A review on the molecular diagnostics of Lynch syndrome: a central role for the pathology laboratory

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- Lynch syndrome
- Identification of patients at risk for Lynch syndrome
- Molecular basis of Lynch syndrome and sporadic MMR-deficient tumours
- Molecular diagnostics of Lynch syndrome
- Description of molecular analyses

- MSI analysis
- Immunohistochemistry
- *MLH1* promoter hypermethylation assay
- *BRAF* mutation analysis
- Limitations of molecular analyses
- Conclusions

Abstract

Lynch syndrome (LS) is caused by mutations in mismatch repair genes and is characterized by a high cumulative risk for the development of mainly colorectal carcinoma and endometrial carcinoma. Early detection of LS is important since surveillance can reduce morbidity and mortality. However, the diagnosis of LS is complicated by the absence of a pre-morbid phenotype and germline mutation analysis is expensive and time consuming. Therefore it is standard practice to precede germline mutation analysis by a molecular diagnostic work-up of tumours, guided by clinical and pathological criteria, to select patients for germline mutation analysis. In this review we address these molecular analyses, the central role for the pathologist in the selection of patients for germline diagnostics of LS, as well as the molecular basis of LS.

Lynch syndrome

Colorectal cancer (CRC) is the most common malignancy within the European Union and ranks second to lung cancer as a cause of cancer-related mortality [1]. CRC results from both genetic and environmental factors. The most common genetic susceptibility for CRC is Lynch syndrome (LS), formerly known as hereditary non-polyposis colorectal cancer (HNPCC). LS accounts for approximately 3% of all CRCs [2, 3], and also for 2% of all endometrial cancers [4]. The burden of LS is considerably greater than these percentages imply, as the cancers are diagnosed at a young age and synchronous or metachronous malignancies occur in 30% of the patients [5, 6].

LS is characterized by a high lifetime risk for the development of CRC (20–70%), endometrial cancer (15–70%) and other extra-colonic cancers (<15%) [7–14]. These extra-colonic malignancies include carcinomas of the small intestine, stomach, pancreas and biliary tract, ovarium, brain, upper urinary tract and skin. LS is caused by germline mutations in mismatch repair (MMR) genes [15], and the definitive diagnosis is currently made by identification of an inactivating germline mutation in one of the MMR genes *MLH1*, *MSH2*, *MSH6* or *PMS2* [16]. Early detection of LS is of great importance, particularly in pre-symptomatic mutation carriers,

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Table 1 Amsterdam criteria I [21]

Families must fulfil all criteria:

1. There should be at least three relatives with a CRC.

2. One should be a first-degree relative of the other two.

3. At least two successive generations should be affected.

4. At least one should be diagnosed before the age of 50 years.

5. Familial adenomatous polyposis (FAP) should be excluded.

6. Tumours should be verified by pathological examination.

Table 2 Amsterdam criteria II [23]

Families must fulfil all criteria:

1. There should be at least three relatives with a LS-associated cancer*.

2. One should be a first-degree relative of the other two.

3. At least two successive generations should be affected.

4. At least one should be diagnosed before the age of 50 years.

5. Familial adenomatous polyposis (FAP) should be excluded in the CRC case(s), if any.

6. Tumours should be verified by pathological examination.

*CRC, cancer of the endometrium, small bowel, ureter or renal pelvis.

Table 3 Original Bethesda Guidelines [28]

Individuals meeting any one of the following should undergo MSI testing:

1. Individuals with cancer in families that meet the Amsterdam criteria.

2. Individuals with two LS-related cancers, including synchronous and metachronous CRCs or associated extracolonic cancers*.

3. Individuals with CRC and a first-degree relative with CRC and/or LS-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45 years, and the adenoma diagnosed at age <40 years.

4. Individuals with CRC or endometrial cancer diagnosed at age <45 years.

5. Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age <45 years ‡.

6. Individuals with signet-ring-cell-type CRC (more than 50% signet ring cells) diagnosed at age <45 years.

7. Individuals with adenomas diagnosed at age <40 years.

*Endometrial, ovarian, gastric, hepatobiliary or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter.
‡Solid/cribriform defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces.

since colonoscopic surveillance has proven to reduce CRC morbidity and mortality by 65–70% [17–19] and prophylactic surgery may prevent endometrial and ovarium carcinoma effectively [20]. Individuals with a predisposing mutation are candidates for participation in surveillance programs.

The diagnosis of LS is hampered by the absence of specific diagnostic features and the first manifestation in many patients is the presence of an advanced cancer. Furthermore, DNA mutation analysis is time consuming and expensive. For these reasons, DNA analysis is generally preceded by a molecular diagnostic work-up to select patients as candidates for genetic tests. This molecular diagnostic work-up may be guided by several clinical and pathological criteria such as the presence of LS associated malignancies, number of malignancies and age at cancer diagnosis, family history as well as histological tumour features such as mucinous or signet-ring differentiation. In this review, we address the central role for the pathologist in the selection of patients for germline diagnostics of LS, the molecular analyses to identify LS as well as the molecular basis of LS.

Identification of patients at risk for Lynch syndrome

Different models and strategies have been developed to identify patients with LS. In 1990, the Amsterdam Criteria I were developed to provide a basis for uniformity in collaborative studies to find the disease-causing gene (Table 1) [21]. These criteria were designed to be highly specific at the expense of the sensitivity [3, 22]. They were criticized because extra-colonic tumours were not taken into account, thereby excluding classical LS families. Therefore, the Amsterdam Criteria II were established in 1999 (Table 2) [23]. However, many families with the syndrome (*i.e.* mutation carriers) do not meet these criteria [24], usually because these families are too small or there is a late onset of the disease. In addition, obtaining a thorough family history is difficult in clinical practice [25] and patients may have limited knowledge of their family history [26, 27].

In 1997, the Bethesda Guidelines were published to select patients whose tumours should be analysed for molecular features associated with LS, *i.e.* microsatellite instability (MSI), to identify potential mutation carriers (Table 3) [28]. The Bethesda Guidelines have been revised in 2004 to make them more suitable for use in clinical practice, and are not only based on family history, but also on age at cancer diagnosis, number of LS-associated carcinomas and certain histological tumour features (Table 4) [29]. These histological tumour features, associated with LS, include the presence of tumour-infiltrating lymphocytes, a Crohn's-like lymphocytic reaction, mucinous or signet-ring cell differentiation and a medullary or undifferentiated and solid growth pattern. The additional value of these pathology characteristics in the selection of tumours for further testing for LS has been described previously [30, 31]. However, these histological

Table 4 Revised Bethesda Guidelines [29]

Individuals meeting any one of the following should undergo MSI testing:

1. CRC diagnosed in an individual under age 50 years.

2. Presence of synchronous, metachronous colorectal, or other LS-associated tumours*, regardless of age.

3. CRC with the MSI-H histology‡, in a patient <60 years of age.

4. CRC in 1 or more first-degree relatives with a LS-related tumour*, with 1 of the cancers being diagnosed under age 50 years.

5. CRC diagnosed in 2 or more first- or second-degree relatives with LS-related tumours*, regardless of age.

*Endometrial, ovarian, gastric, small bowel, pancreas, hepatobiliary tract, renal pelvis or ureter, and brain tumours, sebaceous gland adenomas and keratoacanthomas.
‡Presence of tumour-infiltrating lymphocytes, Crohn's like lymphocytic reaction, mucinous or signet-ring differentiation, or medullary growth pattern.

Table 5 Dutch guideline for MSI testing (www.oncoline.nl)

The pathologist is advised to requests MSI testing (and immunohistochemistry of the MMR proteins) in the following patients:

1. CRC or endometrial carcinoma before the age of 50 years.

2. A second CRC before the age of 70 years.

3. CRC before the age of 70 years AND another synchronous or previous LS-associated tumour*.

*CRC, endometrial, ovarian, gastric, small bowel, pancreas, hepatobiliary tract, renal pelvis or ureter, and brain tumours, sebaceous gland adenomas and keratoacanthomas.

features are related to both microsatellite unstable sporadic tumours as well as LS tumours. Therefore the ability to identify LS patients alone on the basis of these tumour features is limited [32]. In addition, the assessment of these histological tumour features indicating MSI is poorly implemented in daily clinical practice [32].

