Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors

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Chapter 3

2. INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of death because of malignancy in the Western world. The cause of CRC is multifactorial, involving hereditary and environmental factors and somatic genetic changes during tumor progression [1]. A family history of CRC is a clinically significant risk factor and may be found in up to 15% of all patients with CRC [2]. The most common hereditary CRC syndromes are familial adenomatous polyposis coli (FAP), accounting for <1% of CRC cases and HNPCC (hereditary nonpolyposis colorectal cancer), accounting for 1 to 6% of the cases [3]. HNPCC is an autosomal dominantly inherited disorder that is clinically defined by the Amsterdam Criteria [4,5]. In HNPCC, germline mutations have been identified in four DNA mismatch repair (MMR) genes, MSH2 [6], MLH1 [7], PMS2 [8], and MSH6 [9-14]. In 50 to 70% of the families fulfilling the Amsterdam criteria a germline mutation is detected in MLH1 or MSH2 [15,16]. Germline mutations have been found in MSH6 in families with atypical HNPCC, ie not entirely fulfilling the Amsterdam criteria [11-14].

Microsatellite instability (MSI) in colorectal tumors, first reported in 1993 [17-19], is caused by a failure of the DNA MMR machinery to repair errors occurring during DNA replication and leading to length alterations in simple, repetitive microsatellite sequences distributed throughout the genome [20]. According to international guidelines, a panel of five specific microsatellite markers has been recommended for MSI evaluation [20]. If at least two markers show instability, the tumor is referred to as MSI-high (MSI-H), if only one marker is unstable, the tumor is considered MSI-low (MSI-L). MSI is reported in 85 to 92% of CRC associated with HNPCC and in 10 to 15% of sporadic CRC [17,21-23].

In 1996, Leach and colleagues [24] and Thibodeau and colleagues [25] reported the use of monoclonal antibodies directed against
MSH2 and MLH1 in the immunohistochemical analysis of CRCs. Subsequent reports described immunohistochemistry (IHC) of MLH1, MSH2, and MSH6 in sporadic and HNPCC tumors with varying results [26-35]. Some authors suggested that IHC can be used as a prescreening method for the actual mutation analysis of the MMR genes [32,36,37]. Others concluded, however, that IHC cannot replace MSI analysis as a prescreening method, because of a lower sensitivity [35,38,39]. The studies on the value of IHC published so far are hampered by small numbers of tumors associated with a known MMR gene mutation. In addition, most studies focused on tumors associated with MLH1 and MSH2 mutations and not MSH6 mutations.

IHC for diagnostic purposes is usually performed on whole slides. A novel approach that allows high-throughput IHC is provided by the so-called tissue microarrays (TMAs) composed by large numbers of small punched-out tissue cores from different tumors [40]. With this technique up to 1000 different samples can be analyzed in a single immunohistochemical staining experiment [41]. Previous reports concluded that binary immunophenotypes can be reliably investigated on TMAs using two to three representative cores per tumor sample [42]. However, validation of data generated by TMA is needed to determine the minimal amount of tissue cores/tumor required in one TMA and to inventory possible problems of TMA that might influence staining results including technical artifacts such as differences in fixation of archived material or loss of tissue [43].

The aim of the present study was to evaluate whether IHC analysis of colorectal tumors could predict the presence of a MMR mutation in tumors in a large series of HNPCC patients with a known mutation, and to compare these results with the outcome of MSI. In addition, we compared the results of IHC performed on whole slides with the TMA technique. Validation of TMA for IHC has not yet been performed for colorectal tumors.

3. PATIENTS AND METHODS

3.1 Patients
A total of 45 patients (25 males and 20 females) with a known germline mutation in MLH1, MSH2, or MSH6, were selected from 35 HNPCC families. Information on cancer site, age at diagnosis, and location of the colon tumors were collected for all patients (Table 1). The paraffin-embedded tissue blocks from these patients dated back from 1976 until 1999. One tumor from each patient was used for the analysis. In total, 44 CRCs and a single duodenal carcinoma were analyzed.

