Evaluation of Microsatellite Instability Analysis as a Diagnostic Tool to Identify Lynch Syndrome in Endometrial Cancer Patients

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Abstract

Hereditary endometrial cancer (EC) is a Lynch syndrome (LS) related cancer variant and 2-10% of all EC are hereditary. The aim of this study was to develop a method for analysis of microsatellite instability (MSI) as such analysis would assist in identifying potential LS patients with EC at an early state of their disease, before a possible second cancer is developed in another organ.

Twenty-six patients with adenocarcinoma in the endometrium, diagnosed at Uppsala University Hospital in Sweden between 1993 and 2012, were included in the study. Seven of these patients were also diagnosed with LS and the rest were sporadic EC. DNA was extracted from the patients’ formalin-fixed and paraffin-embedded tissues. The extracted DNA was subjected to a multiplex PCR with fluorescently labelled primers and then analysed by using capillary electrophoresis.

Of the sporadic EC, 26% was MSI-High, which correlates well with published data. Of the LS patients, 83% was MSI-High. The outcome of this project resulted in that MSI analysis is now a validated and established method used in the process of identifying potential LS among patients with EC.

Keywords

HNPCC, Hereditary, MSI, Endometrium, Tumour
**Sammanfattning**


Analysmetoden för MSI som utvärderades i detta projekt undersöker små områden i människans DNA som kallas mikrosatelliter. När dessa områden blir instabila så kan det vara en indikation att patienten har LS. Den bakomliggande orsaken till denna instabilitet sitter i cellens DNA-reparationssystem och leder till att cellen inte kan reparera spontana mutationer i DNA som kan uppkomma när nya celler bildas. Dessa mutationer är en av anledningarna till att friska celler kan utvecklas till tumörceller. I denna studie undersöktes operationsmaterial från totalt 26 kvinnor med diagnostiserad livmodercancer varav 7 även var diagnostiserade med LS.

Av LS-patienterna var 83% mikrosatellitinstabila och motsvarande siffra för de patienter med sporadiska livmodercancer var 26% vilket korrelerar väl med andra studier. Detta projekt har lett till att analysmetoden för MSI idag är validerad och etablerad för att undersöka kvinnor med livmodercancer som man misstänker även har LS.
Introduction

Cancer is a disease that can develop in different organs and tissues in the body. The definition of cancer is uncontrolled cell proliferation and cell growth. Cancer cells have abnormal signalling pathways for control of cellular growth and apoptosis. The abnormal cell signalling leads to a form of tissue called neoplasm or tumour. There are two main types of neoplasm; benign and malign. A benign neoplasm grows locally and has well defined margins. In general, patients with benign neoplasm have a very good prognosis as this rarely leads to death. On the other hand, a malignant neoplasm has poorly defined margins and the tumour cells spread by growth into the surrounding tissue. Patients with malignant neoplasm have a poor prognosis and the disease can lead to a patients’ death. The incidence of new cancer diagnosis in Sweden 2010 was approximately 45,000 individuals, of these 21,000 were females.

The endometrium is the inner lining surrounding the uterus, beneath the myometrium. The endometrium changes in thickness throughout the menstrual cycle due to the action of hormones, e.g. estrogen and progesterone. Every year, approximately 150,000 patients are diagnosed with endometrial cancer (EC) worldwide. Approximately 90% of these cases are sporadic and the rest are considered hereditary [1]. However, other studies indicate that only 2% is hereditary [2, 3]. In Sweden approximately 1,400 new patients are diagnosed with EC annually.

Hereditary EC is a Lynch syndrome (LS) related cancer. LS is also known as hereditary nonpolyposis colorectal cancer syndrome (HNPCC). One characteristic feature of LS is early onset of cancer. LS is an autosomal dominant disease, with a reduced penetrance of approximately 65-70% [4, 5]. In LS, cancer can develop in multiple organs such as the stomach, colon, rectum, ovaries, small bowel, urinary tract, kidneys etc. [6, 7]. Therefore patients with LS related EC also have an increased risk of developing cancer in an additional organ [8]. The most common form of LS related cancer is colorectal cancer (CRC), with a lifetime risk of 80% to develop CRC [6]. EC is the second most common form of LS cancer and in one study it was suggested that female LS patients have a 50-60% lifetime risk to

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1 http://www.socialstyrelsen.se/Lists/Artikelkatalog/Attachments/18530/2011-12-15.pdf Date 12-08-16
2 http://www-dep.iarc.fr/nordcan/SW/frame.asp Date 12-05-22
develop EC [7]. However, other studies indicate that female LS patients have higher risk to develop EC than CRC [9, 10].

