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ORIGINAL ARTICLE

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Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression

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Abstract Tissue microarray technology enables the analysis of hundreds of specimens by arranging numerous 0.6-mm tissue core biopsy specimens into a single paraffin block. Validation studies are necessary to evaluate the representativeness of small disks taken from the original tissue. We validated the tissue microarray technology in colorectal carcinoma by analyzing the immunohistochemical expression of proteins involved in the two main pathways of colorectal carcinogenesis: p53 protein for loss of heterozygosity tumors, hMLH1 and hMSH2 proteins for microsatellite instability (MSI) tumors. We compared in 30 colorectal carcinomas (15 MSI^- and 15 MSI^+), 8 microarrays disks, and the whole section of the block from which they were derived. Tumoral tissue was present in 95.7% of the microarray disks. The analysis of three disks per case was comparable to the analysis of the whole section in 99.6% (p53), 98.8% (hMLH1), and 99.2% (hMSH2) of cases. In the second part we applied the tissue microarray technology to 263 consecutive cases of colorectal carcinoma, sampled by three cores. We showed that 48.5% overexpressed p53 and 8.7% lost hMLH1 or hMSH2. Tissue microarray technology, validated in colorectal carcinoma, appears as a useful research tool for rapid analysis of the clinical interest of molecular alterations.

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Introduction

Genome screening tools, such as cDNA microarrays, reveal different levels of expression of numerous genes within normal and pathological tissues. These potential markers need to be tested in hundreds of human specimens to confirm their clinical interest in diagnostic, prognostic or therapeutic fields. This is rapidly achieved by the tissue microarray (TMA), a high-throughput technique described in 1998 by Kononen et al. [21]. Studies combining cDNA microarray and TMA identify new markers with great efficiency [27, 30]. The TMA technique consists of arraying, in a paraffin block, up to 1,000 samples removed from selected areas of donor blocks. Parallel studies of morphology by standard histological staining, nucleic acids by in situ hybridization, and proteins by immunohistochemistry (IHC) can be carried out on serial sections of the array block. The limit of this technique may be the lack of representativeness of heterogeneous tissues by small disks (0.6 mm in diameter in most studies). Validation studies are necessary to compare microarrays disks and standard whole sections, and establish the number of cores required to adequately represent the expression of a marker. We evaluated the TMA in colorectal carcinoma (CRC) by the IHC pattern of three common markers of the two pathways of colorectal carcinogenesis [8, 22]. The loss of heterozygosity (LOH) pathway responsible for 85% of sporadic CRC and the familial adenomatous polyposis (FAP) [14] is characterized by losses of chromosomal segments bearing tumor suppressor genes. Mutation of the second allele leads to the inactivation of these genes, in the early stage of the tumor growth as *APC* (adenomatous polyposis coli) gene or later as *p53* gene. Most mutations in the *p53* gene result in a p53 protein with a long half-life and an ensuing nuclear accumulation detectable on IHC [17].

Immunohistochemistry can also be used in LOH tumors to show abnormal nuclear expression of β-catenin secondary to APC or β-catenin gene mutation [18]. However, a high rate of β-catenin nuclear expression has also been reported by some authors in tumors showing microsatellite instability (MSI) [11, 26]. The latter phenomenon of MSI is present in 15% of sporadic CRC and more than 90% of hereditary nonpolyposis colorectal cancers (HNPCC) [14]. This phenotype is due to a defective mismatch repair (MMR) system of which one gene, in most cases *hMLH1* or *hMSH2*, shows biallelic inactivation [20]. The diagnosis of MSI status is carried out by polymerase chain reaction (PCR) in accordance with the National Cancer Institute recommendations [4] but can also be effected on IHC by showing loss of expression of hMLH1 or hMSH2 proteins by tumoral cells [24].

After validation of the TMA in 30 CRC we applied this technique to 263 consecutive cases of CRC, by analyzing the IHC pattern of p53, hMLH1, and hMSH2 proteins.

