The fluctuation of BRCA1 gene is associated in pathogenesis of familial colorectal cancer type X

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Abstract
Aim: Among families fulfilling the Amsterdam criteria HNPCC, nearly 40% of this cases are microsatellite stable and not DNA mismatch repair gene (MMR) and also these mutations were found in this situation. Correspondingly, these families were nominated as familial colorectal cancer type X (FCCTX). The BRCA1 gene plays a role in main cellular pathways and germ line mutations in this gene could underlie different familial cancers. Material and Method: In this way, samples were selected according to previous study that patients who met Amsterdam criteria for lynch syndrome but did not have defective MMR gene and are known as FCCTX. So, 25 normal tissues have been considered for further studies and HRM method was used in order to detect mutations in 2, 3, 5, 16 and 20 exons of the BRCA1 gene. Results: For all samples, the DNA was tested by HRM for the detection of mutations in BRCA1 exons. After normalization and temperature shifting, a clear difference was evident between the melt curves for each sample. Through the different samples of each group, one was selected for sequencing. Based on our sequencing results deleterious BRCA1 gene mutations were not identified and there was no significant difference between the sequences of the samples with a different melting curve. Discussion: Conclusively, our study showed that although BRCA1 is a critical gene playing role in several CRCs, it maybe plays an insignificant role in this type of CRC.

Keywords
FCCTX; HNPCC; Lynch Syndrome; BRCA1; HRM
Introduction
Colorectal cancer (CRC) is the third most common cancer in males and second most common cancer in females worldwide[1]. Inherited elements have a critical role in at least one third of all CRCs [2]. Hereditary nonpolyposis colorectal cancer (HNPPC) is one of the most common inherited CRC syndromes, due to mutations in a DNA mismatch repair gene [3,4]. Among families fulfilling the Amsterdam criteria HNPPC, nearly 40% of them are microsatellite stable and not DNA mismatch repair gene (MMR) mutations were found among them. These families were nominated as familial colorectal cancer type X (FCCTX) [5]. Individuals with FCCTX display diminished risk for extracolonic neoplasms, and tumorogenesis process tends to occur later in life[6,7]. An ambiguity in the nature of the disease for FCCTX presents considerable challenges in terms of deciding upon the best approach to interrogate its genetic basis [8]. The BRCA1 gene plays a role in numerous main cellular pathways, including checkpoint activation, DNA damage repair, and chromatin remodeling. It seems that the BRCA1 tumor suppression function is related to its function in homologous recombination damage repair [9]. Germline mutations in this gene could underlie different familial cancers[8], for example in addition to breast and ovarian cancer its role in prostate, pancreatic and stomach cancers was seen [10]. A recent prospective study of 7015 women with BRCA1 mutation recognized fivefold increased risk of colorectal cancer among BRCA1 mutation carriers younger than 50 years [11].

In this study, in order to identify new mutations in genes involved in FCCTX, we focus on BRCA1 gene. In the present study, we have analyzed mutations in some hot spot exons of the BRCA1 gene by High Resolution Melting (HRM) technique. HRM consists of the accurate checking of the alteration in fluorescence caused by the release of an intercalating DNA dye from a DNA duplex as it is denatured by increasing temperature [12]. This technique has been developed recently and displays great potential for scanning germline and somatic mutations[13].

Material and Method
Sample collection and preparation
The Samples were collected from Poursina Hakim Research Center, a referral gastroenterology clinic in central part of Iran, Isfahan. Samples were selected according to previous study that patients who met Amsterdam criteria for Lynch syndrome but did not have defective MMR gene [14]. Out of 219 patients who were under 50 years at diagnosis, 45 HNPPC families were selected meeting AC-1 for Lynch syndrome. From those, 25 families that did not have defective MMR gene and were known as colorectal cancer type X were selected. The normal tissues of these patients have been considered for further studies. 10μm thickness sections were prepared from 25 samples using microtome.