In order to identify LS patients the Amsterdam I criteria was published in 1991 and then revised in 1999. All following five criteria should be fulfilled in order to diagnose a patient with LS: I. At least three relatives should have the diagnose LS associated cancer; CRC, EC, cancer in the small bowel, ureter or in the renal pelvis. II. One should be a first degree relative of the other two. III. At least two successive generations should be affected and at least one should have been diagnosed before 50 years of age. IV. Familial adenomatous polyposis should be excluded. V. A pathologist should examine and verify the tumour [11, 12].

One problem with the Amsterdam I and II criteria is that many LS families do not fulfil the inclusion criteria. This can be due to late onset of the disease, small families, adoption etc. [13]. Therefore the Revised Bethesda Guideline was published in 2004 for better possibility to identify LS patients. The Bethesda Guidelines are used to identify patients that should undergo genetic testing for Microsatellite instability (MSI). The Bethesda Guidelines propose that tumours should be tested for MSI in the following five situations: I. CRC diagnosed in patients <50 years old. II. Presence of synchronous, metachronous colorectal or other LS associated tumours for example endometrium, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract and brain regardless of age. III. CRC with the MSI-High histology diagnosed in a patient who is less than 60 years old. IV. CRC diagnosed in one or more first-degree relatives with LS related tumour, with one of the cancers being diagnosed under the age of 50. V. CRC diagnosed in two or more first- or second-degree relatives with LS related tumours, regardless age [14].

There are different genes involved in LS and the most frequently affected genes are the mismatch repair genes MLH1, MSH2, MSH6 and PMS2 [10]. A damaged MLH1, MSH2, MSH6 or PMS2 allele/gene is inherited in a dominant inheritance pattern. The presence of one damaged allele is enough to become afflicted with an autosomal dominant illness and the risk is 50% to inherit the damaged allele. However, not all patients with LS develop cancer, the reason behind the reduced penetrance of cancer in some LS is not yet fully understood, but is believed to be due to a combination of external factors and modified genes [5].
In normal deoxyribonucleic acid (DNA) synthesis, errors occur frequently, and in individuals with a functional Mismatch repair (MMR) system most of these errors will be corrected. The MMR system can recognise and correct insertion-deletions and base substitution mismatches that can occur during DNA synthesis. There are many proteins involved in the MMR system each with a different assignment. The correction of a mismatch sequence starts when the protein-complex MutS binds to the mismatch sequence. There are two homologs of MutS; MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3). Studies indicate that MutSα primarily is involved in repair of insertion-deletion and single base-base mismatches. MutSβ also function as repair of insertion-deletion mismatches and has capacity to repair insertion-deletion of up to 16 nucleotides in one strand [15]. When the MutS binds to the mismatch sequence, another protein-complex, MutL, scans the nearby area of the newly replicated DNA strand for a nick (single-strand break). When MutL finds a nick, the degradation process is activated on the nicked strand and correction of the DNA strand takes place during DNA synthesis.

LS is due to that one or multiple genes in the MMR system are mutated. The most common MMR mutation in EC is in the MSH6 gene. In LS patients MSH6 mutations are five times more common in EC compared with CRC [10].

To diagnose LS in Sweden, a combination of three different methods is used: MSI analysis, immunohistochemistry (IHC) and DNA sequencing. MSI analysis is a method that investigates microsatellites which are short repeats, of one to five bases throughout the genome and varies in length between different individuals [16]. When there is a defect in the MMR system in tumour cells, errors during DNA replication and recombination can not efficiently be corrected. Therefore, the microsatellites can become longer or shorter due to insertions or deletions of nucleotides [17]. When the MMR system is defective and the microsatellites are instable in length, the tumour cells are denoted MSI. In a MSI analysis, DNA from tumour cells is compared to DNA from normal cells from the same individual to see if the tumour is MSI or microsatellite stable (MSS).

At the National cancer institute (NCI) meeting in Bethesda 1998, a panel of five mixed mono- and di-nucleotides was suggested to be used in diagnosis of LS. The panel is called the NCI panel (BAT-25, BAT-26, D2S123, D17S250 and D5S346) and is also known as the Bethesda
panel [18]. Later studies indicate that a panel of mononucleotides (BAT-25, BAT-26, NR-21, NR-22 and NR-24) has a higher sensitivity and specificity than the NCI panel to detect defects in the MMR system [16, 19]. Other studies compared yet another panel of five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) against the NCI panel. These studies indicated the same result; mononucleotide panels have a higher sensitivity and specificity than the NCI panel to detect MSI [20, 21].