Material and methods

Tumor samples

Cases of colorectal adenocarcinomas were retrieved retrospectively from the files of the Department of Pathology of the Hospital Saint Antoine (Paris, France). The specimens had been formalin-fixed, paraffin-embedded and the diagnosis made on hematoxylin and eosin (HE) sections. For the validation study we selected 30 CRC received between 1995 and 2000 and which were tested previously for MSI by PCR [31]. Fifteen CRC were classified MSI^+ in accordance with the National Cancer Institute recommendations [4]. Nine of these were associated with the HNPCC syndrome, and six were sporadic. Fifteen CRC were stable in PCR and classified MSI^-; nine were developed on FAP, and six were sporadic. In the second part of the study we included 263 consecutive cases of CRC received in our department in 2001.

Tissue microarray technique

A map of the receiver block was prepared with coordinates for each sample to correctly identify the tumors. Under a microscope areas of interest and nonnecrotic and rich in tumoral glands were marked out accurately with an indelible pen, on the HE whole section of each donor block. The tissue microarrayer, a custom-made precision instrument (Beecher Instruments, Woodland, USA) was used: cores of 0.6 mm diameter were punched from the donor blocks and positioned in a recipient paraffin array block, in smaller holes, of 0.4 mm for best adhesion of the samples to the array block. The cores were arranged 4 mm from the edges of the array block in an asymmetric way. The array blocks were then incubated 30 min at 37°C to improve adhesion between cores and paraffin of the recipient block. They were cut at room temperature with a standard microtome (Microm, Heidelberg, Germany).

Description of the array blocks of the study

For the validation study two blocks of 124 cores were made: each CRC was punched eight times. In ten cases it was possible to sample the edge (four cores) and the center (four cores) of the tumor. Four cores from a CRC already known as positive for p53, hMLH1, and hMSH2 were added as positive controls for IHC in each array block. Block I represented the 15 MSI^- CRC and block II the 15 MSI^+ CRC. In the second part of the study 263 CRC punched three times in accordance with the results of the validation study were allocated in three array blocks of 273 (block A) and 276 cores (blocks B and C). Twelve samples of normal colorectal mucosa were added in each block.

Immunohistochemistry

Sections of 5 µm from the five tissue arrays and 30 standard blocks were cut onto silane-treated Super Frost slides (CML, Nemours, France) and left to dry at 37°C overnight. The slides were deparaffinized in xylene and rehydrated in pure ethanol. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 30 min. The slides were then placed in a microwave oven in citrate buffer (pH 6.0), for antigen retrieval, for 15 min at 750 W and 15 min at 150 W. All slides were processed using an automatic immunostainer (Optimax Plus 1.5, Biogenex, San Ramon, USA) with a supersensitive biotin-streptavidin-peroxidase technique (Super Sensitive Detection Kit, Biogenex). Mouse anti-human monoclonal antibodies to p53 (dilution 1:800, clone BP53-1, Biogenex), hMLH1 (dilution 1:100, clone G168-728, Pharmingen, San Diego Calif., USA) and hMSH2 (dilution 1:125, clone FE11, Calbiochem, Oncogene Research Products, Cambridge Mass., USA) were used. The slides were developed with amino-ethyl-carbazol, a red chromogen, and counterstained with Mayer's hemalun. Nuclear staining was interpreted for each antibody. A core was classified p53 positive when more than 50% of neoplastic cells showed a nuclear staining [3, 32]. For hMLH1 and hMSH2, a core was considered negative when there was a complete absence of nuclear staining of neoplastic cells in the presence of an internal positive control represented by normal epithelial cells, endothelial cells, fibroblasts, or lymphocytes [7]. In the validation study a case was classified p53 positive if at least one of eight cores was positive and was classified hMLH1 or hMSH2 negative if all the cores available were negative. Concerning the study of 263 consecutive CRC the same criteria were used for three cores per case. Whole sections of donor blocks and tissue array sections were read separately by two pathologists. Discordant cases were reviewed for consensus.

