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UTILITY OF P16 IMMUNOHISTOCHEMISTRY FOR THE IDENTIFICATION OF LYNCH SYNDROME

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Abstract

Immunohistochemistry for mismatch repair proteins has shown utility in the identification of Lynch syndrome, but majority of tumours with loss MLH1 expression are due to sporadic hypermethylation of the *MLH1* promoter. These tumours 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.

METHODS: 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 tumour 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 out 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 tumours with loss of p16 expression showed hypermethylation of *p16* (21/21), 95.2% (20/21) showed *MLH1* methylation and 71.4% (15/21) were mutated for *BRAF* V600E Mutational analysis showed pathogenic germline mutations

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in 8 of the patients, harbouring 10 tumours. All 10 of these tumours showed normal staining of p16 in the immunochemical 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.

Keywords

colorectal cancer; Lynch syndrome; p16; immunohistochemistry; diagnosis

BACKGROUND

Lynch syndrome is an autosomal dominant disorder that accounts for approximately 3-4% of all colorectal cancers (CRC) (1). Lynch syndrome is caused by germline mutations in the DNA mismatch repair (MMR) 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 HNPCC (2). These tumours can also be identified by immunohistochemical loss of MMR proteins (3;4). The presence of MSI may be observed in up to 10-15% of sporadic CRC. In these cases, MMR impairment is caused by epigenetic silencing of MLH1, due to MLH1 promoter methylation (5).

Since 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 fulfilment of revised Bethesda criteria (7), followed by either MSI testing or MMR proteins immunohistochemistry is a sensible approach to pre-select patients for genetic testing (4). Patients having tumours with loss of expression of MSH2 or MSH6 are suspected carriers of germline mutations of any of these genes, but patients whose tumours show loss of MLH1 may either have hereditary or sporadic disease. The majority of sporadic tumours with loss of MLH1 expression belong to a group of colorectal cancers that are hypermethylated at multiple genetic loci. These CRC have been described as displaying the CpG Island Methylator Phenotype (CIMP) (8;9), and a panel of markers has been proposed for its diagnosis (10). One of the loci frequently methylated in these CIMP tumours is *CDKN2A* (*p16*). Presumably, some of the tumours 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 tumours that show loss of MLH1.

METHODS

Subjects

Immunohistochemical analysis of MLH1 was performed in 2401 CRC tumours. Tumour tissue was collected from a series of 2,246 non-selected surgical CRC specimens from the EPICOLON study (n=1.281) (11) and from the Pathology Department of the Hospital General Universitario of Alicante, collected between the years 1999-2007 (n=965). The remaining 155 tumours were collected from patients of the Genetic Counselling in Cancer Department 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 tumours 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 performed in 92 tumours from 88 patients that showed loss of MLH1 immunohistochemical expression. Eighty-three tumours were non selected population-based CRC specimens and 9 were from the Genetic Counselling Unit. Figure 1 shows a flow chart of the molecular analysis performed on the samples.

Immunohistochemistry

Immunohistochemical analysis of MLH1, MSH2, MSH6 and PMS2 was performed in blocks of formalin-fixed paraffin-embedded tumour tissue as previously described (4;12).

Immunohistochemical analysis of p16 expression was performed on tissue microarray (TMA). One of the requirements for inclusion in the study was that enough tumour tissue was present within the block of wax-embedded tissue to facilitate subsequent TMA construction. The representative tumour 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, Durviz). A total of 6 TMAs were constructed for the study. TMAs contained between 30 and 50 cores of 1 mm needle size. For inclusion in the study, at least two evaluable cores of tumour tissue were required per case. Four-micrometer-thick sections were cut from TMAs. The slides were put on a TechMate 500 immunostainer and incubated for 30 minutes 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, USA) (13). The antibody was detected by the Envision+ technique (Dako). Processed immunohistochemical slides were evaluated by two pathologists. A tumour 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 tumour 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 tumour paraffin-embedded tissue blocks. Two tissue cylinders of 1 mm of diameter were punched out with the tissue arrayer from the previously selected tumour 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 performed by real time PCR assay Methylight as previously described (Applied Biosystems, Foster City, CA, USA) (14). Bisulphite conversion was made with the EZ DNA methylation-Gold kit as described by the manufacturer (Zymo Research, Orange, CA, USA). Quantitative PCR was performed by ABI 7500 (Applied Biosystems, Foster City, CA, USA). Primers and a probe, designed to detect bisulphite converted fully methylated MLH1 and p16 DNAs, have been described and used previously (10;15-17). The PCR reactions were performed according to the protocols (16; 18).