At present, the most widely accepted recommendation for the identification of patients with LS is based on the combination of these revised Bethesda Guidelines and MSI testing. This combination has proven to be an effective and efficient strategy for LS identification, with a sensitivity for detection of mutation carriers reported from 72% [3] up to 100% [33–36], and a specificity ranging from 77% to 98% [33, 35, 36]. However, these criteria have been criticized because of the use of broad and complex variables, and families with MSH6 and possibly also PMS2 mutations remain undetected [37]. It has also been shown in several studies that these criteria are poorly implemented in clinical practice [32, 38–40].

In 2005, a Dutch group therefore developed a new strategy for the detection of LS [41]. In this strategy the pathologist selects newly diagnosed patients fulfilling one of the following criteria for MSI analysis; (1) CRC before the age of 50 years, (2) Two LS-associated tumours, including synchronous or metachronous CRCs or LS-associated tumours or (3) adenoma before the age of 40 years. These criteria, known as MIPA criteria, simplify the Bethesda guidelines in such a way that pathologists, without knowledge of family history, can easily apply them. These criteria were found to be effective, efficient and feasible in daily practice [41, 42].

In The Netherlands, the diagnosis of LS is currently based on a nationwide guideline for MSI analysis (Table 5), that was introduced in January 2008 (www.oncoline.nl). This guideline resembles the MIPA criteria. MSI analysis (and immunohistochemistry of the MMR proteins) is requested by the pathologist in patients newly diagnosed with CRC or endometrial carcinoma before the age of 50 years, or patients with two LS-associated tumours (including synchronous and metachronous CRCs or LS-associated tumours) before the age of 70 years. Presence of multiple LS-associated cancers is registered in PALGA, the nationwide network and registry of histopathology and cytopathology in The Netherlands (www.palga.nl). For MSI analysis based on a positive family history, referral to a clinical geneticist is indicated. In those cases MSI analysis will generally be performed when the (revised) Bethesda or Amsterdam Criteria are met and if archival paraffin-embedded tumour tissue can be obtained.

Since clinical criteria do not quantify the likelihood of being a mutation carrier, refined algorithms and multivariable models have been developed to make a quantitative estimation of the risk of carrying a germline MMR-gene mutation, without the requirement of tissue [36]. Several models that combine personal and familial data have been developed, such as the Leiden model, the Edinburgh Model, Premm1,2 and the MMR-pro model [43–46]. One of the advantages of the quantitative models is that the threshold for sensitivity or specificity of the model can be adjusted based upon the clinical situation. However, the role for these models in daily clinical practice remains to be determined.

At present a study (called LIMO and coordinated by the Erasmus MC, Rotterdam, The Netherlands) is performed to determine whether further improvement of LS diagnostics can be obtained by the performance of MSI analysis in CRC patients up to the age of 70 years. MSI analysis is performed in a prospective consecutive series of 1000 newly diagnosed CRC patients ≤70 years, and the results are expected in 2010.

Molecular basis of Lynch syndrome and sporadic MMR-deficient tumours

LS is caused by a germline mutation in one of the MMR genes, most commonly *MLH1* and *MSH2* (±90%) [47, 48], but also *MSH6* and *PMS2* [37, 49, 50]. LS patients are born with a germline mutation in one of these MMR genes, and acquire inactivation of the second wild-type allele in their tumours, fulfilling Knudson's two hit hypothesis for inactivation of tumour suppressor genes [51]. Because of the high chance of inactivation of the
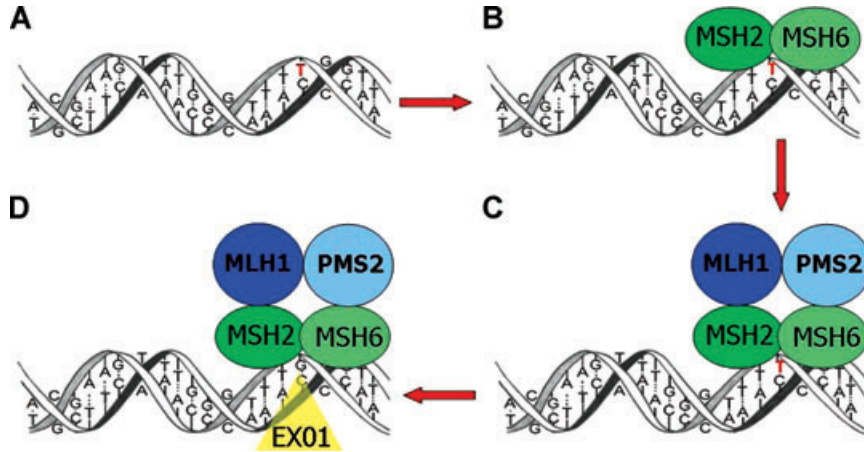
Fig. 1 The MMR system. During DNA replication, insertions or deletions of one or more nucleotides and single nucleotide mismatches may occur. For example: (A) A single nucleotide mismatch occurs (G>T in red). (B) MSH2 and MSH6 form a heterodimer and recognize the mismatch. (C) MLH1 and PMS2 dimerize and bind to the MSH2-MSH6 complex. (D) The complex of four proteins activates an exonuclease to perform the DNA repair.
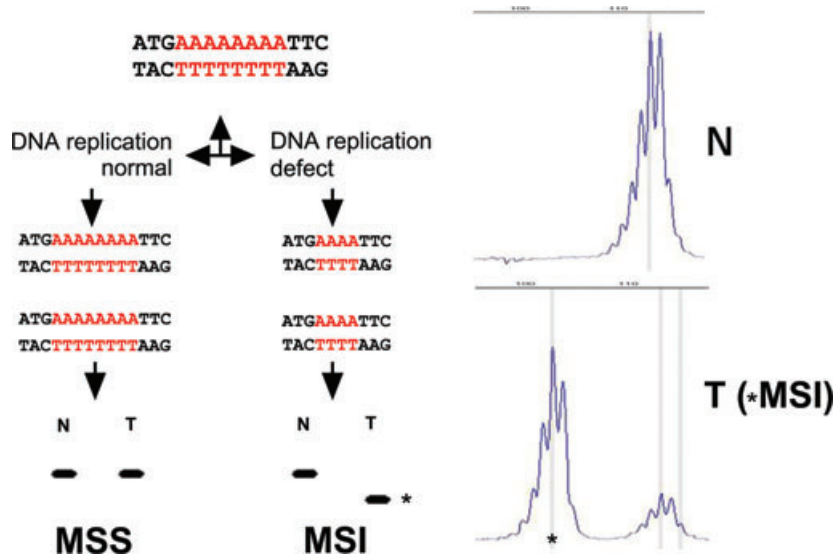

Fig. 2 MSI. A schematic microsatellite is indicated (poly A track). When the tumour cells have an intact MMR system the size of the microsatellite will be the same in DNA isolated from normal (N) and from tumour (T) cells: microsatellite stable (MSS) tumour. In case of a defect in MMR the size of the microsatellite (number of repeat units) can change (in most cases becomes shorter) when comparing N with T DNA: microsatellite unstable (MSI) tumour. Asterisks indicate the microsatellite unstable tumour DNA fragment.

homologous wild-type allele during life, LS transmits phenotypically in an autosomal dominant fashion. The somatic inactivation of the corresponding wild-type allele occurs almost exclusively by small mutations or (partial) gene loss, and bi-allelic inactivation then leads to complete abolition of the protein function. This results in a defective DNA MMR system, since the protein products of the MMR genes are involved in correction of nucleotide base mismatches and small insertions or deletions that arise during DNA replication [52–54].