Statistical analysis

In the validation study, we calculated the percentage of cases correctly classified by the TMA, by comparing the cores to the whole section. We started from the theory that incorrectly classified cases (n) were independently arranged among the eight tissue cores, for each of the 30 tumors, according to Poisson distribution: percentage=$1-(n/30\times1/8)$. The percentage was calculated for each antibody according to the number of cores made by tumor, from one to eight. The 95% confidence interval was given by the Poisson distribution [13]. In the second part of the study differences between the IMC results recorded for CRC MSI^- and CRC MSI^+, were examined by χ^2 probability test [13]. Differences with P values less than or equal to 0.05 were regarded as statistically significant.

Results

Validation study

A disk was informative if at least 10% of the disk area contained tumoral glands (Figs. 1, 2): 94.5% for hMLH1, 96% for p53, and 96.7% for hMSH2. Eight disks per tumor could be interpreted in 78.8% of cases and seven in 90%. The lowest number of disks interpretable per case was four, only in one case. The loss of information was

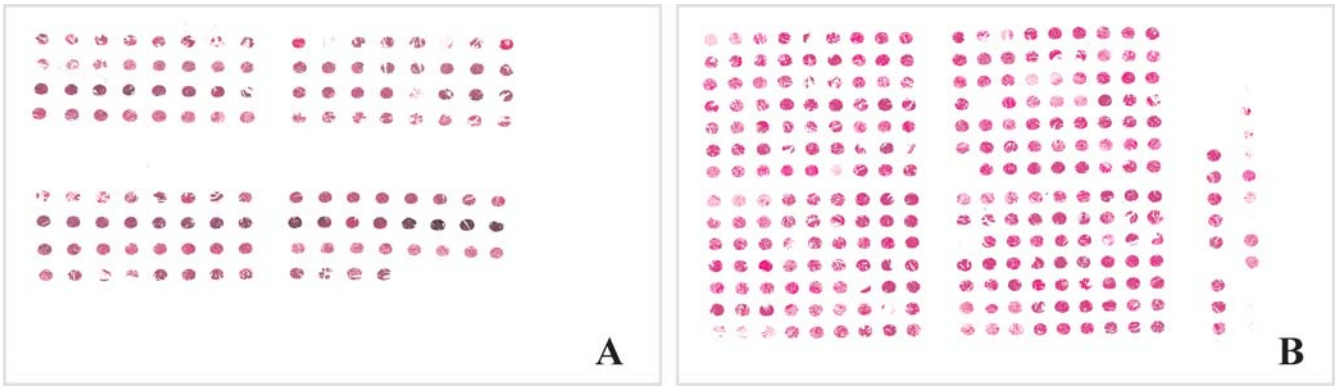

Fig. 1 Overview of hematoxylin and eosin stained sections of tissue array blocks of colorectal cancers; ×2.5. **A** Block I with 124 tissue cores. **B** Block A with 273 tissue cores