In order to calculate the percentage of methylated reference (PMR) we established the dichotomization threshold at PMR= 4, to obtain a bimodal distribution in the MLH1 and CDKN2A methylation loci. Methylation positive (PMR > 4) MLH1 and CDKN2A samples could be distinguished from negative (PMR ≤ 4) ones.

BRAF V600E Mutation

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

MLH1 germline genetic testing

Germline genetic alteration studies were performed on genomic DNA isolated from peripheral blood leukocytes or from non-tumour colon tissue as previously described (4). Point mutation analysis of *MLH1* gene was done by polymerase chain reaction (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 (MLPA) according to the manufacturer protocols (Salsa MLPA Kit P003 and P008; MRC-Holland, Amsterdam, The Netherlands).

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 Chi^2 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 non-normally distributed continuous variables. A p value of less than 0.05 was considered significant.

RESULTS

p16 immunohistochemistry was performed in 92 tumours with loss of MLH1 expression (Figure 2) from 88 patients. In 13 of the tumours, 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 out of 79 samples (26,6%) (Fig.2). Characteristics of tumours 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 (<0.005). All tumours with loss of p16 expression showed hypermethylation of p16 (21/21), 95.2% (20/21) showed MLH1 methylation and 71.4% (15/21) were mutated for BRAF V600E (table 1). However, 20 out of 41 (50%) of the tumours with p16 methylation retained p16 expression (Table 1). Tumours with loss of p16 expression showed more frequently poor differentiation. p16 immunohistochemistry was also performed in 60 sporadic tumours with normal expression of MLH1 and microsatellite stability, loss of p16 expression was seen in only 5 of these tumours (3%).

Mutational analysis of MLH1 was performed in 52 out of 88 patients whose tumours 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, harbouring 10 tumours. All 10 of the tumours 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 tumours from patients with germline mutations showed non-mutated BRAF and non-methylated 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, 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 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 tumour shows high MSI and loss of MMR protein expression, the likelihood of germline mutation detection is approximately 70-80% (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 microsatellite instability prompted to use these markers as a first pre-screening modality. Then, the demonstration of the role of the immunohistochemistry and its equivalence to microsatellite instability 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 tumours 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 have had some detractors, because a number of patients with Lynch syndrome might not fulfil 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 underutilization 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 tumours (26), and the different response to 5-fluorouracil based chemotherapy that these tumours 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.

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 tumours with MSI and this mutation is not detected in tumours from patients with germline mutations in MLH1 or MSH2 genes (30;31). Several studies have demonstrated 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), that are time consuming and not available for the majority of clinical

centresAberrant promoter hypermethylation associated with transcriptional silencing of multiple tumour suppressor genes has been proposed as a mechanistic component in the evolution of multiple cancers (35). Tumours with a critical degree of aberrant methylation have the CpG island methylator phenotype (CIMP). CIMP tumours 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 $p15^{INK4a}$, $p16^{INK4b}$ and ARF play widespread and independent roles in tumour 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 vascularisation 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 1α. Using *in vitro* models, Gonzalgo et al (1998) observed that p16 expression could occur in the presence of a relatively heavily methylated coding domain (exon 1α, named as region D). Methylation of certain regions upstream of the *p16* exon 1α may be more critical for transcription activity (particularly region C) (40). 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 tumours 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.

STATEMENT OF TRANSLATIONAL RELEVANCE

The main contribution of this manuscript is the utility of p16 immunohistochemistry in the identification of patients with colorectal cancer and high level of suspicion of Lynch syndrome. Patients with tumours showing loss of MLH1 expression can be hereditary or sporadic. In this study we demonstrate that p16 immunohistochemistry is a good surrogate marker for both p16 and MLH1 hypermethylation. Patients whose tumours have loss of both MLH1 and p16 expression have hypermethylated colorectal cancer and, therefore, their tumours 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.

