

FISH analysis of PTEN in endometrial carcinoma. Comparison with SNP arrays and MLPA

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Aims: To check the usefulness of a standardized protocol of *PTEN* FISH in 31 endometrial carcinomas (ECs) in comparison with SNP array (SNPA), multiplex ligation-dependent probe amplification (MLPA), and immunohistochemistry.

Methods and results: Fluorescence *in-situ* hybridization analysis showed two *PTEN* copies in 17 cases, three copies in nine cases, hemizygous deletion in two cases, and diverse cell populations with different *PTEN* copy number in three cases. A good correlation was seen between FISH and SNPA, particularly in cases with three copies. FISH identified two cases with entire deletion of chromosome 10, but did not identify a focal deletion of *PTEN*. Five cases with

PTEN deletion and duplication of the second allele by SNPA were interpreted as normal by FISH. Concordance between FISH and MLPA was seen in 15 cases with two copies, and in two cases with *PTEN* deletion. Six cases were interpreted as amplified by MLPA, but showed polyploidy by FISH. FISH was superior to SNPA and MLPA in assessing the tumours with diverse cell populations with different *PTEN* copies.

Conclusions: The results show good concordance between FISH, SNPA and MLPA. SNPA was superior in tumours with deletion of one copy and duplication of the second allele. FISH was superior in assessing tumour heterogeneity.

Keywords: deletions, fluorescence *in-situ* hybridization, polysomy, preanalytical variables, protocol, *PTEN*

Introduction

PTEN (phosphatase and tensin homologue deleted on chromosome 10) has been identified as a tumour suppressor gene,¹ and is mutated in a large number

of cancers.^{2–10} It is rather than cancers, it is located at 10q23.31. *PTEN* is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase containing a tensin-like domain as well as a catalytic domain similar to that of the dual-specificity protein tyrosine phosphatases.¹¹ Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates.¹² It negatively regulates the PI3K–Akt signalling pathway.¹³

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Somatic alterations of *PTEN*, including point mutations, insertions, and deletions, occur throughout the whole gene in a significant proportion of sporadic cancers. In some cases, *PTEN* deletion occurs in the setting of large chromosomal losses involving the whole of chromosome 10.¹⁴ In other instances, deletions are restricted to the entire *PTEN* gene, or to a few exons.¹⁵ Loss of heterozygosity (LOH) at 10q23 occurs in 40% of endometrial carcinomas (ECs).¹⁶ Deletions may be hemizygous or homozygous. *PTEN* being a tumour suppressor gene, two hits are common for *PTEN* loss of function, and deletion of *PTEN* is commonly the second hit. However, more recently, it has become clear that one alteration may be sufficient for *PTEN* inactivation.^{17,18} Interestingly, when a tumour has chromosomal instability, three copies of *PTEN* can be seen, as a result of polysomy of chromosome 10.¹⁹ The biological significance of chromosome 10 polysomy, and the consequences for *PTEN* levels, have not been fully elucidated.

In some types of cancer, *PTEN* alteration is an early event, being involved in the initial steps of the process of neoplastic development.²⁰ In these cases, alterations of *PTEN* are seen to be homogeneously distributed in the tumours. However, in other cases, *PTEN* abnormalities occur in the process of tumour progression,^{8,21} and *PTEN* alterations can be detected in specific regions of the tumour. The heterogeneous involvement of *PTEN* in a tumour may be demonstrated by immunohistochemistry.²² However, decreased *PTEN* expression does not necessarily reflect the presence of LOH at 10q23, as it may result from *PTEN* point mutations, and also from promoter hypermethylation, transcriptional repression, microRNA regulation, disruption of competitive endogenous RNA networks, or post-translational modification.^{19,23}

Fluorescence *in-situ* hybridization (FISH) may be used to detect cytogenetic aberrations,²⁴ such as deletions,²⁵ duplications,²⁶ amplifications²⁷ and structural abnormalities of specific genes, loci, or chromosomal sequences.²⁸ The regions assessed with FISH are typically larger than those studied with polymerase chain reaction (PCR), but smaller than those visualized microscopically with standard cytogenetics. Studies have demonstrated that FISH analysis of *PTEN* can identify groups of tumours with different prognoses among prostatic carcinomas²⁸ and rectal carcinomas.²⁹

In the present study, we developed a standardized protocol for *PTEN* FISH to detect *PTEN* copy number alterations (see Supporting Information) in a series of ECs in comparison with results obtained by single nucleotide polymorphism array (SNPA) and multiplex ligation-dependent probe amplification (MLPA). We

used the fast IQ hybridization buffer technology (IQ-FISH; Dako, Glostrup, Denmark).³⁰ We assessed the role of FISH in evaluating the heterogeneous presence of *PTEN* copy number alterations in the tumours. Finally, we compared the molecular results with the levels of *PTEN* protein determined by immunohistochemistry, in an attempt to correlate *PTEN* copy number alterations with *PTEN* loss.

Materials and methods

CASE SELECTION

A series of 31 ECs were studied. Formalin-fixed paraffin-embedded (FFPE) blocks were available for FISH and immunohistochemistry. Matched frozen tissue samples were selected for SNPA and MLPA. Haematoxylin and eosin (H&E) sections were obtained to confirm that frozen samples corresponded to the same areas as the FFPE material. The tumours were obtained from the files of Hospital Universitari Arnau de Vilanova, Lleida, Spain (HUAV). The local Ethics Committee approved the study. Informed consent was obtained from each patient. Pathological features of the tumours (histological type, grade, and stage) are summarized in Table 1.

For evaluation of preanalytical variables, two additional surgical specimens were used, obtained from the surgical pathology files of HUAV. They corresponded to ECs removed by hysterectomy and bilateral salpingoophorectomy. Surgical specimens were obtained from the operating room, immediately after resection. Cases were recruited because the size of the tumours allowed the selection of different small fragments to test the high number of variables. Tissue subjected to preanalytical factor evaluation of *PTEN* FISH was obtained from the excess material after selection of tissue samples for appropriate diagnosis.

FLUORESCENCE IN-SITU HYBRIDIZATION

Formalin-fixed paraffin-embedded blocks were sectioned at a thickness of 3 µm, dried for 1 h at 65°C, deparaffinized, and rehydrated. A pretreatment was performed in a microwave oven with a sensing capability for 10 min at 95°C, and the sections were then left for 15 min at room temperature (20–25°C). Pepsin digestion was performed using pepsin RTU (ready to use) at 37°C. The *PTEN* IQFISH probe used for hybridization was made as a mixture of a DNA-based Texas Red (TxR) probe specific for *PTEN* and a PNA-based, fluorescein isothiocyanate (FITC)-labelled refer-