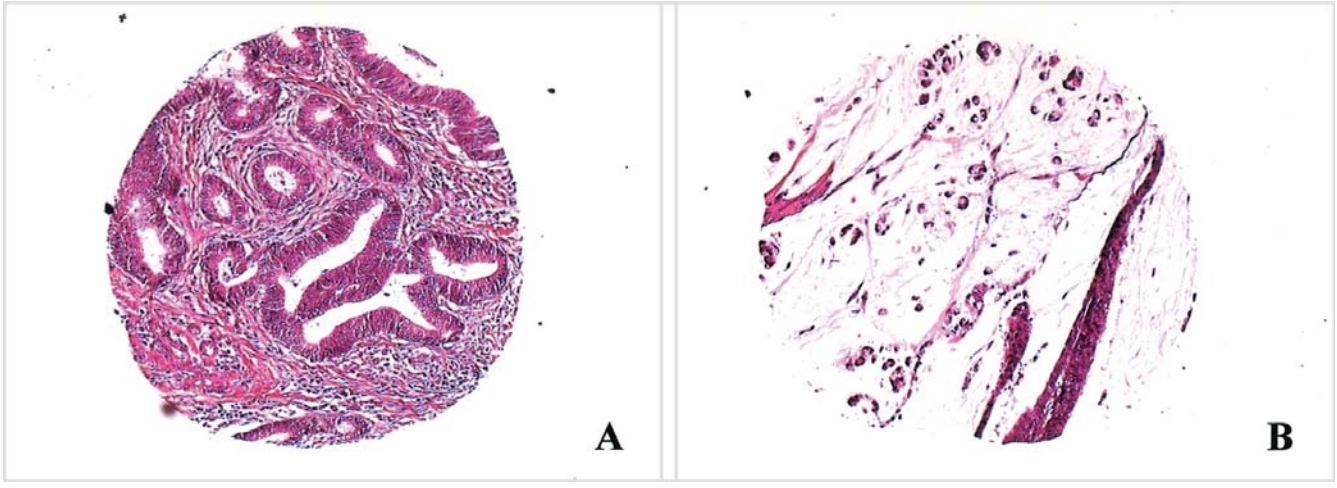

Fig. 2 Hematoxylin and eosin stained tissue microarray samples; ×100. **A** Well differentiated colorectal adenocarcinoma. **B** Mucinous colorectal adenocarcinoma

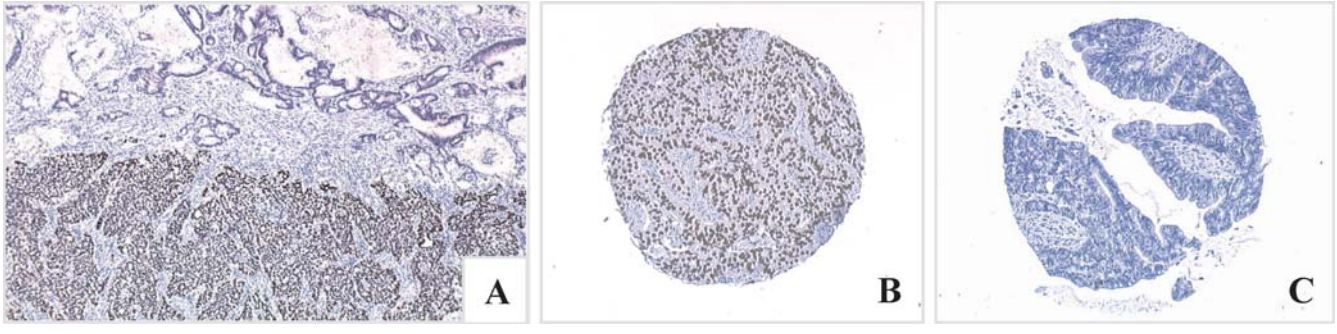

Fig. 3 **A** Heterogeneous immunohistochemical staining of p53 protein in a colorectal adenocarcinoma with varied differentiation patterns; ×50. **B** Tissue microarray sample of the p53 protein positive poorly differentiated area; ×100. **C** Tissue microarray sample of the p53 protein negative well differentiated area; ×100

due to the presence of normal tissue (muscle, connective tissue or mucosa) in 67.7% of cases, stroma without tumoral cells in 6.5% of cases and damaged disks in 25.8% of cases. An "interdisk" disagreement, that is, the presence of at least one disk classified differently from the other seven from the same tumor, was observed in 4.4% of cases. One case was due to varied differentiation patterns of the tumor, with overexpression of p53 limited to a poorly differentiated area (Fig. 3). In two cases for hMSH2 and one case for hMLH1, cores punched at the

