

Laser Pressure Catapulting (LPC): Optimization LPC-System and Genotyping of Colorectal Carcinomas

VIVIANA BAZAN,¹ GASPARE LA ROCCA,¹ SIMONA CORSALE,¹ VALENTINA AGNESE,¹
MARCELLA MACALUSO,^{1,4} MANUELA MIGLIAVACCA,² VALTER GREGORIO,¹ SANDRA CASCIO,¹
PASQUA SANDRA SISTO,¹ GAETANA DI FEDE,¹ MARIA BUSCEMI,² EUGENIO FIORENTINO,¹
RITA PASSANTINO,³ VINCENZA MORELLO,³ ROSA MARIA TOMASINO,³ AND ANTONIO RUSSO^{1*}

¹Department of Oncology, Università di Palermo, Palermo, Italy

²Institute of Experimental Medicine, Università di Palermo, Palermo, Italy

³Institute of Pathology, Università di Palermo, Palermo, Italy

⁴Sbarro Institute for Cancer Research and Molecular Medicine,
College of Science and Technology, Temple University, Philadelphia, Pennsylvania

Genotype analysis is becoming more and more useful in clinical practice, since specific mutations in tumors often correlate with prognosis and/or therapeutic response. Unfortunately, current molecular analytical techniques often require time-consuming and costly steps of analysis, thus making their routine clinical use difficult. Moreover, one of the most difficult problems arising during tumor research is that of their cell heterogeneity, which depends on their clear molecular heterogeneity. SSCP analysis discriminates by means of aberrant electrophoresis migration bands, mutated alleles which may represent as little as 15–20% of their total number. Nevertheless, in order to identify by sequencing the type of alteration revealed by this technique, only the mutated allele must be isolated. The advent of laser microdissection is a procedure which easily solves these problems of accuracy, costs, and time. The aims of this study were to perfect the system of laser pressure catapulting (LPC) laser microdissection for the assessment of the mutational status of *p53* and *k-ras* genes in a consecutive series of 67 patients with colorectal carcinomas (CRC), in order to compare this technique with that involving hand-dissection and to demonstrate that since the LPC system guarantees more accurate biomolecular analyses, it should become part of clinical routine in this field. The LPC-system was perfected with the use of mineral oil and the LPC-membrane. To compare the techniques of hand- and LPC-microdissection, alcohol-fixed, paraffin-embedded tissue from 67 cases of CRC were both hand- and laser-microdissected. In either case, dissected samples were analyzed by SSCP/sequencing and direct sequencing for *k-ras* and *p53* gene mutations. LPC-microdissection made it possible to pick up mutations by direct sequencing or SSCP/sequencing, whereas hand-microdissection mutations were identified only by means of SSCP followed by sequencing; direct sequencing did not reveal any mutation. In the 67 patients examined by either method, 36% (24/67) showed *p53* mutations, 32 of which identified. Seventy-eight percent (25/32) were found in the conserved areas of the gene, while 12% (4/32) were in the L2 loop, 50% (16/32) were in the L3 loop, and 12% (4/32) in the LSH motif of the protein. Moreover, of the 67 cases examined, 40% (27/67) showed mutations in *k-ras*, with a total of 29 mutations identified. Of these, 14 (48%) were found in codon 12 and 15 (52%) in codon 13. The modifications which we brought to the LPC system led to a vast improvement of the technique, making it an ideal substitution for hand-microdissection and guaranteeing a considerable number of advantages regarding facility, accuracy, time, and cost. Furthermore, the data obtained from the

The authorship is shared equally by Viviana Bazan and Gaspare La Rocca.

Contract grant sponsor: CNR-MIUR (Strategic Project in Oncology (PSO); Contract grant number: 02.00334-ST97; Contract grant sponsor: Italian Association for Cancer Research (AIRC).

*Correspondence to: Antonio Russo, Via Veneto 5, 90144 Palermo, Italy. E-mail: Lab-oncobiologia@usa.net

Received 17 March 2004; Accepted 4 May 2004

DOI: 10.1002/jcp.20150

mutational analyses performed confirm that the LPC system is more efficient and rapid than hand-microdissection for acquiring useful information regarding molecular profile and can therefore be used with success in clinical routine. J. Cell. Physiol. 202: 503–509, 2005. © 2004 Wiley-Liss, Inc.

