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CHAPTER 7

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Mass Spectrometry-Based Loss of Heterozygosity Analysis of Single-Nucleotide Polymorphism Loci in Paraffin Embedded Tumors Using the MassEXTEND Assay

Single-Nucleotide Polymorphism Loss of Heterozygosity Analysis of the Protein Tyrosine Phosphatase Receptor Type J in Familial Colorectal Cancer

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As the number of identified single-nucleotide polymorphisms (SNPs) increases, high-throughput methods are required to characterize the informative loci in large patient series. We investigated the feasibility of MassEXTEND LOH analysis using Sequenom's MassArray RT software, a mass spectrometry method, as an alternative to determine loss of heterozygosity (LOH). For this purpose, we studied the c.827A>C SNP (1176A>C p.Gln276Pro) in protein tyrosine phosphatase receptor type-J (*PTPRJ*), which is frequently deleted in human cancers. In sporadic colorectal cancer (CRC), c.827A>C showed allele-specific LOH of the c.827A allele, which is important because LOH of *PTPRJ* may be an early event during sporadic CRC. To elucidate the impact of this low-penetrance gene on familial CRC, we studied c.827A>C in 222 familial CRC cases and 156 controls. In 6.2% of the A/C genotyped CRC samples, LOH of c.827A was observed with MassEXTEND LOH analysis and confirmed by conventional sequencing. Furthermore, a case with LOH of c.827A showed no LOH in 22 synchronously detected adenomas, including one with malignant transformation. The importance of the *PTPRJ*- c.827A>C SNP appears to be limited in familial CRC. We conclude that MassEXTEND LOH analysis (using Sequenom's MassARRAY RT software) is a sensitive, high-throughput, and cost-effective method to screen SNP loci for LOH in formalin-fixed paraffin-embedded tissue. *(J Mol Diagn 2005, 7:623–630)*

Loss of heterozygosity (LOH) analysis has been commonly used to provide (indirect) evidence for the presence of a tumor suppressor gene within a genomic region.[1] Standard LOH studies with polymorphic microsatellite markers compare individual allele intensities of normal and tumor DNA. LOH analysis of specific single-nucleotide polymorphism (SNP), however, requires a different approach such as allele-specific amplification or direct sequencing. The former requires thorough optimization of PCR protocols (especially in cases of A/T polymorphisms), whereas the latter is not quantitative. Furthermore, direct sequencing is labor intensive and expensive, with relatively low throughput. For the characterization of the increasing number of informative SNPs in large patient series, high-throughput methods are required. Moreover, for many such series only formalin-fixed paraffin-embedded (FFPE) tissue is available for retrospective testing.

In this study, we used a novel form of LOH analysis, MassEXTEND LOH analysis based on matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).[2,3] This method is less labor intensive and expensive than sequencing with potential for enormous throughput. MALDI-TOF MS has been used to solve a variety of biochemical and molecular genetic questions.[4] The inherent high-molecular weight resolution of MALDI-TOF MS gives high specificity and good signal-to-noise ratio to perform accurate quantification.

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The MassEXTEND LOH analysis introduced here is based on such quality.[5]

In FFPE tissue from familial colorectal cancer (CRC) cases, we have studied LOH of the c.827A >C SNP in protein tyrosine phosphatase receptor type-J (*PTPRJ*). Recently, MALDI TOF MS genotyping of *PTPRJ* was published including limited LOH analysis. No validation for LOH was done, and the spectra were not automatically analyzed.[6]

PTPRJ is a member of the receptor protein tyrosine phosphatases, which play specific and active roles in setting the levels of tyrosine phosphorylation in cells, and as such, they are important in the regulation of many physiological processes.[7] Furthermore, recent mutation analysis in human colorectal cancer suggests that tyrosine phosphatases may function as "true" tumor suppressor genes regulating a wide variety of pathways, which may be susceptible for therapeutic intervention.[8]. In the mouse, *Ptprj* has been identified as a colon cancer susceptibility gene.[9]. Frequent LOH of the *PTPRJ* locus was shown in human sporadic colorectal, breast, and lung tumors[9] and in human thyroid carcinomas.[10] Additionally, Ruivenkamp et al[11] concluded that LOH of *PTPRJ* frequently occurs in the adenoma stage of sporadic human CRC.

