Characterization of New Founder Alu-Mediated Rearrangements in MSH2 Gene Associated with a Lynch Syndrome Phenotype

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Abstract

It has been reported that large genomic deletions in the MLH1 and MSH2 genes are a frequent cause of Lynch syndrome in certain populations. Here, a cohort has been screened and two new founder rearrangements have been found in the MSH2 gene. These mutations have been characterized by break point determination, haplotype analysis, and genotype-phenotype correlation. Mutations have been identified in the MLH1, MSH2, and MSH6 genes in 303 subjects from 160 suspected Lynch syndrome unrelated families. All subjects were tested using heteroduplex analysis by capillary array electrophoresis. Multiplex ligation-dependent probe amplification was used to detect rearrangements in mutation-negative index patients and confirmed by reverse transcriptase PCR. The break point of the deletions was further characterized by the array comparative genomic hybridization method. Immunohistochemical staining and microsatellite instability were studied in tumor samples. Hereditary nonpolyposis colorectal cancer-related phenotypes were evaluated. More than 16% (24 of 160) of the families had pathogenic mutations (8 MLH1, 15 MSH2, and 1 MSH6). Twelve of these families (50%) are carriers of a novel mutation. Seven of the 15 positive MSH2 families (47%) are carriers of a rearrangement. The exon 7 deletion and exon 4 to 8 deletion of MSH2 are new founder mutations. The segregation of a common haplotype, a similar phenotype, and anticipation effects were observed in these families. These findings will greatly simplify the diagnosis, counseling, and clinical care in suspected Lynch syndrome families and not just in specific geographic areas, so wide distribution may be explained by migration patterns. Cancer Prev Res; 4(10); 1546–55. ©2011 AACR.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPPC) syndrome, or Lynch syndrome (MIM 120435), is probably the most common form of inherited colorectal cancer (CRC), accounting for 1% to 5% of cases (1). Affected individuals have a family history of CRC at an early age, characterized by tumor predominance in the proximal colon and an association with extracolonic tumors. Germ-line mutations in at least 5 mismatch repair (MMR) genes (MLH1, MSH2, MSH6, PMS1, and PMS2) have been identified in families fulfilling international criteria for the syndrome, namely, Amsterdam criteria I or II (2–4), or less stringent criteria referred to as the Bethesda guidelines (5), which lead to tumors characterized by widespread microsatellite instability (MSI).

Identification of inherited predisposition is important because it enables targeted clinical surveillance, which significantly reduces cancer morbidity and mortality in Lynch syndrome families (6).

Nearly 90% of the mutations in databases (Leiden Open Variation Database, LOVD) affect either MLH1 (MIM 120436) or MSH2 (MIM 609309; refs. 7, 8). The vast majority of mutations are nonsense, missense, splicing, or frameshift mutations, but a recent report (9) indicates that a substantial percentage of HNPPCs are caused by gross genomic rearrangements in MMR gene alterations undetectable with traditional methods of mutation analysis. They account for up to 15% of all pathogenic mutations in MSH2 and MLH1 (10). The frequency of large rearrangements in MSH2 as compared with MLH1 depends on the studied population (11). Different prescreening methods have been proposed.
Here, a combined method of heteroduplex analysis by capillary array electrophoresis (HA-CAE) and multiplex ligation-dependent probe amplification (MLPA) has been used to screen our HNPCC population (12). Using this protocol, numerous different mutations have been detected and a high proportion has rearrangements, all in the MSH2 gene.

Two founder mutations in the MSH2 gene are presented and characterized here. A similar break point was found in all index cases of the carrier families. These mutations seem to be associated with founder effects, as a common haplotype was associated with each; besides, the novel exon 4 to 8 deletion seems to be associated with anticipation.

Our findings have important implications in the diagnosis and management of such families, and these results will help to simplify genetic testing for Lynch syndrome.

