Utility of p16 Immunohistochemistry for the Identification of Lynch Syndrome

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Abstract

Purpose: Immunohistochemistry for mismatch repair proteins has shown utility in the identification of Lynch syndrome, but majority of tumors with loss of MLH1 expression are due to sporadic hypermethylation of the MLH1 promoter. These tumors can also show epigenetic silencing of other genes, such as p16. The aim of our study is to evaluate the utility of p16 immunohistochemistry in the prediction of MLH1 germline mutations.

Experimental Design: p16 immunohistochemistry was appropriately evaluated in 79 colorectal cancers with loss of MLH1 expression. Methylation of MLH1 and p16 were quantitatively studied using real-time PCR assay Methylight. BRAF V600E mutation in tumor tissue was also investigated. Genetic testing for germline mutation of MLH1 was made on 52 patients.

Results: Loss of p16 expression was seen in 21 of 79 samples (26.6%). There was found statistically significant association between p16 expression and p16 methylation (P < 0.001), MLH1 methylation (P < 0.001), and BRAF mutation (P < 0.005). All tumors with loss of p16 expression showed hypermethylation of p16 (21 of 21), 95.2% (20 of 21) showed MLH1 methylation, and 71.4% (15 of 21) were mutated for BRAF V600E. Mutational analysis showed pathogenic germline mutations in 8 of the patients, harboring 10 tumors. All 10 of these tumors showed normal staining of p16 in the immunochaemical analysis.

Conclusions: p16 immunohistochemistry is a good surrogate marker for p16 and MLH1 epigenetic silencing due to hypermethylation, and is useful as screening tool in the selection of patients for genetic testing in Lynch syndrome.

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Background

Lynch syndrome is an autosomal dominant disorder that accounts for ~ 3% to 4% of all colorectal cancers (CRC, ref. 1). Lynch syndrome is caused by germline mutations in the DNA mismatch repair genes, mainly MLH1, MSH2, MSH6, and PMS2 (1). Defects in this pathway lead to changes in the length of nucleotide repeat sequences, a phenomenon called microsatellite instability (MSI), which constitutes the molecular hallmark of Lynch syndrome (2). These tumors can also be identified by immunohistochemical loss of mismatch repair proteins (3, 4). The presence of MSI may be observed in up to 10% to 15% of sporadic CRC. In these cases, mismatch repair impairment is caused by epigenetic silencing of MLH1, due to MLH1 promoter methylation (5).

Because molecular characterization of Lynch syndrome was established, the identification of gene carriers has become a critical issue. Identification of patients with Lynch syndrome has important clinical implications because surveillance for CRC and other cancers in this population is able to reduce cancer mortality and is cost effective (6). A previous study from our group established that fulfillment of revised Bethesda criteria (7), followed by either MSI testing or mismatch repair proteins immunohistochemistry, is a sensible approach to pre-select patients for genetic testing (4). Patients having tumors...
Translational Relevance

The main contribution of this article is the use of p16 immunohistochemistry in the identification of patients with colorectal cancer and high level of suspicion of Lynch syndrome. Patients with tumors showing loss of MLH1 expression can be hereditary or sporadic. In this study, we show that p16 immunohistochemistry is a good surrogate marker for both p16 and MLH1 hypermethylation. Patients whose tumors have loss of both MLH1 and p16 expression have hypermethylated colorectal cancer and, therefore, their tumors are sporadic. These patients can be confidently excluded for genetic testing of MLH1. P16 immunohistochemistry is easy to perform and available for every pathology department, taking advantage over other more exigent techniques such as BRAF mutation.

with loss of expression of MSH2 or MSH6 are suspected carriers of germline mutations of any of these genes, but patients whose tumors show loss of MLH1 may either have hereditary or sporadic disease. The majority of sporadic tumors with loss of MLH1 expression belong to a group of CRCs that are hypermethylated at multiple genetic loci. These CRC have been described as displaying the CpG Island Methylator Phenotype (8, 9), and a panel of markers has been proposed for its diagnosis (10). One of the loci frequently methylated in these CpG island methylator phenotype tumors is CDKN2A (p16). Presumably, some of the tumors with loss of MLH1 caused by epigenetic silencing through aberrant methylation should also have a silenced p16 and, therefore, immunohistochemical loss of staining of this protein. The aim of our study is to evaluate the value of p16 immunohistochemistry in the prediction of MLH1 germline mutations in patients with tumors that show loss of MLH1.