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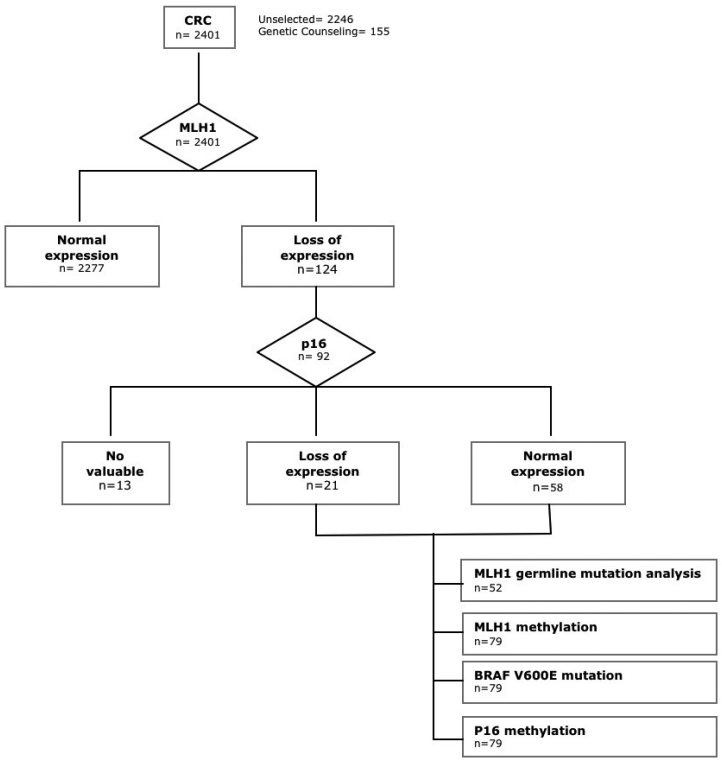