Among the 35 families with a known MMR defect, 27 different germline mutations have been identified by denaturing gradient-gel electrophoresis or Southern blotting [44-47]: 14 in MLH1, 11 of which were pathogenic (nonsense, frameshift, or splice site mutants) and 3 unspecified variants; 8 in MSH2 (seven pathogenic, one unspecified variant); five in MSH6 (three pathogenic, two unspecified variants) (Table 1). Twenty-four of the 45 patients in our cohort, originating from 20 families, carry an MLH1 mutation. The same mutation was identified in six different families (1852_1854del, K618del). Thirteen of the 45 patients, originating from 10 families, were carriers of a mutation in MSH2. Two common mutations have been identified in two different families. Seven patients from five families were carriers of five different MSH6 mutations.

The average age at cancer diagnosis of the carriers of MLH1 (n = 21), MSH2 (n = 12), and MSH6 (n = 4) pathogenic mutations was 44 years (range, 28 to 68 years), 41 years (range, 23 to 61 years), and 54 years (range, 26 to 84 years), respectively.
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**Table 1.** MLH1, MSH2, and MSH6 Mutations

M, Male; F, female. IHC: 0, no nuclear staining; +, nuclear staining; na, not analysed. MSI: H, MSI-H; L, MSI-L; S, MSS; eg, 3/5: 3 of the 5 Bethesda markers tested instable, +, ++, +++: respectively 1, 2, or 3 of the 3 additional (BAT40, MSH3, and MSH6) markers are instable; na, no MSI analysis performed. Family diagnosis 1, Amsterdam II; 2, suspected HNPCC; 3, late onset; 4, sporadic young age.
We defined four categories of clinical diagnoses (Table 1). The first category includes families that fulfilled the revised Amsterdam criteria (AII+) [5]. The second category includes suspected HNPCC families, ie, familial cases fulfilling the Bethesda criteria (B+) [48]. The third category encompasses late onset families consisting of three CRC patients within two or three generations, with no diagnosis made at younger than the age of 50 years. The fourth category includes sporadic patients diagnosed at younger than the age of 40 years.

3.2. MSI
MSI analysis was performed on paired tumor-normal tissue DNA samples using the Bethesda panel of microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26) [20]. This panel was extended with the additional markers BAT 40, MSH3, and MSH6, as previously reported [49]. Tumors were scored as MSI-H (high) if at least two of the five Bethesda markers showed instability, MSI-L (low) if only one of these markers showed instability, or MSS (stable) if none of the Bethesda markers showed any shift in mobility.

The annotations +, ++, or +++ were used to indicate if, respectively, one, two, or three of the additional (BAT40, MSH3, and MSH6) mononucleotide markers showed instability.

3.3. Tissue Microarray (TMA)
TMAs were assembled from formalin-fixed, paraffin-embedded tissues as previously described [40] using a 0.6-mm-diameter punch (Beecher Instruments, Silver Spring, MD). The arrays encompass 362 tissue cores from colorectal tumors derived from 129 (suspected) HNPCC patients, including the 45 tumors from MMR gene mutation carriers. These tumor samples dated back from 1974 until 2000. Also, we included three tissue cores from normal colonic mucosa and one core of lung tissue (for orientation purposes).

Using a tape-transfer system (Instrumedic, Hackensack, NJ), 4 µm sections were transferred to glass slides. We were unable to analyze, because of tissue loss during processing, 52 (14%), 41 (11%), and 56 (15%) of the punches for MLH1, MSH2, and MSH6, respectively. Because two to three punches were taken per tumor this meant that for six (5%), five (4%), and five (4%) of the tumors, respectively, we were unable to analyze the staining of these proteins.