IHC can be used to demonstrate protein-expression in tissues. In order to diagnose LS in Sweden, the expression of four mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2) are usually analysed. If the result from IHC analysis shows a loss of expression in any of these proteins, this indicates a defective MMR system. However, expression loss of a MMR protein is not always due to a defective gene. Methylation in the promoter region of the gene can also lead to down-regulation of protein expression. Methylations occur in a cytosine-guanine dinucleotide rich region in the DNA called CpG islands. If a methyl group binds to a cytosine in the CpG Island, the gene expression can be affected [13].

DNA sequencing is a method to determine the sequence of nucleotides. Primers specific for different parts of the DNA decide which region that is going to be analysed. In Sweden, LS is analysed by looking at three different MMR genes: MLH1, MSH2 and MSH6. Depending on which MMR gene that demonstrates expression loss in the IHC analysis that gene will be sequenced because this indicates that the specific protein is not functional.

For MSI analysis, the use of polymerase chain reaction (PCR) is essential. When multiple DNA regions are amplified in the same reaction, the method is called multiplex PCR. The primers that are used in the multiplex PCR are labelled with different fluorescent probes. Therefore, the different PCR products can directly be analysed by using capillary electrophoresis.

Capillary electrophoresis, which can be used for fragment analysis, is a form of electrophoresis that is performed in a thin capillary tube filled with a polymer. The labelled
PCR products are injected into the capillaries and since DNA is negatively charged the PCR products migrate through the capillaries towards a positively charged anode. The PCR products are separated by size and when they pass through a laser beam the fragments fluoresce and an optical detector register the fluorescent signals. Signals of the PCR products are analysed together with a ladder to define the length of the DNA fragments and a software is then converting the fluorescent signals to digital data and the result is interpreted using a software that visualise the length of the DNA fragment and the intensity in relative fluorescence units (RFU).

The aim of this study was to investigate and validate a method that can help identify potential LS among EC patients in an early state of the disease, before a possible second cancer has developed in another organ. An additional aim was to identify MSI positive DNA samples that can be used as positive controls in the clinical MSI diagnosis of EC patients. Methods utilised in this project were DNA extraction, multiplex PCR and fragment analysis.
Materials and Methods

Patient samples

Patients with adenocarcinoma in the endometrium, diagnosed at the Uppsala University Hospital in Sweden between 1993 and 2012 were identified. To identify potential patients that could be included in the study, pedigrees showing the patients’ family history of cancer, were obtained from the department of Clinical Genetics at Uppsala University Hospital and clinical patient information were obtained from the database at the department of Clinical Pathology at Uppsala University Hospital. Of the identified patients, 26 were enrolled in this study cohort. The samples belong to different groups; patients diagnosed with EC and with \( n=7 \) or without LS \( n=19 \). Moreover the patients with EC were divided into different subgroups depending on the median age (see Table 1). All tissues used were formalin-fixed and paraffin-embedded (FFPE).

DNA extraction

Glass slides with hematoxylin-eosin stained tumour tissues from the patients in the cohort were reviewed by a pathologist to identify the most suitable tissues for analysis and estimate the tumour cell content. The corresponding FFPE tumour tissues were carefully dissected to obtain parts of tissues with at least 10% tumour cells. DNA was then extracted from the patients tumour and corresponding normal tissues using QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturers’ instruction (Qiagen). The DNA concentration was measured using a NanoDrop® 2000/2000c spectrophotometer according to the instruction from the manufacturer (Termo Fisher Scientific, Waltham, MA, USA).

Multiplex PCR for microsatellite analysis

The MSI analyses were carried out using multiplex PCR with fluorescently labelled primers, included in the MSI Analysis System kit, Version 1.2 (Promega Corp. Madison, WI, USA), for amplification of five mononucleotide repeat markers (NR-21, BAT-26, BAT-25, NR-24, MONO-27). The PCR reactions were performed in a 10µl volume containing 6ng DNA, 1 x Gold ST*R buffer (Promega), 1 x MSI AnalysisSystem PrimerPair Mix (Promega), 0.075U/µl Platinum™ Taq (Invitrogen, Life Technologies Corp. Carlsbad, CA, USA) and nuclease free H₂O (Promega). The PCR was performed using a GeneAmp® System 9700 Thermal Cycler
(Perkin-Elmer, Waltham, MA, USA) and the following PCR program: 95°C 11min, 96°C for 1min, 10 x (ramp 100% to 94°C 30s, ramp 29% to 58°C 30s, ramp 23% to 70°C 1min), 20 x (ramp 100% to 90°C 30s, ramp 29% to 58°C 30s, ramp 23% to 70°C 1min) and 60°C for 30min. Five samples were rerun using 25 cycles instead of 20 cycles in order to gain sufficient PCR products. One sample was once again rerun using 25 cycles and the double amount of DNA (12ng) to gain a sufficient PCR product.

