An American founder mutation in MLH1

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Mutations in the mismatch repair genes cause Lynch syndrome (LS), conferring high risk of colorectal, endometrial and some other cancers. After the same splice site mutation in the MLH1 gene (c.589-2A>G) had been observed in four ostensibly unrelated American families with typical LS cancers, its occurrence in comprehensive series of LS cases (Mayo Clinic, Germany and Italy) was determined. It occurred in 10 out of 995 LS mutation carriers (1.0%) diagnosed in the Mayo Clinic diagnostic laboratory. It did not occur among 1,803 cases tested for MLH1 mutations by the German HNPCC consortium, while it occurred in three probands and an additional five family members diagnosed in Italy. In the U.S., the splice site mutation occurs on a large (~4.8 Mb) shared haplotype that also harbors the variant c.2146G>A, which predicts a missense change in codon 716 referred to here as V716M. In Italy, it occurs on a different, shorter shared haplotype (~2.2 Mb) that does not carry V716M. The V716M variant was found to be present by itself in the U.S., German and Italian populations with individuals sharing a common haplotype of 280 kb, allowing us to calculate that the variant arose around 5,600 years ago (225 generations; 95% confidence interval 183–272). The splice site mutation in America arose or was introduced some 450 years ago (18 generations; 95% confidence interval 14–23); it accounts for 1.0% all LS in the Unites States and can be readily screened for.

Germline mutations that occur in more than one or just a few individuals are of two distinct types. The first type, here referred to as “recurrent” arises repeatedly de novo, usually because of a sequence peculiarity that predisposes to an abnormal event at meiosis. The second type, referred to here as a “founder” mutation, arose in a single ancestor and is subsequently inherited by numerous descendants.

Lynch syndrome (LS), previously known as hereditary nonpolyposis colorectal cancer (HNPCC), is caused by germline mutations in the mismatch repair genes MSH2, MLH1, MSH6 and PMS2. LS is characterized by an extremely high risk of colorectal and endometrial cancer and to a lesser degree, several other cancers. Both recurrent and founder mutations in the mismatch repair genes have been amply described. As an example, a prototype recurrent mutation in MSH2 is an A to T transversion in the third nucleotide of the donor splice site of intron 5 (abbreviated c.942+3A>T) leading to the skipping of exon 5 resulting in loss of MSH2 function. The sequence peculiarity predisposing to this recurrent mutation is apparently the fact that the mutated A is the first of a tract of 26 adenines, which predisposes to misalignment during meiotic pairing.1,2 This recurrent mutation occurs worldwide and accounts for 5–10% of all MSH2 mutations. A prototype LS founder mutation is the c.1906G>C transversion in MSH2 that leads to a missense amino acid

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substitution of alanine to proline, A632P. As is the case with many founder mutations, this mutation occurs only in one ethnic population, in this case the Ashkenazi Jews, where it may account for approximately one-third of all LS cases.

Recurrent and founder mutations are medically important because they can facilitate diagnostic approaches. In populations where they occur with appreciable frequency, mutation testing can begin by a simple test for the main recurrent or founder mutations. If a mutation is found, further testing can be avoided thus resulting in cost savings. Founder mutations typically occur in isolated, ethnically distinct populations. The U.S. population is ethnically heterogeneous; therefore widespread founder mutations are not common. A remarkable exception is the deletion of exons 1–6 of the MSH2 gene that is referred to as the American Founder Mutation. In this communication, we describe a splice site mutation in MLH1. We show that this mutation is relatively common in the U.S. where it either arose in or was brought in by an early immigrant. Moreover, we show that the mutation occurred in an allele that already carried another more common founder sequence change in MLH1 (missense variant V716M) so that the mutated allele contains both changes. We discuss the implications of these findings.

Material and Methods

Study samples

Our study included cases from Italy, Germany and the United States carrying either the deleterious MLH1 splice site mutation (c.589-2A>GT) or the innocent MLH1 V716M variant or both. Informed consent, approved by our respective Institutional Review Boards to conduct genetic experiments, was obtained from the study subjects. The Italian samples belonged to families A-AV23 and A-AV24. The newly acquired probands for families A-TN5 and A-TN6 were selected in Presidio Ospedaliero Santa Chiara in Trento (Italy) and sent to Centro Riferimento Oncologico in Aviano for genetic testing.