One of the problems involved in the understanding of tumor progression is how to integrate the information obtained in molecular analysis with the morphological and structural results of histological examinations. The number of tumor cells may often represent less than 50% of the total tumor tissue cell population, which means that normal molecular techniques do not always offer analytical accuracy and may not correspond with the histological results.

The more homogeneous the sample examined is, the more accurate the molecular analysis will be. In the analysis of mRNA or protein expression, it is absolutely essential to start off with homogeneous material; in fact, an examination performed on different, mixed cell populations with potentially different expression values of any particular gene, would give a result presenting only the arithmetic mean of the real values of each single cell type, and the data obtained would have very little significance (Paweletz et al., 2001). Furthermore, genomic analysis of homogeneous samples might require more detailed information about a tissue's specificity for a particular genetic alteration. Different mutational patterns might be present in the endothelial tissue and in the adjacent fibroblasts, and the neighboring glandular epithelium of colorectal carcinoma (CRC), at various stages of differentiation, might present a different mutational status in specific genes (Kinzler and Vogelstein, 2002).

Moreover, the molecular profile may be of great help in refining diagnostic and prognostic assessment. The mutational status of a small group of selected genes may, in fact, be useful in determining tumor relatedness and the profile of certain proteins involved in therapeutic response may help in the clinical definition of morphological parameters of prognosis (Sirivatanakorn et al., 1999). Unfortunately, the molecular screening assays used up till now involve time-consuming and costly steps of analysis, so that their routine clinical use is impractical.

For all the above reasons, in the last few years there have been several attempts to devise a rapid technique which might make it possible to obtain information regarding genes and/or proteins on a fairly large amount of good quality, extremely homogeneous sample material (Ellsworth et al., 2003; Judex et al., 2003). At the present time, laser microdissection would seem to be the most rapid and efficient system for the selection of cells from complex normal and diseased tissues (Moskaluk and Kern, 1997; Dillon et al., 2001; Grant and Jerome, 2002; Greene et al., 2003).

In the light of this, therefore, the aims of this study were to perfect the laser pressure catapulting (LPC) laser microdissection system and use it for the assessment of the mutational status of *p53* and *k-ras* genes in a consecutive series of 67 patients with CRC in order to compare this method with the hand-microdissection technique and demonstrate that the use of the LPC

system greatly improves the accuracy of biomolecular analyses and should therefore be included in routine clinical practice.

MATERIALS AND METHODS

Patient features. A prospective study was performed on paired tumor and normal colon tissue samples from a consecutive series of 67 patients undergoing resective surgery for primary operable CRC at a single institution (Department of Oncology, University of Palermo).

Eligibility criteria used were: (a) electively resected primary CRC, (b) processing of fresh paired normal mucosa-tumor samples within 30 min after tumor removal, (c) available DNA from normal and tumor tissue for biomolecular analyses, and (d) access to accurate follow-up information. Briefly, the following exclusion criteria were used: (a) history of previous neoplasias, (b) patients from families with familial adenomatous polyposis or hereditary nonpolyposis CRC with a highly penetrant genetic predisposition to CRC, (c) synchronous or metachronous CRC, and (d) chemotherapy or radiation therapy prior to surgery. A resection of the primary CRC was performed in all cases. In order to avoid evaluator variability in the patients, all resection specimens and microscopic slides were meticulously examined by two independent pathologists (RMT and VM) who were not aware of the original diagnosis and of the results of the molecular analyses. The complete excision of the primary tumor was proven by the histopathological examination on 3–5 consecutive 5 μm -thick sections of the proximal and distal resected margins, respectively. All tumors were histologically confirmed to be CRC. Written informed consent was obtained from all patients included in this study. Tissues were fixed in 70% ethyl alcohol and paraffin-embedded for biomolecular examination.

Tissue preparation

Five-micrometer sections, prepared using a microtome, were mounted on the supporting LPC membrane already placed on the slide. Subsequent dehydration was achieved using graded alcohol and xylene treatment as follows: 95% ethanol for 30 sec (times 2), 100% ethanol for 30 sec (times 2), and xylene for 5 min (2 times). The slides were dried under a laminar flow for 10 min and then stained with hematoxylin and eosin.