The mechanism of MMR has been largely elucidated (Fig. 1). MSH2 (mutS homologue 2) forms a heterodimer with MSH6 (mutS homologue 6), sliding along the DNA as a clamp to identify single nucleotide mispairs and small insertions and deletions [55, 56]. MLH1 (mutL homologue 1) dimerizes with PMS2 (post-meiotic segregation 2) and binds to the MSH2-MSH6 complex. Together this group of four proteins recruits an exonuclease to

perform the DNA repair [57, 58]. If any of the four major proteins (MSH2, MLH1, MSH6, or PMS2) is functionally inactive, mismatches are not repaired. A defective DNA MMR system increases the mutation rate and makes the cell vulnerable to mutations in genes controlling cell growth (including tumour suppressor genes and oncogenes), resulting in an elevated cancer risk.

In case of a defective MMR system, mutations occur frequently in small (usually mononucleotide or dinucleotide) repetitive DNA sequences, known as microsatellites [59, 60]. In MMR deficient tumour cells the number of nucleotide repeat units of microsatellites can deviate from the corresponding normal DNA; the number of repeats is usually decreased, but occasionally increased (Fig. 2). This variation in repeat units and thus length or size of microsatellites is called MSI. MSI (formerly referred to as MIN, another abbreviation for MSI, or replication error abbreviated as RER) is the molecular hallmark of LS since approximately 95% of all

LS-associated cancers show MSI [61–63]. MSI thereby serves as a reliable phenotypic marker of MMR deficiency which is easy to evaluate in order to pre-select patients for germline mutation analysis of the MMR genes.

Despite the fact that tumour MSI is a reliable marker for MMR deficiency, it is a marker for LS with limited specificity since 15% of sporadic CRCs also demonstrate a MSI phenotype. This is mainly caused by somatic hypermethylation of the *MLH1*-gene promoter [64, 65]. DNA methylation is an epigenetic DNA modification that specifically targets cytosine residue at CpG dinucleotides. Genomic regions that contain a high frequency of CpG dinucleotides are called CpG islands, present in the promoters of about 40% of all human genes, including the *MLH1*-gene [66]. Hypermethylation of CpG islands in the *MLH1* promoter causes severe inhibition of gene transcription thereby functionally mimicking an inactivating gene mutation. If both copies of the gene are inactivated (mainly by bi-allelic hypermethylation), the DNA MMR function of *MLH1* is lost. This leads to microsatellite unstable cancers, especially in older patients [65]. MLH1 deficient microsatellite unstable tumours can be assessed for *MLH1* hypermethylation to distinguish sporadic CRCs from LS-related cancers. Theoretically, sporadic hypermethylation of the other MMR genes is possible but has not yet been demonstrated.

Specific activating mutations in the *BRAF* oncogene, usually V600E missense mutations (formerly reported as V599E), can be detected in 40–87% of all sporadic microsatellite unstable tumours. An oncogenic *BRAF* mutation has been described only once [67] in numerous investigated LS tumours [68–75, 76]. These results indicate that *BRAF* mutations are closely correlated with *MLH1* methylation in sporadic CRCs [69–72, 76, 77]. Therefore, *BRAF* mutation status can be used to identify sporadic microsatellite unstable tumours, although it has been demonstrated that determination of hypermethylation of the *MLH1* gene promoter is more sensitive to detect sporadic MSI tumours [69].

In addition to sporadic forms of *MLH1* promoter hypermethylation, germline epimutations of *MLH1* (soma-wide mono-allelic hypermethylation of the gene promoter) have also been reported [78–85]. Germline *MLH1* hypermethylation, often showing some degree of mosaicism, is functionally equivalent to an inactivating mutation and produces a clinical phenotype that resembles LS. Inheritance of epimutations is weak as the methylation can be cleared on passage through the germline (germline *MLH1* promoter epimutations are reversible during meiosis) and so can display non-Mendelian inheritance. Heritability of epimutations might also be explained by the inheritance of an unknown predisposition to epimutations, rather than the inheritance of the epimutation itself [86]. Although very rare, germline *MLH1* promoter methylation should be considered in younger individuals or individuals with multiple LS-associated tumours without a family history who present with an MSI tumour showing loss of MLH1 expression [81, 82].

Besides germline *MLH1* hypermethylation, a new mechanism of germline *MSH2* hypermethylation has recently been discovered [87]. Ligtenberg *et al.* showed that a germline deletion of the last two exons of *TACSTD1*, the gene just upstream of *MSH2* encoding epithelial cell adhesion molecule (EpCAM), leads to inactivation of the *MSH2* gene by promoter hypermethylation exclusively in tissues expressing EpCAM (mosaic pattern). This mechanism may cause LS in patients with MSH2-deficient microsatellite unstable tumours with an undetectable *MSH2* germline mutation. Identification of these cases is possible by the determination of the methylation status of the *MSH2* gene promoter in the tumour and in EpCAM expressing normal tissues (*e.g.* normal colorectal mucosa). In addition, evidence for the presence of *MSH2* methylation can be obtained by detection of deletions in the 3′ end of the *TACSTD1* gene.

Molecular diagnostics of Lynch syndrome

The molecular diagnostics of LS usually starts with MSI analysis. MSI analysis is traditionally performed with a panel of five microsatellite markers proposed by a NCI (National Cancer Institute) sponsored consensus conference, also known as the Bethesda panel [29]. With these markers, microsatellites in tumour DNA are compared to microsatellites in corresponding DNA from normal tissue. Tumours with more than one unstable marker (or ≥40% of markers) are categorized as having a high degree of MSI (MSI-H), which is suspect for LS or epigenetic *MLH1* silencing [88–91]. Those with one unstable marker (20–40% of markers) are categorized as having a low degree of MSI (MSI-L) and tumours with no instability (≤20%) are categorized as being microsatellite stable (MSS), seen in sporadic carcinomas [92]. Although there are no clear differences in clinical or pathological features between MSI-L and MSS tumours, it has been speculated that MSI-L tumours comprise an independent phenotype [93]. However, there is nowadays no role for separating MSI-L from MSS tumours in the diagnostic work-up. Furthermore, MSI testing seems not only important for recognition of LS, but may in the future also improve the clinical management of CRC patients. This is because patients with microsatellite unstable CRCs appear to have a better prognosis than patients with MSS tumours [94–97] and they do not seem to benefit from adjuvant chemotherapy with 5-fluorouracil [98–100].

The Bethesda panel, comprising two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D2S123, D5S346 and D17S250) [62], does have some limitations, mainly caused by the dinucleotide repeats. These repeats are highly polymorphic and less sensitive and specific in the identification of MSI-H tumours than mononucleotide repeats. Their use in MSI screening requires analysis of corresponding germline DNA [101] and the interpretation of size alterations in dinucleotide repeats is more difficult due to stutter, a PCR artefact. Their use can result in misclassification of MSI-L tumours as MSI-H [102, 103]. Furthermore, MSH6 mutation carriers may develop tumours (predominantly endometrial cancer) without alteration in these dinucleotide repeats leading to false MSI-L or MSS results [104, 105].

Table 6 Immunohistochemical expression patterns associated with MMR-gene mutations

		MMR-gene mutation			
		MLH1	*MSH2*	*MSH6*	*PMS2*
Protein expression	MLH1	−*	+	+	+
	MSH2	+	−	+	+
	MSH6	+	−	−	+
	PMS2	−	+	+	−

* Absent MLH1 protein expression can be associated with either *MLH1* germline mutations as well as epigenetic *MLH1* silencing by promoter methylation.

+ = present nuclear protein expression in tumour cells (as well as in normal cells).

− = absent nuclear protein expression in tumour cells (and present staining in normal cells, thus serving as internal positive control).

The limitations of the Bethesda panel have lead to the development of a pentaplex panel, which comprises five quasi-monomorphic mononucleotide repeats (see below). This panel shows less variation in size among different ethnic populations and has been shown to be superior to the Bethesda panel for the detection of MSI-H tumours [102, 106]. Because the pentaplex analysis is carried out in a single multiplex PCR, this method is simple to use and is free of errors due to mixing samples.

