sequences: MLH1 - U07343, version U07343.1, CDS 22. 2292 and MSH2 - U03911, version U03911.1, CDS4.2808.
Rows in bold – genetic variations not found in other populations.

tion, due to this epigenetic change, to exclude the sporadic cases. Hypermethylation was considered to be one of the "hits" in the Knudson hypothesis [5]. Methylation of the hMLH1 promoter region has recently been described in many MSI tumours [13–15]. To prove the role of this event, we also examined the expression pattern of the MLH1 protein. All methylated tumors failed to express the MLH1 protein and these patients were not further analysed. On the other hand, the treatment of cell lines derived from such tumours with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-azad-C) leads to demethylation of the MLH1 promoter, reaccumulation of the MLH1 protein and restoration of MMR activity [13]. It should therefore be possible to reverse the cancer phenotype by the use of methylation inhibitors [16]. Many clinical approaches have recently been trying to demonstrate target gene demethylation, and the success in this area of pharmacogenetics is probably forthcoming.

Our mutation search found pathogenic hMLH1 mutations in four families (13,3%). This low frequency may be explained with the fact that in the group of MSI, only 5 patients responded to the Amsterdam criteria and this resulted in an 80% detection rate for HNPCC. One other possible explanation is that the sensitivity of the SSCP method is about 80-85%. Higher detection rates can be achieved by direct sequencing, and it was undertaken for two HNPCC MSS cases. No mutation was found in these two patients. Some possible explanations might be mutations in other MMR genes or large deletions, which this method misses. To improve the effectiveness of our screening, we examined the MLH1 protein expression. The tumours of the four mutation carriers failed to express this protein. Lack of expression was also detected in one LOH patient from the family group. In this patient, the mutation c.655 A>G was homozygous. No other mutation has been detected in the hMLH1 gene that may explain this finding. We supposed that homozygosity of the mutation may lead to stronger protein alterations leading to this

phenotype or that one of the gene copy is mutation carrying while the second one is deleterious that made the wrong impression of homozygous condition. A MLPA analysis for large genomic deletions needs to be developed to prove this suggestion.

We did not perform a MSH2 IHC analysis and it was not clear how to interpret the finding for the c.965 G>A carrier patient according to Bethesda criteria 2 and 3. The role of this mutation in the etiology of CRC is still under discussion. One of the reasons to accept it for polymorphism is the high frequency of this alteration in the general population (1-6%) and its presence in some sporadic cases. However it was found in HNPCC pedigrees in which no other mutation has been detected. Drotschmann et al. found that this mutation does not affect the MSH2- MSH6 binding, but the mutation rate in the cell increased dramatically [17]. This study proved the hypothesis for the predisposing effect of some allelic variants or the possible relation with other MMR genes or factors.

In summary, we developed an efficient diagnostic strategy for HNPCC testing and the mutation status of 80% MSI HNPCC cases that fulfill the Amsterdam criteria could be detected. In the whole group, 28% were proved as sporadic cases. The PCR- based HpaII method for detection of the MLH1 promoter region hypermethylation is a simple and applicable method in the diagnostic algorithm for CRC. We propose this method in the diagnostics of CRC as a step that follows the analysis for MSI and prior to the screening for mutations in the MMR genes, especially in sporadic cases, because in most cases they are mutation negative. The use of IHC analysis improves the detection rate and might be very helpful tool in the investigation of some unclear molecular variations.

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