Table 1. Summary of pathological features of the tumours and analysis by FISH

No	PTEN/CEN10 ratio	%	FISH result	Histological type	Grade	Stage
1	0.97	72	Polysomy (three copies)	SC	III	IA
2	1.02	76	Normal (two copies)	EEC	II	IB
3	1.13	81	Normal (two copies)	EEC	II	IB
4	0.84	77	Normal (two copies)	EEC	I	IA
5	1.1	86	Normal (two copies)	EEC	I	IA
6	1.13	73	Polysomy (three copies)	EEC	II	IB
7	1.05	79	Normal (two copies)	EEC	II	IA
8	0.96	71	Polysomy (three copies)	SC	III	IB
9	0.91	83	Normal (two copies)	EEC	III	IB
10	Clone 1: 0.87 Clone 2: 0.98	44 56	Heterogeneous (C1, polysomy; C2, normal)	SC	III	IA
11	0.93	74	Normal (two copies)	EEC	II	IB
12	1.02	85	Polysomy (three copies)	EEC	II	IB
13	1.05	76	Normal (two copies)	EEC	II	IA
14	0.98	78	Normal (two copies)	EEC	II	IA
15	Clone 1: 0.66 Clone 2: 1.10	42 58	Heterogeneous (C1, hemizygous deletion; C2, normal)	EEC	I	IA
16	1.1	74	Polysomy (three copies)	EEC	II	IA
17	1.12	79	Normal (two copies)	EEC	III	IB
18	0.95	71	Normal (two copies)	EEC	III	IB
19	0.63	81	Hemizygous deletion (one copy)	EEC	III	IA
20	1.08	73	Polysomy (three copies)	EEC	II	IB
21	0.95	77	Normal (two copies)	EEC	III	IB
22	Clone 1: 1.06 Clone 2: 0.89	40 60	Heterogeneous (C1, polysomy; C2, normal)	EEC	II	IA
23	0.94	83	Normal (two copies)	SC	III	IB
24	0.98	81	Normal (two copies)	EEC	I	IA
25	1.12	74	Polysomy (three copies)	EEC	II	IB
26	0.56	77	Hemizygous deletion (one copy)	EEC	III	II
27	1.04	78	Normal (two copies)	EEC	III	II
28	0.92	75	Normal (two copies)	EEC	II	IA
29	1.13	72	Polysomy (three copies)	EEC	III	IB
30	1.1	74	Normal (two copies)	EEC	III	IB
31	0.97	82	Polysomy (three copies)	EEC	I	IB

EEC, Endometrioid carcinoma; SC, serous carcinoma.

ence probe specific for the centromeric region of chromosome 10 (CEN10); the probe was a gift from Dako. We used the fast IQ hybridization buffer technology (IQFISH), which utilizes ethylene carbonate rather than formamide as the DNA-destabilizing component. Consequently, the IQFISH buffer is non-toxic, and also reduces the time needed for hybridization to genomic targets. The total turn-around time for FISH staining with an IQFISH-based probe is therefore <4 h. The IQFISH probe was incubated in Dako Hybridizer in two steps: denaturation at 66°C for 10 min, and hybridization at 45°C for a maximum of 2 h. After hybridization, a stringent wash was performed for 10 min at 63°C with a stringent wash buffer. Finally, samples were dehydrated, and nuclei were stained with DAPI. All reagents (wash buffer, pretreatment buffer, stringent buffer, and pepsin RTU) were obtained from the Histology FISH Accessory Kit (K5799; Dako). Signals were counted under a microscope equipped with a $\times 100$ objective, with a tetramethylrhodamine isothiocyanate (TRITC) philtre, an FITC philtre, and a TxR/FITC double philtre.

PTEN FISH SCORING

For evaluation of the results of FISH, several factors were taken into account to select the optimal tumour sections for *PTEN* FISH scoring, as rigorous quality criteria for suitability for FISH analysis. They included: preservation of tissue architecture, fixation artefacts, tumour cellularity within the section, and the absence of truncated nuclei and irregular glandular distortions. Areas selected for FISH evaluation were marked on an H&E-stained section that was directly adjacent to the section being used for FISH. Tissue areas were considered to be appropriate for FISH analysis after meeting rigorous criteria: (i) at least 85% of all nuclei in the target area were easily enumerable; (ii) the FISH signal was consistently greater than background intensity; (iii) areas where the borders of individual nuclei were not clearly identifiable were avoided; (iv) areas with excessive nuclear overlap were disregarded; (v) similar signal staining in the nuclei; and (vi) uniform DAPI staining. Staining of adjacent normal tissue was checked as reference. We required ~100 tumour cell nuclei to be analysed for each sample, depending upon the density of cancer cells. In complex tissue where there was more than one type of aberration, each of them was scored individually (scoring 100 cells for each).

We established five different statuses for *PTEN* FISH. First, normal *PTEN* copy number was characterized by the presence of two green (CEN10) and

two red (*PTEN*) signals in a minimum of 70% of nuclei, and a *PTEN*/CEN10 ratio (number of *PTEN* signals/number of CEN10 signals) between 0.8 and 1.2. Second, *PTEN* hemizygous deletion was characterized by the presence of two green (CEN10) signals and one red (*PTEN*) signal in a minimum of 70% of nuclei, and a *PTEN*/CEN10 ratio of <0.8. Third, *PTEN* gene homozygous deletion was characterized by the presence of two green (CEN10) signals and no red (*PTEN*) signal in a minimum of 70% of nuclei, and a *PTEN*/CEN10 ratio between 0 and 0.2. Fourth, whole chromosome 10 deletion was characterized by the presence of one green (CEN10) and one red (*PTEN*) signal in a minimum of 70% of nuclei, and a *PTEN*/CEN10 ratio between 0.8 and 1.2. Fifth, increased *PTEN* copy number (polysomy) was characterized by the presence of three green (CEN10) and red (*PTEN*) signals in a minimum of 70% of nuclei, and a *PTEN*/CEN10 ratio between 0.8 and 1.2.³¹ The criteria for scoring *PTEN* by FISH were obtained after taking into account the previous experience of the research team and data from the literature.³² The scoring system was validated with 25 cases of normal endometrial tissue. In all cases, the *PTEN* score fitted within the ranges of normal *PTEN* copy number.

SNP ARRAY

The Human CytoSNP array (Illumina; San Diego, CA, USA) was used to detect 240 000 SNPs evenly distributed across the genome, according to the manufacturer's protocol. ASCAT (allele-specific copy number analysis of tumours) was used to dissect the allele-specific copy numbers of solid tumours. On the basis of the observed B-allele frequency and log_R ratio of each SNP, chromosomal amplifications, deletions and copy-neutral loss of heterozygosity were detected at the genome-wide level.

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

We used SALSA MLPA probe-mix P225-C1 *PTEN* (MRC-Holland; Amsterdam, The Netherlands). This probe-mix contains at least two probes for each of the nine exons; the total number of *PTEN* probes included is 20. It also contains: 13 probes located elsewhere on chromosome 10 that can help to distinguish focal *PTEN* deletions in tumour-derived DNA samples from loss of the complete 10q arm or a chromosome 10 aneuploidy; two probes for copy number detection of the pseudogene *PTENP1* (at 9p13.3); and 15 reference probes. A DNA extraction was used (DNeasy Blood and

Tissue Kit; Qiagen, Hilden, Germany). The MLPA assay was performed according to the manufacturer's protocol (MLPA DNA protocol version MDP-v002). After fragment separation by capillary electrophoresis (Applied Biosystems; Foster City, CA, USA), an evaluation of the raw data was performed using GeneMapper. The results were always normalized and compared with strict external controls such as blood samples and non-tumour samples.

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Formalin-fixed paraffin-embedded sections were evaluated by applying our optimized PTEN staining immunohistochemistry assay.³³ Sections were obtained at a thickness of 3 µm, dried for 1 h at 65°C, deparaffinized, rehydrated, and subjected to target retrieval in the pre-treatment module PT Link (Dako) at 95°C for 20 min in Target Retrieval Solution (pH 9) (Dako). Endogenous peroxidase was blocked using peroxidase-blocking reagent (Dako), and this was followed by incubation with primary PTEN antibody (clone 6H2.1; Dako). The incubation time for the primary antibody was 40 min. EnVision FLEX+/HRP (Dako) (20 + 15 min) was used to amplify signal, and detection was done using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen (Dako). Slides were counterstained with haematoxylin, dehydrated, and mounted. PTEN expression was nuclear and cytoplasmic. Appropriate negative controls were also tested: these involved omission of the primary antibody, and the inclusion of tissue samples known to show *PTEN* silencing. We used a histological staining score (Hscore) that takes into consideration the intensity of the staining, and the percentage of positive cells, by applying the formula: $Hscore = (1 \times \% \text{ light staining}) + (2 \times \% \text{ moderate staining}) + (3 \times \% \text{ strong staining})$. This gives a score ranging from 0 (no immune reaction) to 300 (maximal immunoreactivity).