Materials and Methods

Subjects. Immunohistochemical analysis of MLH1 was done in 2,401 CRC tumors. Tumor tissue was obtained from a series of 2,246 nonselected surgical CRC specimens from the EPICOLON study (n = 1,281; ref. 11) and from the Pathology Department of the Hospital General Universitario of Alicante, collected between the years 1999 to 2007 (n = 965). The remaining 155 tumors were obtained from patients of the Genetic Counselling Unit of the Hospital General Universitario of Elche. Demographic, clinical, and tumor-related characteristics of probands, as well as a detailed family history, were obtained using a pre-established questionnaire, as described elsewhere (4). Loss of MLH1 expression was found in 124 tumors (5.2%), from 120 patients. All these tumors showed normal expression of MSH2 and MSH6. In 32 cases, there was not enough tissue to perform immunohistochemical or molecular studies and they were excluded from this study. Finally, the study was done in 92 tumors from 88 patients that showed loss of MLH1 immunohistochemical expression. Eighty-three tumors were nonselected population-based CRC specimens and nine were from the Genetic Counselling Unit. Figure 1 shows a flow chart of the molecular analysis done on the samples.

Immunohistochemistry. Immunohistochemical analysis of MLH1, MSH2, MSH6, and PMS2 was done in blocks of formalin-fixed paraffin-embedded tumor tissue as previously described (4, 12). Immunohistochemical analysis of p16 expression was done on tissue microarray. One of the requirements for inclusion in the study was that enough tumor tissue was present within the block of wax-embedded tissue to facilitate subsequent tissue microarray construction. The representative tumor regions were identified and marked on the H&E-stained slides and subsequently identified on the corresponding tissue blocks. Tissue cylinders of diameter of 1 mm were punched out from the marked areas of each block and incorporated into a recipient paraffin block using a precision instrument—the tissue arrayer (Beecher Instruments). A total of six tissue microarrays were constructed for the study. Tissue microarrays contained between 30 and 50 cores of 1-mm needle size. For inclusion in the study, at least two evaluable cores of tumor tissue were required per case. Four-micrometer-thick sections were cut from tissue microarrays. The slides were put on a TechMate 500 immunostainer and incubated for 30 min at room temperature with the mouse monoclonal antibody JC2, which recognizes the first ankyrin repeat of p16 (provided by Dr. Jim Koh, Duke University, Durham, NC; ref. 13). The antibody was detected by the Envision-technique (Dako). Processed immunohistochemical slides were evaluated by two pathologists. A tumor was considered to have normal expression for p16 when unequivocal nuclear staining was seen in some neoplastic epithelial cells, with or without cytoplasmatic staining. Cases with loss of expression included those cases with lack of expression in tumor cells in presence of internal positive control (stromal cells or blood vessels). Samples were considered not scored when no staining of internal control was seen.

MLH1 and CDKN2A methylation analysis. Genomic DNA was extracted from tumor paraffin-embedded tissue blocks. Two tissue cylinders of 1 mm of diameter were punched out with the tissue arrayer from the previously selected tumor areas. QiaAmp DNA Mini kit for DNA extraction was used according to the manufacturer’s protocol after removal of paraffin by xylene.

The MLH1 and CDKN2A (p16) methylation analysis was done by real-time PCR assay Methylight as previously described (Applied Biosystems; ref. 14). Bisulfite conversion was made with the EZ DNA methylation-Gold kit as described by the manufacturer (Zymo Research). Quantitative PCR was done by ABI 7500 (Applied Biosystems). Primers and a probe, designed to detect bisulfite converted fully methylated MLH1 and p16 DNAs, have been described and used previously (10, 15–17). The PCR reactions were done according to the protocols (16, 18). To calculate the percentage of methylated reference, we established the dichotomization threshold at percentage of methylated reference of 4, to obtain a bimodal distribution in the MLH1 and CDKN2A methylation loci. Methylation-positive (percentage of methylated reference, >4) MLH1 and CDKN2A samples could be distinguished from negative (percentage of methylated reference, ≤4) ones.

BRAF V600E mutation. V600E BRAF mutation was detected using specific TaqMan probes by real-time PCR (ABI PRISM 7500; Applied Biosystems) and the allelic discrimination software (Applied Biosystems) as previously described by Benlloch et al. (19).