The German cases were counseled and underwent genetic testing at the facilities of the German HNPCC-Consortium as described elsewhere. They were then entered into the German mutation database. The first description of the splice site mutation was in a colorectal cancer patient. We have studied North American samples from Boston, Ohio, Michigan and the Mayo Clinic Molecular Diagnostic Laboratory. Since the Mayo Clinic is a major reference laboratory in the U.S., the samples obtained from them were from patients undergoing clinical genetic testing for LS from around the U.S. These samples were anonymized to retain confidentiality. The Michigan family was identified in a clinical series tested at the University of Michigan Cancer Genetics Clinic.

Controls genotyped for haplotype analysis were Caucasian (n = 78) and African American (n = 6) from the Ohio State University Medical Center’s Human Genetics Sample Bank. They are derived from the Columbus-area population. This collection of control samples is approved by the Biomedical Sciences Institutional Review Board at Ohio State University Medical Center.

Haplotype analysis

To characterize the haplotypes associated with the MLH1 splice site and V716M changes we used 14 out of 15 microsatellite markers previously reported that span the MLH1 locus. We added three additional single nucleotide polymorphisms (SNPs) present in the MLH1 gene. All available probands and family members and 84 controls were typed for these MLH1 markers.

Microsatellite markers were typed utilizing a labeled M13 primer in conjunction with an M13-tailed, amplicon specific, primer in a three primer PCR. Each 15-μl PCR reaction contained 7.5 μl of AmpliTaq Gold master mix (PE Applied Biosystems, Foster City, CA), 25 ng of genomic DNA, 10 pmol of untailed primer, 5 pmol of M13-tailed primer and 10 pmol of the FAM-labeled M13 primer. Reactions were cycled using the following profile: 96°C for 10 min, 36 cycles of 96°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and final extension at 72°C for 5 min. The PCR product was sized using an ABI 3730 DNA Analyzer.

For the genotyping of SNPs, we used the same PCR conditions as above in the presence of 10 pmol forward and reverse primers. The PCR product containing the SNP was subjected to the SNAPSHOT reaction (PE Applied Biosystems, Foster City, CA). The sequences for the primers are listed in Supporting Information Table 1.

Estimating the age of the variants

The DMLE+2.3 software developed by Reeve and Rannala was used to estimate the age of the two variants. The program, which is freely available from www.dmle.org, uses a Bayesian approach to compare differences in linkage disequilibrium between the mutation and flanking markers, among DNA samples from mutation carriers and unrelated controls.

This software uses genotype data for cases and controls, marker locations, population growth rates and an estimate of the proportion of the mutation bearing chromosomes being analyzed.

Marker locations were obtained from the human genome reference sequence (Human build 37.2). The deCODE genetic positions were known for most of the microsatellite markers. For the markers and SNPs not in the deCODE map, genetic distances were obtained with spline interpolation using known genetic positions of adjacent deCODE markers.

The age of the V716M variant in Europeans was calculated using German (n = 13) and Italian (n = 2) probands. For the population growth rate we used 1.05 fold per generation. We have estimated the number of the V716M variant bearing chromosomes for the Italian and German populations using the frequency of the variant found in the samples tested, assuming that an innocent variant should have a similar frequency in controls as in cases. Based on the
population sizes (Italy ~ 60 million, Germany ~ 82 million) the numbers of chromosomes were 402,791 and 907,399, respectively.

The age of the splice site mutation in the American population was calculated using a growth rate of 1.65 fold.6 To estimate the proportion of disease bearing chromosomes studied, we used the following data: 5.5% lifetime risk for CRC; 2.8% of CRCs are LS and 1.0% of LS cases are due to this splice site mutation.

Additionally, we applied the Estiage program17 to estimate the age of the most recent common ancestor carrying the V716M variant, since the shared haplotype is relatively small (280 kb), and it has been densely genotyped. We were unable to use the same method for the estimation of the most recent common ancestor carrying the splice site mutation. The shared haplotype is ~17-fold longer (~4.8 Mb) and the number of markers used increases only from 5 to 15, resulting in significant distances between the markers that is not suitable for the program.17

Results and Discussion
The MLH1 intron 7 splice site mutation
The c.589-2A>G mutation, first published by Luce et al.,10 disrupts the acceptor splice site of intron 7 and results in the skipping of exon 8. This predicts loss of MLH1 function and LS, both of which are amply confirmed by the data presented here. This mutation is one of several hundred different MLH1 mutations that are known today based on two major databases (www.insight-group.org/mutation; http://www.med.mun.ca/mmrvariants/). Subsequently Syngal et al.11 found the same mutation in a LS proband of a family that met the Amsterdam I criteria.