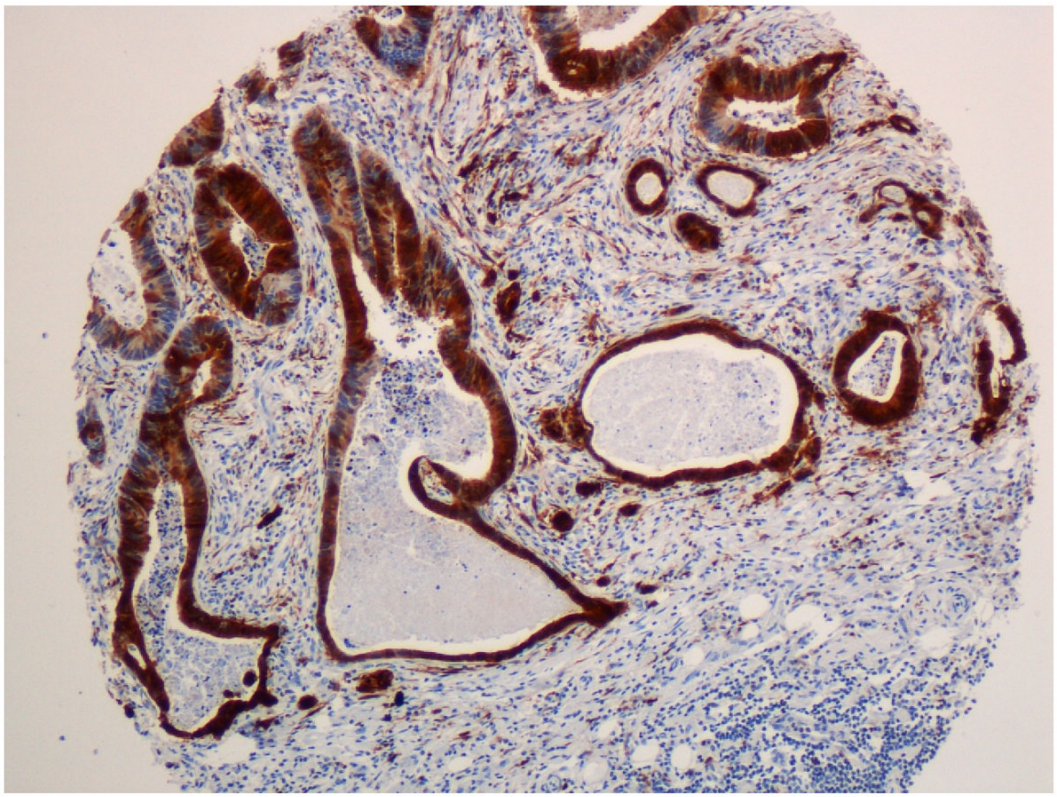
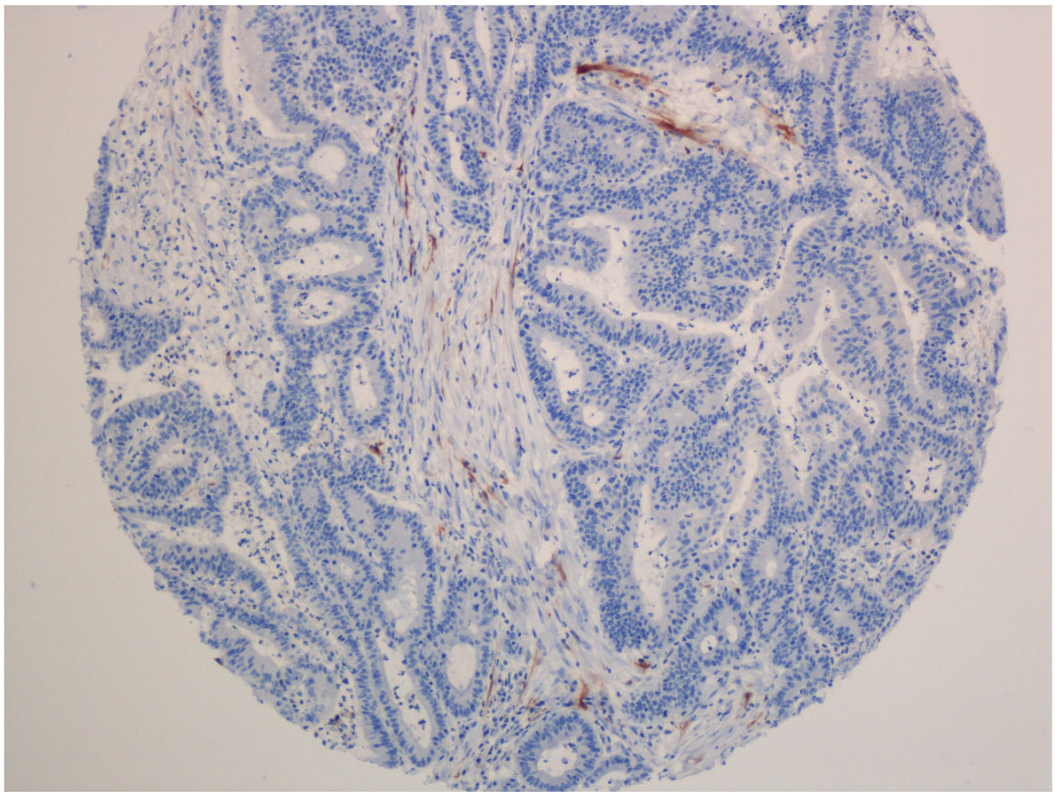
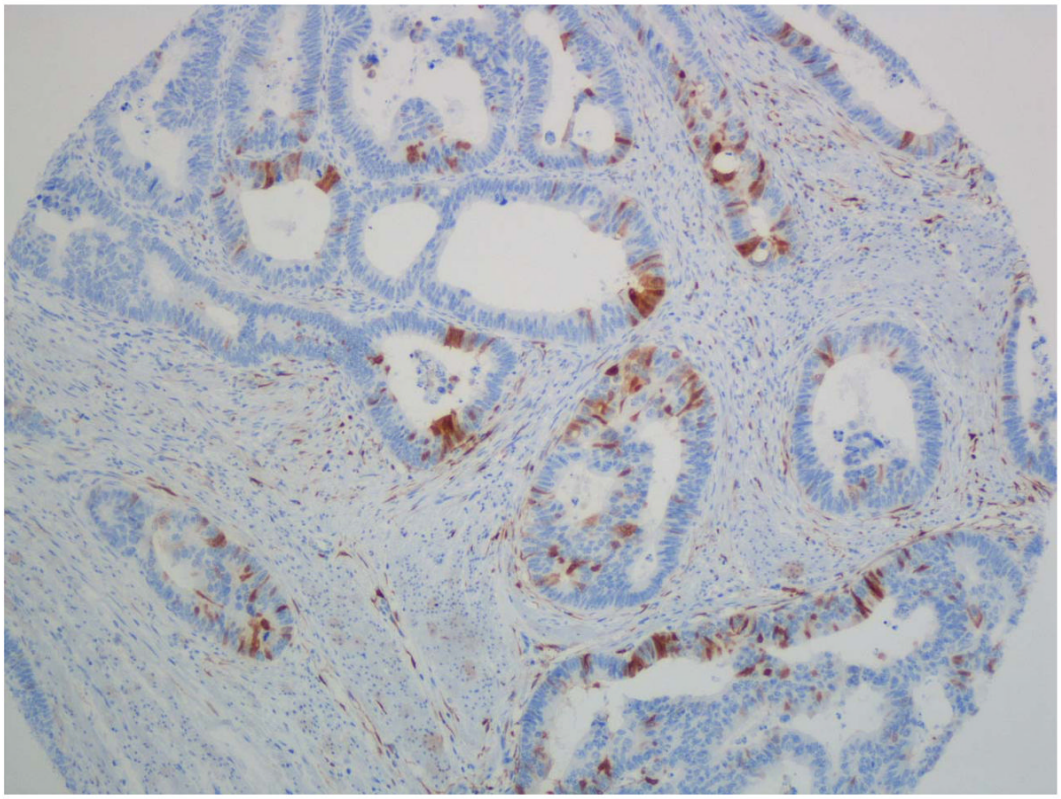