Hand-microdissection

The different regions of the tumor were identified and marked on 5 μm sections and were carefully scraped with a sterile scalpel from the adjacent 20 μm unstained paraffin sections.

LPC

LPC was performed using a Zeiss inverted microscope PALM Laser Micro-Beam System UV laser at 337 nm, linked to a PC with the required software programs.

Before performing microdissection, 1 μ l of mineral oil was dripped onto the CRC samples. The areas to be dissected were selected by means of extremely high-precision microcuts (the specimens may range from as little as 1 to 1,000 μ m in diameter). After cutting, the high photonic pressure force of the laser beam was used to impress a certain amount of kinetic energy onto the isolated material—the beam hits the tissue from below and catapults it upwards at high speed for several millimeters within the appropriately-placed capture support (the cap of a common Eppendorf-tube). LPC dissection was performed using a few shots each of 100 μ m in diameter. After the catapulting the material was removed from the cap for analysis.

DNA extraction

Genomic DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) with the standard protocol from primary CRC and normal colorectal specimens.

Detection of *k-ras* and *p53* gene mutations by SSCP/sequencing

Mutations within the *k-ras* and *p53* genes were detected by SSCP analysis following PCR amplification of the exons 1 for *k-ras* and of the exons 5–8 for *p53* respectively, performed as previously described (Bazan et al., 2002; Russo et al., 2002). In every instance, negative (DNA was replaced with water) controls were amplified by PCR and included in the experiment. In all PCR assays aerosol-resistant pipette tips were used to avoid cross-contamination. The quality and the concentration of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium staining. One hundred nanogram aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA) were denatured and analyzed by SSCP analysis. DNA of normal colon tissue from each patient was also amplified and run in parallel with matched tumoral DNA samples on SSCP gels, to evaluate the occurrence of germ-line mutations or polymorphisms. Individual ssDNA fragments with shifted mobilities, compared to normal control, were electroeluted from polyacrylamide gel, as described previously (Albanese et al., 1997), reamplified and sequenced. Automated sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the model 3100 DNA sequencer (Perkin-Elmer, Foster City, CA).

Detection of *k-ras* and *p53* gene mutations by sequencing

Mutations within the *k-ras* and *p53* genes were detected by direct sequencing following PCR amplification. The same exons of *k-ras* (exon 1) and of *p53* (exons 5–8) were amplified as previously described, and automated sequencing was performed using the same sequencing kit and the same sequencer.

RESULTS

Optimization of the LPC-system

The addition of 1 μ l of mineral oil dripped onto 5 μ m sections of mucosa and of CRC mounted on a supporting membrane greatly improved visibility (Fig. 1), thus

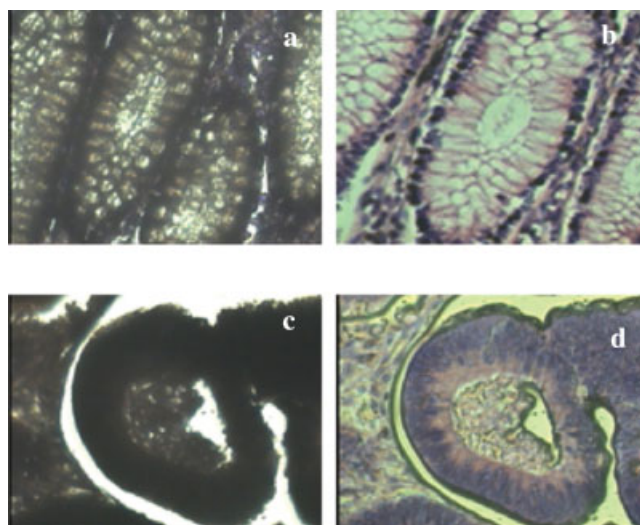


Fig. 1. Typical image of the progress of the oil spreading throughout normal tissue and throughout tumoral tissue (H&E staining; magnification: $\times 400$). **a**: Normal human mucosa without oil; **b**) note the good quality of the image after the oil has spread in the tissue; **c**) tumoral tissue without oil; **d**) note the good quality of the image after the oil has spread in the tissue.

guaranteeing a clearer discrimination of the different zones under observation and also facilitating the cutting and subsequent catapulting procedures (Fig. 2).