3.4. IHC
Conventional IHC on whole tumor sections was performed for all tumor samples. Immunohistochemical staining was performed on 4 µm sections of formalin-fixed, paraffin-embedded tissues. Whole tissue slides and TMA slides were stained with antibodies against MLH1 (clone 14; Calbiochem, Cambridge, MA), MSH2 (clone GB12) and MSH6 (clone 44; Transduction Laboratories/Becton Dickinson, Lexington, KY) in a DAKO Techmate 500+ (Glostrup, Denmark) automated tissue stainer using standard protocols [49] and procedures as indicated by the manufacturer. We initially tested the influence of different fixation intervals on the results of IHC for MLH1, MSH2, and MSH6. Therefore tumor parts of two control cases (one colon carcinoma and one rectal carcinoma) were fixated in buffered formalin for 1, 7, and 40 days, respectively. Overall the results were comparable. When fixation was performed with ethanol (for 1 and 4 days, respectively) in comparison with fixation in buffered formalin (also for 1 and 4 days) staining results were extremely poor after fixation with ethanol. For two initially frozen tissues, subsequent fixation in buffered formalin for 1 and 4 days seems to give less strong staining than what we normally experience after immediate fixation in formalin. Furthermore, when testing
IHC and TMA-IHC in hereditary colon tumors

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4. RESULTS

4.1. MSI Analysis

MSI analysis was performed in 33 of the 45 tumors derived from HNPCC mutation carriers (Table 1). Although the majority of the cases showed a high frequency of instability (28 MSI-H), 3 MSI-L and 2 MSS tumors (one of which was MSS+), were found. One of the MSI-L tumors was found in a carrier of a pathogenic MLH1 mutation (1852_1854del, K618del, exon 16). In this tumor, too, the BAT40 and MSH6 markers showed instability. IHC showed positive staining for MSH2 and MSH6 and absent staining for MLH1. Moreover, a CRC from an additional family member was MSI-H. The other two MSI-L tumors occurred in carriers of an unclassified variant. The MSS tumor occurred in a carrier of a pathogenic MLH1 mutation (677G>A, R266Q, splice donor, exon 8) and could not be tested for the three additional markers BAT40, MSH3, and MSH6. IHC indicated a mutation in MLH1. Furthermore, two tumors from additional carriers of the same mutation were scored as MSI-H. In the MSS+ tumor, found in a carrier of a pathogenic MSH6 mutation (1784delT, L595fsX609, exon 4), the BAT40 marker additionally showed instability. IHC showed absent staining for MSH6 while staining was positive for MLH1 and MSH2. Again, another tumor from an affected family member was MSI-H.

The BAT40 marker showed instability in 26 of the 29 tumors in which it was tested. MSI analysis, if both patients with MSI-H and with MSI-L tumors are considered candidates for mutation analysis, gives a sensitivity of 93% (25 of 27) in predicting an MMR pathogenic mutation if the five standard Bethesda markers are used and a sensitivity of 96% (26 of 27) using the BAT40, MSH3, and MSH6 markers in addition to the standard markers. The increase of sensitivity is mainly because of the use of the BAT40 marker.

In addition, 71 of 84 colorectal lesions from (suspected) HNPCC patients without known mutations were tested for MSI. The vast majority (53 of 71, 74%) of these tumors were classified as MSI-H, whereas 6% (4 of 71) were MSI-L and 20% (14 of 71) MSS.

4.2. Whole Slide Immunohistochemical Analysis of Tumors from Mutation Carriers

4.2.1 Individual Staining of the MMR Proteins

Twenty of the 25 (80%) tumors derived from MLH1 mutation carriers did not stain for the MLH1 protein. When unspecified variants are excluded, this figure rises to 18 of 21 (86%) (Table 2). The remaining three (14%) MLH1-positive tumors were found in carriers of small in-frame deletions or splice mutations. Notably, two of the MLH1-positive tumors were part of a series of seven tumors from carriers of the 1852_1854del
mutation in exon 16. The remaining five stained negative. Contrasting MLH1-staining patterns were also obtained with tumor samples from different carriers of the 277A>G, S93G missense mutation. The third MLH1-positive case was found in a carrier of a splice donor (677 + 1delG) mutation (according to the splice site prediction program, BDGP splice site prediction by Neural Network, the value decreased from 0.98 to 0.14).

Eleven of 13 tumors (85%) from \textit{MSH2} mutation carriers show no MSH2 staining. Again, this percentage increases when unspecified variants are excluded (11 of 12, 92%) (Table 2). MSH2-positive staining was observed in only one of three tumors from carriers of an in-frame exon 3 deletion.