**Microsatellite fragment analysis**

A loading cocktail was prepared by mixing 9.5µl Hi-Di™ Formamide (Applied Biosystems, Foster City, CA, USA) with 0.5µl of an internal lane standard, ILS600 (Promega), for each sample. After vortexing the loading cocktail it was aliquoted into a 96-well plate. Subsequently, 1µl of each PCR product was added to each well with loading cocktail. The 96-well plate was then centrifuged briefly to remove air bubbles followed by denaturation of the samples at 95°C for 3min and then put in a cooling block in the freezer for 3min. The 96-well plate was centrifuged briefly to remove condense in the lid and loaded for capillary electrophoresis in an ABI 3130xl instrument (Applied Biosystems) for fragment analysis according to the instructions from the manufacturer (Promega). Data was analysed using GenMapper® Software Version 3.7 (Applied Biosystems). According to the NCI guideline, samples were denoted MSI-High if two or more markers showed instability, MSI-Low if one marker showed instability and Microsatellite stable (MSS) if no marker displayed instability.

**Statistical analysis**

The Fisher’s exact test was used for analysing categorical data and a p<0.05 was considered significant.
Result

DNA extraction

DNA was extracted from FFPE tissues and the concentration was determined on a NanoDrop® 2000/2000c spectrophotometer (Termo Fisher Scientific). The amount of DNA obtained varied between 0.1-46.3µg.

Microsatellite fragment analysis

The PCR products were analysed using GenMapper® Software Version 3.7 (Applied Biosystems) and typical fragment analysis data are shown in Figure 1 to 3.

Figure 1. Fragment analysis result of a MSS patient. All five markers (NR-21, BAT-26, BAT-25, NR-24 and MONO-27) were stable and no shifts in the lengths of the microsatellites were found. The x-axis represents the size in bp (base pair) of the PCR products and the y-axis shows the RFU. The red peaks are the size standard. A=Tumour tissue and B=Normal tissue. The black and blue peaks, in the range 160-200bp are control peaks for patient identification.
Figure 2. Fragment analysis result of a MSI-High patient. The figure shows a shift in lengths of all five markers (NR-21, BAT-26, BAT-25, NR-24 and MONO-27). The x-axis represents the size in bp of the PCR products and the y-axis shows the RFU. The red peaks are the size standard. A=Tumour tissue and B=Normal tissue. The black and blue peaks, in the range 160-200bp are control peaks for patient identification.

Figure 3. Fragment analysis result of a MSI-High patient. A small shift is shown in BAT-26 and probably in NR-24 and MONO-27. The x-axis represents the size in bp of the PCR products and the y-axis shows the RFU. The red peaks are the size standard. A=Tumour tissue and B=Normal tissue. The black and blue peaks, in the range 160-200bp are control peaks for patient identification.
Analysed data

In the study cohort \((n=26)\), microsatellite status could be defined for 96% of the patients and of these patients 36% displayed MSI in DNA from the tumour tissue. Six of the patients for which microsatellite status could be defined were previously diagnosed with LS. Of note, 83% \((n=5)\) of these patients had MSI (see Table 1).

Nine patients were <45 years of age and without a LS diagnose, 11% of them displayed MSI and of the sporadic EC \((n=19)\), 26% were MSI (see Table 1).

The association between MSI and LS was statistically significant \((p=0.02)\) moreover the sensitivity of the MSI method used in this study was 83%.

Table 1. Result of analysed data and summary of patient data.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>MSI-High</th>
<th>MSS</th>
<th>Median age</th>
<th>Diagnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7</td>
<td>83% ((n=5))</td>
<td>17% ((n=1))</td>
<td>53</td>
<td>LS(^1) with EC(^2)</td>
</tr>
<tr>
<td>8-16</td>
<td>11% ((n=1))</td>
<td>89% ((n=8))</td>
<td>41</td>
<td>EC</td>
</tr>
<tr>
<td>17-26</td>
<td>40% ((n=4))</td>
<td>60% ((n=6))</td>
<td>79</td>
<td>EC</td>
</tr>
</tbody>
</table>

*One sample, in patient group 1-7, was not determined and was therefore excluded in the calculation.