Patients and Methods

Participants

Patients were recruited through the Regional Hereditary Cancer Prevention Program of Castilla y León (Spain). Informed consent was obtained from 303 subjects belonging to 160 unrelated suspected Lynch syndrome families. The control population used was from the National DNA Bank, a collection of representative DNA samples of the Spanish population. Available DNA, RNA, and tumor blocks were obtained for at least one affected person in each family.

Mutation analysis

General screening of point mutation of the MLH1, MSH2, and MSH6 genes was carried out using HA-CAE. This method was developed in our laboratory (13), and the validation for MMR genes has recently been published (12).

Figments showing an HA-CAE–altered pattern were sequenced with the BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems) with unlabeled forward and reverse primers on the ABI 3100 DNA Sequencer (4 capillaries; Applied Biosystems).

Genomic rearrangement screening

Negative samples by HA-CAE were tested using MLPA. Genomic rearrangements were evaluated by MLPA (MRC.). MLPA test kits P003 for MLH1/MSH2 and P008 for MSH6/PMS2 were used according to the supplied protocol. Fragment analysis of the PCR products was carried out on the ABI 3130 Genetic Analyzer and gene dosage calculation and analysis were done using Genemapper software (Applied Biosystems).

Reverse transcriptase PCR

All the alterations detected by MLPA were confirmed by reverse transcriptase PCR (RT-PCR). Three microliters of total RNA was used to synthesize cDNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) using random primers. RT-PCR was carried out with specific primers designed for the coding sequences flanking the putative mutation. To confirm deletions, short amplicons from RT-PCR were sequenced with the following primers:

- Exon 4 to 8 deletion: forward in exon 3, 5'-ggttggagtgg-gggtgggttgatt-3'; reverse in exon 9, 5'-ggttggacctctcctttc-3'
- Exon 7 deletion: forward in exon 6, 5'-ggtgaaagttga-aacccc-3'; reverse in exon 8, 5'-ggagaagtgaagagatcg-3'

Immunohistochemical and tumor MSI testing

Immunohistochemical (IHC) staining for MLH1, MSH2, and MSH6 genes in tissue from at least one individual of the genomic rearrangement families was analyzed by a pathologist in the General Yagüe Hospital, Burgos, Spain. Paraffin-embedded tumors from these cases were received in our laboratory for MSI study. Paired colon tumor tissues and normal tissues were microdissected and DNA was extracted using the DNAeasy Tissue Kit (Qiagen). A fluorescence multiplex PCR to amplify BAT25, BAT26, D2S123, D5S346, and D17S250 was carried out as described before (14, 15) with slight modifications. Fluorescently labeled PCR products were detected using the ABI 3130 Genetic Analyzer and the GeneScan software. The tumor was categorized as microsatellite stable if none of the 5 markers showed instability, as MSI-low if one of the 5 markers showed instability, and as MSI-high if 2 or more markers showed instability (14).

Break point characterization

An array comparative genomic hybridization (aCGH) strategy was used to confirm the deletions identified by MLPA and to identify the location of deletion break points.

One positive sample, from each different family with a rearrangement, was tested by a human aCGH 44K specially designed by Nimgenetics for coverage of chromosome 2: 47419322-47580004 (NCBI 36).

On the basis of the information obtained from aCGH, primers were designed spanning the putative break points for each case and used in long-range PCR.

Haplotype analysis

Index cases and their relatives were genotyped with 8 microsatellite polymorphic markers surrounding the MSH2 gene and 2 single-nucleotide polymorphisms in the MSH2 gene. As controls, 96 unrelated DNA samples from the general population of Castilla y Leon (National DNA Bank) were also genotyped, and allele frequencies were estimated.

The 10 markers used were localized in an approximately 3.6-Mb region encompassing MSH2 (locus order: cen-D2S123-D2S1248-D2S1247-Clen30-rs3771278-rs2162123-D2S2227-D2S391-Clen27-D2S119-tel) on chromosome 2: 47419322-47580004. Fluorescently labeled primers were used to amplify the microsatellite polymorphic regions. PCR products were analyzed on the Genetic Analyzer 3130 using Genemapper 3.7 software (Applied Biosystems). The 2 intragenic single base substitutions located within intron 1 and intron 9 of MSH2 (rs2162123 and rs3771278)
were screened through high-resolution melting technology (LightCycler 480 Instrument), and samples with altered curves were sequenced.