The c.827A>C (also known as 1176A>C) SNP in exon 5 of PTPRJ encodes the p.Gln276Pro amino acid change. Preferential loss of the c.827A versus c.827C allele was described, which suggests that the putative "cancer resistance" A allele is lost whereas the (potential less active) C allele is retained in sporadic colorectal cancer.[9]. In this study, we focused on the feasibility of using MassEXTEND LOH analysis to determine LOH of the c.827A>C SNP in FFPE tumor tissue.

We show that the results obtained with the MassEXTEND LOH analysis (using Sequenom's MassARRAY RT software) are as reliable as conventional sequence methods and document the utility of this new technique to detect LOH of a specific SNP in a sensitive, cost-effective manner in FFPE tissue from archival samples. Furthermore, our results suggest limited importance of the c.827A>C polymorphism in familial CRC, including (suspect) Hereditary Non–Polyposis Colorectal Cancer (HNPCC) cases.

Materials and Methods

Cases

At the Unit Molecular Diagnostics, Department of Pathology, Leiden University Medical Center, The Netherlands, 222 cases recorded as familial-CRC (fulfilling either Amsterdam II criteria for HNPCC, Bethesda criteria, or being registered as late onset familial [three or more cases of CRC all diagnosed at age >50 years]) were registered between November 1999 and December 2002. These cases were analyzed following the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (*www.fmwv.nl/gedragcodes/goedgebruik/CodeProperSecondaryUseOfHumanTissue.pdf*).

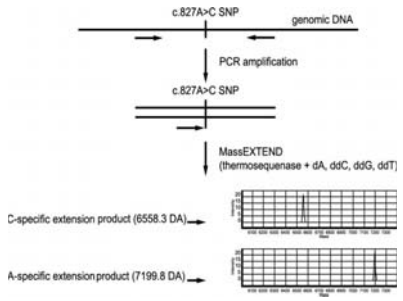

Figure 1. Design of the MassEXTEND genotyping of c.827A>C SNP assay. 1) PCR amplification generated a product including the c.827A>C SNP. 2) MassEXTEND reaction that results in two products with different mass: c.827C allele, 6558.3 d, and c.827A allele, 7199.8 d.

The mean age of diagnosis of the 222 patients was 54 years. Appearance of tumor sites was distributed as follows: coecum, 27; left colon, 15; colon transversum, 3; right colon, 38; sigmoid, 30; recto-sigmoid, 19; and rectum, 35. In 55 cases, the location was unspecified. One hundred and thirty-one cases showed a microsatellite stable phenotype, 88 cases had a microsatellite (MSI) instable (MSI-high, 71; MSI-low, 17) phenotype, and in three cases, the phenotype was unknown. As a control group, lymphocyte DNA of 156 healthy Dutch blood donors was used. Before analysis, MassEXTEND analysis of c.827A>C was validated with a standard control panel of 96 human genomic DNAs (BD Biosciences Clontech).

DNA Isolation

Normal colon, carcinoma tissue was collected as 0.6-mm-diameter punches with a tissue microarrayer (Beecher Instruments, Inc., Sun Prairie, WI) based on evaluation of hematoxylin- and eosin-stained slides. Conventional microdissection (dissection with a needle of selected areas from a 10 μm hematoxylin-stained paraffin slide under microscopic examination with an inverted microscope.) was performed on the 22 adenomas of case 02031. Furthermore, flow sorting was carried out in three carcinomas containing <60% tumor cells (case 02031, 02395, and 01362) and one metastasis (02031).[12] Genomic DNA was isolated from FFPE using a chelex extraction method as described by De Jong et al.[13]