\(^1\)LS=Lynch Syndrome, \(^2\)EC=Endometrial cancer (Adenocarcinoma)
Discussion

Every year approximately 1,400 women are diagnosed with EC in Sweden and some of these women have the hereditary form of the disease (LS). It is important to be able to identify these women before a possible additional cancer develops. For identification process of these women with potential LS it is important to establish MSI analysis for EC. The benefit of using a multiplex PCR system to analyse MSI is that it is a fast and simple system compared to performing five separate PCR with different primers. The multiplex PCR is based on one reaction, a subsequent fragment analysis and then a result that is ready to be analysed. The disadvantages is the cost of the analysis, it is more expensive than IHC. However, a publication indicates that using only IHC analysis for identification of LS patients could lead to false negative results in 5% of cases where MSI-High is present. The mononucleotide multiplex PCR system has higher sensitivity and specificity than the IHC method [20]. However, using the two analyses MSI and IHC together leads to higher sensitivity and specificity if the results are interpreted together.

The method used in this study, multiplex PCR with fluorescence labelled primers, is an established method for analysis of MSI in CRC, both in its sporadic and hereditary form [22, 23]. Previously published studies indicates that the method is also suitable for EC tumours [1, 10, 24, 25], but MSI analysis in EC is not evaluated as much as in CRC.

In this study, five of the samples were rerun because of poor amplification of their PCR products. For four samples, the amplification could be improved by adding additional PCR cycles and sufficient PCR products were gained. For one sample additional PCR cycles had to be used in combination with higher amount of DNA with the aim to get sufficient DNA amplification. However, this did not have an effect on the amplification of the DNA. The poor amplification of some samples could be due to fragmented DNA (poor DNA quality) of the FFPE sample.

It has been previously described that the fixation process and embedding of the tissue have a negative effect on DNA quality and a prolonged storage time of FFPE blocks also has a negative effect on the DNA [26, 27]. Working with FFPE tissues always generate problems in clinical diagnostics as described above. The amount of DNA obtained by the extraction of the
tissues was between 0.1-46.3µg. The difference in the amount of DNA could be due to difference in size of the tissues used in the extraction and various amounts of necrotic cells in the tissues.

When the MMR system has a defect, deletions, insertions or nucleotide changes in the DNA can not be corrected during DNA synthesis. All patients in this cohort that were MSI-High had deletions of nucleotides in the different markers. A previous report demonstrates that all EC patients with MSI-High status showed deletions in mononucleotide markers [28]. Interestingly, in LS EC appears to have smaller size deletions compared to CRC, for example one study presented that BAT-25 had an average deletion of 4.1bp in EC compared to 6.7bp in CRC [29]. In average, size difference in deletions between EC and CRC is 1-5bp [28, 29]. Because of the small shift in the microsatellites that can occur in EC, it is important to include normal tissue in the analysis of the tumour tissue so the mononucleotide markers in DNA from normal and tumour samples can be directly compared. One sample had a difficult mononucleotide pattern to interpret because of the small shifts that had occurred (see Figure 3). Without the normal sample it would have been even more difficult to decide if the tumour was MSI or MSS.

In theory all the LS patients were expected to present a MSI genotype. In the group of patients that had a LS diagnosis 17% (n=1) were found MSS and the remaining samples were MSI. This could be due to low tumour cell content in the sample that was MSS (20% tumour cells) and perhaps a tumour cell content of 20% is too low to be able to identify MSI. In a publication from 2010, the sensitivity and specificity to find LS patients with CRC using the mononucleotide markers was found to be 95.6% and 100%, respectively indicating that by using MSI analysis not all patients with LS could be identified [22]. The sensitivity of the MSI analysis in this study was 83%, the low percentage could be due to the small number of patients with LS in the cohort. The association between MSI and LS was statistically significant (p=0.02), although this requires further validation using a larger cohort.

At this moment, the percentage of tumour cells needed to detect MSI in a sample is not defined for EC or CRC tissues. To improve MSI analysis a correct cut off of the tumour cell content needs to be established. To define the lowest percentage of tumour cells that is needed
to correctly identify MSI, the next step would be analysis of a dilution series of a MSI-High cell line or samples.

Nine patients in this cohort were <45 years of age and without a LS diagnose, 11% of them displayed a MSI genotype and that could be an indication of an undiagnosed LS. Therefore it is important to have a method to identify MSI among patients with EC and to have the ability to analyse these women before a possible secondary form of cancer emerge. A recent publication describes a similar study as ours were the aim was to implement a “universal” screening method using MSI/IHC for all EC to detect LS before a possible secondary cancer develops [30]. Of the sporadic EC in this study (n=19), 26% were MSI which correspond well to another study in which the incidence of MSI in sporadic EC was 28% [31].

The result of this study has led to that MSI analysis is now validated and established at the Molecular Pathology laboratory in Uppsala, Sweden as a method to help identify LS patients among patients with EC.
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References