To gain insight into what gene might be affected in patients with MSI-H tumours, MLH1, MSH2, MSH6 and PMS2 protein expression can be assessed by immunohistochemistry. The combination of MSI analysis and MMR protein immunostaining is generally considered as the superior strategy for the identification of suspected LS patients [107]. Absence of MMR protein nuclear staining within the tumour cells can be compared to nuclear staining in the normal cells within the same tumour specimen (and same histological section). The latter then serve as internal positive control.

Due to their heterodimeric nature, different immunohistochemical staining patterns of the MMR proteins can be observed (Table 6). Loss of MLH1 protein due to *MLH1* gene mutation or promoter hypermethylation is usually accompanied with absence of PMS2 in the tumour (Fig. 3). Similarly, absence of MSH2 due to *MSH2* mutations results in absence of MSH6 (Fig. 3), since MSH6 and PMS2 will disintegrate without their obligatory partners MSH2 and MLH1, respectively. A mutation in either *PMS2* or *MSH6* does not lead to loss of MLH1 and MSH2 protein, respectively (Fig. 3), because of the formation of other heterodimers than MLH1-PMS2 and MSH2-MSH6. MLH1 can for instance dimerize with either MLH3 or PMS1 [108, 109] and MSH2 can also bind to MSH3 [110]. Due to the binding of MLH1 and MSH2 to other MMR proteins in the absence of PMS2 or MSH6, there is no concurrent loss of MLH1 and MSH2 [111]. To date no bona fide involvement of

PMS1, *MLH3* or *MSH3* (inactivating mutations) has been demonstrated in LS.

In general, absent MSH2, MSH6 or PMS2 expression in tumour cells with present staining in normal cells is suspect for underlying LS and calls for germline testing. Absent MLH1 (and PMS2) expression can indicate either LS or a sporadic tumour with epigenetically silenced *MLH1* [111]. If epigenetic *MLH1* silencing has been excluded by the analysis of *MLH1* hypermethylation and/or *BRAF* mutation analysis, *MLH1* germline mutation testing is indicated. Furthermore, it might theoretically be possible that immunohistochemical absence of PMS2 or MSH6 without concomitant absence of MLH1 or MSH2, respectively, is due to mutations in *MLH1* or *MSH2*. These mutations then will not lead to decreased MLH1 and MSH2 immunostaining, while binding to and expression of PMS2 and MSH6, respectively, is abrogated. Therefore, absent PMS2 or MSH6 immunostaining without detectable mutations in the *PMS2* or *MSH2* gene, asks for mutation analysis of *MLH1* or *MSH2* respectively.

At our institution, MSI analysis and immunohistochemistry are requested either by the pathologist when patients fulfil the criteria as depicted in Table 5, or by the clinical geneticist (or clinician) when individuals meet the Bethesda Guidelines. The flowchart of the molecular diagnostics of LS in The Netherlands is depicted in Fig. 4. All these different molecular diagnostic procedures will be described in more detail in the next paragraphs.

Description of molecular analyses

MSI analysis

From routine formalin fixed and paraffin embedded (FFPE) tumour tissue specimens 10 to 20 consecutive sections of 4 μm are cut and routinely glued on microscope glass slides. The number of sections is determined by the size of the tissue fragments that need to be isolated for DNA analysis. All sections are deparaffinized and the first and the last section of the series are routine Mayer haematoxylin and eosin stained. These sections are used as reference for the isolated tissue parts. The intermediate sections are stained in haematoxylin and rinsed in distilled water. The indicated tumour and normal tissue fragments are then manually scraped in distilled water from the glass slide and transferred to Eppendorf vials. From the remaining tissue fragments on the glass slides, routine microscopic preparations are made after additional staining with eosin. With these preparations the isolated tissue fragments can be verified (Fig. 5). Occasionally, when large and easy recognizable tissue fragments can be isolated, scraping is performed from paraffin sections on glass slides without deparaffinization. Furthermore, when the tissue fragments to be isolated are too small for manual isolation, laser microdissection is used on haematoxylin and eosin stained sections glued on membrane containing glass slides (PALM Membrane Slides, P.A.L.M. Microlaser Technologies AG, Bernried, Germany) (Fig. 5).
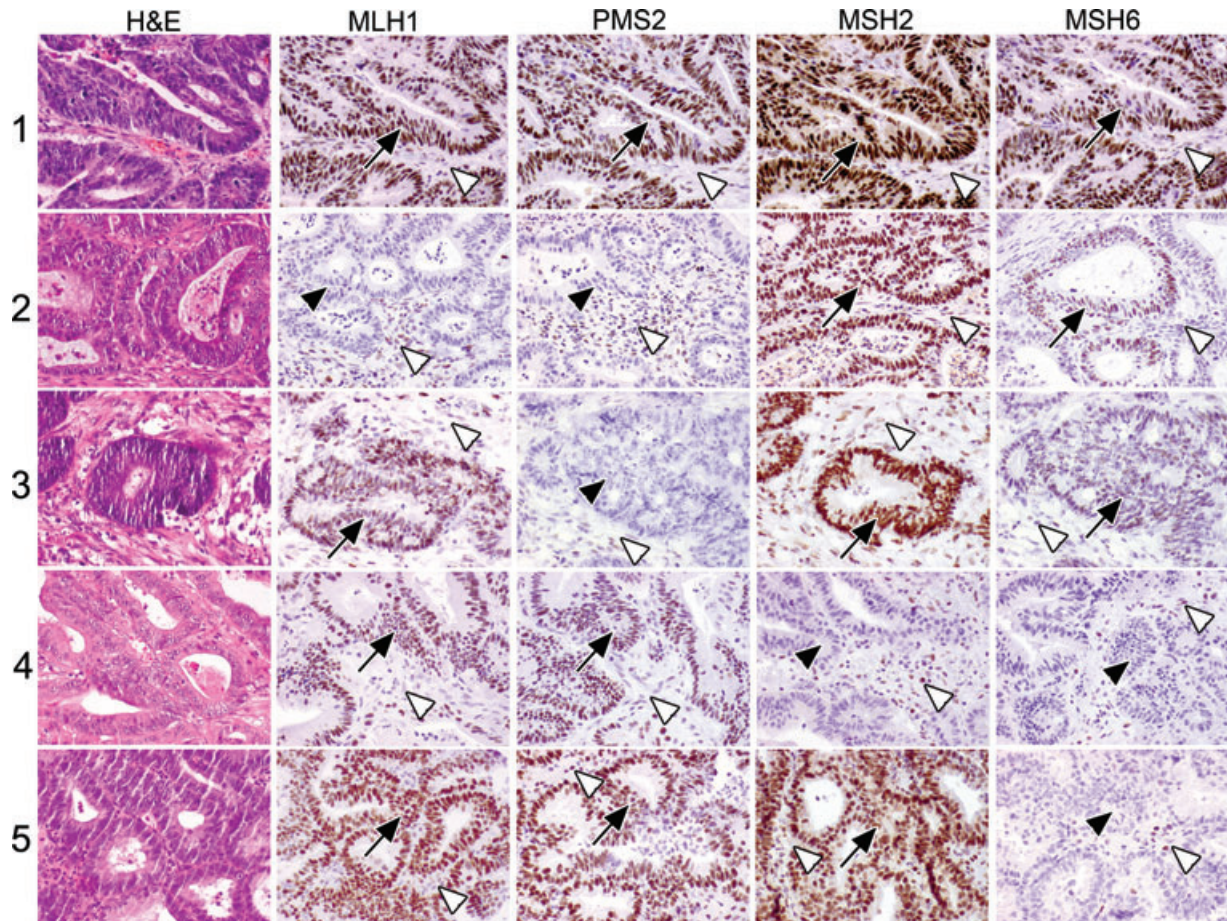
Fig. 3 Five CRC cases; haematoxylin and eosin staining and MLH1, PMS2, MSH2 and MSH6 immunohistochemistry (IHC) results. Case 1: normal IHC in the tumour cells. Case 2: absence of MLH1 and PMS2 in the tumour cells. Case 3: absence of PMS2 in the tumour cells. Case 4: absence of MSH2 and MSH6 in the tumour cells. Case 5: absence of MSH6 in the tumour cells. Arrows point to IHC$^+$ tumour cells, filled arrow heads point to IHC$^-$ tumour cells and open arrow heads point to IHC$^+$ stromal cells.