Mutation analysis of *p53* gene

Mutation analysis of exons 5–8 of the *p53* gene was performed on genomic DNA from primary CRCs of 67 patients by PCR-SSCP/sequencing and by PCR/direct sequencing. Tumor tissue was obtained by hand- and by LPC-microdissection. Aberrantly migrating bands were found in 36% (24/67) of the cases (Fig. 3) with a total of 32 mutations. The presence of abnormal bands was assessed in at least two independent SSCP analyses. Sequence analysis of the DNA fragments with altered electrophoretic mobility made it possible to establish the exact site and nature of the genetic alteration. The features of the *p53* mutations are summarized in Table 1. Of the 32 mutations, 32% (10 of 32) were in exon 5, 6% (2 of 32) in exon 6, 47% (15 of 32) in exon 7, and 16% (5 of 32) in exon 8. Furthermore, 25% (8/32) of the mutations were frameshift, of which 7 were deletions and one was insertion; 1.75% (24/32) were single-nucleotide substitutions distributed as follows: 23 missense and 1 silent mutations in codon 249 previously identified as the site of polymorphism. Moreover, transitions were of 56% (18/32) and transversions of 19% (6/32). No germ-line mutations were found, indicating that in every case the change was somatic. Twenty-five mutations (78%) were in the conserved areas, 2 of which in area II, 3 in area III, 15 in area IV, and 5 in area V. In addition, by taking into account the specific functional and structural domains of *p53* affected by the mutations, the cases were also classified as follows: 12% (4/32) in the L2 loop, 50% (16/32) in the L3 loop, 12% (4/32) in the LSH motif of the protein, and 26% (8/32) with mutations outside L2, L3, and LSH.

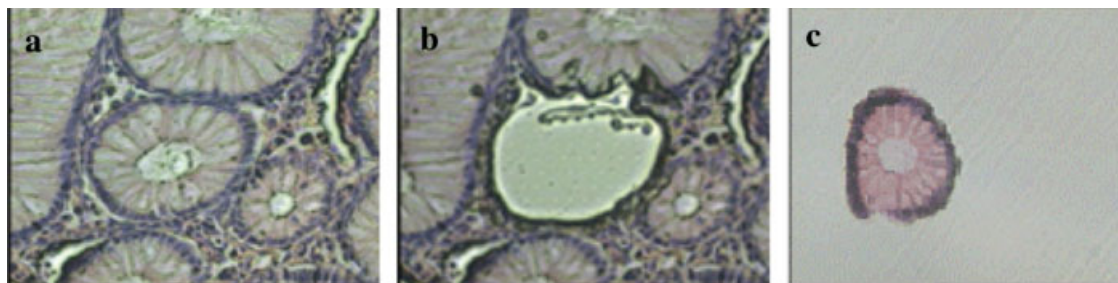


Fig. 2. Typical image of the laser pressure catapulting (LPC) process involving an epithelial gland of tissue placed on a slide together with a membrane after the addition of 1 μ l of mineral oil. **a:** Improvement of tissue visibility after the addition of oil on the slide with the membrane; **(b–c)** cutting and catapulting of the gland; **(c)** catapulted gland on the Eppendorf cap.

Hand-microdissection did not make it possible to pick up any mutation, which could only be identified by means of subsequent SSCP and sequencing. The use of the LPC microdissection technique revealed mutations with both the SSCP and PCR/direct sequencing techniques (Fig. 4).

Mutation analysis of the *k-ras* gene

Mutation analysis of exon 1 of the *k-ras* gene was performed on genomic DNA from primary CRCs of 67 patients by PCR-SSCP/sequencing and by PCR/direct sequencing. Tumor tissue was obtained by hand- and by LPC-microdissection. Aberrantly migrating bands were found in 40% (27/67) of the cases with a total of 29 mutations. The presence of abnormal was assessed in at least two independent SSCP analyses. Sequence analysis of the DNA fragments with altered electrophoretic mobility made it possible to establish the exact site and nature of the genetic alteration in all the tumor samples. The features of the *k-ras* mutations are summarized in Table 2. Of the 29 mutations, 14 (48%) were found in codon 12, and 15 (52%) in codon 13; 25/27 tumors presented single mutations, 2/27 a mutation in codon 12 and one in codon 13. No mutations were detected at any other site of the first exon. The most frequent mutation in codon 12 was GGT to GAT (Gly \rightarrow Asp), which was observed in 5/14 mutations (36%). The other mutations observed in the same codon resulted in replacement of glycine with valine (GGT to GTT, 4/14 cases, 29%), or cysteine (GGT to TGT, 3/14 cases 21%), or serine (GGT to