STATISTICAL ANALYSIS

Fisher's exact test was used to correlate PTEN expression as detected by immunohistochemistry with *PTEN* copies as detected by SNPA, MLPA, and FISH.

Results

FISH SCORING

Results from the analysis of the 31 ECs are shown in Table 1. Seventeen cases showed a normal *PTEN* copy number, nine showed three copies (polysomy 3),

and two cases had hemizygous deletion of *PTEN* (one copy). The presence of more than one tumour cell population with differences regarding *PTEN* copy number was seen in three cases. In one case (case 10), 44% of tumour cells showed three copies of *PTEN* (polysomy), and 56% showed a normal copy number. In the second case (case 15), 42% of tumour cells showed hemizygous deletion of *PTEN*, and 58% showed a normal copy number. In the third case (case 22), 40% of tumour cells showed three copies of *PTEN* (polysomy), and 60% showed a normal copy number. Representative pictures are shown in Figures 1–7.

SNP ARRAY

Table 2 shows the correlation between FISH and SNPA after exclusion of the three cases that showed heterogeneous populations by FISH, which will be presented separately. A normal *PTEN* copy number was seen in 11 of 31 cases. Three copies of *PTEN* were identified in nine cases. An absolute correlation between FISH and SNPA was achieved in cases showing three copies of *PTEN*. Hemizygous deletion of the whole of chromosome 10 (one copy of chromosome 10) was seen in two cases. A hemizygous deletion restricted to *PTEN* was seen in one case. In five cases, SNP analysis showed hemizygous deletion of one *PTEN* allele with duplication of the second allele. Overall, deletion of *PTEN* was seen in eight of 31 cases (26%), although, in five of them, there was also duplication of the second allele. Fluorescence *in situ* hybridization identified the two cases with deletion of the whole of chromosome 10. However, FISH did not identify the only case with focal deletion of *PTEN*. The five cases of *PTEN* deletion and duplication of the second allele were interpreted as normal by FISH.

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

Table 3 shows the correlation between MLPA results and those obtained by FISH; and Table 4 the correlation between MLPA results and those obtained by SNPA. In both Tables the three cases that showed heterogeneous populations by FISH have been excluded. A normal *PTEN* copy number was seen in 17 cases, four cases showed one copy of *PTEN*, six cases showed *PTEN* amplification, and one case showed a complex *PTEN* status (amplification and deletion). Fifteen cases had a concordant normal diploid pattern by FISH and MLPA, and two had

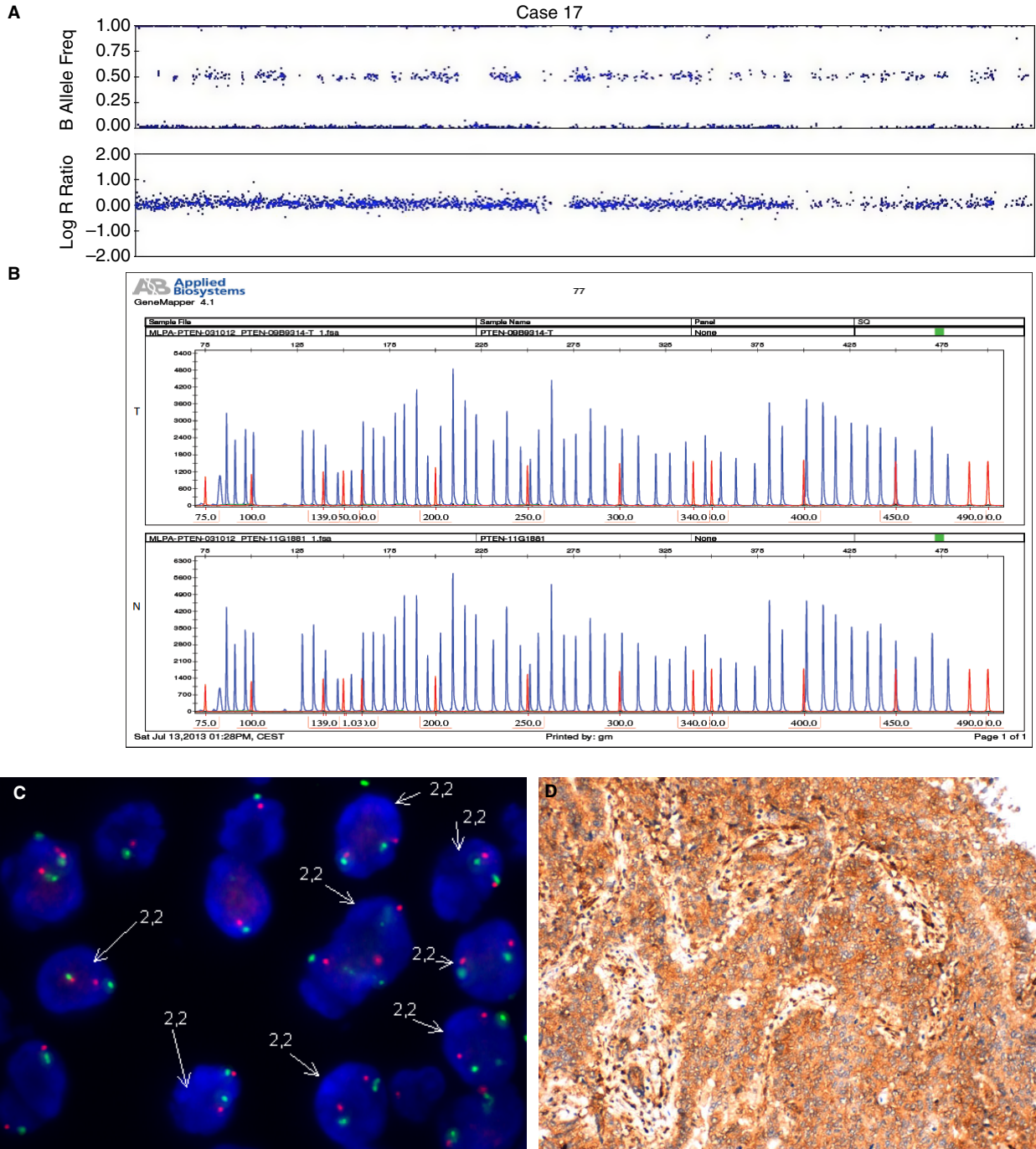


Figure 1. An endometrial carcinoma with normal *PTEN* status (case 17). **A**, SNP image showing normal *PTEN*. **B**, MLPA with normal *PTEN* in tumour tissue (T) as compared with normal tissue control (N). **C**, FISH image showing cells with two copies of *PTEN*. **D**, Positive immunostaining for *PTEN*.

two copies by MLPA and three copies by FISH. Two cases had a concordant pattern of *PTEN* deletion by FISH and MLPA, whereas results were discordant in two additional cases. Six cases were interpreted as

amplified by MLPA, but showed polyploidy by FISH. One case with a complex MLPA pattern (amplification and deletion) was interpreted as normal by FISH.