Although MSI can be reliably detected even when DNA is isolated from a tissue fragment composed of only 10% neoplastic cells (unpublished data), tumour DNA is isolated preferably from a tissue fragment with a high percentage (>70%) of tumour cells. DNA isolated from tissue with a high percentage of tumour cells can also be used for reliable additional investigations (*BRAF* mutation and *MLH1* hypermethylation). In the case of an adenoma the fragment with the highest grade of dysplasia should be used for DNA isolation. For isolation of normal DNA a tissue fragment composed of normal cells, preferably from the normal epithelial counterpart of the tumour (*e.g.* normal colorectal or normal endometrial mucosa), is used to circumvent heterogeneity problems that can be caused by mosaicism (*e.g.* mosaic *MLH1* promoter germline hypermethylation or *MSH2* promoter hypermethylation only in Epcam expressing cells). However, since these mosaic phenomena are very rare, other normal tissue fragments (*e.g.* a tumour-negative lymph node) can be used for normal DNA isolation, in cases where there is no, or not easy to isolate, normal mucosa available.

From the microdissected FFPE tissue fragments DNA is extracted by addition of 100 to 200 μl (when very small tissue fragments are used digestion is performed in a volume down to 25 μl) lysis buffer (10 mM Tris/HCL pH 8.0, 1 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 0.01% Tween 20) containing 2 mg/ml proteinase K and 5% Chelex 100 resin. Following overnight incubation at 56°C, proteinase K is inactivated at 100°C for 10 min. Next, dissolved DNA is separated from cell debris by centrifugation at maximum speed in a microcentrifuge for 5 min. The DNA-containing supernatant is carefully pipetted from the Chelex resin-containing pellet (Chelex resin inhibits polymerase activity) and transferred to another Eppendorf vial. In case undeparaffinized sections were used for DNA isolation, the DNA-containing supernatant is collected by carefully poking the pipette tip through the solidified paraffin layer on top of the supernatant.
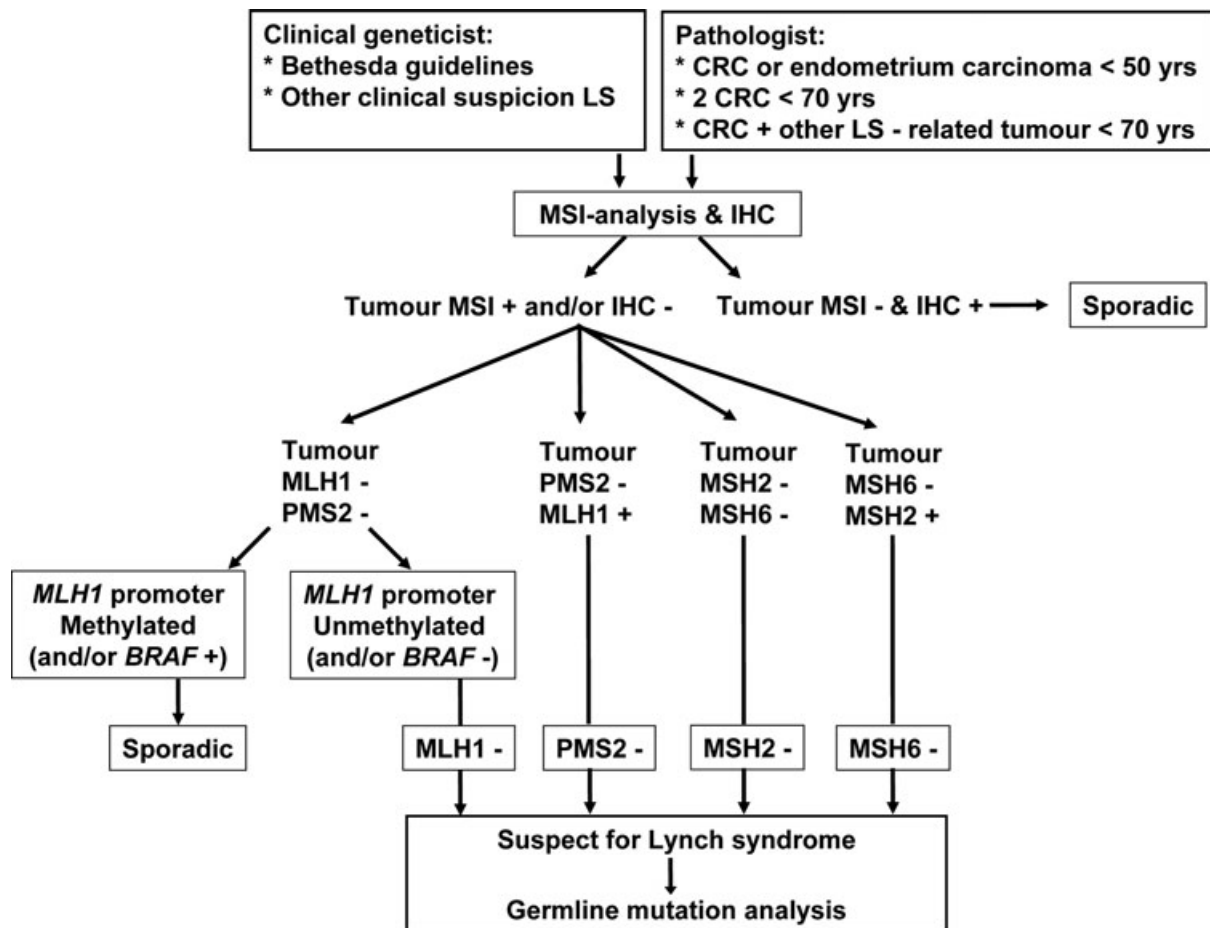
Fig. 4 Flowchart for molecular diagnostics of LS in The Netherlands.

Different methods for MSI analysis are currently available. In our laboratory we use the MSI analysis system of Promega (Promega, Madison, WI, USA) [103]; a fluorescent multiplex PCR-based assay in which the PCR products are separated by capillary electrophoresis using an ABI PRISM 3130*xl* genetic analyser (Applied Biosystems, Foster City, CA, USA). PCR is performed according to the kit instructions in a total volume of 10 μl including 2 μl of an about 80-fold dilution of the isolated DNA solution. The output data are analysed with GeneMarker software (SoftGenetics, State College, PA, USA) to determine MSI status of tumour samples. This system includes fluorescently labelled primers for co-amplification of five quasi-monomorphic mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24 and MONO-27. In addition, 2 pentanucleotide markers (Penta C and Penta D) characterized by a high level of polymorphism have been added to provide information on possible sample mix-up or contamination. Because of the low size variation in the population of the selected mononucleotide markers this analysis allows, in most cases, that only tumour DNA is investigated for MSI. DNA from a

MSS cell line suffices as normal DNA reference. If inconclusive results are obtained, for example due to the infrequent occurrence of bi-allelic variation or borderline shifts of the marker peaks, the assay is repeated with both tumour and patient matched normal DNA. Furthermore, additional mononucleotide MSI markers such as BAT-40 can be used in the case of a MSS tumour with a strong clinical suspicion for underlying LS. Results of MSS and microsatellite unstable tumours are shown in Fig. 6.