MassEXTEND Genotyping of c.827A>C

DNA samples isolated from normal tissue of the patient and control group were genotyped with the MassEXTEND assay (Sequenom, Inc., San Diego, CA). This SNP scoring is based on the mass difference of allele-specific primer extension products. Design of the assay Figure 1. First, a 110-bp amplicon was generated using a standard PCR protocol with a forward primer, 5′-ACGTTGGATGGTTCAATACAACATCAACCCG-3′, and a reverse primer, 5′-ACGTTGGATGTTGTAACTCACCCAAGCCAC-3′. Note that

the PCR primers incorporate a 10-nucleotide-long generic tag at their 5′ end. Second, the PCR was treated with shrimp alkaline phosphatase (SAP) to remove the dNTPs subsequently. SAP was, in turn, heat inactivated at 80°C for 5 minutes. The primer extension reaction was initiated by the addition of a primer, 5′-ACATCAACCCGTATCTTCTAC-3′, that matches with the target sequence adjacent to the interrogated SNP. Thermosequenase and a substrate mix consisting of dATP and the dideoxynucleotides G, C, and T substrate mix was chosen to maximize the mass difference between all possible extension products, thus facilitating automated calling of the genotypes. Forty rounds of primer extension were performed by temperature cycling. The resulting reactions were treated with a cation-exchange resin (SpectroCLEAN; Sequenom, Inc., San Diego, CA) to remove extraneous salts that interfere with the mass spectrometrical analysis. The amplification, SAP treatment, primer extension reaction, and cleaning step were all performed in a single well of a 384 microtiter plate. Finally, ~15 nl of each reaction was spotted onto the pads of a 384-format SpectroCHIP and subjected to MALDI-TOF MS (Bruker-Sequenom Biflex III array mass spectrometer). In addition to the unextended primer (6285.2 d), a C-specific extension product (5′-ACATCAACCCGTATCTTCTAC-ddC-3′; 6558.3 d), an A-specific extension product (5′-ACATCAACCCGTATCTTCTAC-AAddT-3′; 7199.8 d), as well as two possible polymerase pausing products (5′-ACATCAACCCGTATCTTCTAC-A-3′, 6598.4 d; and 5′-ACATCAACCCGTATCTTCTAC-AA-3′, 6911.6 d) are discernable in the mass spectra. The genotypes were called in real-time using Sequenom's MassARRAY RT software. The assay protocol was validated by means of a commercially available human genomic DNA preparation as well as four representative FFPE samples.

MassEXTEND LOH Analysis of the c.827A>C SNP in PTPRJ

The MassEXTEND assay described above was also used to determine loss of heterozygosity for 64 heterozygous cases. The quantification of the allele-specific mass signals generated in a MassEXTEND assay has previously been exploited to assess SNP allele frequencies in DNA pools.[14] The use of the MassEXTEND assay to measure LOH at the c.827A>C SNP was validated by means of a control experiment among 48 independent measurements of the c.827A>C SNP allele frequencies in a pool of samples (unrelated to the samples of the present study). In 64 cases, paired normal/tumor DNA samples were assayed in triplicate. The analysis of the spectra and the automated quantification of the alleles by comparison of the peak areas were performed with Sequenom's MassARRAY RT software. The C/A frequency ratios for tumor samples were divided by the C/A frequency ratio of the corresponding "normal" tissue. To obtain an allelic imbalance factor, the threshold for LOH was defined as 40% reduction of one allele, equating to a allelic imbalance factor of ≥ 1.7 or ≤ 0.59; the threshold for retention ranged from 0.76 to 1.3; for so-called gray areas with ratios of 0.58 to 0.75 and 1.31 to 1.69, no definitive decision was made.[15,16]

Sorting/Fluorescence Activated Cell Sorter (FACS) Flow Cytometry

On three tumors and one metastasis, with <60% tumor cells, flow sorting was performed following procedures as described previously.[12] For each measurement, data from 10,000 single-cell events were collected using a FACSCalibur (BD Biosciences, San Jose, CA). Propidium iodide fluorescence (DNA stain) was pulse-processed for FL3-area versus FL3-width that enabled us to discriminate single cells from debris (nuclear fragments) and cell aggregates. Simultaneous staining for keratin with anti-keratin antibody AE1/AE3 (Chemicon International, Inc., Temecula, CA), enabled discrimination between keratin-positive tumor cells and keratin-negative stromal and infiltrating inflammatory cells. Data were analyzed using WinList 5.0 and ModFit LT 3.0 software packages (Verity Software House, Inc., Topsham, ME). Cell fractions were sorted using a FACSVantage flow-sorter (BD Biosciences).