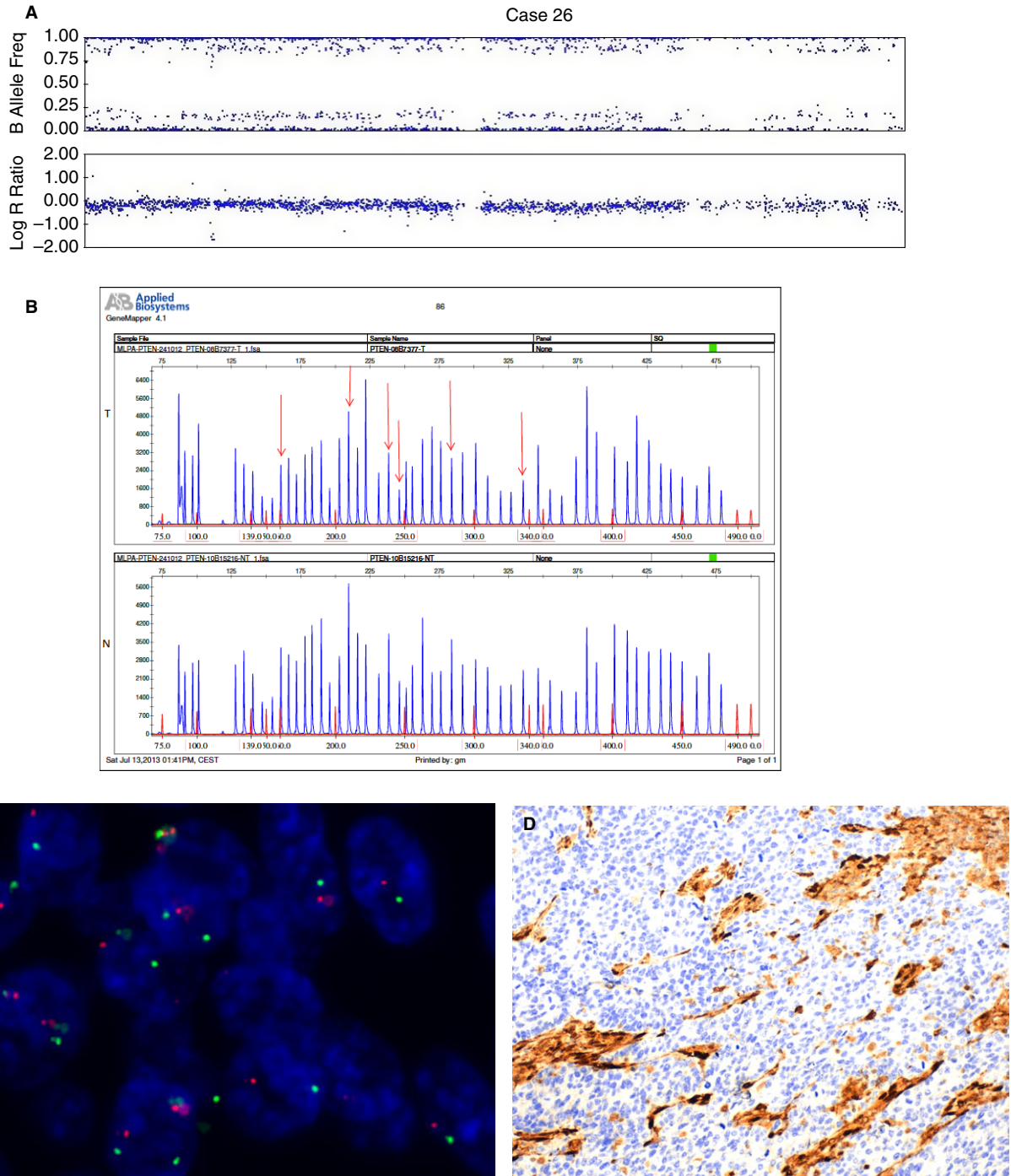


Figure 2. An endometrial carcinoma with a *PTEN* deletion (case 26). **A**, SNPA image showing deletion of chromosome 10. **B**, MLPA with deleted *PTEN*; arrows indicate deleted areas in tumour tissue (T) as compared with normal tissue control (N). **C**, FISH image showing one copy of chromosome 10. **D**, Negative immunostaining for *PTEN*.

A concordant normal pattern (two copies) was seen in 11 cases with SNPA and MLPA. Six cases showed amplification by MLPA and polysomy by SNPA, but discordant results were obtained in three additional cases, which

were interpreted as polyploid by SNPA. Five cases showed deletion and duplication of the second allele by SNPA, and four of them were interpreted as normal by MLPA and one as having a *PTEN* deletion.

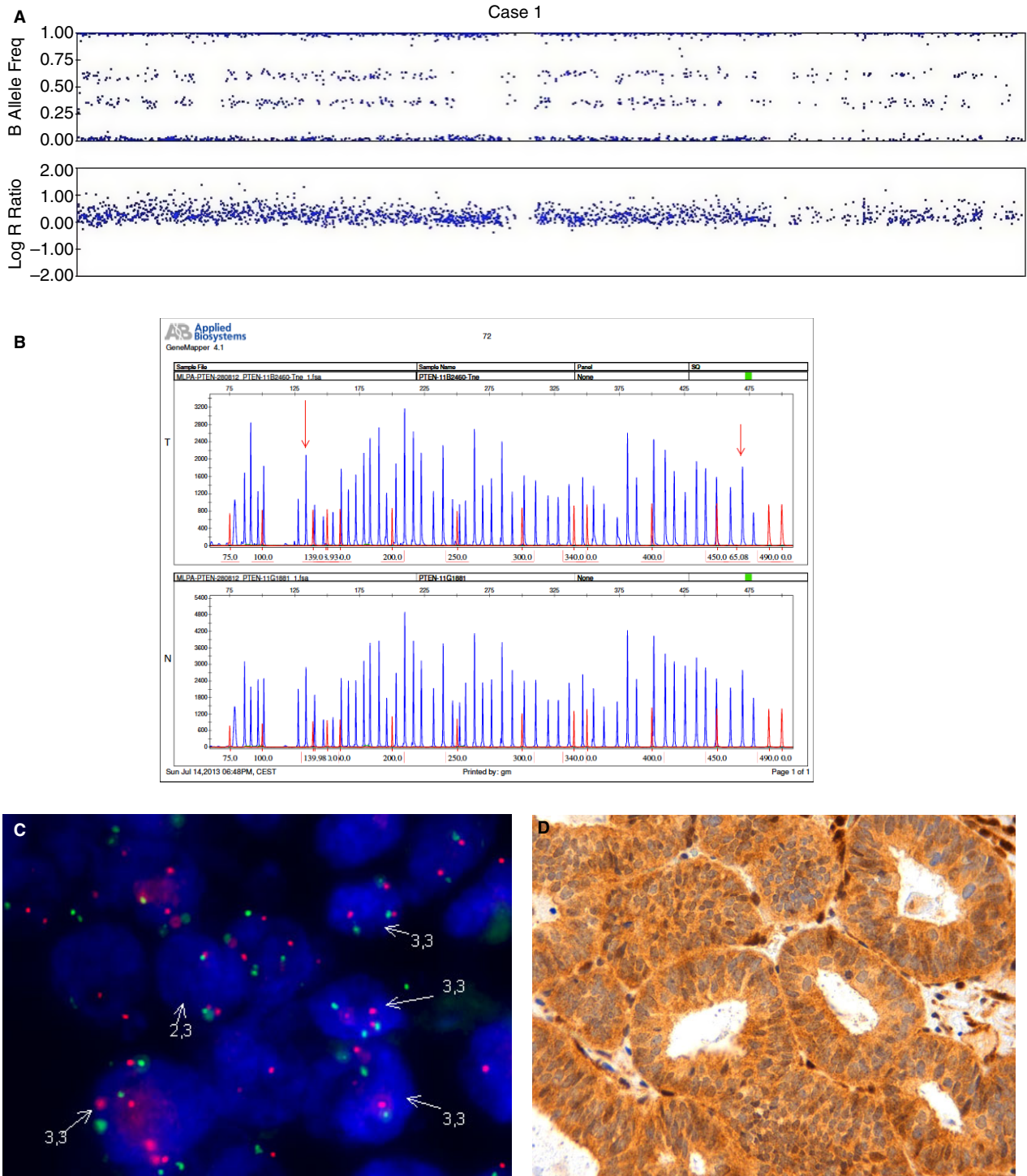


Figure 3. An endometrial carcinoma with polysomy of chromosome 10 (case 1). **A.** SNPA image showing three copies of *PTEN*. **B.** MLPA with amplification of exons 1 and 9 in tumour tissue (T) as compared with normal tissue control (N). **C.** FISH image showing cells with three copies of chromosome 10. **D.** Positive immunostaining for *PTEN*.