**Phenotypic characterization**
Genealogic data and phenotype characteristics (gender, age at onset, cancer history, and characteristic feature of tumors) were evaluated in all rearrangement carrier families.

**Statistical method**
Comparisons between MMR mutation types (point mutations vs. deletions) in the MSH2 gene were assessed using the Web resource GraphPad Software. The variables related to the proband family members included type and number of CRC, endometrial cancer, and/or other Lynch syndrome–related cancers and the corresponding ages of diagnosis.

The number and type of cancers were treated as a categorical variable. Categorical data were reported as absolute values (n) and relative frequencies (%), whereas groups were compared by analyzing a 2 × 2 contingency table using Fisher’s exact test. Age was treated as a continuous variable and thereafter dichotomized to less than 50 or 50 years or more. Continuous data were reported in mean values with their corresponding SD, and groups were compared using Student’s t test. A 2-sided \( P < 0.05 \) was considered statistically significant.

**Results**

**Molecular mutation identification**
A total of 24 families with a pathogenic germline mutation were detected in MSH2, MLH1, and MSH6 by combined HA-CAE–MLPA analysis. Clinicoopathologic features, molecular findings of the index patients, and sample numbers are listed in Table 1. Twelve of these families (50%) are carriers of a novel mutation. Seven of them (29.2%) have a rearrangement, all in MSH2. Two new rearrangements encompassing exon 7 and exon 4 to 8 deletions were detected in 3 and 4 nonrelated families, respectively.

**Identification of the MSH2 recurrent mutations**
One family had previously been detected with exon 7 deletion and 3 families with exon 4 to 8 deletion. (The MLPA assay, RT-PCR products, and sequencing pattern are shown in ref. 12). New cases of this detection are presented here, and the recurrence of these mutations and the founder effect has been investigated. In total, 4 families (VA17, VA20, VA32, and VA134) were studied for exon 4 to 8 deletion and 3 families (VA4, VA169, and VA247) for exon 7 deletion. Table 1 shows that 58 patients (19% of our tested population) were analyzed for a rearrangement, 60% of whom are carriers.

A high frequency of MSH2 exon 4 to 8 deletion was observed in MSH2 mutation carrier families, which accounted for more than 26% (4 of 15).

In total, 33 probands were analyzed, 22 of which are carriers and 11 are not (double the number of carriers). IHC and Mls were analyzed in 2 families and both presented no staining of the MSH2 protein and MSI-H (see Table 1).

**Break point identification**
A customized 4 × 44 Agilent platform was used to map somatic rearrangements (designed by NimGenetics). The aCGH assay provides a prediction of rearrangement break points for the convenient design of primers and sequencing. Eight hundred twenty-two oligonucleotide probes were used to cover MSH2. A median distance between nonoverlapping array probes of 500 bp was obtained.

The break points predicted from aCGH are shown in Figure 1. For exon 7, aCGH predicts a 0.01 Mb deletion at the position 47507393-47515906 and presents 71 probes lost (Fig. 1A); for the rearrangement of encoding exons 4 to 8, a 0.04 Mb deletion is predicted between positions 47492237-47527926, with 284 probes lost (Fig. 1B).

PCR primers were designed in the first probe before and after the deletion. To confirm common deletions, PCR was carried out for several index cases of each family with exon 7 deletion and exon 4 to 8 deletion. These resulted in a deletion product of 36.7 kb in exon 4 to 8 deletion (g.13272_49953del36681; NG_007110.1.gl) and 9.4 kb in exon 7 deletion (g.28106_37472del9366; NG_0071-10.1.gl), exclusively observed in carriers of the common deletion and not in deletion-negative controls (see Fig. 1A and B).