LOH Analysis at the PTPRJ Locus with Microsatellite Markers

Four tumors, one metastasis, and one adenoma with malignant transformation with LOH calling using MassEXTEND LOH analysis were tested for conventional LOH at the *PTPRJ* locus using five microsatellite markers: D11NKI01, D11S4117, D11S4183, D11S1350, and D11S1326.[9] The density of the tumor cells varied from 60 to 100% per case. PCR was performed under conditions recommended by the manufacturer (Applied Biosystems, Inc.) with 2 pmol of the primer pairs as mentioned above with exception of D11S1350 from which 10 pmol was used. The following PCR conditions were used in Gene Amp 9700 thermocycler (Applied Biosystems, Inc.): initial denaturation step, 5 minutes at 96°C, followed by 33 cycles of 45 seconds at 94°C, 1.5 minutes at 58°C, and 45 seconds at 72°C thereafter; and a final elongation step of 7 minutes at 72°C was performed. Mixtures of 24 μl of deionized formamid, 1 μl of TAMRA 500 size standard (Applied Biosystems Inc.), and 1.0 μl of PCR product were run on an ABI 310 Genetic Analyzer (Applied Biosystems, Inc.) for 24 minutes with run profile GS STR POP 4 (1.0 ml) C and analyzed with Gene Scan. A threshold characterizes conventional LOH, comparing normal and tumor DNA; this threshold was defined as described under MassEXTEND LOH analysis of the c.827A>C SNP in *PTPRJ*.

PTPRJ Sequencing

Sequencing analysis of PCR products was done at the Leiden Genome Analysis Center. Sequencing reactions were run on an ABI3730 (Applied Biosystems, Inc.) and analyzed with chromas 1.5. (*www.technelysium.com.au/chromas.html*).

Table 1. Distribution of the Genotype c.827A>C SNP in Exon 5 of *PTPRJ*: A/C, A/A, and C/C Genotypes in 222 Familial CRC and Suspected HNPCC Cases Compared with 156 Healthy Blood Donors Showed Comparable Frequencies

Genotype	A/C	A/A	C/C
Control (n = 156)	47	103	6
Normal CRC (n = 222)	64*	149	9
Tumor CRC			
–/C*	4	–	–
A/C	60	–	–
A/–	0	–	–

*In 4 of 64 tested tumors from patients with an c.827A>C genotype, loss of the A allele was detected.

Results

Genotyping of the c.827A>C Polymorphism in PTPRJ Using the MassEXTEND Analysis

The c.827A>C SNP in PTPRJ was genotyped in 156 healthy blood donors and normal DNA from 222 patients with familial CRC (including cases with HNPCC) using the MassEXTEND analysis. The distribution of the three possible genotypes (A/A, A/C, and C/C) was the same in the two groups analyzed (Table 1). The A/A (Figure 2A) genotype was present in 66% of the control cases versus 67% in CRC cases; the A/C genotype (Figure 2C) was present in 30% of the control cases versus 29% in CRC cases; whereas the C/C genotype (Figure 2B) was found in 4% of the control and CRC cases. Among the cancer cases, no significant difference was found among the three genotypes with regard to distant metastases, tumor size, tumor site, age, or MSI status.