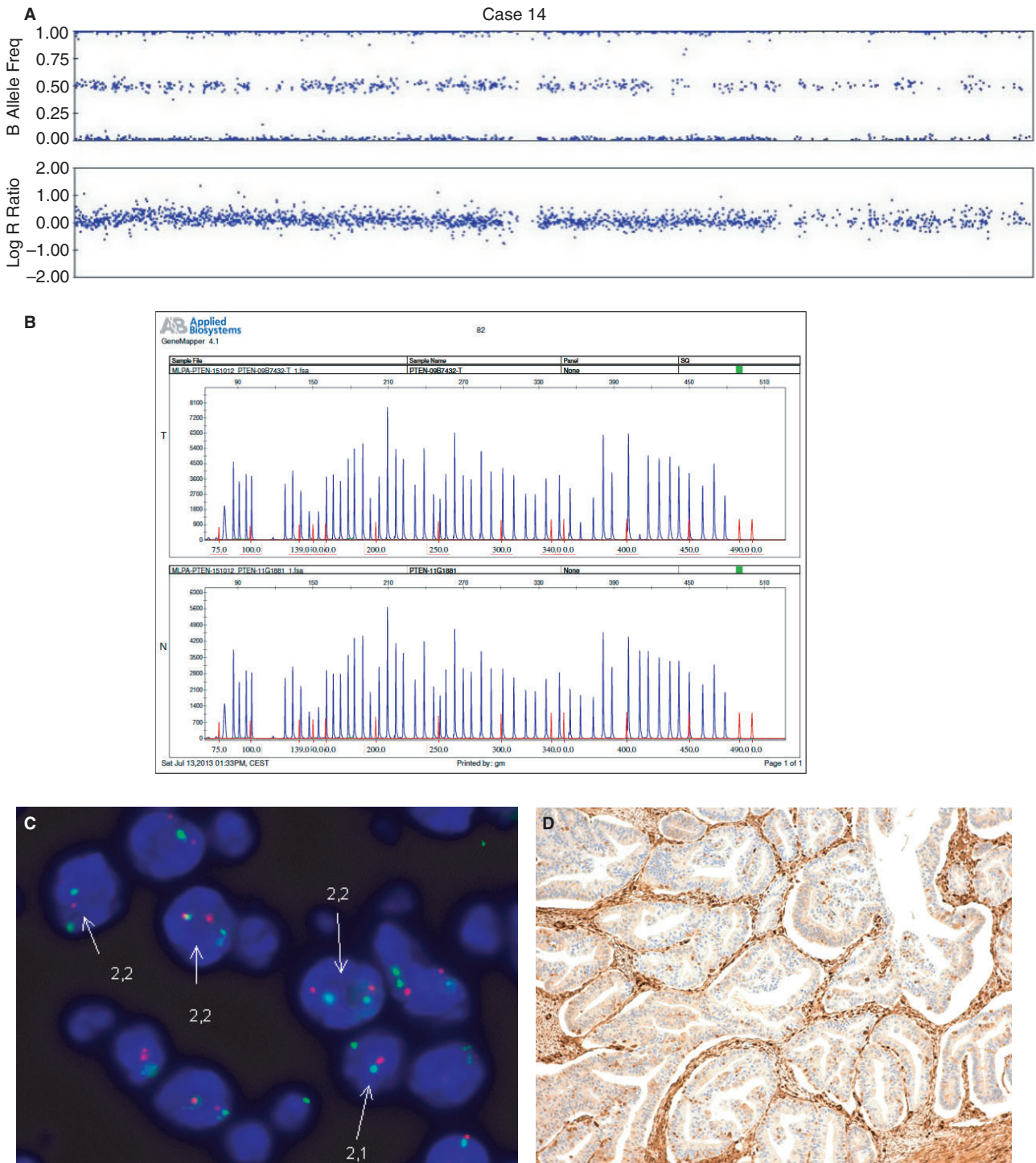


Figure 4. An endometrial carcinoma with deletion of *PTEN* and duplication of the second allele (case 14). **A**, SNPA image showing one *PTEN* allele deleted and the second allele duplicated. **B**, MLPA with normal *PTEN* status in tumour tissue (T) as compared with normal tissue control (N). **C**, FISH image showing cells with two copies of *PTEN*. **D**, Negative immunostaining for *PTEN*.