Seventy-five percent (three of four) of the tumors from carriers of a pathogenic \textit{MSH6} mutation show no staining for the corresponding protein. Of the three tumors from patients with an \textit{MSH6} unspecified variant, only one (G670R) tumor could be analyzed: no MSH6 staining was found, thus indicating, but not proving, pathogenicity of this mutation. When unspecified variants are included 80% (four of five) of the tumors from \textit{MSH6} mutation carriers show absent staining of the corresponding protein.

To determine sensitivity of IHC in detecting MSI in general we considered all tumor samples that showed abrogation of at least one of the three proteins tested to be positive for MMR deficiency. In 86% (18 of 21), 100% (12 of 12), and 75% (3 of 4) of tumors from carriers of a \textit{MLH1}, \textit{MSH2}, or \textit{MSH6} pathogenic mutation, respectively, absent staining for at least one of the three proteins was shown. MMR deficiency would thus have been detected in 89% (33 of 37) of the cases.

### 4.2.2 Staining Patterns

MMR-IHC analysis in carriers of pathogenic \textit{MLH1} mutations revealed that in only 48% of the cases was an MLH1-negative staining accompanied by normal MSH2 and MSH6 staining patterns (Table 3). In these cases, the IHC results clearly direct the mutation analysis to a single gene, namely \textit{MLH1}. However, in another subset (24%) of the \textit{MLH1}-mutant tumors, an MSH6-negative staining pattern accompanied the loss of MLH1

<table>
<thead>
<tr>
<th>IHC</th>
<th>MMR</th>
<th>MLH1</th>
<th>MSH2</th>
<th>MSH6</th>
</tr>
</thead>
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<tr>
<td>1+/2+/6+</td>
<td>3 (14%)</td>
<td>1 (25%)</td>
<td></td>
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<tr>
<td>1-/2+/6+</td>
<td>10 (48%)</td>
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<tr>
<td>1+/2+/6-</td>
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<td>3 (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-/2+/6-</td>
<td>11 (92%)</td>
<td></td>
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<td>3 (14%)</td>
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<tr>
<td>1-/2-/6-</td>
<td>21</td>
<td>12</td>
<td>4</td>
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</table>

**Table 3.** IHC Staining Pattern in Carriers of a Pathogenic Mutation

1, MLH1; 2, MSH2; 6, MSH6; +, nuclear staining; -, no nuclear staining.
IHC and TMA-IHC in hereditary colon tumors

4.3. Tissue Microarray

Immunohistochemical Analysis (TMA-IHC)

A TMA encompassing the total cohort of 129 colorectal tumors was generated. We evaluated TMA-IHC staining for the presence or absence of the three main MMR proteins in the (suspected) HNPCC tumors and compared these with the results obtained by whole tumor section IHC when available (Table 4). An example of the staining pattern in a tumor from an MLH1 mutation carrier (1744 C>T, L582F; Table 1a) is shown in Figure 1 for the MLH1 (Figure 1A), MSH2 (Figure 1B), and MSH6 (Figure 1C) protein, respectively; MLH1 is abrogated, whereas MSH2 and MSH6 are present in the nuclei of the tumor cells.

Staining was concordant in 71 of 84 (85%) cases tested for MLH1, and in 77 of the 81 (95%) for MSH2. A somewhat lower level of concordance was found for MSH6: only 49 of 65 (75%) tumors showed similar results, mainly because of a high number [13] of positive staining results in TMA, scored as negative on whole slides. Of the latter samples six belonged to MLH1 mutation carriers (all AII+), two to MSH2 mutation carriers (all AII+), one to an MSH6 mutation carrier and four samples belonged to individuals in whom no mutation was identified (2 times AII+, 2 times AII-, B+).