We found the splice site mutation in the proband of a large LS family identified in Ohio. The proband was enrolled in a population-based study of LS in 563 unselected newly diagnosed patients with endometrial cancer of whom 14 had LS (Refs. 12,18 and unpublished data). To assess the proportion of cases carrying the splice site mutation in the Ohio series, we can use additional data from a study of LS in 1,566 consecutive colorectal cancer patients, of whom 44 had LS.19 Thus, the prevalence of the splice site mutation among probands with LS is 1/58 or 1.7%. The mutation occurs in six further members of the Ohio family, four of whom have been diagnosed with LS cancer. Of note, the splice site mutation is not mentioned in the original reports12,19 because it was not called by the sequencing software and missed when the testing was first performed and was only recognized later in a collaboration with and thanks to our colleagues in Germany. Instead, the V716M missense variant was originally found in the proband and those family members in whom the splice site mutation was later detected (Pedigree in the Supporting Information Fig. 3). To conclusively show that the splice site mutation and the V716M variant were on the same chromosome, we used the method of conversion to haplody20 confirming that the two changes are in cis (see Supporting Information for description). We thus had evidence of the splice site mutation in three US families; a fourth family was communicated to us by Dr. Gruber, and it appeared that the proportion of all LS having the splice site mutation might be high enough to warrant further investigation. In our study, it was of interest to answer two questions. First, how common is the mutation in the US and elsewhere? Second, is it recurrent or of founder type?

Disease causing mutations are usually too rare to search for in the general population. Instead it is meaningful to ask how common the mutation is among LS probands. The data from individual laboratories are almost never extensive enough for this purpose, so we turned to three larger sources, the molecular diagnostic laboratory of the Mayo Clinic, the German HNPCC consortium and a collaborative group in Italy. Italy was studied because reports of the mutation had been published.8,21

The archives of a large molecular diagnostic laboratory (the Mayo Clinic) were searched for the splice site mutation. It had been seen in totally 10 individuals, 8 of whom were ostensibly unrelated, likely representing different families. During the same time period, a total of 995 cases with LS had been diagnosed in this laboratory. Thus, the proportion of carriers of the splice site mutation in the US was 1.0% (10/995), but none was found in Germany. While it is clearly desirable to study further cohorts from various geographic regions, we hypothesize that the mutation is indeed rare in most other populations than the US and Italy because it has not been reported to the databases quoted above. In total, we are presently aware of 22 individuals carrying the splice site mutation in the US (Table 1).

The MLH1 exon 19 missense variant
In the splice site mutation carrier, Syngal et al.11 noted the presence of the V716M variant in exon 19, termed missense mutation, caused by the c.2146G>A sequence change. This change has been reported numerous times in smaller and larger series of patients tested for LS. For instance, the INSIGHT database (www.insight-group.org/mutations) states that it has been “cited 58 times” but due to double reporting the real number may be smaller. The amino acid in position 716 is only weakly conserved among species but resides in the MLH1 domain that binds PMS2. The wild type amino acid, valine is neutral and hydrophobic while methionine is also hydrophobic, but contains sulfur.

Typically, missense mutations can be either deleterious or nondeleterious, and it is almost impossible to predict the pathogenicity of missense changes based solely on the functional domain and the nature of the amino acids involved. Several groups of investigators have used a variety of strategies to investigate the variant’s effect on mismatch repair. Raeaara et al.22 examined protein expression, stability, subcellular localization, PMS2 interaction and mismatch repair efficiency and concluded that V716M is functionally normal. The same result emerged from functional assays in yeast.23,24
from a cell free complementation assay and from a recent study combining several methods. Thus, ample functional evidence strongly suggests that V716M does not affect mismatch repair.

Existing evidence from the case-report type publications points in the same direction. V716M has been reported numerous times, but it is not well established how many cases totally were studied for MLH1. In the Mayo Clinic series, V716M (without the splice site mutation) occurred in at least 7 individuals out of 1,385 in whom MLH1 was studied for mutation (~0.5%). In the German series, 20 V716M carriers were found among 1,803 patients in whom MLH1 was studied (1.1%).

Several authors report having searched for V716M in controls and no V716M mutation carriers were found in a total of some 400 controls reported in these studies. In a study of primary antibody deficiency syndromes in Sweden, 991 control individuals were tested for V716M. The allele frequency was 3/1904 (i.e., a carrier frequency of approximately 3/952, 0.3%).