Figure 1.
Flow diagram for the immunohistochemical and molecular analysis performed in tumors.







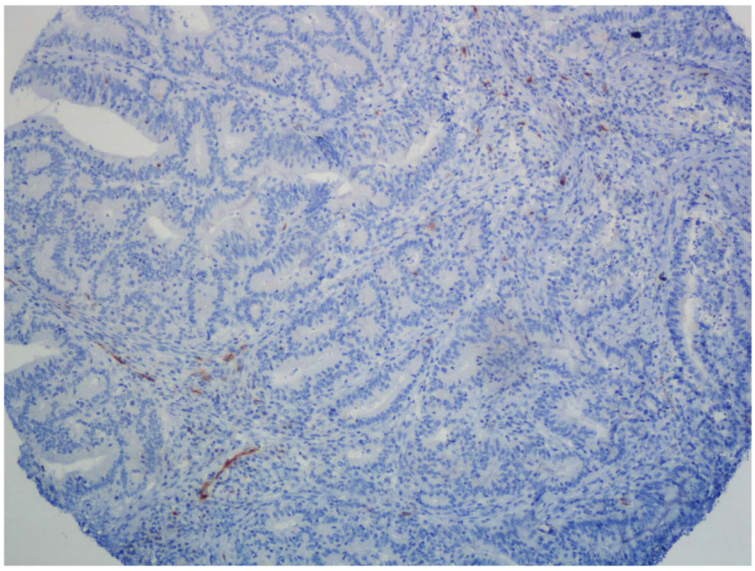


Figure 2.
p16 immunohistochemistry. **A-C.** Positive cases with nuclear and cytoplasmatic staining of tumour cells. **B-D.** Negative cases with stromal staining as a internal control

Table 1

Characteristics of tumors regarding p16 expression

	P16 normal expression (n=58)		P16 loss of expression (n=21)		TOTAL (n=79)		Odds ratio	p
	me	**P_{25}-P_{75}**	**Me**	**P_{25}-P_{75}**	**me**	**P_{25}-P_{75}**		
Age at diagnosis	73	52-80	77	69-79	75	50-80		*N.S.*
	n	%	N	%	n	%		
Age at diagnosis								
<50 years	14	*24.1*	2	*9.5*	16	*20.3*	*3.0 (0.6-14.6)*	*N.S.*
>50 years	44	*75.9*	19	*90.5*	63	*79.7*		
Sex								
Male	27	*46.6*	8	*38.1*	35	*44.3*	*1.4 (0.5-3.9)*	*N.S.*
Female	31	*53.4*	13	*61.9*	44	*55.7*		
Revised Bethesda guidelines								
Fulfilling	31	*53.4*	5	*23.8*	36	*45.6*	*3.7 (1.2-11.4)*	*<0.05*
Not fulfilling	27	*46.6*	16	*76.2*	43	*54.4*		
Amsterdam II criteria								
Fulfil	11	*19.0*	1	*4.8*	12	*15.2*	*4.7 (0.6-38.7)*	*N.S.*
No fulfil	47	*81.0*	20	*95.2*	67	*84.8*		
Tumour location								
Right-sided	47	*81.0*	15	*71.4*	62	*78.5*	*1.8 (0.6-5.8)*	*N.S.*
Left-sided	11	*19.0*	6	*28.6*	17	*21.5*		
Grade								
Poorly differenciated	37	*63.8*	9	*42.9*	46	*58.2*	*2.3 (0.9-6.5)*	*N.S.*
Other	21	*36.2*	12	*57.1*	33	*41.8*		
Methylation p16								