MLH1 germline genetic testing. Germline genetic alteration studies were done on genomic DNA isolated from peripheral blood leukocytes or from nontumor colon tissue as previously described (4). Point mutation analysis of MLH1 gene was done by PCR amplification and direct sequencing of the entire coding region and the exon-intron boundaries. PCR primers and conditions have been described elsewhere (20–22). Large genomic rearrangements (insertions and/or deletions) in MLH1 loci were screened by multiplex ligation-dependent probe amplification according to the manufacturer protocols (Salsa multiplex ligation-dependent probe amplification kit P003 and P008; MRC-Holland).

Data management and analysis. Data were collected and entered into the computer using MICROSOFT ACCESS software for storage and initial analysis. Further analysis was done using SPSS software (SPSS 15.0). For continuous variables, relevant measures of central tendency (means for normally distributed data and medians and interquartile ranges for skewed data) were used to explore data. The χ² test was used for comparison of qualitative variables. A Student’s t test was used for comparison of normally distributed continuous variables and a Mann-Whitney U test was used for unpaired comparison of
nonnormally distributed continuous variables. A P value of <0.05 was considered significant.

Results

p16 immunohistochemistry was done in 92 tumors with loss of MLH1 expression (Fig. 2) from 88 patients. In 13 of the tumors, p16 immunohistochemistry could not be confidently assessed and was classified as not scored, due to absence of clear staining in stromal cells, which served as internal positive controls. Loss of p16 expression was seen in 21 of 79 samples (26.6%; Fig. 2). Characteristics of tumors according to p16 expression status can be seen in Table 1. There was a statistically significant association between p16 expression and p16 methylation ($P < 0.001$), MLH1 methylation ($P < 0.001$), and BRAF mutation ($P < 0.005$). All tumors with loss of p16 expression showed hypermethylation of p16 (21 of 21), 95.2% (20 of 21) showed MLH1 methylation, and 71.4% (15 of 21) were mutated for BRAF V600E (Table 1). However, 20 of 41 (50%) of the tumors with p16 methylation retained p16 expression (Table 1). Tumors with loss of p16 expression showed more frequently poor differentiation. p16 immunohistochemistry was also done in 60 sporadic tumors with normal expression of MLH1 and microsatellite stability, loss of p16 expression was seen in only 5 of these tumors (3%).

Mutational analysis of MLH1 was done in 52 of 88 patients whose tumors showed loss of MLH1 staining. Fifty-four CRC from these 52 patients were analyzed. Thirty of these patients fulfilled some of the revised Bethesda criteria, and 11 fulfilled Amsterdam II criteria. Mutational analysis showed pathogenic mutations in 8 of the patients, harboring 10 tumors. All 10 of the tumors analyzed from the 8 patients with germline pathogenic mutations in MLH1 showed normal staining of p16. All patients with germline mutations met Bethesda criteria. Moreover, all tumors from patients with germline mutations showed non-mutated BRAF and nonmethylated MLH1 (Table 2).

Table 3 shows values of sensitivity, specificity, positive and negative predictive value, and positive Likelihood ratio for Bethesda criteria, BRAF mutation, MLH1 methylation, and p16 immunohistochemistry. Different combinations of these variables for the prediction of germline MLH1 mutation can also be seen in Table 3. Values for p16 immunohistochemistry and BRAF mutation are similar, and combination of these techniques improves separately the obtained results.
Discussion

Selection of patients for genetic testing in Lynch syndrome is frequently difficult in clinical practice. The use of Amsterdam criteria is capable to detect Lynch Syndrome with a high specificity but with very low sensitivity. When clinical presentation and family history are most compelling, the yield of mutational testing is often no better than 50%, and even in the best case-scenario, when Amsterdam criteria are met and a tumor shows high MSI and loss of mismatch repair protein expression, the likelihood of germline mutation detection is ~70% to 80% (23). Other strategies, such as the revised Bethesda criteria (7), improve the sensitivity but with a high lack of specificity. With this approach, a high number of patients are sent for genetic testing based only on clinical criteria, with the subsequent expending of resources and the consequent generation of anxiety to patients and their families.

Several approaches have been used for refining the selection of patients for genetic testing. The observation that patients with Lynch syndrome show a characteristic phenotype with MSI prompted to use these markers as a first prescreening modality.