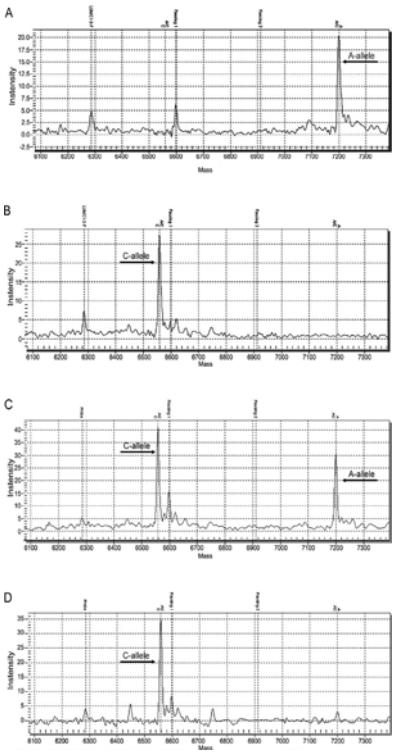

Figure 2. Mass spectra of the three c.827A>C SNP genotypes (**A,** A/A; **B,** C/C; **C,** A/C) and tumor 02031 with loss of the A allele (**D**). The alleles are indicated with thick horizontal **arrows**. Pausing and probe peaks are indicated above the graph.

LOH Analysis of the c.827A>C SNP in PTPRJ with a MassEXTEND LOH Assay and Its Validation

In a control experiment (see Materials and Methods), among 48 independent measurements, the c.827A allele was observed with a frequency of 0.766 ± 0.02 and 0.234 ± 0.02 for the c.827C allele (0.02 is the SD). In the 64 patients with an A/C genotype (Table 1), LOH using the MassEXTEND LOH assay was determined in triplicate (Table 2). The dropout rate was ~5%, and there were no discrepancies among the replicate measurements. In 4 of 64 (6.2%) cases, LOH with selective loss of the A allele was found with Sequenom's MassARRAY RT software; the mean allelic imbalance factors (AIFs) were 6.09, 13.3, 4.72, and 3.60 (A, B, C, and D) (Table 2).

In four carcinomas, LOH was validated using conventional LOH analysis at the *PTPRJ* locus with flanking polymorphic markers. These were two cases with an AIF of, respectively, 6.09 (A) and 13.3 (B) and two cases with ambiguous (gray value) AIFs of 0.65 (E) and 0.59 (F) (Table 2). In tumors (A and B), conventional LOH analysis showed high allelic imbalance in 22 of 23 informative markers with a mean value of 4.78. In those tumors (E and F) with ambiguous MassEXTEND LOH, limited allelic imbalances with conventional markers was seen in enriched tumor cell populations. In all six cases (A through F) (Table 2) and in an additional seven heterozygous tumors without apparent MassEXTEND LOH, the c.827A>C SNP was analyzed by sequencing. Cases A through D clearly show loss of the A allele in tumor cells. In the two tumors (E and F) with ambiguous MassEXTEND LOH values, an A/C heterozygote sequence is identified indicating retention of both the A and C alleles. Seven tumors, with a mean AIF of 1.03, all showed retention of the A and C alleles (G).

Interestingly, all four tumors showing loss of the A allele were microsatellite stable and located in the recto-sigmoid. Case 02031 (A) (Figure 2) demonstrating LOH of the c.827A allele (AIF of 6.09) concerns a 37-year-old female patient with a Dukes C rectal carcinoma and synchronously one separate adenoma with malignant transformation and at least 21 other adenomas (*APC* and *MYH* germline mutation analysis proved negative; C.M. Tops and M.M. Weiss, unpublished results). Flow cytometry analysis of this rectal carcinoma showed two aneuploid keratin-positive tumor cell fractions (one hypo- and one hypertetraploid fraction; Figure 3). Only the hypertetraploid tumor cell fraction was present in one of the lymph-node metastases analyzed (Figure 3). DNA sequencing of the sorted tumor cell fractions confirmed the

Table 2. Validation of the *MassEXTEND* LOH Analysis of the c.827A>C SNP of *PTPRJ* in Tumors with Conventional LOH of the PTPRJ Locus and Sequence Analysis