<table>
<thead>
<tr>
<th></th>
<th>ws +, tma+</th>
<th>ws +, tma-</th>
<th>ws -, tma+</th>
<th>ws -, tma-</th>
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<th>Sensitivity</th>
<th>Specificity</th>
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<td>84</td>
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<tr>
<td>MSH6</td>
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<td>13</td>
<td>65</td>
<td>75%</td>
<td>88%</td>
<td>67%</td>
</tr>
</tbody>
</table>

Table 4. Validation of TMA for Mismatch Repair Proteins

Staining results of the array compared to results of staining of whole slides from the same patients for MLH1, MSH2, and MSH6. Staining was scored as either positive or negative, as described above. Tma, tissue microarray; ws, whole slide; +, positive nuclear staining; -, negative nuclear staining; conc, percentage concordance; sensitivity (percentage of true positives), specificity (percentage of true negatives).
5. DISCUSSION

The identification of MMR gene mutations in suspected HNPCC families is of great relevance for allowing the identification of mutation carriers for whom surveillance of the colon is required and has been proven to lower the risk to develop and to die of colorectal carcinoma [50]. A potential problem in the everyday clinical practice is that MMR genetic testing is expensive and time-consuming. In this study, we first evaluated the sensitivity of conventional whole section IHC analysis of MLH1, MSH2, and MSH6 in colon tumors from 45 established carriers of a MMR gene mutation and compared it with MSI analysis.

The sensitivity of IHC in predicting a pathogenic mutation was 89% (33 of 37), only slightly lower than that of MSI analysis using the Bethesda panel of five markers (93%, 25 of 27), or using the additional three markers (96%, 26 of 27). For IHC these results are remarkable because the paraffin blocks dated back from 1976 until 1999, with 35% of the samples older than 10 years, and fixation until now not fully standardized. We argue that intratumor heterogeneity will not be a problem in hereditary cases because of the fact that loss of MMR, and consequently often abrogation of MMR protein expression, is such an early event that it is present in all tumor cells. Should IHC become a standard of care in unselected cases heterogeneity is an issue that still needs further investigation, although in colorectal tumors with a MMR defect because of somatic abrogation of MLH1, this feature seems to be a dominant characteristic, as

![Figure 1. Immunoreactivity in a TMA of mainly (suspected) HNPCC patients. A tissue core with a colon carcinoma from a patient with a germline hMLH1 missense mutation (1744C>T, L582F) is shown, stained for MLH1 (A), MSH2 (B), and MSH6 (C). MLH1 is abrogated, whereas MSH2 and MSH6 are present in the nuclei of the tumor cells. Slides were stained with antibodies against MLH1 (clone 14, Calbiochem, Cambridge, MA), MSH2 (clone GB12, Calbiochem), and MSH6 (clone 44, Transduction Laboratories/Becton Dickinson, Lexington, KY). Original magnifications, x100.](image)
can be interpreted from a study on such heterogeneity [51].

An important advantage of IHC compared to MSI analysis is represented by the prediction of the specific MMR gene mutated in the germline of the corresponding patient. In tumors from most MLH1 mutation carriers (80%), staining of the MLH1 protein was absent, as expected. However, in only half (48%) of the tumors associated with a MLH1 mutation, the staining pattern (MLH1-, MSH2+, MSH6+) would have predicted unequivocally a pathogenic mutation in the MLH1 gene. In the future, the inclusion of PMS2 staining, which is often negative in tumors associated with MLH1 germline mutations [52], will most likely lead to a further increase of IHC sensitivity.

Negative staining for both MSH2 and MSH6 was found in tumors from MSH2 mutation carriers. Tumors from MSH2 mutation carriers, showed a lack of MSH6 staining only. These findings are most likely because of the failure of MSH6 to form a stable heterodimer in the absence of MSH2 [53]. On the other hand, if MSH6 is absent, a heterodimer can still be formed between MSH2 and MSH3, thus resulting in stabilization and positive staining of the MSH2 protein [49,54]. In our study, the specific staining pattern (MLH1+, MSH2-, MSH6-) of MSH2 mutated tumors would have correctly predicted the mutated MMR gene in all but one (92%) of the cases, while the specific staining pattern of tumors from MSH6 mutation carriers (MLH1+, MSH2+, MSH6-) would have predicted the presence of a MSH6 mutation in 75% of the cases.

In previous smaller studies, the sensitivity of IHC and MSI analysis has been evaluated in colorectal tumors of carriers of specified MLH1, MSH2 [25,30,32,35,55], and MSH6 [27,54,56] mutations. A problem we cannot solve is the possibility that variable outcomes of IHC analyses might be because of differences in staining protocols and antibodies used. Furthermore, a considerable number of mutations included in these studies are unclassified variants in which pathogenicity is by definition uncertain. In the present study, only pathogenic mutations were included in the determination of the sensitivity of IHC on whole slides.