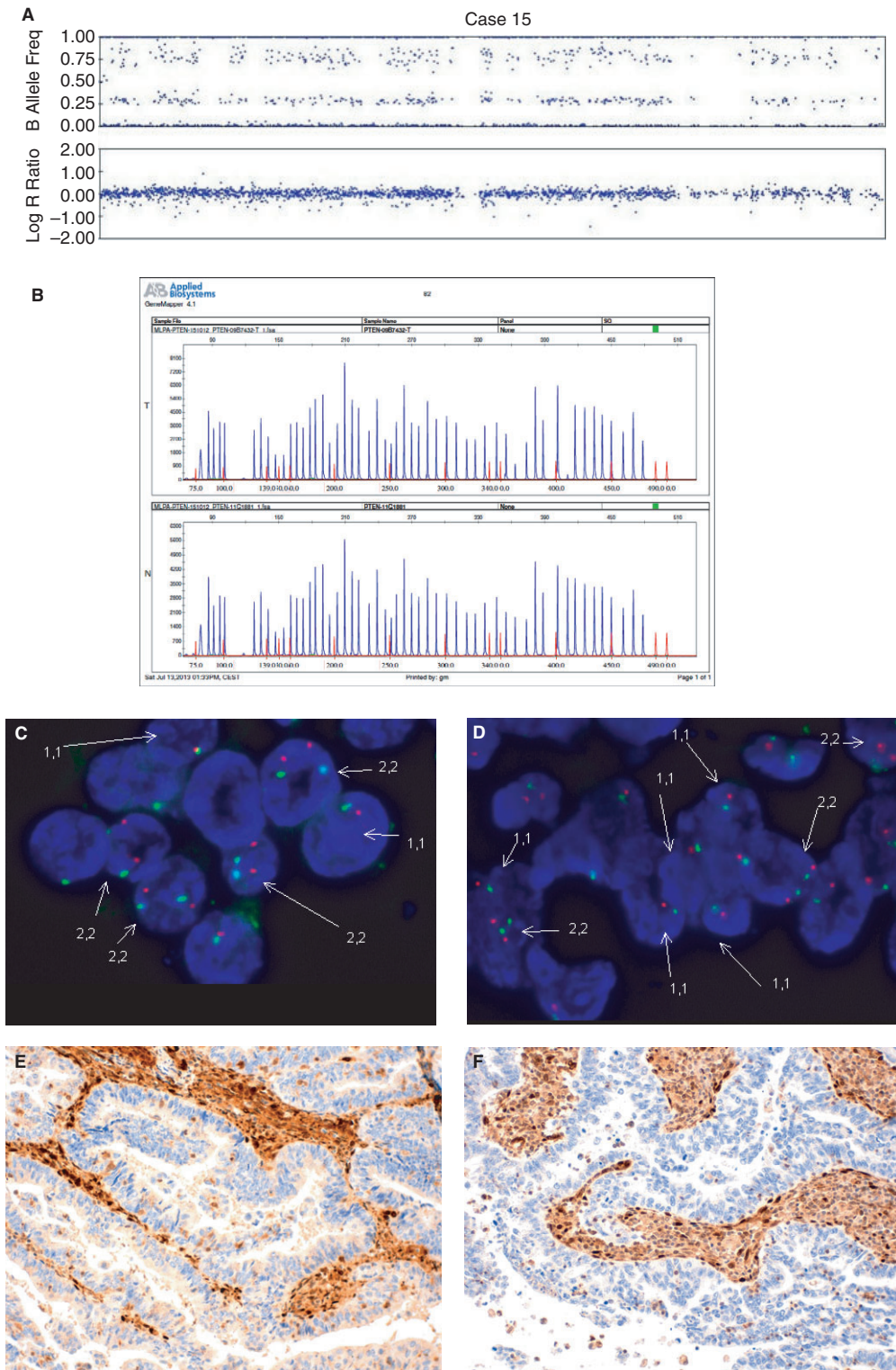


Figure 6. An example of an endometrial carcinoma with heterogeneous distribution of *PTEN* copy number (case 15). **A**, SNPA showing one allele of *PTEN* deleted and the second allele duplicated. **B**, MLPA with normal *PTEN* in both tumour tissue (T) and normal tissue control (N). **C**, **D**, FISH image showing a group of cells with two copies of *PTEN* (**C**) and a group of cells with complete deletion of chromosome 10 (**D**). **E**, **F**, Negative *PTEN* immunostaining in the two areas.

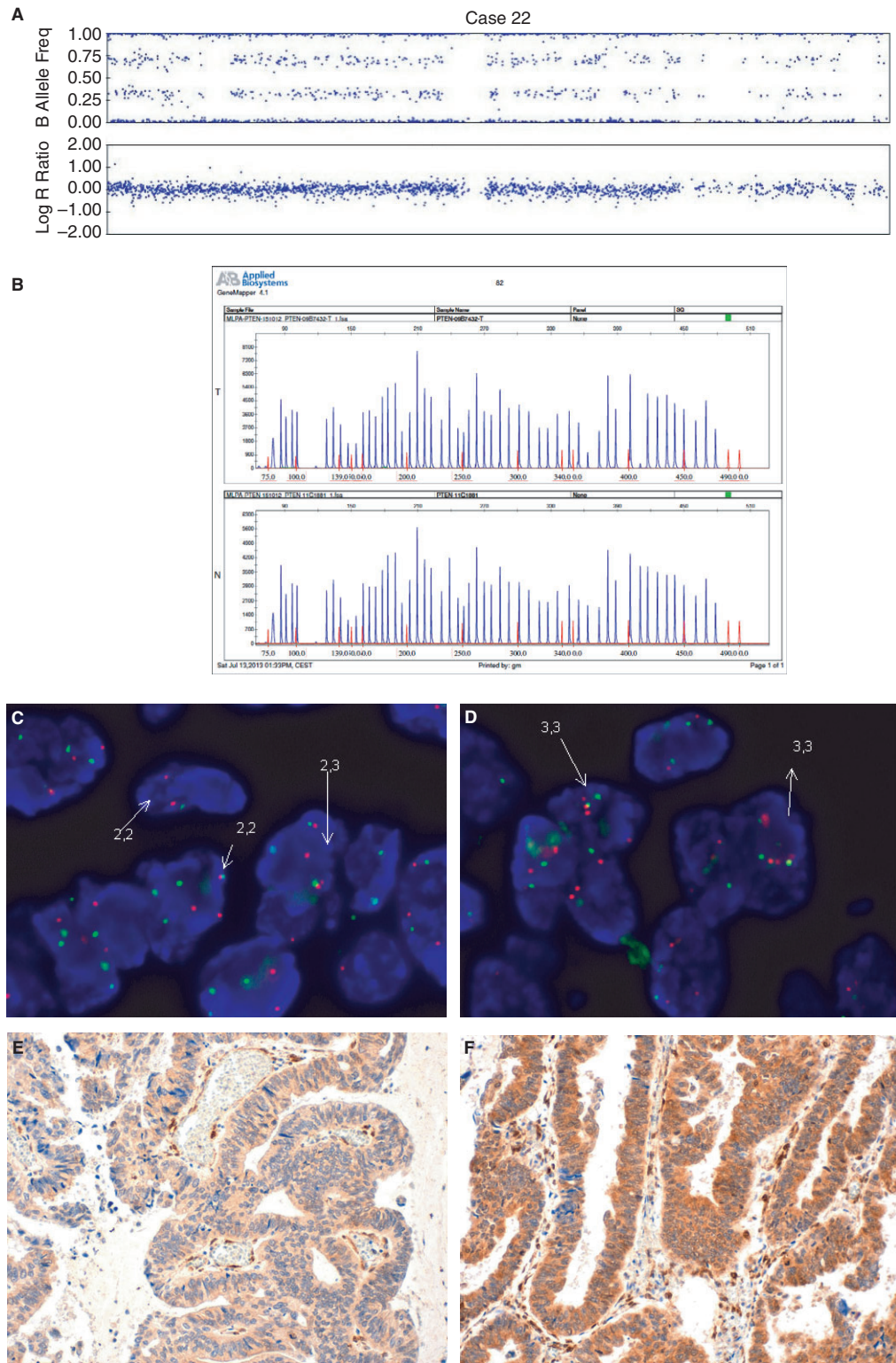


Figure 7. An example of an endometrial carcinoma with heterogeneous distribution of *PTEN* copy number (case 22). **A**, SNPA showing one allele of *PTEN* deleted and the second duplicated. **B**, MLPA with normal *PTEN* in both tumour tissue (T) and normal tissue control (N). **C**, **D**, FISH image showing a group of cells with two copies of *PTEN* (**C**) and a group of cells with three copies of *PTEN* (**D**). **E**, **F**, Immunohistochemistry shows cells with negative staining (**E**) and positive staining (**F**) for *PTEN*.

Table 2. Correlation between FISH and SNP array in 28 cases; three cases with heterogeneous alterations of *PTEN* are excluded

SNP array	FISH		
	Normal (two copies)	Polysomy (three copies)	Deletion (one copy)
Two copies	11		
Three copies		9	
One copy			2
Focal deletion	1		
<i>PTEN</i> deletion plus duplication of the second allele	5		

Table 3. Correlation between multiplex ligation-dependent probe amplification (MLPA) and FISH in 28 cases; three cases with heterogeneous alteration of *PTEN* by FISH are excluded

MLPA	FISH		
	Normal (two copies)	Polysomy (three copies)	Deletion (one copy)
Normal	15	2	
Deletion	1	1	2
Amplification		6	
Amplification plus deletion	1		

Table 4. Correlation between SNP array and multiplex ligation-dependent probe amplification (MLPA) in 28 cases; three cases with heterogeneous alteration of *PTEN* by FISH are excluded

SNP array	MLPA			
	Normal	Deletion	Amplification	Amplification plus deletion
Two copies	11			
Three copies	2	1	6	
One copy		2		
Focal deletion				1
Deletion plus duplication of the second allele	4	1		

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Tables 5, 6 and S1 show the results of analysis of the relationship between immunohistochemistry and the results obtained with the three molecular techniques (FISH, SNPA, and MLPA), after exclusion of the three cases that showed heterogeneous populations by FISH. Interestingly, tumours showing polysomy by FISH and SNPA (interpreted as amplified by MLPA) showed similar *PTEN* expression as tumours with a normal *PTEN* copy number ($P = 0.68$ and $P = 1$, respectively). In contrast, cases showing deletion of *PTEN* by FISH, SNPA or MLPA showed decreased expression of *PTEN*, even in the presence of duplication of the second allele, although statistical significance was not reached.