The same mutation was found in exon 7 deletion carrier families and in exon 4 to 8 deletion carrier families. The identical break points are positioned within 2 Alu elements (Fig. 1A and B). Thus, the deletion is likely to have arisen through an Alu-mediated recombination. The presence of identical break point sequences in all cases (ID proband in Table 1) is suggestive of a founder mutation because a frequently recurring recombination event would likely result in at least a few single-nucleotide differences.

**Haplotype analysis**
Haplotype analysis was carried out to confirm the common genetic origin of the deletion rearrangements (Fig. 2). Most of the positive families for the deletion are from a small area in Castilla y León (in central of Spain), ancestors of families carrying the exon 4 to 8 deletion came from Valle de las Navas and exon 7 deletion ancestors came from Lerma, both in the province of Burgos.

A shared haplotype was observed cosegregating with the mutation (Fig. 2), which was absent in noncarriers of these families. It provides evidence for a common ancestry among these families.

Figures 2A and B represent the pedigrees of the 4 Del4_8 families and the 3 Del_7 families. The phenotype observed in these families is shown, as are the results of the common haplotype segregation.
Table 1. Pathogenic mutations, clinicopathologic features, and molecular findings of the 24 carrier families

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<th>Gene</th>
<th>Family code</th>
<th>ID proband</th>
<th>Gender (male, female)</th>
<th>Cancer site and age at diagnosis</th>
<th>Mutation designation</th>
<th>Exon/intron</th>
<th>Mutation type</th>
<th>Mutation status</th>
<th>Loss of protein expression</th>
<th>MSI status</th>
<th>No. of carrier</th>
<th>No. of noncarrier</th>
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<td>Colon-resectum, 43–63</td>
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<td>Frameshift</td>
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<td>deletion</td>
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(Continued on the following page)
Ninety-six unrelated samples were also genotyped as controls and allele frequencies were estimated; these are also shown in Figure 2A and B.

**Screening of founder rearrangement deletion in MSH2**

A PCR test was designed to screen these deletions in first-degree relatives. A routine PCR procedure was optimized. Three primer sequences were used: 1 forward and 2 reverse. The product is a multiplex PCR with the presence of 1 band in the wild type and 2 bands in deletion carrier samples (Fig. 3). This procedure is faster, cheaper, and easier than MLPA.

**Genotype–phenotype correlation**

Taking into account the fact that 62.5% of mutations we have found are in \( \text{MSH2} \), largely due to the existence of 2 recurrent mutations in this gene that represent approximately half the mutations in \( \text{MSH2} \); we have correlated the type of mutation (punctual or rearrangement) with the occurrence of extracolonic tumors and the age at diagnosis (Table 2).

The results of Table 2 show no differences between the 2 groups in the number of tumors developed \( (P = 1.0000) \) or the age at diagnosis, using the threshold of 50 years \( (P = 1.0000) \), unlike the tumor type where the prevalence of endometrial and urinary system tumors is higher in founder mutation carriers than in point mutation carriers \( (P = 0.47279 \text{ and } 0.1247) \).

On the other hand, it must be emphasized that the median age at onset is different in both groups; 46.7 versus 40.67 years in CRC males and 50 versus 33 years in CRC females. These data, however, were not statistically significant.

**Discussion**

Lynch syndrome is a heterogeneous disorder with respect to its molecular basis as well as its phenotypic expression.

A variety of point mutations, as well as large genomic rearrangements, have been reported in LOVD (ref. 16). Here, 160 families were screened using a combined method previously published by our group (12). The overall mutation detection rate in our study is 15% (24 of 160 positive families).

We have found 8 \( \text{MLH1} \) mutation carrier families (Table 1), 3 of which have the \( c.306+5G>A \) mutation, which has been described as a founder mutation in Spain, and our group has participated in the work (17). Two truncated mutations in exon 1 at \( \text{MLH1} \) are novel, both appearing in a proband with colon cancer before 50 years of age, whereas the \( \text{MLH1}_{0}1285 \) mutation has been described by us before (18).