Immunohistochemistry

Our method of immunohistochemistry was described in detail previously [112]. Briefly, FFPE tissue sections (4 μm) are dewaxed, and antigen retrieval is performed in 10 mM Tris-EDTA buffer, (pH 9.0) in a microwave oven for 45 min. at 100°C. Primary antibodies anti-MLH1 (Pharmingen BD, Alphen aan den Rijn, The Netherlands; clone G168–728; dilution, 1:20), anti-MSH2 (Pharmingen BD; clone G219–1129; dilution, 1:300), anti-MSH6
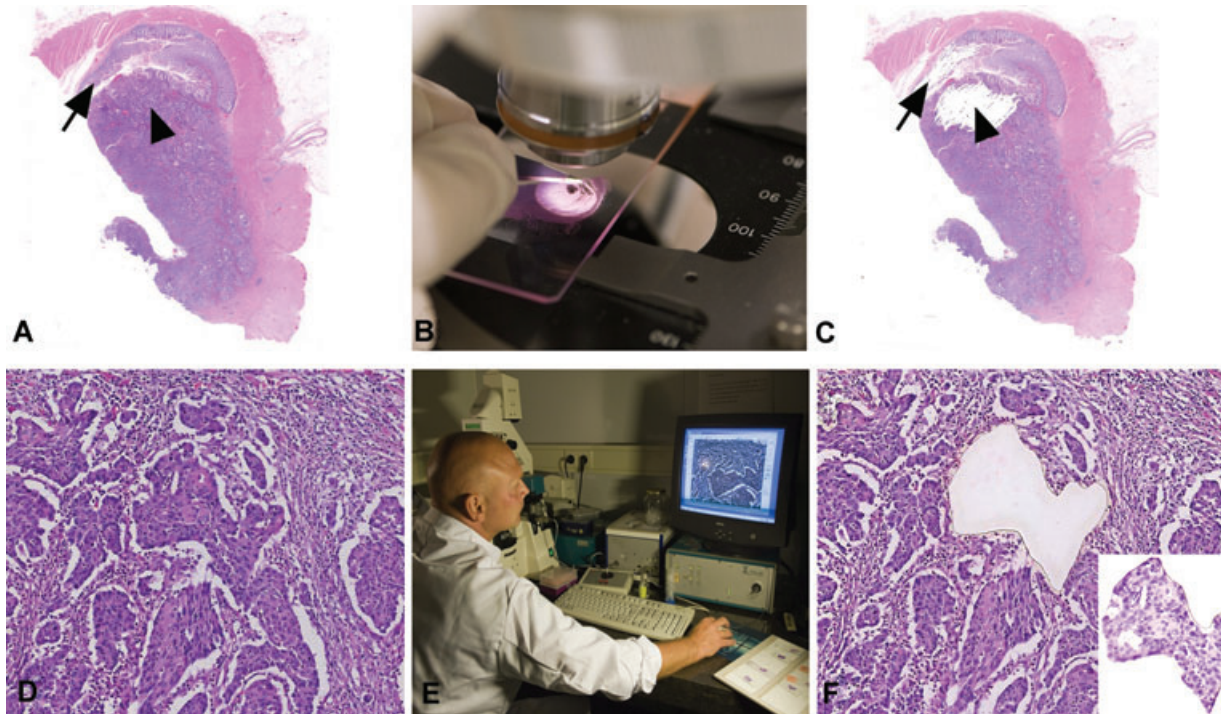
Fig. 5 (**A**, **B**, **C**) Manual macrodissection of a normal tissue fragment (arrow) and a region of the tumour composed of a high percentage of tumour cells (arrow head), before, during and after macrodissection, respectively. (**D**, **E**, **F**) Laser capture microdissection of a small tumour tissue fragment surrounded by abundant stromal cells, before, during and after laser capture microdissection, respectively. Insert in (**F**) shows the microdissected fragment.

(Pharmingen BD; clone 44; dilution, 1:100) and anti-PMS2 (Pharmingen BD; clone A16–4; dilution, 1:50) are applied for 1 hr at room temperature. After washing, immunoreactivity is visualized with the Envision kit (Dako, Glostrup, Denmark). Subsequently, the sections are counterstained with Mayer haematoxylin and evaluated under a light microscope (Fig. 3).

MLH1 promoter hypermethylation assay

In case of absent MLH1 expression in tumour cells, the methylation status of the *MLH1* promoter can be determined by different methods such as methylation-specific PCR [113] and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) [114]. MS-MLPA is performed with the SALSA MS-MLPA Kit ME011-A1 for MMR genes (MRC-Holland, Amsterdam, The Netherlands). The analysis is performed according to the kit instructions with 3 μl undiluted DNA solution as input. The assay takes advantage of methylation-sensitive endonuclease *Hha*I, which only cleaves unmethylated DNA fragments. The MS-MLPA kit contains 8 control probe sequences and 21 methylation-sensitive probes of which 5 recognize CpG dinucleotides within the *MLH1* promoter. The methylation-sensitive probes contain a restriction site for *Hha*I. Comparison of a *Hha*I-digested DNA sample (yielding only signal of methylated DNA) to its undigested counterpart (yielding signal of both methylated and unmethylated DNA) provides insight into the degree of methylation. Details of the MS-MLPA protocol are freely available on the website of the manufacturer (http://www.mrc-holland.com).

Basically, tumour DNA is hybridized to the probe mix. After hybridization, half of the sample is subjected to a ligation step joining both adjacently hybridized fragments of a probe set, whereas the other half of the sample is subjected to both ligation and *Hha*I digestion, leaving only methylated sequences intact. Subsequent PCR amplification exponentially amplifies all ligated, but undigested, probes. The signal generated with the part of the sample that has undergone both ligation and digestion represents the amount of methylated DNA present in the tumour. For fragment analysis, PCR products are separated by capillary gel electrophoresis using an ABI PRISM 3130*xl* genetic analyser (Applied Biosystems) and quantified with GeneMarker software version 1.7 (SoftGenetics). The MS-MLPA results are normalized by dividing the peak height of each *MLH1* probe signal by the mean peak height of the eight control fragments obtained with the same sample (Fig. 7). The degree of methylation for individual *MLH1* probes can be assessed by dividing normalized values of each *MLH1* probe within digested DNA samples by normalized values of the probe in corresponding undigested samples. The MS-MLPA assay is performed with both tumour and normal mucosal DNA to detect possible germline *MLH1* promoter hypermethylation.
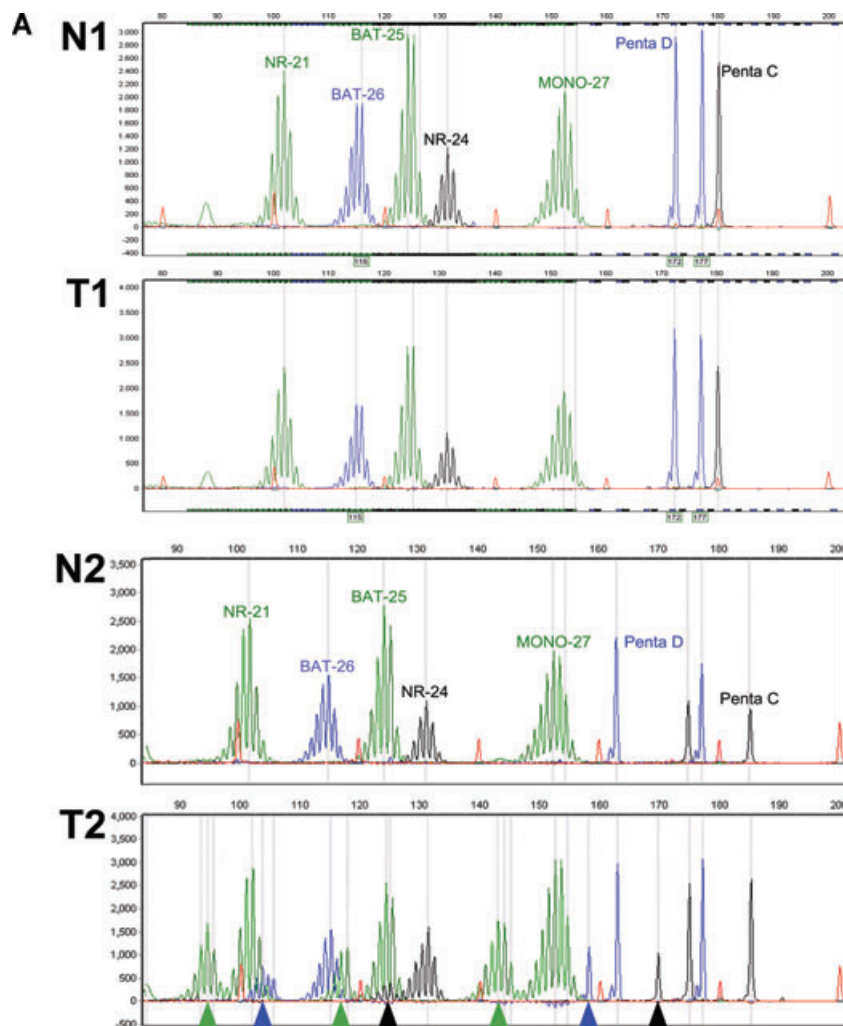
Fig. 6 MSI analysis of four cases of paired normal (N) and tumour (T) DNA. (**A**) Case 1: N1 and T1, CRC no MSI: MSS. Case 2: N2 and T2, CRC demonstrates clear MSI. (**B**) Case 3: N3 and T3, endometrial carcinoma with MSI (subtle microsatellite shifts). Case 4: N4 and T4, CRC without MSI but with heterozygous NR-21 microsatellite alleles (present in normal and tumour DNA). Arrow heads indicate the MSI shifts, arrows indicate the variant NR-21 allele.