				MassEXTEND c.827A>C LOH	LOH *PTPRJ* locus					
ID	Sample ID	FACS sorting	Tumor percentage (%)	C/A ratio (AIF)	D11NKI01	D11S4117	D11S4183	D11S1350	D11S1326	PTPRJ sequencing
A*	02031 n.†	No								A/C
	02031 ad. M. transform.‡	No		0.97 (0.89–1.03)	–§	–	–	–	–	A/C
	02031 ca.¶	No	50	6.09 (5.39–6.81)	+‖	+	+	+	+	/C
	02031 ca. (fr1)(ker+)	Yes			+	+	+	±**	+	/C
	02031 ca. (fr2)(ker+)	Yes			+	+	+	+	+	/C
	02031 metastasis (ker+)	Yes			+	+	+	+	+	/C
B	02327 n.	No								A/C
	02327 ca.	No	60	13.3 (11.4–14.9)	+	NA	+	+	+	/C
C	02034 n.	No								A/C
	02034 ca.	No	60	4.72 (3.91–6.04)	NA	NA	NA	NA	NA	/C
D	00040 n.	No								A/C
	00040 ca.	No	60	3.60 (3.50–3.77)	NA	NA	NA	NA	NA	/C
E	02395 n.	No								A/C
	02395 ca.	No	40	0.65 (0.60–0.70)						A/C
	02395 ca.	Yes			–	NA	+	–	NA	A/C
F	01362 n.	No								A/C
	01362 ca.	No	50	0.59 (0.56–0.63)						A/C
	01362 ca.	Yes			+	±	+	±	+	A/C
G	7 ca.	No	>60	1.03 (0.82–1.27)	NA	NA	NA	NA	NA	A/C

*From carcinoma case 02031 with loss of the A allele, 21 additional adenomas were tested with sequence analysis; no loss of the A allele was found in any of these 21 samples.
†n., normal.
‡ad. M. transform., adenoma with malignant transformation.
§Retention AIF 0.76 to 1.3.
¶ca., carcinoma.
‖LOH AIF ≥1.7 or ≤0.59.
**Gray area AIF 0.58 to 0.75 and 1.31 to 1.69.

loss of the c.827A allele in both aneuploid tumor fractions, implying that loss of the A allele most likely was an early event during tumorigenesis. However, sequence analysis and conventional LOH analysis of the 22 adenomas (including MassEXTEND LOH of the adenoma with malignant transformation; Table 2) did not identify LOH of flanking microsatellite markers nor of the c.827 *PTPRJ* alleles (data of the 21 additional adenomas not shown).

Cost-Comparison MassEXTEND LOH Analysis versus Sequencing Analysis

A cost comparison between the on mass spectrometry bases MassEXTEND LOH analysis and sequencing analysis was made in Table 3 on the basis of our facilities. In our setting, the MassEXTEND LOH analysis is ninefold less expensive and the throughput is 10 times higher than conventional sequencing.

Discussion

We have shown that the MassEXTEND (LOH) assay is a reliable and cost-effective method for typing SNPs and detecting LOH of SNP loci using formalin-fixed paraffin-embedded (FFPE) tissue. The automated analysis of the spectra is made possible by Sequenom's MassARRAY RT software. Genotyping with MALDI TOF has already been described by Haff and Smirnov[2] as a high-volume application. Recently, MALDI TOF genotyping of *PTPRJ* is also published including limited LOH analysis, although no validation for LOH was done, and the spectra were not automatically analyzed.[6] For FFPE material, the MassEXTEND (LOH) assay is significantly less labor intensive than direct sequencing analysis (the main alternative for detecting LOH at specific SNP loci in tumors). Furthermore, in our setting, the MassEXTEND LOH assay is ninefold less expensive, and the throughput is 10 times higher than conventional sequencing. Lately, high-throughput SNP tools have become available for mass screening of leukocyte DNA and frozen tumor tissue. Such tools will lead to the identification of new markers for cancer susceptibility, tumor behavior, and prediction of treatment response. When selected markers need to be tested in FFPE, the MassEXTEND (LOH) assay may appear to be an excellent option.