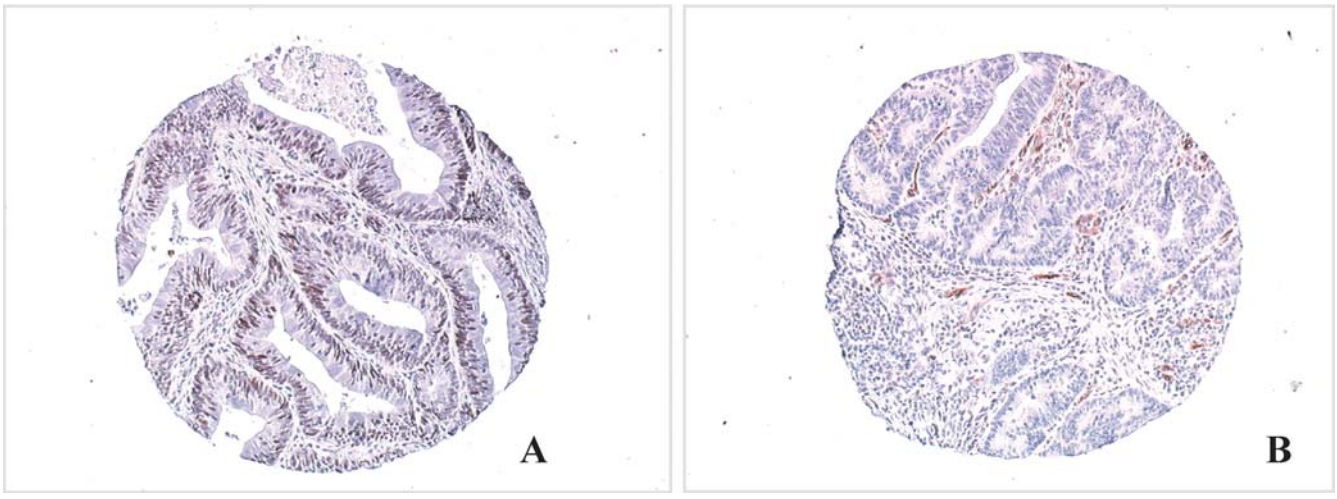

Fig. 4 hMLH1 immunohistochemical staining of tissue microarray samples; ×100. **A** hMLH1 positive colorectal adenocarcinoma. **B** hMLH1 negative colorectal adenocarcinoma with positive internal control represented by endothelial cells and lymphocytes of the stroma

Table 1 Immunohistochemical results of p53, hMLH1, and hMSH2 proteins expression in colorectal carcinoma (*CRC*) (*MSI* microsatellite instability)

	Overexpression of p53	Loss of expression of hMLH1	Loss of expression of hMSH2
Validation study of 30 CRC[a]			
15 MSI^-	5	0	0
15 MSI^+	1	8	7
Study of 263 consecutive CRC[b]	126	17	6

[a] Study of the whole section and eight tissue microarray cores
[b] Study of three tissue microarray cores

Table 2 Percentage of cases correctly classified on tissue microarray, in comparison with the whole section, in relation to the number of cores (*n*) analyzed by case (Poisson distribution)

Antibody	Number of cores punched by case							
	1	2	3	4	5	6	7	8
p53	97.1	97.9	99.6	99.6	100	100	100	100
hMLH1	96.7	98.3	98.8	99.2	100	100	100	100
hMSH2	96.7	98.4	99.2	99.6	100	100	100	100

surface of the tumor were positive while they were negative in deeper areas. The ten CRC sampled in the center (four cores) and edge (four cores) of the tumor had a perfect "interdisk" agreement. Overall the final result with eight disks was similar to the corresponding whole section in all cases (Table 1). Overexpression of p53 was observed in 33.3% of MSI^- cases and in 6.6% of MSI^+ cases. Loss of expression of hMLH1 or hMSH2 proteins was observed in 100% of MSI^+ cases and no MSI^- cases (Fig. 4). Then we established the number of cases correctly classified according to the number of cores made per case. For one core 96.7% of cases (hMLH1, hMSH2) and 97.1% (p53) were correctly evaluated. This concordance increased with the number of cores, reaching 98.8–99.6% for three cores and 100% for five cores and more (Table 2).