Mutation screening by HRM
DNA extraction was performed using DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. DNA concentration and purity were measured by UV spectrophotometry using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

In this study, we used HRM method to detect mutations in some exons of the BRCA1 gene. We investigated five BRCA1 hot spot exons included 2, 3, 5, 16 and 20 exons. Designing primer for HRM needs certain special attention for instance, because DNA derived from FFPE is often degraded. Primers were designed by beacon designer software. Primer sequences and amplicon size are given in table 1.

PCR conditions and HRM assay
PCR and HRM analysis was performed in 0.1ml tubes on the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). All samples were tested in duplicate and one negative DNA control was included in each experiment. 10 ng of DNA was amplified in a final volume of 10 ml containing Type-it HRM PCR Kit (Qiagen, Hilden, Germany), forward primer, reverse primer and nuclease free H2O.

PCR cycling parameters were according to the following conditions: initial denaturation at 95°C for 5 min; 45 cycles of 95°C for 15 seconds, 60°C for 25 seconds and 72°C for 25 seconds. The final melting program was melting from 70°C to 90°C, with a ramp of 0.2°C per second. The cycling and melting conditions for all exons were the same. These experiments have been done on all of 25 normal tissues. Results were analyzed as fluorescence versus temperature graphs by accompanying Gene Scanning software (v1.7). With normalized, temperature-shifted melting curves displayed as difference plot. Those samples showed a different melting curve were sequenced.

Ethical approval
This study was conducted on samples utilized in previous study and ethical approval process has been already explained in the introduction.

Table 1. Primer sequences for HRM

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>Forward Primer</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Forward Primer</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward Primer</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>16-1</td>
<td>Forward Primer</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>16-2</td>
<td>Forward Primer</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Forward Primer</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Melting curve and Normalized HRM difference graph of exon 2
shown that mice deficient in colon cancer in the animal models, for example, it has been associated with a mutation was 2.03 [19,20].

Based on our sequencing results deleterious BRCA1 gene mutations were not identified. There was no significant difference between the sequences of the samples with a different melting curve. The result of one of the investigated exons (exon2) was shown in figure 1. The results of other exons were similar (data not shown). Other information available.

Discussion
Since the genetic etiology for FCCTX is largely unknown, genetic confirmation of the diagnosis can help direct surveillance recommendations for the patient and their at-risk family members [15]. BRCA1 gene has high tumor heterogeneity and the role of its mutations in numerous cancers specially in CRCs is a probable event [16,17].

For our purpose HRM has been used as a rapid screen for identifying BRCA1 mutations, finally, after comparing melting curves and analyzing sequencing results, our study showed that there is not any association between the BRCA1 exons 2, 3, 5,16 and 20 mutations and FCCTX. This finding is in contrast to same studies which done about the role of BRCA1 mutations in other CRCs. For example, in a study by Phelan C et al 7015 women with a BRCA1 mutation were followed in order to detect new cases of colorectal cancer. They showed that twenty-one incident colorectal cancer cases were discovered among all mutation carriers and there was a substantial fivefold increased risk of colorectal cancer among BRCA1 mutation carriers younger than 50 years [18]. Also in the studies done in the Breast Cancer Linkage Consortium the authors reported modest but significant increases in colorectal cancer risk in family members of families with a BRCA1 or BRCA2 mutation. The relative risk associated with a BRCA1mutation was 2.05 [19,20].

There are also some studies, showing a probable role for BRCA1 in colon cancer in the animal models, for example, it has been shown that mice deficient in BRCA1 will be affected by a varied range of carcinomas, frequently breast and lung but also gastric, endometrial and colon cancer [21]. In the investigated exons we did not find any mutation in FCCTX patients. It can be due to the limited number of samples. Our samples obtained from the screening of 219 CRC patients and only 25 samples remained, which represents FCCTX. On the other hand the mutations in other exons of the BRCA1 gene may be involved in causing disease. We investigated hot spot exons and it is recommended to check other exons. Finally, although BRCA1 is a critical gene playing role in several CRCs, it maybe plays an insignificant role in this type of CRC and other studies confirm this subject [22].

One of the most notable aspects of our study is that, this is the first time that the role of BRCA1 mutations has been analyzed in this type of colorectal cancer and suggest that other BRCA1 exons investigate.

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Competing interests
The authors declare that they have no competing interests.

References


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