For the *PTPRJ* c.827A>C SNP, we observed a similar distribution in familial CRC patients as in healthy blood donors, not supporting this polymorphism as an evident risk modifier in familial CRC. Recently, preferential loss of

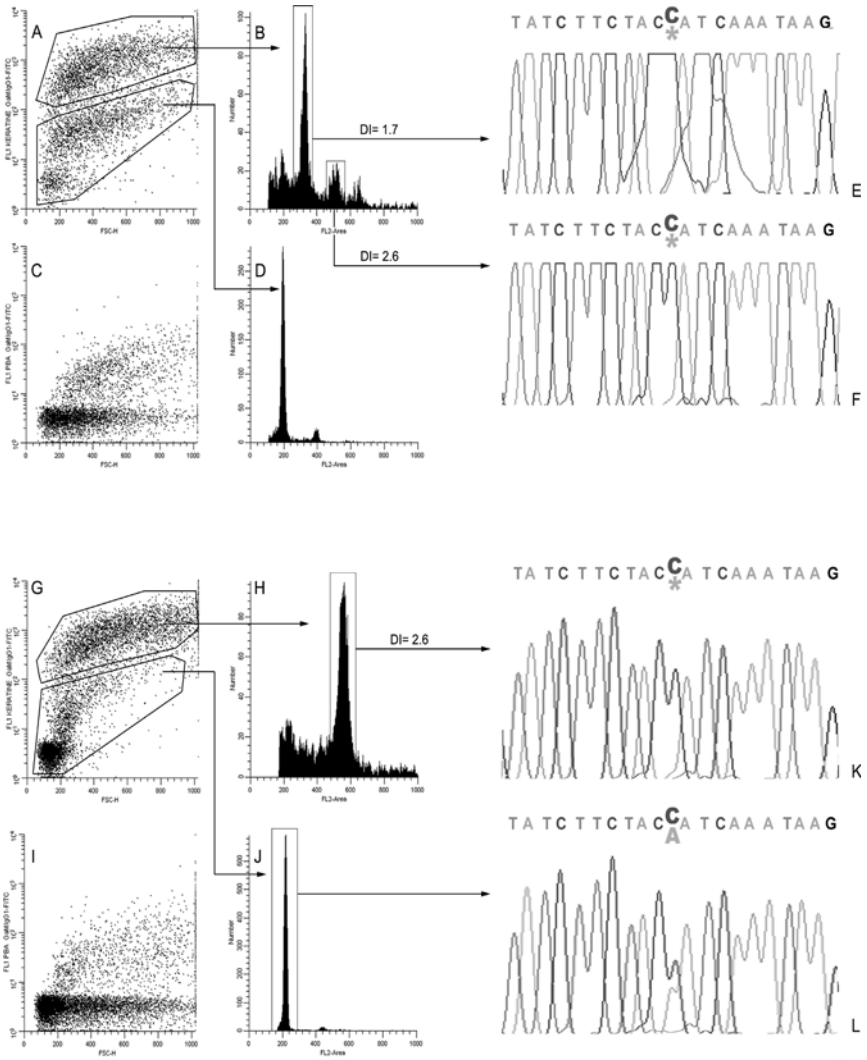

Figure 3. Sequence analysis of the c.827A>C SNP of *PTPRJ* of flow-sorted cell populations. Distinct cell populations were flow-sorted from the formalin-fixed paraffin-embedded primary tumor and lymph-node metastasis of case 02031. **A–F:** Primary tumor. **A:** Keratin positive (K pos.) cells can be clearly identified in the forward scatter versus keratin dot plot, compared with a negative control (**C**). **B:** After gating on the K pos. cells, a bimodal DNA histogram can be observed with two dominant cycling populations with a DNA index of 1.7 and 2.6, respectively. **D:** The Keratin negative (K neg.) cells, comprising inflammatory and stromal cells, revealed an unimodal DNA diploid histogram.[17] **E** and **F:** Sequence analysis of fraction ID 1.7 and fraction ID 2.6 showed loss of the A allele in both populations. **G–L:** Lymph-node metastasis. **G:** Forward scatter versus keratin dot plot. **I:** Negative control. **H:** Gating on the K pos. cells shows an unimodal DNA histogram with a DNA index of 2.6. These cells probably branched from the second DNA aneuploid population (DI = 2.6) of the primary tumor. **J:** Unimodal DNA diploid histogram of the K neg. cells. **K:** Sequence analysis of ID 2.6 fraction showed loss of the A allele. **L:** The K neg. cells are diploid and show the normal A/C genotype.

