Characterization of new founder Alu-mediated rearrangements in MSH2 gene associated with a Lynch Syndrome phenotype.

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**Running head:** New founder rearrangements in MSH2.

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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 breakpoint 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 (HA-CAE). Multiplex Ligation-Dependent probe amplification (MLPA) was used to detect rearrangements in mutation-negative index patients and confirmed by RT-PCR. The breakpoint of the deletions was further characterized by the Array Comparative Genomic Hybridization (CGH array) method. Immunohistochemical (IHQ) staining and microsatellite instability (MSI) were studied in tumor samples. Hereditary non-polyposis colorectal cancer (HNPCC)-related phenotypes were evaluated. More than sixteen percent (24/160) 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 fifteen positive MSH2 families (47%) are carriers of a rearrangement. The exon 7 deletion and exons 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, counselling and clinical care in suspected Lynch syndrome families; not only in specific geographical areas, so wide distribution may be explained by migration patterns.
INTRODUCTION

Hereditary Nonpolyposis Colorectal Cancer syndrome (HNPCC), or Lynch Syndrome (MIM# 120435), is probably the most common form of inherited colorectal cancer, accounting for 1%-5% of cases (1). Affected individuals have a family history of colorectal cancer (CRC) at an early age, characterized by tumor predominance in the proximal colon and an association with extra-colonic tumors. Germline mutations in at least five 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 (2,3,4), 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) affect either MLH1 (MIM 120436) or MSH2 (MIM 609309) (7, 8). The vast majority are nonsense, missense, splicing or frameshift mutations, but a recent report (9) indicates that a substantial percentage of HNPCCs 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 to MLH1 depends on the studied population (11). Different pre-screening methods have been proposed. Here a combined method of HA-CAE and 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 breakpoint was found in all index cases of the carrier families. These
mutations appear to be founder effects, as a common haplotype was associated
with each; besides, the novel exons 4-8 deletion appears 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

Point mutation general screening of the MLH1, MSH2 and MSH6 genes was performed using heteroduplex analysis by capillary array electrophoresis (HA-CAE). This method was developed in our laboratory (13) and the validation for MMR genes has recently been published (12).

Fragments showing an HA-CAE-altered pattern were sequenced with the BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems) with unlabelled forward and reverse primers on an 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 multiplex ligation-dependent probe assay (MRC-Holland, Amsterdam, The Netherlands). MLPA test kits P003 for MLH1/MSH2 and P008 for MSH6/PMS2 were performed according to the supplied protocol. Fragment analysis of the PCR products was carried out on an
ABI 3130-Genetic Analyzer and gene dosage calculation and analysis was done using GeneMapper software (Applied Biosystems, Foster City, CA).

**Reverse Transcription PCR (RT-PCR)**

All the alterations detected by MLPA were confirmed by RT-PCR. Three microliters of total RNA were used to synthesize complementary DNA (cDNA) with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers. RT-PCR was performed 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 4-8 deletion**: Forward in exon 3: 5´-gttggagtgggtagttggatt -3´ and Reverse in exon 9: 5´-tgttgactgcatctttctttc -3´.
- **Exon 7 deletion**: Forward in exon 6: 5´-tgctgaataagtgtaaaacccc -3´ and Reverse in exon 8: 5´-ggagaagtcagaacgaagatcag -3´.

**Immunohistochemical (IHC) and tumor microsatellite instability (MSI) testing.**

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 extracted using the DNAeasy Tissue kit (Qiagen). A fluorescence multiplex polymerase chain reaction 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 five markers showed instability, as MSI-low if one of the five markers showed instability, and as MSI-high if two or more markers showed instability (14).

**Breakpoint characterization**

A CGH array strategy was done to confirm the deletions identified by MLPA and to identify the location of deletion breakpoints.

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

Based on the information obtained from the CGH array, primers were designed spanning the putative breakpoints 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 SNPs 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 ten markers used were localized in a ~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 in a Genetic Analyzer 3130 using the GenMapper 3.7 software (Applied Biosystems).

The two intragenic single base substitutions located within intron 1 and intron 9 of MSH2 (rs2162123 and rs3771278) were screened through High Resolution
Melting (HRM) technology [LightCycler® 480 Instrument (Roche)] and samples with altered curves were sequenced.