Only one family is an \( \text{MSH6} \) mutation carrier, this is a novel truncated variant. The carrier shows a late-onset endometrial cancer with the absence of any staining of the \( \text{MSH6} \) protein and MSS tumor. Similar results have been reported in several studies (19).
The higher rate of mutations in this study appears in the MSH2 gene (nearly double that in MLH1); this is probably because of the high number of recurrent mutations in this gene.

Two mutations that affect the splicing are reported here and which we have described in LOVD. The c.1661G>A mutation is present in family VA6, which is a large family; we have studied 44 members, of which 18 are carriers (Table 1). This alteration is associated with the absence of MSH2/MSH6 staining, MSI-H tumor, and a phenotype with colon rectum cancer before the age of 50. The mutation c.2634G>A in exon 15 of MSH2 caused...
Figure 2. A, a, marker localization and allele frequency in control population of the MSH2 gene in chromosome 2. b–d, representative pedigrees of exon 7 deletion carrier families. Legend and tumor phenotype are included. B, a, marker localization and allele frequency in control population of the MSH2 gene in chromosome 2. b–e, representative pedigrees of exon 4 to 8 deletion carrier families. Legend and tumor phenotype are included.
endometrial cancer in a mother and her daughter at 43 and 40 years of age, respectively.

Two recurrent rearrangements have been detected in 7 of 15 MSH2 carrier mutation families (>46%). Genome rearrangements represent a significant proportion of all pathogenic mutations in the MMR genes of patients with colon cancer (20).

An exon 7 deletion was found in 3 nonrelated families, the deletion producing a change in the reading frame and a truncated protein. Other rearrangements in this exon have been described previously (9), but here we exactly characterize this mutation in every index case of the 3 families and all of them have the identical mutation and a shared haplotype (Fig. 2A). The identification of the break points, within the Y and Sq Alu elements, supports the hypothesis that the recurrent exon 7 deletion is due to an Alu repeat–mediated recombination event. These 3 families had a high prevalence of CRC, followed by endometrial cancer and prostate tumor, with the majority of cancers diagnosed before the age of 50 (Fig. 2A). In recent years, recurring mutations for various hereditary cancer syndromes have been identified around the world (21).

The most important evidence in our study is a big deletion that includes 5 exons in the MSH2 gene, an in-frame mutation that produces a shorter protein and appears in 4 nonrelated Amsterdam criteria fulfilled families.

The study of the break point in index cases of each of the 4 families and the haplotype analyzed confirms the same mutation in all families (Fig. 2B).

We identified that another Alu repeat event that was involved in the exon 4 to 8 deletion (Fig. 2B).

The significant proportion of families in our population with one of these founder rearrangements (near 50% of the families) suggests the need to design a simpler, faster, and cheaper method to detect these mutations. Thus, our sample study protocol could be changed and a prescreening of founder mutations can be started.

We have carried out a multiplex PCR (Fig. 3) that is cheaper than the MLPA method.

Table 2 describes the cancer types in affected carriers and the average age of onset. Most of them developed colon cancer before the age of 45, and the women developed endometrial cancer before the age of 50.

Our data indicate that the anticipation phenomenon is associated with exon 4 to 8 MSH2 deletion. This is a subjective observation based on evidence from the age of onset and an exhibition of a more aggressive disease severity (number of cancers/tumors or the stage of the tumor) over successive generations (Fig. 2B).

The anticipation phenomenon has been described in other syndromes (22). The presence of anticipation in HNPPC is more controversial, and some reports provide significant evidence that MHL1 mutations, in particular, may be associated with anticipation (23). Our data suggest that MSH2 deletions may well be associated with anticipation effects. The median age at colon cancer diagnosis was 44 years in the 4 families (Table 2), but in family VA20, we observed that the difference at onset over 2 generations was 20 years. In family VA17, the number of cancers increased over the generations.