p53, hMLH1 and hMSH2 proteins expression in 263 consecutive CRC evaluated on TMA

According to the validation study, we applied the TMA technique in 263 consecutive CRC sampled by three cores. Three cases (1.1%) could not be interpreted for p53, the three disks being noninformative. In 8.7% of cases the expression of one MMR protein was lost, either hMLH1 (74%) or hMSH2 (26%). No case lost the expression of the two proteins. In 48.5% of cases there was overexpression of p53 protein; 17.4% of CRC losing an MMR protein and 51.5% of hMLH1/hMSH2 positive CRC overexpressed p53 ($P<0.005$; Table 3).

Table 3 Immunohistochemical results of p53, hMLH1, and hMSH2 proteins expression in 263[a] consecutive CRC ($P<0.005$)

p53	hMLH1 and hMSH2	
	Positive	One negative
Positive	122	4
Negative	115	19

[a] Three cases could not be interpreted because the three cores were noninformative

Discussion

The first concept of a multicore tissue block was described in 1986 by Battifora [2] to test new antibodies by IHC. A maximum of 100 samples of 1 mm thickness were tied up and embedded perpendicularly in a paraffin block. The TMA, described by Kononen et al. in 1998 [21], has many advantages on this technique. The tissue arrayer instrument enables to punch very small cores, of 0.6 mm diameter, and to array up to 1,000 samples into a single block. Moreover, specimens are arrayed in accordance with precise coordinates and are therefore correctly identified.

In our study, after a selection of donor blocks which is of great importance, it took 4 h to prepare a block of 273 cores. Moreover, as one section of one block enables the analysis of hundreds of cases with the amount of reagents usually used for one case, this technique allows a major reduction in cost. In our experience the interpretation of small areas of 0.6 mm was easier than for a whole section, which heterogeneity and staining artifacts at the borders can make it difficult to analyze. The latter phenomenon, frequently observed on large whole sections, was not encountered at the periphery of TMA blocks. Another advantage was that punches of 0.6 mm minimize tissue damage inflicted to the donor blocks. These blocks can still be used for other research studies or to test new markers of clinical interest for one patient.

Overall we attempted to answer the following question: are the small TMA samples representative of their potentially heterogeneous donor tumor? We conducted a validation study in CRC which is exceedingly common in most Western countries. We compared the IHC pattern of p53, hMLH1, and hMSH2 proteins between eight disks and the whole section of the block from which they were derived, in 15 MSI^- and 15 MSI^+ tumors. To our knowledge, we performed the first IHC study of hMLH1 and hMSH2 expression by the TMA technology. This technique was of great efficiency as only 4.3% of disks were not informative, a low rate in comparison with most validation studies [6, 10, 15, 19, 28, 29, 33] (Table 4). In our experience, the damage caused to a few disks was probably due to the IHC technique, especially to the antigen-retrieval step, as the corresponding cores were intact on HE-stained slides. The main reason for the loss of material was the absence of tumoral glands because of an abundant stroma or normal tissue. The selection of the donor blocks is crucial to minimize nonevaluable disks: large tumoral areas rich in glands must be selected. MSI^+ CRC are often mucinous, and it can be difficult to sample neoplastic cells in such cases. Moreover, the donor blocks must be chosen thick to obtain long cores that allow multiple studies on the same array block.