BRAF mutation analysis

BRAF alterations of mutational hotspot codon V600 are determined by bi-directional cycle sequencing of PCR-amplified fragments. PCR amplification is performed by M13-tailed forward primer 5′-TGT AAA ACG ACG GCC AGT AAA CTC TTC ATA ATG CTT GCT CTG -3′ and M13-tailed reverse primer 5′-CAG GAA ACA GCT ATG ACC GGC CAA AAA TTT AAT CAG TGG AA-3′. PCR products are generated in a 15 μl reaction mixture including 1.0 μl undiluted DNA solution, 10 μmol of each primer, 25 mM MgCl$_2$, 10 mM dNTPs and 1 U Taq polymerase (Promega). The PCR reaction is performed with a thermocycler (Biometra, Gˆttingen, Germany) with an initial denaturating step (95°C) for 3 min., followed by 35 cycles consisting of denaturation (95°C) for 30 sec., annealing (60°C) for 45 sec. and extension (72°C) for 45 sec. After the final cycle, an extension period of 10 min. at 72°C is performed. The PCR products are sequenced with M13 forward primer 5′-TGT AAA ACG ACG GCC AGT-3′ and M13 reverse primer 5′-CAG GAA ACA GCT ATG ACC-3′ using the ABI PRISM *BigDye* Terminator v3.1 kit (Applied Biosystems). Sequence analyses are performed on an ABI PRISM 3130*xl* genetic analyser (Applied Biosystems). Samples are analysed using Mutation Surveyor software (SoftGenetics) and are compared with the public sequence of GenBank (NT_007914). Examples of *BRAF* mutation analysis results are shown in Fig. 8.

Limitations of molecular analyses

Over the last decade, the diagnostics of LS have improved considerably. Nevertheless, there still remain some limitations that need to be addressed. It has to be taken into account that the described procedures provide information on the chance that a
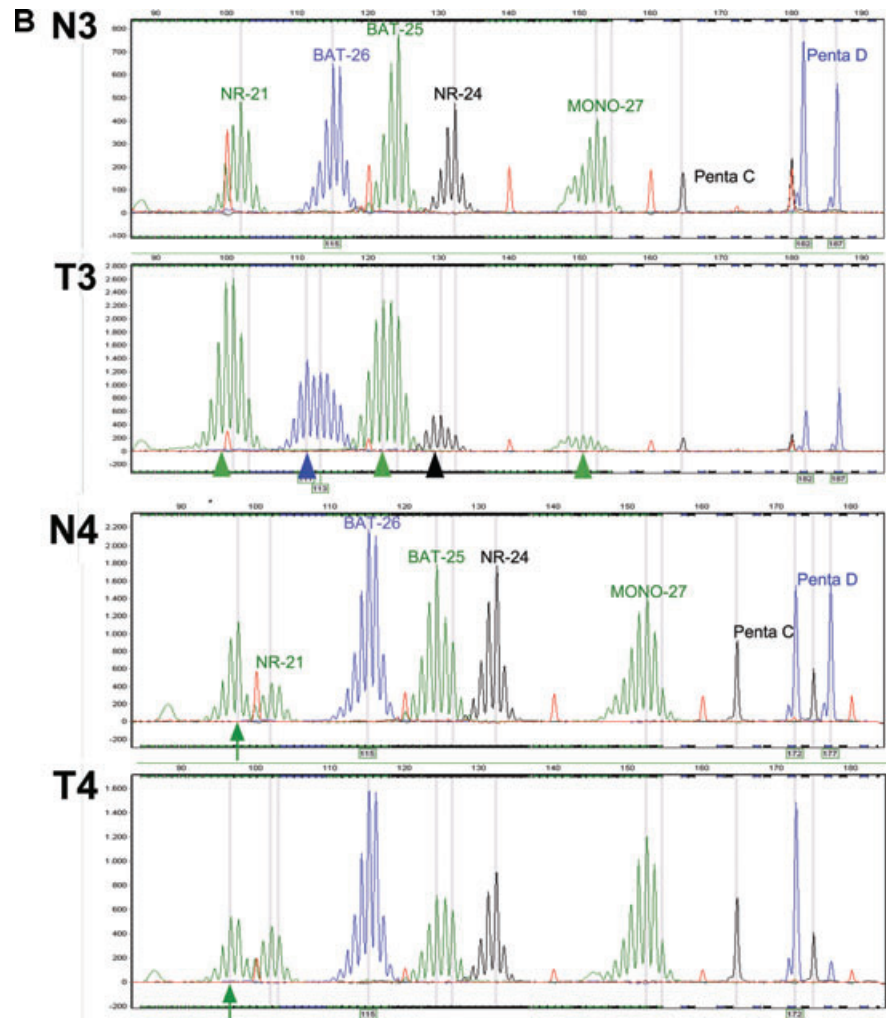
Fig. 6 Continued

certain tumour arose in the context of LS and are not diagnostic for LS in an absolute sense. The false negative rate of MSI analysis is very low (<5%) but cannot be completely ruled out. MSI can be very subtle or escape detection particularly in low grade lesions as adenomas, in endometrial carcinomas (Fig. 6B, panel N3/T3) and in samples with a low percentage of neoplastic cells [115]. These false-negative results may lead to the exclusion of LS patients (and affected family members) from necessary surveillance programs and subsequent failure to detect (secondary) cancers in an early stage. In addition, although rare, sporadic MSS tumours can occur in LS patients and MSI analysis then fails to indicate LS. To exclude false-negative MSI results as much as possible it is necessary to isolate DNA from a tissue fragment with a high percentage of tumour cells. For this, laser microdissection might be preferable instead of manual microdissection. However, laser microdissection is a time consuming and labour-intensive procedure to obtain sufficient

tissue fragments for DNA isolation [116]. In general, it is recommended to refer patients with a high clinical or familial suspicion of LS to a clinical genetics department, irrespective of the MSI status. In addition, other hereditary CRC syndromes such as attenuated familial adenomatous polyposis, MYH-associated polyposis, Cowden syndrome or Peutz-Jeghers syndrome might need to be excluded.