**Phenotypic Characterization**

Genealogical 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 mismatch repair mutation types (point mutations versus 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 colorectal cancer, 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), relative frequencies (%), while groups were compared by analyzing a 2x2 contingency table using Fisher’s exact test. Age was treated as a continuous variable and thereafter dichotomized to <50 or ≥50 years. Continuous data were reported in mean values with their corresponding standard deviation (SD) and groups were compared using the Student’s t test. A two-sided P value <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. Clinicopathological 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 exons 4-8 deletions were detected in three and four non-related families respectively.

**Identification of the MSH2 recurrent mutations**

One family had previously been detected with exon 7 deletion and three families with exons 4-8 deletion. (The MLPA assay, RT-PCR products and Sequencing pattern are in Perez-Cabornero et al. Eur J Cancer, 2009). 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 exons 4-8 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 4-8 exons deletion was observed in MSH2 mutation carrier families, which accounted for more than 26% (4/15).

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

**Breakpoint identification**

A customized 4x44 Agilent platform was used to map somatic rearrangements (Designed by NimGenetics, Spain). The aCGH assay provides a prediction of rearrangement breakpoints for the convenient design of primers and sequencing. 822 oligonucleotide probes were used to cover MSH2. A median distance between non-overlapping array probes of 500 bp was obtained.

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

PCR primers were designed in the first probe before and after the deletion. In order to confirm common deletion, PCR was performed for several index cases of each family with exon 7 deletion and exons 4-8 deletion. These resulted in a deletion product of 36.7Kb in exons 4-8 deletion (g.13272_49953del36681; NG_007110.1.gb) and 9.4Kb in exon 7 deletion (g.28106_37472del9366; NG_007110.1.gb), exclusively observed in carriers of the common deletion and not in deletion-negative controls (See Figures 1.A and 1.B).

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

**Haplotype analysis**

Haplotype analysis was performed to confirm the common genetic origin of the deletion rearrangements (Figure 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 4-8 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 (Figure 2) which was absent in non-carriers of these families. It provides evidence for a common ancestry among these families.

Figures 2.1 and 2.2 represent the pedigrees of the four Del4_8 families and the three Del_7 families. The phenotype observed in these families is shown as are the results of the common haplotype segregation.

96 unrelated samples were also genotyped as controls and allele frequencies were estimated; these are also shown in figures 2.1 and 2.2.

**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: one forward and two reverse. The product is a multiplex PCR with the presence of one band in the wild type and two bands in deletion carrier samples (Figure 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 MSH2, largely due to the existence of two recurrent mutations in this gene which represent approximately half the mutations in 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 two 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 are higher in founder mutation carriers than point mutation carriers ($p=0.47279$, $p=0.1247$).

On the other hand, it must be emphasized that the median age at onset is different in both groups; 46.7 years versus 40.67 years in CRC males and 50 years 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 the Leiden Open Variation Database (LOVD) (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 positive families out of 160).

We have found 8 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 MLH1 are novel, both appearing in a proband with colon cancer before 50 years of age; while the MLH1_01285 mutation has being described by us before (18).

Only one family is an MSH6 mutation carrier, this is a novel truncated variant. The carrier shows a late onset endometrial cancer with absence of any staining of the 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 due to the high number of recurrent mutations in this gene.

Two mutations which affect the splicing are reported here and which have been described by us in LOVD. The c.1661G>A mutation is present in family VA6, which is a large family, and 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 endometrial cancer in a mother and her daughter at 43 and 40 years of age, respectively.
Two recurrent rearrangements have been detected in seven out of fifteen MSH2 carrier mutation families (more than 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 three non-related 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 three families and all of them have the identical mutation and a shared haplotype (Figure 2.1). The identification of the breakpoints, within the Y and Sg Alu elements, supports the hypothesis that the recurrent exon 7 deletion is due to an Alu repeat mediated recombination event. These three families had a high prevalence of colorectal cancer, followed by endometrial cancer and prostate tumor, with the majority of cancers diagnosed before the age of 50 (Figure 2.1). 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 five exons in the MSH2 gene, an in-frame mutation that produces a shorter protein and appears in four non-related Amsterdam criteria fulfilled families. The study of the breakpoint in index cases of each of the four families and the haplotype analyzed confirms the same mutation in all families (Figure 2.2). We identified that another Alu repeat event that was involved in the exon 4-8 deletion (Figure 2.2).