We analyzed eight cores per tumor and rare cases had some "interdisk" disagreement, showing that it is necessary to sample different areas in an heterogeneous tumor, as in one case, the expression of p53 protein depended on the tumor differentiation. If a marker is known to have an heterogeneous expression, it is crucial to sample the appropriate area, as the surface of the tumor for hMLH1 and hMSH2 proteins. On the other hand, we found no difference in expression of the three markers whether the cores were punched from the center or the edge of the tumor, contrary to the results obtained by Camp et al. [6] for hormonal receptors and Her2/neu in breast carcinoma. We then compared the results obtained with eight TMA disks to those obtained on the whole section: the results were similar in all cases. We statistically determined the minimal number of disks necessary to represent correctly the expression of the markers. One core enabled us to classify correctly 96.7–97.1% of cases, and more than four cores 100% of cases. We decided to make three cores in a large series to increase the concordance rate (98.8–99.6%) and decrease the risk of lost cases. Until now all TMA validation studies have confirmed the representa-

Table 4 Results of tissue microarray validation studies published in the literature and of the present study (*ER* estrogen receptor, *PR* progesterone receptor, NA not available)

Reference	Type of tumor	Number of cases	Number of disks by tumor	Number of array blocks	Immunohisto-chemical markers	Percentage of noninformative disks
Mucci et al. 2000 [28]	Prostate carcinoma	50	5–10	1 block of 458 disks	Synaptophysin, chromogranin A	27
Gillett et al. 2000 [15]	Breast carcinoma	157	23	10 blocks of 35 disks	ER, PR	11
Camp et al. 2000 [6]	Breast carcinoma	38	10	1 block of 380 disks	ER, PR, Her2/neu	NA
Torhost et al. 2001 [33]	Breast carcinoma	553	4	4 blocks of 553 disks	ER, PR, p53	23.5–29.7
Hoos et al. 2001 [19]	Fibroblastic tumors	59	3	1 block of 177 disks	Ki67, p53, pRb	10–20
Nocito et al. 2001 [29]	Bladder carcinoma	2317	4	NA	Ki67	11–14.6
Fernebro et al. 2002 [10]	Rectal carcinoma	20	3–6	1 block of 69 disks	Ki67, p53	17
Present study	Colorectal carcinoma	30	8	2 blocks of 124 disks	p53, hMLH1, hMSH2	4.3

tiveness of the technique and also suggested triplicate cores [6, 10, 15, 19]. However, it is important to realize that the TMA technology still remains a research tool. If a marker with a potential value is identified by this technique, for any given patient tests must be carried out on a large section. The IHC results of our validation study were similar to those obtained in previous studies: hMLH1 and hMSH2 antibodies enabled the diagnosis of MSI^+ CRC with a sensibility and specificity of 100% [24]; p53 overexpression was frequent in MSI^- CRC.

In the second part of the study we showed that TMA can be used efficiently to examine large series of tumors after a step of validation. In accordance with the results of our validation study we made three cores per case to study 263 consecutive CRC. We obtained the IHC pattern of the series of CRC for three markers of interest. There was a loss of expression of either hMLH1 or hMSH2 proteins in 8.7% of cases. Therefore we rapidly showed that almost 10% of the CRC operated on during 1 year in our center were MSI^+. As these tumors have a better prognosis [12, 16] and a better response to some chemotherapies [9], this result is potentially of great interest. In 17.4% of MSI^+ CRC and 51.5% of hMLH1/hMSH2 positive CRC there was overexpression of p53, corresponding to the inverse relationship between *p53* gene alterations and MSI [32]. The clinical significance of *p53* mutation and overexpression is controversial, with an influence on clinical outcome and therapeutic response still debated in the literature [1, 23, 25]. An interesting application of TMA would be the use of a "prognosis array block" containing CRC with available clinical follow-up data, to evaluate prognostic value of p53 and other prognostic markers.

In conclusion, we validated the TMA technology in CRC. The selection of donor blocks and sampling areas is a crucial step. The number of replicates must be adapted to the tissue type and the marker expression if it is known [5]. We confirmed by the rapid analysis of three IHC markers in 263 CRC that TMA is a high-throughput technology.

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