The results of our study show that both IHC and MSI are sensitive prescreening methods to identify patients for mutation analysis. At present, IHC cannot completely replace MSI analysis until the sensitivity of MLH1 staining is improved, as recently discussed by de la Chapelle [57], and as long as the role of other putative MMR genes in hereditary CRC has not been elucidated. Because of its gene predictive value, and because of its speed and low cost, we would recommend IHC as a first diagnostic step in families fulfilling the revised Amsterdam criteria in which the probability of detecting a MMR gene mutation is relatively high [58] and MSI analysis is likely to give superfluous information. If a negative staining pattern is found, mutation analysis of the respective gene(s) is the next step. In case of doubtful interpretation or positive staining of all MMR proteins, MSI analysis should be performed. In the case of the absence of microsatellite instability (MSS), the analysis of a second tumor from the same family is recommended, to exclude intrafamilial variability in MSI analysis and IHC results, as shown in this study, and/or the presence of phenocopies.

In families not fulfilling the Amsterdam criteria, we would recommend MSI analysis as the first step. In these cases, the probability of detecting a MLH1 or MSH2 mutation is low [58] and IHC is less likely to be informative. MSH6 families, predominantly found not to comply with the Amsterdam criteria, represent exceptions. In the total group of Amsterdam-negative families MSI analysis is expected to provide
global information on loss of MMR function, including pathogenic missense mutations and alterations in MMR genes other than the known ones. In MSI-H or MSI-L (if the unstable marker is a mononucleotide marker) cases, IHC of all four MMR proteins should be performed as second step. In the case of MSS, IHC for MSH6 is recommended as it was shown that tumors from MSH6 mutation carriers are characterized by a variable MSI phenotype [54,59]. If no IHC abnormality is found, examination of a second tumor could be considered depending on the family history and age of the patient already tested. A scheme for clinical use, summarizing our current approach to patients from families with suspected HNPCC, is given in Figure 2.

The generation of a tissue array encompassing microsatellite unstable tumors has provided us with a powerful tool to quickly characterize the immunohistochemical staining patterns of MMR proteins in hereditary colorectal tumors for research purposes. We found a high level of concordance for MLH1 and MSH2 (85% and 95%, respectively). A somewhat lower concordance level was found for MSH6 (75%), primarily because of positive staining within the TMA and negative staining with the whole slide IHC. Six of the 13 tumors with discordant results for MSH6 originated from patients in whom an MLH1 mutation has been identified, where positive staining for MSH6 is expected. Two and one samples originated from carriers of a MSH2 and MSH6 mutation, respectively, where negative staining for MSH6 is expected. The other four samples originate from individuals in whom to date no mutation is identified and therefore no golden standard is available. Our first goal is to rapidly characterize the staining of other candidate MMR proteins particularly in tumors from (suspected) HNPCC patients in whom to date

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**Figure 2.** Approach of patients with familial clustering of colorectal cancer (CRC). 1: IHC for MLH1, MSH2, MSH6, PMS2. 2: If the tumor is MSI-H, mutation analysis is the next step. 3: If the tumor is MSI-H and mutation has already been performed, research is the next step.
no mutation was detected, to direct mutation analysis. Problems relative to differences in fixation standardization, age of tissues, punching outside the tumor area, and loss of tissue still represent serious obstacles. For fixation standardization we tend to suggest fixation for 1 day in buffered formalin. Use of ethanol fixation and acetone pretreatment should be avoided (see Patients and Methods). However, our study shows the general validity of this approach in the molecular diagnosis of familial CRC. Accordingly, recent IHC-TMA analysis has enabled us to identify a number of patients with abrogated staining of PMS2 with or without MLH1 staining, thus providing direction to mutation analysis of PMS2.

In conclusion, we have demonstrated the value of IHC using both whole slides and TMA as prescreening tools in selecting patients eligible for mutation analysis of MMR genes, in diagnostic and research settings respectively.

6. ACKNOWLEDGEMENTS

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7. REFERENCES


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