Both MSH2 rearrangements observed here occurred between intron 4 and 8. It has been described that this is an Alu-rich region because approximately 84% of Alu sequences in MSH2 are located between the promoter and exon 9 and it is possible that these sequences mediate the recombination observed in this gene (24, 25).

Estimating the age of founder mutations is generally an inexact task (26). It was therefore impossible to estimate
whether all our founder carrier families share the same haplotype. The fraction of haplotype can be inadequate for recombination events, as we have a small number of carrier families and these show a short genealogy length. The evidence suggests that the origin of these mutations is a recent event.

Given the high proportion of rearrangements, we have evaluated the phenotype in \( MSH2 \) mutation carriers. A more severe phenotype (more different tumors, less onset, etc.) in rearrangement carriers was expected (25), but differences were not found. We only noticed a less primary CRC tumor onset in rearrangement carriers than in other \( MSH2 \) mutation carriers.

We detected rearrangements more often in families with endometrial or urinary cancer. These data are similar to those published by Geray and colleagues (27). A possible explanation could be that both rearrangements occur in the \( MSH2 \) gene and both are founder mutations in our population.

We detected rearrangements more often in families with endometrial or urinary cancer. These data are similar to those published by Geray and colleagues (27). A possible explanation could be that both rearrangements occur in the \( MSH2 \) gene and both are founder mutations in our population.

The differences found are not statistically significant, and this does not allow us to orient the diagnosis based on mutation type (punctual or rearrangement) in clinicopathologically suspect patients of \( MSH2 \) mutation.

In conclusion, we have provided genetic evidence that the exon 7 deletion and exon 4 to 8 deletion are both pathogenic founder mutations involved in causing HNPCC in a territory in central Spain. Our data show that large genomic rearrangements occur in these genes with a high frequency and emphasize the need to incorporate techniques to routinely detect them. This should facilitate the genetic diagnosis of Lynch syndrome in our population. The origin of the \( MSH2 \) founder rearrangement can be linked to specific geographic areas, and their current distribution is compatible with the presumed migration pattern in our country. In fact, members of our families have been studied in other cities of Spain (Madrid, Barcelona, and Seville), and it is for this reason that our results are very important.

Our findings will greatly simplify the diagnosis, counseling, and clinical care in suspected families.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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### Table 2. Genotype–phenotype correlation in \( MSH2 \) mutation carriers

<table>
<thead>
<tr>
<th></th>
<th>Punctual (truncating)</th>
<th>Rearrangements (founder)</th>
<th>Statistical comparison</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of families</td>
<td>8</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of affected</td>
<td>19</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males, ( n ) (%)</td>
<td>10 (52.6)</td>
<td>6 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 tumor</td>
<td>11 (57.9)</td>
<td>8 (63.3)</td>
<td>Several tumors (yes/no)</td>
<td>1.0000</td>
</tr>
<tr>
<td>&gt;1 tumor</td>
<td>8 (42.1)</td>
<td>7 (46.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon (CRC)</td>
<td>17 (58.6)</td>
<td>13 (50.0)</td>
<td>CRC/EC</td>
<td>0.4727</td>
</tr>
<tr>
<td>Endometrial cancer (EC)</td>
<td>4 (13.8)</td>
<td>6 (23.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary system (US)</td>
<td>2 (6.9)</td>
<td>7 (26.9)</td>
<td>CRC/US</td>
<td></td>
</tr>
<tr>
<td>Other cancers</td>
<td>6 (20.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>15 (78.9)</td>
<td>11 (73.3%)</td>
<td>Age at diagnosis &lt;50 (yes/no)</td>
<td>1.0000</td>
</tr>
<tr>
<td>&gt;50</td>
<td>4 (21.1)</td>
<td>4 (26.7%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** *Standard deviation.*
References


Characterization of New Founder Alu-Mediated Rearrangements in MSH2 Gene Associated with a Lynch Syndrome Phenotype

Lucia Pérez-Cabornero, Ester Borràs Flores, Mar Infante Sanz, et al.


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