Then, the demonstration of the role of the immunohistochemistry and its equivalence to MSI analysis in the diagnostic algorithm of Lynch syndrome (4) improved the availability of these tools and its generalization in clinical practice, due to the possibility of performing immunohistochemistry in any pathology department. Moreover, patients with tumors showing MSH2 or MSH6 lack of expression should be directly sent for genetic testing because it is a strong indicator for mutation in these genes (23). However, this clinical-molecular strategy has had some detractors because a number of patients with Lynch syndrome might not fulfill revised Bethesda criteria (24). Sometimes family history is difficult or even impossible to obtain. Furthermore, recent studies show that, even among patients with a known high risk for Lynch syndrome, there is a marked under utilization of MSI analysis (25). For these reasons, some authors advocate for routine molecular screening of patients with CRC for Lynch syndrome using immunohistochemistry (24). Another fact that can support routine immunohistochemical study of CRC is the recognized better prognosis of mismatch repair deficient tumors (26), and the different response to 5-fluorouracil–based chemotherapy that

Fig. 2. p16 immunohistochemistry. A and C positive cases with nuclear and cytoplasmatic staining of tumor cells. B and D negative cases with stromal staining as an internal control.
these tumors have (27–29). In our study, we included only patients with MLH1 loss of expression, and compared molecular only with clinical-molecular approaches for diagnosis, showing that combinations of only molecular tests are at least as good as strategies that include clinical data (Table 3). Our results show that p16 immunohistochemistry can improve the results of this strategy, avoiding germline genetic testing in approximately a third of patients with loss of MLH1 expression.

Table 1. Characteristics of tumors regarding p16 expression

<table>
<thead>
<tr>
<th></th>
<th>P16 normal expression (n = 58)</th>
<th>P16 loss of expression (n = 21)</th>
<th>Total (n = 79)</th>
<th>Odds ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (y)</td>
<td>Me 73 P25-P75: 52-80</td>
<td>Me 77 P25-P75: 69-79</td>
<td>Me 75 P25-P75: 50-80</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis &lt;50 y</td>
<td>14 (24.1)</td>
<td>2 (9.5)</td>
<td>16 (20.3)</td>
<td>3.0 (0.6-14.6)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Age at diagnosis &gt;50 y</td>
<td>44 (75.9)</td>
<td>19 (90.5)</td>
<td>63 (79.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male 27 (46.6)</td>
<td>8 (38.1)</td>
<td>35 (44.3)</td>
<td>1.4 (0.5-3.9)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Female 31 (53.4)</td>
<td>13 (61.9)</td>
<td>44 (55.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Revised Bethesda guidelines</td>
<td>Fulfilling 31 (53.4)</td>
<td>5 (23.8)</td>
<td>36 (45.6)</td>
<td>3.7 (1.2-11.4)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Not fulfilling 27 (46.6)</td>
<td>16 (76.2)</td>
<td>43 (54.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amsterdam II criteria</td>
<td>Fulfil 11 (19.0)</td>
<td>1 (4.8)</td>
<td>12 (15.2)</td>
<td>4.7 (0.6-38.7)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>No fulfil 47 (81.0)</td>
<td>20 (95.2)</td>
<td>67 (84.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td>Right sided 47 (81.0)</td>
<td>15 (71.4)</td>
<td>62 (78.5)</td>
<td>1.8 (0.6-5.8)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Left sided 11 (19.0)</td>
<td>6 (28.6)</td>
<td>17 (21.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>Poorly differentiated 37 (63.8)</td>
<td>9 (42.9)</td>
<td>46 (58.2)</td>
<td>2.3 (0.9-6.5)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Other 21 (36.2)</td>
<td>12 (57.1)</td>
<td>33 (41.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylation p16</td>
<td>Not methylated 38 (65.5)</td>
<td>0 (0)</td>
<td>38 (48.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methylated 20 (34.5)</td>
<td>21 (100)</td>
<td>41 (51.9)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BRAF V600E mutation</td>
<td>Not mutated 41 (70.7)</td>
<td>6 (28.6)</td>
<td>47 (59.5)</td>
<td>5.6 (1.9-17.1)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>Methylated 17 (29.3)</td>
<td>15 (71.4)</td>
<td>32 (40.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylation MLH1</td>
<td>Not methylated 28 (48.3)</td>
<td>1 (4.8)</td>
<td>29 (36.7)</td>
<td>18.7 (2.3-148.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Methylated 30 (51.7)</td>
<td>20 (95.2)</td>
<td>50 (63.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Odds ratio of p16 cannot be calculated.
Abbreviation: Me, median.