Thus, in summary, the proportion of V716M carriers in patients studied for LS (~0.8%) appears to be similar to the

### Table 1. Clinical features of c.589–2A>G mutation carriers

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age</th>
<th>Cancer History</th>
<th>Relationship</th>
</tr>
</thead>
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<tr>
<td>U.S. Cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGN 6291–00</td>
<td>Female</td>
<td>50</td>
<td>EC;48, Ovarian;48</td>
<td>Proband</td>
</tr>
<tr>
<td>1229–01.SV</td>
<td>Female</td>
<td>50</td>
<td>EC;39</td>
<td>Proband</td>
</tr>
<tr>
<td>1229–02.BV</td>
<td>Female</td>
<td>75</td>
<td>CRC;41, EC;63</td>
<td>Mother</td>
</tr>
<tr>
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<td>Female</td>
<td>48</td>
<td>Unaffected</td>
<td>Sister</td>
</tr>
<tr>
<td>1229–07.2JV</td>
<td>Male</td>
<td>31</td>
<td>Unaffected</td>
<td>Son</td>
</tr>
<tr>
<td>1229–11.2PS</td>
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<td>72</td>
<td>Urothelial;60</td>
<td>Maternal Uncle</td>
</tr>
<tr>
<td>1229–25.1MS</td>
<td>Male</td>
<td>d.43</td>
<td>Small bowel;41</td>
<td>Maternal first cousin (son of 1229–11.2PS)</td>
</tr>
<tr>
<td>1229–11.1MS</td>
<td>Male</td>
<td>d.51</td>
<td>8–10 Colon polyps</td>
<td>Maternal Uncle</td>
</tr>
<tr>
<td>1229–24.1IH</td>
<td>Female</td>
<td>40</td>
<td>CRC;28</td>
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<tr>
<td>Mayo 3</td>
<td>Female</td>
<td>42</td>
<td>EC; by 42, CRC by 42; Ovarian by 42</td>
<td>Proband</td>
</tr>
<tr>
<td>Mayo 4</td>
<td>Female</td>
<td>48</td>
<td>EC; 48</td>
<td>Proband</td>
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<td>50</td>
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<td>Male</td>
<td>50</td>
<td>Synchronous CRC; 45</td>
<td>Proband</td>
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<td>Mayo 9</td>
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<td>33</td>
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<td>Son of proband (not tested at Mayo)</td>
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<tr>
<td>Mayo 10</td>
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<td>65</td>
<td>CRC; age unknown</td>
<td>Relative of proband (not tested at Mayo)</td>
</tr>
<tr>
<td>Mayo 11</td>
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<td>78</td>
<td>CRC; 49, 51, 61</td>
<td>Proband</td>
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<tr>
<td>Mayo 12</td>
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<tr>
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<td>23</td>
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<td>Grandaughter of Mayo 11</td>
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<td>Mayo 14</td>
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<tr>
<td>Female</td>
<td>d.61</td>
<td>Breast;39, CRC;47, CRC;59</td>
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</tr>
<tr>
<td>Male</td>
<td>54</td>
<td>CRC;53</td>
<td>Son of DF 1751</td>
<td></td>
</tr>
</tbody>
</table>

| Italian Cases |
| CFS1 (A-AV24) | Female | 70 | EC;52 | Proband |
| CFS87 (A-AV24) | Male | 51 | CRC;30 | Cousin of CFS1 |
| CFS88 (A-AV24) | Female | 59 | CRC;52, EC;57 | Cousin of CFS1 |
| CFS507 (A-AV24) | Female | 46 | EC;44, Ovarian;44 | Daughter of CFS1 |
| CFS629 (A-AV24) | Female | 28 | CRC;26 | Granddaughter of CFS1 (daughter of CFS507) |
| CFS728 (A-TN5) | Female | 63 | EC;56, CRC;60 | Proband |
| CFS802 (A-TN6) | Female | 46 | CRC;27, Stomach;43 | Proband |
| CFS803 (A-TN6) | Male | 64 | CRC;56 | Brother of CFS802 |

Abbreviations: CRC, colorectal cancer; EC, endometrial cancer; d, died. Current age/Age at death. Numbers refer to age at diagnosis.
proportion in controls (~0.6%), supporting the notion that the missense change should be viewed as a harmless polymorphism. Our haplotype data (see below) suggest that V716M is an ancestral founder variant. Such changes are known to vary in frequency between populations; hence, minor biases in the choice of cases and controls may underlie the differences reported.