TUMOURS WITH HETEROGENEOUS *PTEN* ALTERATIONS

The three cases that showed a heterogeneous distribution of *PTEN* by FISH were interpreted separately (Table 7; Figures 5–7). The first case (case 10) contained one cell population with three copies of chromosome 10 (44%) and a second population with a normal copy number (two copies) (56%). The case was interpreted as containing three copies by SNPA and containing two copies by MLPA. Interestingly, immunohistochemical expression of *PTEN* was heterogeneous in the tumour. Although there was not an absolute correlation, the areas with polysomy showed increased *PTEN* immunostaining in comparison with tumour areas with a normal *PTEN* copy number. The second case (case 15) contained a tumour cell population with a *PTEN* deletion (42%), and a population with a normal copy number (58%). The tumour was interpreted as having one allele deleted and duplication of a second allele by SNPA, and as having a

Table 5. Correlation between FISH and immunohistochemistry (IHC) in 28 cases; three cases with heterogeneous alteration of *PTEN* are excluded

IHC Hscore	FISH		
	Normal (two copies)	Polysomy (three copies)	Deletion (one copy)
0–40	9	4	2
41–100	4	4	
101–200	2		
201–300	2	1	

normal copy number by MLPA. Immunohistochemical expression of *PTEN* was also homogeneously negative in the two components. The third case (case 22) contained one cell population with three copies of chromosome 10 (40%) and a second population with a normal copy number (two copies) (60%). The tumour was interpreted as having one allele deleted and

duplication of a second allele by SNPA, and as having a normal copy number by MLPA. Immunohistochemical expression of *PTEN* was also heterogeneous.

Discussion

Tumour suppressor genes (TSGs) such as *PTEN* are important in tumorigenesis. Although inactivation of TSGs may take place at the transcriptional and protein levels (enhanced protein degradation, or decreased expression because of promoter hypermethylation), it usually results from partial loss of the gene, or even loss of the entire gene. According to Knudson,³⁴ the inactivation of a TSG takes place in two steps. The first is usually a point mutation, or a small deletion, whereas the second typically consists of a large deletion of part of a chromosome that involves the gene. Although it has recently been demonstrated that monoallelic inactivation may be sufficient,³⁵ demonstration of deletions is still quite informative regarding assessment of the role of a specific TSG in a particular type of tumour. Searching for specific areas of the genome where gene deletions (LOH) commonly occur in a determinate type of

Table 6. Correlation between SNP array and immunohistochemistry (IHC) in 28 cases; three cases with heterogeneous alteration of *PTEN* are excluded

IHC Hscore	SNP array				
	Two copies	Three copies	One copy	Focal deletion	Deletion plus duplication of the second allele
0–40	6	4	2		3
41–100	2	4			2
101–200	2				
201–300	1	1		1	

Table 7. Correlation between FISH, SNP array and multiplex ligation-dependent probe amplification (MLPA) in three cases with heterogeneous *PTEN* FISH patterns

	SNP array	FISH	MLPA	IHC staining
Case 10	Polysomy (three copies)	Polysomy (44%) Normal (56%)	Normal	Heterogeneous
Case 15	One allele deleted plus duplication of the second allele	Deleted (42%) Normal (58%)	Normal	Negative
Case 22	One allele deleted plus duplication of the second allele	Polysomy (40%) Normal (60%)	Normal	Heterogeneous

tumour is important, because it may lead to the recognition of TSGs that are involved in the development or progression of such a tumour. *PTEN* is among the most frequently lost or mutated TSGs, with a frequency of monoallelic mutations estimated at 50–80% in EC, glioblastoma, and prostate cancer, and at 30–50% in breast, colon and lung tumours.¹⁴ Complete loss of *PTEN* is observed at the highest frequencies in EC and glioblastoma.⁷ Detection of gene deletions not only helps in understanding the molecular mechanisms underlying the development of cancer, but also provides important information for disease diagnosis and prognosis.^{20,25} Identification of deletions (LOH) may be carried out by different techniques, including karyotyping,³⁶ microsatellite polymerase chain reaction (PCR),³⁷ comparative genomic hybridization,³⁸ FISH,³⁹ MLPA,⁴⁰ and SNPA.⁴¹

Fluorescence *in-situ* hybridization is a good method for identifying deletions in tumour cells.²⁵ It usually requires the identification of the deleted target gene with a locus-specific probe, in combination with preservation of the corresponding chromosomal centromere, with the appropriate centromeric probe. It has limited value in detecting small deletions.⁴² FISH analysis has some advantages over molecular analysis, particularly regarding correlation with morphology and assessment of tumour heterogeneity.⁴³ However, one of the major limitations of detecting deletions in tissue sections by FISH is that part of the cell can be lost during the sectioning process, leading to artificially induced deletions in a significant percentage of nuclei.⁴² To decrease the false-positive rate, the literature recommends using centromeric probes as controls, as well as evaluation of a substantial number of cells.³² The usefulness of FISH in the evaluation of *PTEN* has been evaluated in carcinomas of the prostate,²⁵ rectum,⁴⁴ and stomach.⁴⁵ In prostate cancer, *PTEN* alterations, as analysed by FISH, have been shown to have prognostic importance.²⁵

In the MLPA technique, genomic DNA is hybridized in solution to probe sets, each of which consists of two halves. One half consists of a target-specific sequence (20–30 bp),⁴⁰ and the other half also has a target-specific sequence at one end (25–43 bp) and a universal primer sequence at the other, but has a variable-length random fragment in between (19 and 370 bp) to generate the size differences in the probes that are necessary to allow electrophoretic resolution. The amounts of ligated probe produced will be proportional to the target copy number, and, after PCR amplification, the relative peak heights indicate deletion or duplication of the target sequence. MLPA has

proven to be a highly sensitive and accurate tool for detecting copy number changes.

Single nucleotide polymorphism array enables simultaneous detection of a large number of DNA polymorphic loci in a simple way. Single nucleotide polymorphism array provides a high-throughput, high-resolution approach to genotypic analysis that includes assessment of LOH of chromosomal regions based on paired normal and tumour samples from the same patients.⁴¹ The application of informatics tools to this technique may allow the identification of LOH in many loci, and also the hierarchical clustering of cancers on the basis of shared LOH patterns. Resolution varies according to the type of array, but LOH may be resolved at least to approximately 3 Mb, or 100 SNPs, in 100K arrays. Further technical developments make SNPA capable of analysing both signal intensity variations and changes in allelic composition in parallel. Single nucleotide polymorphism array can also detect both copy number changes and copy-neutral LOH events. The Human CytoSNP array (Illumina) was used in this study, and is able to detect 240 000 SNPs evenly distributed across the genome.