Table 2. Characteristics of tumors in patients with germline mutation

<table>
<thead>
<tr>
<th>Germline mutation</th>
<th>Yes (tumors n = 10; patients n = 8)</th>
<th>No (tumors n = 44; patients n = 44)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC p16</td>
<td>Normal expression 10 (100)</td>
<td>30 (69.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Loss of expression 0 (0)</td>
<td>14 (30.2)</td>
<td></td>
</tr>
<tr>
<td>Methylation p16</td>
<td>Not methylated 8 (80)</td>
<td>20 (46.5)</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Methylated 2 (20)</td>
<td>24 (53.5)</td>
<td></td>
</tr>
<tr>
<td>Methylation MLH1</td>
<td>Not methylated 10 (100)</td>
<td>9 (20.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Methylated 0 (0)</td>
<td>35 (79.1)</td>
<td></td>
</tr>
<tr>
<td>BRAF V600E mutation</td>
<td>Not mutated 10 (100)</td>
<td>25 (55.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Mutated 0 (0)</td>
<td>19 (44.2)</td>
<td></td>
</tr>
<tr>
<td>Bethesda guidelines</td>
<td>Fulfilling 10 (100)</td>
<td>21 (46.5)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>No fulfilling 0 (0)</td>
<td>23 (53.5)</td>
<td></td>
</tr>
<tr>
<td>Amsterdam II criteria</td>
<td>Fulfilling 7 (70)</td>
<td>4 (9.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>No fulfilling 3 (30)</td>
<td>40 (90.7)</td>
<td></td>
</tr>
</tbody>
</table>
Instruments for the refinement of the selection of patients with loss of MLH1 for genetic testing have been proposed. Mutation V600E in the oncogene BRAF has been suggested as characteristic of sporadic colorectal tumors with MSI, and this mutation is not detected in tumors from patients with germline mutations in MLH1 or MSH2 genes (30, 31). Several studies have shown that detection of BRAF V600E mutation could simplify the selection of CRC patients for genetic testing for Lynch syndrome (32, 33). However, the use of BRAF mutational analysis in clinical practice has been limited, probably due to the need of molecular biology resources for its implementation. The main strength of p16 immunohistochemistry for clinical use in selection of suspected Lynch syndrome patients for genetic testing is its feasibility, in contraposition to other methylation markers such as V600E BRAF mutation or MLH1 methylation (34), which are time consuming and not available for the majority of clinical centers.

Aberrant promoter hypermethylation associated with transcriptional silencing of multiple tumor suppressor genes has been proposed as a mechanistic component in the evolution of multiple cancers (35). Tumors with a critical degree of aberrant methylation have the CpG island methylator phenotype. CpG island methylator phenotype tumors show promoter hypermethylation in multiple genes, including p16, p14, MGMT, and MLH1 among others. Loss of the INK4a/ARF/INK4b locus is among the most frequent cytogenetic events in human cancer. The products of this locus p15INK4a, p16INK4b, and ADP ribosylation factor play widespread and independent roles in tumor suppression (36). Specific somatic loss of p16, through point mutation or small deletion, has been reported in human cancer (37), but epigenetic silencing through aberrant promoter methylation is the most common mechanism of inactivation (36, 38). p16 loss of expression provokes increase in proliferation and vascularization in colon cancer cells (13, 39).

Limitations of our study are the small number of patients with MLH1 germline mutations that we included. However, the excellent sensitivity of p16 expression for MLH1 methylation, with virtually all the cases with loss of p16 expression being methylated, makes p16 immunohistochemistry a robust marker for this event. Most samples include abundant stromal components that stain for p16, providing an internal positive control to verify adequate tissue preservation and technical success of the staining. Another limitation is the existence of cases with p16 hypermethylation that showed normal p16 staining. This fact may be caused, at least in part, by the target region analyzed for the p16 methylation. The Methylight system (primers and probe) used here has been described elsewhere, being useful to characterize the CpG island methylator phenotype (18). The amplicon sequence analyzed is located at exon 1a. Using in vitro models, Gonzalgo et al. (40) observed that p16 expression could occur in the presence of a relatively heavily methylated coding domain (exon 1a, named as region D). Methylation of certain regions upstream of the p16 exon 1a may be more critical for transcription activity (particularly region C). Exonic CpG islands are more susceptible to de novo methylation than promoter islands. The cancer-specific promoter methylation might be a result of spreading from exonic foci and selection of cells whose growth is deregulated by the gene inactivation (41).

In conclusion, our results suggest that the immunohistochemical study of p16 could improve the selection of patients for genetic testing of germline mutations in MLH1. Patients with CRC and MLH1 loss of expression, whose tumors also show loss of p16 expression can be reasonably excluded for genetic testing because this loss of expression indicates, with high possibility, aberrant hypermethylation and epigenetic silencing of both p16 and MLH1 genes.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References
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