As quoted above, several extensive studies have now concluded that V716M is clinically innocent. However, at the time we detected V716M in a young proband with endometrial cancer and a family history suggesting LS, the benign nature of V716M had not been definitively established. After the patient and her family had been counseled and tested, it became clear that V716M segregated with the disease in the family as shown in the pedigree (Supporting Information Fig. 3). Later a more penetrant splice site mutation was found in one member of the family that had simply been missed when the proband’s DNA had been sequenced. Given the frequent association of V716M with the splice site mutation in the US, we suggest that the sequencing results for intron 7 be carefully evaluated in individuals displaying V716M.

Haplotype analyses and mutation age estimation
We genotyped 3 SNPs and 14 microsatellite markers in carriers of both the splice site mutation and the V716M variant, and constructed haplotypes by the PHASE method in mutation carriers (Fig. 1 and Supporting Information Fig. 2). Based on data from one individual each from 11 unrelated American families carrying both sequence changes (Fig. 1a) a shared haplotype was found which covers a genomic region of some 4.8 Mb around MLH1. Based on these data the DMLE+2.3 program predicts an age of the splice site mutation of some 18 generations, which is the equivalent of around 450 years (confidence interval 340–585 years) since founding (Fig. 2).

Figure 1. Genotype data spanning the MLH1 locus in 33 probands. Haplotypes associated with the mutations are highlighted in gray, with the mutation allele bolded. (a) US samples with both the splice site mutation and the V716M variant. (b) Italian samples carrying only the splice site mutation. (c) Italian (1, 2), German (3–15) and US (16–20) samples with the V716M variant. The sizes of the shared haplotypes are indicated with empty bars below each group. Underlined is the allele associated with the V716M variant in Italian proband 1, which was determined based on genotyping of 2 relatives of the proband (see Supporting Information Fig. 2). *Frequencies in controls of the bolded alleles (in a).
Based on data from one individual in each of the three Italian families with the splice site mutation (without V716M) an unequivocally determined shared haplotype (Fig. 1b) suggests a founder mechanism, and the haplotype is entirely different from that of the American cases (Fig. 1a). It is also smaller, comprising some 2.2 Mb. These data imply that the splice site mutation arose at least twice, once in an early American immigrant (or in an ancestor of an immigrant) and once elsewhere (perhaps Italy) a long time ago.

All of the US, Italian and German carriers of V716M without the splice site change (Fig. 1c) share a short haplotype comprising some 280 kb. Importantly, this haplotype is identical with the central part of the haplotype seen in US carriers of both the splice site mutation and V716M (Fig. 1a). These haplotypes suggest the possibility that the V716M in all or most cases represents a single, ancient mutational event. Moreover, the splice site mutation seen in the US, but not the one seen in Italy, arose more recently in a chromosome carrying the ancestral V716M. Under this assumption we performed the age calculation for the V716M variant for the group of Italian and German carriers of the V716M and the age was estimated to be some 225 generations (95% CI: 183–272) or around 5,600 years (Fig. 2b).

Applying another method, the Estiage program (Ref. 17; see Material and Methods), we estimated the age of the most recent common ancestor carrying the V716M variant to be some 219 generations (95% CI: 152–317). This is similar to the age of the mutation estimate obtained using DMLE+2.3 program but with a wider confidence interval. We were not able to apply the Estiage program to estimate the age of the splice site mutation reliably due to low density of genotyped markers in the larger shared region (see Material and Methods).

Examples of other MLH1 founder mutations are the two described in Finland that together account for up to 50% of all LS in Eastern Finland. Another example is the one-base pair insertion in exon 13 of MLH1 (c.1489_1490insC) which has been seen frequently in Germany, and a few times in surrounding countries, but not elsewhere. The authors suggested a founder mutation. As the founding of the US mutation is relatively recent its proportion of all LS is modest. We nevertheless consider it worthwhile to suggest screening cancer patients with immunohistochemical loss of staining for MLH1 for the splice site mutation as a first or early step in the mutation detection procedure.

In our study, we estimated the age of both changes. These estimates tend to have broad margins of error based on what parameters are used in the calculation (see Materials and Methods). We are confident that the age of the splice site mutation is considerably younger than the age of the V716M variant based on the size of the shared haplotype and as a result of mutation age estimation. However, the age for the innocent variant could be significantly younger if the frequency of the mutation in the population turns out to be smaller (Supporting Information Fig. 4).

Finally, it is worth considering whether the presence of V716M might predispose to the splice site change. Since both
changes are rare, the a priori likelihood of them being found together is small, but the absence of the V716M variant in Italian cases with the splice site mutation speaks (weakly) against this hypothesis.

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References