Although the assessment of MMR-protein expression by immunohistochemistry is a fast and simple procedure, the interpretation of the results can be difficult. Interpretation may be impeded by absence or low intensity of the nuclear staining in tumour and normal tissue due to fixation artefacts, especially in old archival specimens [117]. In case of missense mutations, the inactive protein may be (partly) expressed and detectable by immunohistochemistry. The interpretation is also hampered by some degree of observer-variation and the value of immunohistochemistry partially depends on the experience of the pathologist
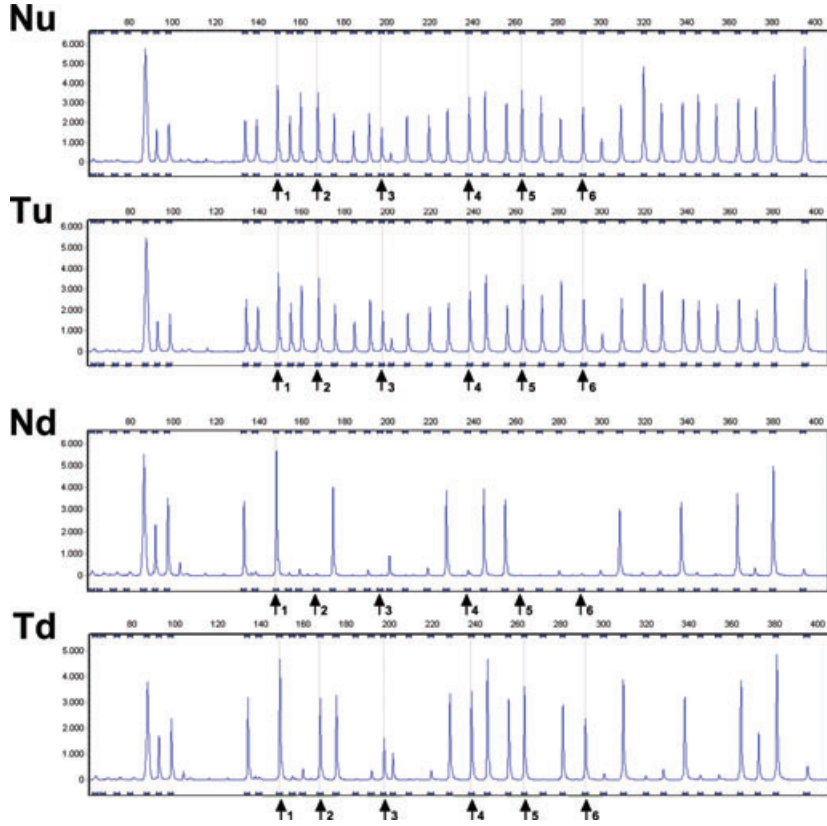
Fig. 7 *MLH1* promoter hypermethylation assay; MS-MLPA analysis of a MSI CRC with absence of MLH1 and PMS2 expression. Results are shown of paired undigested normal (Nu) and undigested tumour (Tu) DNA and of the methylation-sensitive endonuclease *HhaI* digested normal (Nd) and tumour (Td) DNA. Arrows indicate six *MLH1* promoter probes, nr 1 representative for a fragment without and nrs 2–6 representative for fragments with a *HhaI* restriction site. In the *HhaI* digested DNA, probes 2–6 are clearly present in the tumour DNA (Td) and not in the paired normal DNA (Nd) indicating tumour-specific methylation of the *MLH1* promoter fragments 2–6.
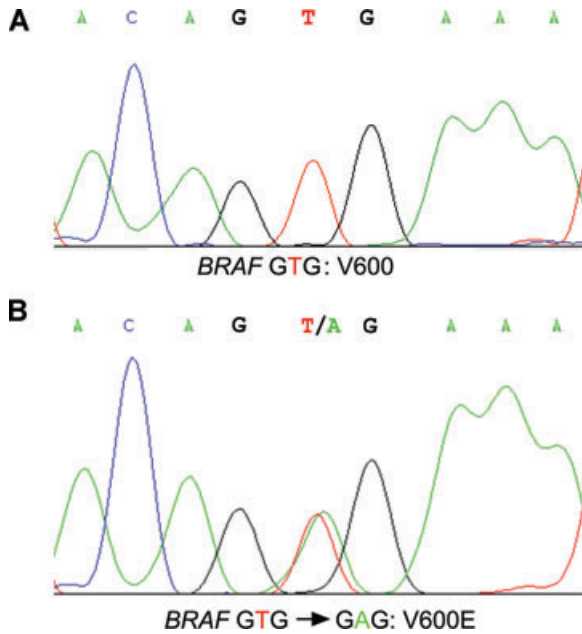

Fig. 8 Mutation analysis of codon 600 of the *BRAF* gene. (**A**) Tumour DNA without *BRAF* codon 600 mutation. (**B**) Tumour DNA with a heterozygous oncogenic mutation GTG-GAG, leading to a V600E amino acid substitution.

[118, 119]. For these reasons immunohistochemistry cannot replace MSI testing to detect LS, and this underlines the importance of the combined application of MSI analysis and MMR protein immunostaining to detect LS.

In the evaluation of *MLH1* promoter methylation, it is important to study the correct promoter regions since MLH1 expression only correlates with methylation of the proximal promoter regions (mainly region C, but also region D) [113, 120, 121]. Nevertheless, there are still studies published in which the distal *MLH1* promoter regions were analysed, which are not or only poorly associated with gene silencing. Moreover, epigenetic inactivation of the second normal *MLH1* allele by promoter methylation (second hit), may also play a role in individuals with LS [122, 123], and it should be realized that the detection of *MLH1* promoter methylation can not completely rule out LS. In the case of a strong clinical suspicion, referral to a clinical geneticist is indicated. The exact frequencies of *MLH1* promoter methylation in LS patients (either as a second inactivating event, or as a heritable germline epimutation), are unknown. It has been reported that in tumours from MLH1 mutation carriers, the wild-type allele is hypermethylated in 0–46% of the tumours [65, 76, 80, 122, 124–129]. However, only one study evaluated the proximal promoter region (region D) associated with gene silencing in 55 CRCs and endometrial cancers of *MLH1* germline mutation carriers [129]. Hypermethylation was seen in 7.3% of all tumours (16% of CRCs). In the other

studies, promoter regions not associated with *MLH1* silencing were investigated (*i.e.* the distal promoter regions) [65, 76, 80, 122, 124–128].

There are some other points of concern in the molecular diagnostics of LS. First, the value of MSI testing and immunohistochemistry in other LS-related tumours than CRC is largely unknown [130]. In endometrial tumours, the second most common malignancy in LS, MSI can escape detection by the occurrence of only subtle shifts in the size of the markers [131]. Therefore, MSI analysis in endometrial cancers is performed with patient matched normal DNA as the reference, and molecular pre-screening has been found feasible [4, 132, 133] (Fig. 6B, panel N3/T3). Furthermore, the quality of DNA extracted from FFPE tumours can occasionally be poor and therefore not suitable for MSI analysis [130]. And last but not least, some individuals might have ethical objections against MSI testing or immunohistochemistry, since the diagnosis of LS can be very likely after the described molecular examinations, which might have negative social consequences and raise concerns *e.g.* about insurance risks. Therefore, we believe that the clinician should inform the patient about the fact that the pathological examinations may not only give information about the nature of the tumour, but may also indicate an elevated risk of an underlying hereditary disorder.

Conclusions

Different diagnostic strategies have been developed for LS as discussed in this review and the optimal method for the identification of LS patients is still debated and in flux. In the previous paragraphs the molecular diagnostic approach of LS in The Netherlands (Erasmus MC, University Medical Center, Rotterdam) has been described (Fig. 4). This approach combines MSI analysis and MMR protein immunostaining and is in our opinion a productive way of pre-selecting patients for germline mutation analysis, with a central role for the pathologist. Nevertheless, if the clinical suspicion for LS is very high, *e.g.* because of a positive family history for LS-associated cancers or a LS-associated malignancy diagnosed at a very young age, referral to a clinical geneticist is strongly recommended, even in the case of tumours without MMR deficiency (*i.e.* MSS).

Acknowledgements

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