	P16 normal expression (n=58)		P16 loss of expression (n=21)		TOTAL (n=79)		Odds ratio	p
	me	**P_{25}-P_{75}**	**Me**	**P_{25}-P_{75}**	**me**	**P_{25}-P_{75}**		
Not methylated	38	*65.5*	0	*0*	38	*48.1*	-	*<0.001*
Methylated	20	*34.5*	21	*100*	41	*51.9*		
BRAF V600E mutation								
Not mutated	41	*70.7*	6	*28.6*	47	*59.5*	*5.6 (1.9-17.1)*	*<0.005*
Mutated	17	*29.3*	15	*71.4*	32	*40.5*		
Methylation MLH1								
Not methylated	28	*48.3*	1	*4.8*	29	*36.7*	*18.7 (2.3-148.4)*	*<0.001*
Methylated	30	*51.7*	20	*95.2*	50	*63.3*		

Me: median;

- Odds ratio of p16 cannot be calculated.

Table 2

Characteristics of tumours in patients with germline mutation.

Germline mutation	Yes (tumours n=10) (patients n=8)		No (tumours n=44) (patients n=44)		
	n	**%**	**n**	**%**	**P**
IHC p16					
Normal expression	10	*100*	30	*69.8*	*<0.05*
Loss of expression	0	*0*	14	*30.2*	
Methylation p16					
Not methylated	8	*80*	20	*46.5*	*0.056*
Methylated	2	*20*	24	*53.5*	
Methylation MLH1					
Not methylated	10	*100*	9	*20.9*	*<0.001*
Methylated	0	*0*	35	*79.1*	
BRAF V600E mutation					
Not mutated	10	*100*	25	*55.8*	*<0.01*
Mutated	0	*0*	19	*44.2*	
Bethesda guidelines					
Fulfilling	10	*100*	21	*46.5*	*<0.005*
No fulfilling	0	*0*	23	*53.5*	
Ámsterdam II criteria					
Fulfilling	7	*70*	4	*9.3*	*<0.001*
No fulfilling	3	*30*	40	*90.7*	

Me: median

Table 3

Values of different strategies for detecting germline mutation in tumours with MLH1 loss of expression.

	GERMLINE MUTATION					
Strategies without clinical information	**Sen**	**Spe**	**PPV**	**NPV**	**+LR**	**NNT**
p16 IHC	100	30.2	25	100	1.4	3.1
BRAF mutation	100	44.2	29.4	100	1.8	2.3
p16 IHC + BRAF mutation	100	51.2	32.3	100	2.0	1.9
MLH1 methylation	100	79.1	52.6	100	4.8	1.4
BRAF mutation + MLH1 methylation	100	79.1	52.6	100	4.8	1.3
p16 IHC + MLH1 methylation	100	79.1	52.6	100	4.8	1.2
p16 IHC + BRAF mutation + MLH1 methylation	100	79.1	52.6	100	4.8	1.2
Strategies needing clinical information						
BETHESDA	100	53.5	33.3	100	2.2	1.9
p16 IHC + BETHESDA	100	62,8	38,5	100	2.7	1.6
BRAF mutation + BETHESDA	100	67.4	41.7	100	3.1	1.5
p16 IHC + BRAF mutation + BETHESDA	100	69.8	43.5	100	3.3	1.4
BRAF mutation + BETHESDA + MLH1 methylation	100	83.7	58.8	100	6.1	1.2
BETHESDA + MLH1 methylation	100	83.7	58.8	100	6.1	1.2
p16 IHC + BRAF mutation + BETHESDA + MLH1 methylation	100	83.7	58.8	100	6.1	1.2

Sen: Sensitivity, **Spe:** Specificity, **PPV:** Positive Predictive Value, **NPV:** Negative Predictive Value, **+LR:** Positive Likelihood Ratio, **NNT**: Number Needed to Test for detecting one germline mutation