the putative cancer resistance allele c.827CA versus the potentially less active c.827C allele was shown in sporadic CRC of heterozygote c.827A>C patients.[9] Our study demonstrates that also in familial CRC, the A allele is preferentially lost, however, only 4 of 64 heterozygotes (6.25%) lost the A allele. The C allele was retained in all cases. Interestingly, loss of the A allele was only found in patients with microsatellite stable tumors that were located in the recto-sigmoid. The percentage of loss of c.827A>C in our study is much lower than the percentages published for CRC of 49 and 71%, respectively.[9,11] This discrepancy might partly be explained by technical reasons; we used a more stringent threshold for LOH, 40% instead of a 20 to 30% reduction of one allele when comparing normal and tumor DNA.[15,16] An additional explanation is that the tumors analyzed for LOH of c.827A>C by Ruivenkamp et al[9] had been preselected for LOH using flanking polymorphic markers. Further-

Table 3. Cost Comparison between the Mass Spectrometry Bases MassEXTEND LOH Analysis and Sequencing Analysis

	Mass spectrometry	Sequencing
Equipment	Brucker-Sequenom Biflex III array mass spectrometer	ABI prism 3730 genetic analyzer
Hands on time per sample	7 s (~6 hours for 3072 samples)	72 s (~6 hours for 288 samples)
Turn around time per sample	30 s (~24 hours for 3072 samples*)	300 s (~24 hours for 288 samples*)
Data analysis per sample	Negligible	30 s
Cost per reaction industrial laboratory	€ 2.00	€ 18.00
Cost per reaction academic laboratory	€ 0.30	€ 3.96
Instrument throughput (samples per day)	7680	960

*The number of samples that can be done in 1 day, assuming there are no limitations in equipment (PCR machines, etc.) and in people.
s, seconds.

more, we studied LOH of the c.827A allele in a cohort of familial CRC cases compared with sporadic colorectal cancer in other studies. Our results suggest that the c.827A>C plays a limited role in familial CRC and (suspect) HNPCC.

We did not detect any LOH of the *PTPRJ* locus using flanking markers or loss for the A1176 SNP allele in 21 early adenomas and 1 adenoma with malignant transformation, from one single case, having loss of the c.827A allele in a synchronous rectal carcinoma. This would appear to be in contrast with previous findings, suggesting loss of PTPRJ to be an early event in colon tumor development, ie, in the adenomatous stage.[11] Additionally, we conclude that in this case, the loss of the c.827A allele must be a relatively early event although only to have occurred in an early carcinoma phase. This conclusion is based on the observation that in all carcinoma cell fractions (a hypotetraploid and a hypertetraploid cell fraction, the latter of which was also found in a metastasis analyzed), loss of the c.827A allele was found. However, we cannot rule out that the clone with LOH could propagate so rapidly that it might have completely wiped out all non-LOH clones.

We show that the results obtained with the MassEXTEND LOH analysis are as reliable as conventional sequence methods, and we document the utility of this new technique to detect LOH of a specific SNP in a sensitive and automated manner in FFPE tissue from archival samples. Furthermore, our results suggest limited importance of the c.827A>C polymorphism in familial CRC, including (suspect) HNPCC cases.

The practical feasibility of the MassEXTEND LOH analysis in a basic molecular diagnostic laboratory on a routine day-to-day basis is limited and must be placed in verification of data in large series of cases. Examples might be the analysis of SNP profiles that, eg, determine chemosensitivity of all sorts of tumors that could be translated in use for daily practice.

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