ECs can be divided into two subtypes with different clinicopathological and molecular characteristics.⁴⁶ Endometrioid carcinomas (EECs) (85%) are usually low-grade, oestrogen-dependent tumours.⁴⁷ Serous carcinomas (SCs), which represent the prototype of non-endometrioid carcinomas (15%), are more aggressive, are independent of oestrogen, and develop in postmenopausal women. The molecular alterations of EEC and SC are different; abnormalities in *PTEN* occur in 40% of EECs. LOH at 10q23 frequently coexists with somatic *PTEN* mutations, which are also found in 37–61% of EECs.⁴⁸ Assessment of *PTEN* by immunohistochemistry or FISH may help in classifying difficult cases.

In the present study, we developed a standardized protocol for evaluation of *PTEN* by FISH, and we also assessed *PTEN* status by FISH in a series of 31 ECs. The same cases were subjected to SNPA, MLPA, and immunohistochemistry. The results show good concordance between FISH and SNPA or MLPA for ECs with a normal *PTEN* copy number (all 11 cases with two copies of *PTEN* by SNPA had a normal copy number by FISH, and a concordant diploid pattern between FISH and MLPA was seen in 15 of 17 cases). Moreover, there was good correlation between FISH and SNPA in assessment of the presence of large deletions (two cases), if we exclude the tumours in which a deletion of *PTEN* coexisted with duplication of the second allele (five cases). In these cases, SNPA was

superior to FISH and MLPA, because FISH could not identify one of the two alleles as a duplicated one. Finally, FISH was superior to SNPA and MLPA in detecting the presence of gene copy number variation, in the context of morphology. This was particularly obvious for the three tumours with a heterogeneous distribution of tumour cells with different pattern of *PTEN* copy number variation. In a previous study,³³ a heterogeneous distribution of *PTEN* staining was seen in 27% of cases, and correlated with different expression levels of putative targets of AKT (BAD, p27, and NFκB; data not shown).

Interestingly, nine ECs had three copies of *PTEN* (polysomy), and in this group there was complete agreement between FISH and SNPA. Polysomy was more frequent among SCs (three of four cases, one of them heterogeneous) than among EECs (nine of 27 cases, one of them heterogeneous), probably reflecting the frequent presence of chromosomal instability in SCs. Six of these nine tumours were interpreted as showing *PTEN* amplification by MLPA. The results suggest that FISH and SNPA are superior to MLPA in assessing *PTEN* triploidy. Interestingly, the *PTEN* levels in the nine tumours with an extra copy of *PTEN*, as measured by immunohistochemistry, were similar to the levels in tumours with a normal *PTEN* copy number ($P = 0.68$). This suggests that the presence of an additional copy of *PTEN* does not have an impact on protein production. However, it is worth mentioning that the area that contained three copies of *PTEN* had a greater correspondence with the area showing increased *PTEN* immunostaining than the area with two copies in two of the three heterogeneous tumours. The biological role of polysomy in altering the function of oncogenes or TSGs is under debate. Polyploidy at chromosome 17, where *HER2* is located, occurs in one-third of breast carcinomas. It has been shown that polysomy 17 is mechanistically distinct from *HER2* amplification. Polysomy at chromosome 17, without *HER2* amplification, does not seem to significantly increase *HER2* mRNA or *HER2* protein levels in many cases, although there is a low to moderate increase in *HER2* level in some cases. Breast cancers with extra copies of chromosome 17 resemble *HER2*-negative tumours according to standard pathological criteria, including tumour grade, and hormonal status. Even more perplexing is the presence of extra copies of TSGs, such as *PTEN*, which are typically inactivated by loss of function molecular events. In ECs, polysomy at chromosome 10 has been described previously. It may occur separately, or may coexist with polysomy in other chromosomes, such as chromosome 1 and chromosome

8. Several studies have shown that polysomy at chromosome 10 is more frequent in EC than in endometrial hyperplasia, and that it is more frequent in tumours with higher histological grade.⁴⁸ Whether polysomy at chromosome 10 is related to disturbance of *PTEN* expression, or simply reflects the presence of chromosomal instability, is still unknown. However, the results of our study show that, as for *HER2* in breast carcinoma, polysomy at the *PTEN* loci does not result in increased *PTEN* protein levels.^{19,36} Interestingly, polysomy at chromosomal areas involving well-known TSGs or genes involved in DNA repair (*RBI*, *p53*, *APC*, *BRCA1*, *BRCA2*, and *NF1*) has been reported.⁴⁹

It is worth mentioning that five tumours showed deletion of one copy of *PTEN* and duplication of the second allele. Although not statistically significant, perhaps because of the small number of cases, these tumours showed a trend for decreased protein expression levels (eight of nine had Hscores of <100), suggesting that the biological significance of the deleted copy is more important than that of duplication of the second allele. Interestingly, one of the heterogeneous tumours showed a *PTEN* deletion in one area and a normal *PTEN* number in another area by FISH (a *PTEN* deletion with duplication of the second allele by SNPA), and *PTEN* immunostaining was negative in both. We may speculate about the molecular basis of this interesting subgroup of tumours. Polysomy is usually a consequence of mis-segregation during mitosis, which happens in tumours with chromosomal instability, and there are many findings showing that ECs with *PTEN* alterations have genomic instability. We may hypothesize that, in the presence of *PTEN* alteration, chromosomal instability can lead to the development of polysomy at some chromosomal loci, chromosome 10 among them. In this regard, it is worth noting that deletion of *PTEN* was seen in eight of 31 cases (26%), and in five of these eight cases there was duplication of the second allele. In other words, the polysomy at chromosome 10 was seen in more than half of the tumours showing deletion of *PTEN*.

In summary, a combination of these techniques is helpful for assessment of *PTEN* copy number changes in ECs. The combination of FISH and SNPA seems to be particularly helpful. Fluorescence *in-situ* hybridization allows much better assessment of tumour heterogeneity, whereas SNPA is helpful in identifying tumours with deletion of *PTEN* and duplication of the second allele, which appear as normal diploid by FISH. The combination of all of these techniques, together with immunohistochemistry, is helpful in

interpreting the biological significance of polysomy at the *PTEN* locus in chromosome 10. Discrepancies between the results obtained with these techniques and those obtained with immunohistochemistry may be explained by the presence of *PTEN* mutations and alterations in *PTEN* translation. Our results suggest that a combination of FISH and immunohistochemistry may be of some help in assessing *PTEN* status in routine practice.

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Author contributions

X. Matias-Guiu and F. Amant were responsible for the study design. X. Matias-Guiu, O. Maiques, D. Cuevas and A. Velasco performed and interpreted FISH assays. O. Maiques, S. Müller and H. C. Pedersen worked on optimization of the protocol. O. Maiques, D. Cuevas, A. Velasco and M. Romero performed and interpreted MLPA assays. X. Matias-Guiu, O. Maiques, M. Santacana and S. Gatus performed and interpreted immunohistochemical staining. D. A. G. Dios, L. Coenegrachts, D. Lambrechts and F. Amant performed and interpreted SNPA assays. X. Matias-Guiu, O. Maiques and S. Gatus reviewed the pathological data. X. Matias-Guiu and O. Maiques were involved in drafting the manuscript. All authors reviewed the Results and Discussion sections.

Conflicts of interest

The study was performed in a research collaboration with Dako Denmark A/S. The sponsor did not participate in the interpretation of results. Two authors (S. Müller and H. C. Pedersen) work for Dako, and were

involved in optimization of the protocol. The other authors do not have any conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Preanalytical variables.

Figure S2. Optimization of *PTEN* FISH

Figure S3. Preanalytical variables did not have a significant influence on *PTEN* FISH staining.

Table S1. Correlation of MLPA with IHC in 28 cases.