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 pre-screening of founder mutations can be started.

We have performed a multiplex PCR (Figure 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 exons 4-8 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 (Figure 2.2).

The anticipation phenomenon has been described in other syndromes (22). The presence of anticipation in HNPCC 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 four families (Table 2), but in family VA20, we observed that the difference at onset over two 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 intron 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 mutations carriers.

We detected rearrangements more often in families with endometrial or urinary cancer. These data are similar to those published by Geray et al. (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 exons 4-8 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 geographical 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 Sevilla), 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.
REFERENCES


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**Figure legend**

**Figure 1.A:** Exon 7 deletion rearrangement. a) the breakpoints predicted from aCGH; b) rearrangement characterization: PCR amplification, sequencing and break point sequence; c) break point location and identification of Alu elements.

**Figure 1.B:** Exons 4-8 deletion rearrangement. a) the breakpoints predicted from aCGH; b) rearrangement characterization: PCR amplification, sequencing and break point sequence; c) break point location and identification of Alu elements.

**Figure 2.1:** A) marker localization and allele frequency in control population of the MSH2 gene in Chromosome 2. B), C) and D) are representative pedigrees of exon 7 deletion carrier families. Legend and tumor phenotype are included.

**Figure 2.2:** A) marker localization and allele frequency in control population of the MSH2 gene in Chromosome 2. B), C), D) and E) are representative pedigrees of exons 4-8 deletion carrier families. Legend and tumor phenotype are included.

**Figure 3:** multiplex PCR of screening rearrangements. A) Exon 7 deletion, the wild type samples show one 586 bp band and deletion samples show two bands: 586bp and an extra-band of 861bp. B) Exons 4-8 deletion, the wild type samples show one 1175 bp band and deletion samples show two bands: 1175 bp and an extra-band of 751bp.
Table legend

**Table 1.** Pathogenic mutations, clinicopathological features and molecular findings of the 24 carrier families.

**Table 2.** Genotype-Phenotype correlation in MSH2 mutation carriers.
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<td>C.1661G&gt;A; p.Ser554Thr</td>
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*Mutations described for us in LOVD
In grey: mutations not described before
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<td></td>
<td>n (%)</td>
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<td>&lt;50 years</td>
<td>15 (78.9)</td>
<td>11 (73.3%)</td>
<td>Age at diagnosis</td>
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<td>&gt;50 years</td>
<td>4 (21.1)</td>
<td>4 (26.7%)</td>
<td>&lt;50 years(yes/no)</td>
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<td>Age at diagnosis</td>
<td>Average (SD)*</td>
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<td>Total (Males and Females)</td>
<td>46.7±10.75 years</td>
<td>40.13±10.46 years</td>
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<td>CRC (Males)</td>
<td>46.71±6.24 years</td>
<td>40.67±9.05 years</td>
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<td>CRC (Females)</td>
<td>50.00±23.72 years</td>
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<td>Median age</td>
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<td>Endometrial (Females)</td>
<td>43.00±1.41 years</td>
<td>50.75±7.37 years</td>
<td>Median age</td>
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Figure 1.8

Intron3 AluSq T6CCACCTCAGCCTCCAGTAGCTGGATTAGGCAATAGCCACGTGCCCCGC
DeletionEx-8 T6CCACCTCAGCCTCCAGAT0CTGGATTAGGCAATAGCCACATGCCCGGC
Intron8 AluSp CACCTGGCTGCTGCTTCCTAAAATTGGAGCGGTAGCCACATGCCCGGC

36.7 Kb
Characterization of new founder Alu-mediated rearrangements in MSH2 gene associated with a Lynch Syndrome phenotype.

Lucia Perez-Cabornero, Ester Borras, Mar Infante, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-11-0227

Supplementary Material  Access the most recent supplemental material at: http://cancerpreventionresearch.aacrjournals.org/content/suppl/2011/07/21/1940-6207.CAPR-11-0227.DC1
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2011/09/28/1940-6207.CAPR-11-0227.DC2

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