AGT, 2/14 cases 14%). The only mutation in codon 13 was GGC to GAC (Gly \rightarrow Asp) which was detected in all cases (100%). Overall, transitions (81%, 22 of 27) were far more frequent than transversions (19%). No germline mutations were found, indicating that in every case the change was somatic. Once again in this case hand-microdissection did not permit direct sequencing to pick up any mutations, which were only identified by means of SSCP followed by sequencing, whereas LPC microdissection made it possible to find mutations with both techniques (SSCP or PCR/direct sequencing).

DISCUSSION

An extremely important advantage of the LPC system is that it cuts out all contact between the operator and the prepared sample, which means an enormous reduction in human error and eliminates the risk of damage being caused by possible contamination or infection (Bernsen et al., 1998; Schindler, 1998; Hunt et al., 2003).

One of the basic problems of the LPC system is ensuring clear visibility of the prepared specimen. Since laser microdissection does not permit the use of a cover-slide, which would prevent the selected cells from rising up towards the capture cap, in most cases the specimen appears dark and opaque. This fact is due to non-specific scattering of light, that is, to the lack of refractive index matching between the desiccated tissue and the interstitial air. This means that it is more or less impossible to identify particular cytological features. In order to improve visibility in the LPC system, a membrane can be placed on the dissected tissue, thus mimicking the presence of a cover-slide, without, however, blocking the catapulting procedure, since the membrane can be cut and transferred into the capture cap together with the tissue (Nagasawa et al., 2000). Nevertheless, this procedure, which is intended to improve visibility, has a serious disadvantage; once the selected specimen has been cut, air may get between the membrane and the tissue, once again reducing visibility of the adjacent areas which are thus no longer suitable for the subsequent microdissection. Moreover, the procedure followed for mounting the membrane must be performed extremely carefully in order not to destroy the tissue and to avoid the formation of air bubbles.

Since the mineral oil permits a specific scattering of light, we also dripped a few drops onto the specimen itself in order to improve visibility even more. After a minute or two, the oil spreads throughout the whole

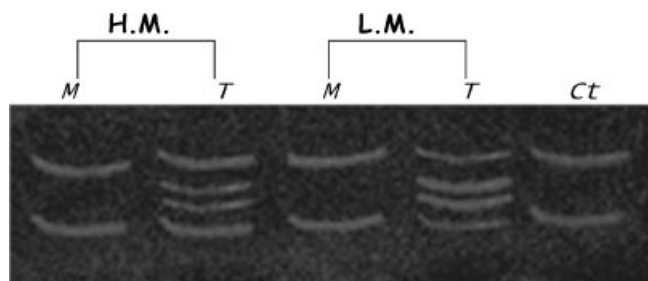


Fig. 3. SSCP analysis of exon 5 of *p53* gene in colon mucosa (M) and colorectal carcinomas (CRC) (T) from 67 samples. The sample extracted by hand microdissection (H.M.) can be seen in lanes 1 and 2, while lanes 3 and 4 show the samples extracted by LPC microdissection (L.M.). Lane 5 shows a control sample.

TABLE 1. Distribution of 32 mutations identified in *p53* gene exons 5–8 in 67 CRCs

Sample	Mutated exon	Mutated codon	Nucleotide change	Aminoacid change	Type mutation	CD	Zn-BD
13 K	8	273	cgt → cat	arg → his	Missense	Y	LSH
26 K	7	238+	tgt → tat	cys → tyr	Missense	Y	L3
		249	agg → ag-	arg →	Frameshift	Y	L3
27 K	5	155	acc → ac-	thr →	Frameshift	N	
28 K	5	174	agg → aag	arg → lys	Missense	Y	L2
36 K	5	158	cgc → cac	arg → his	Missense	N	
70 K	7	244+	ggc → agc	gly → ser	Missense	Y	L3
		249	agg → ag-	arg →	Frameshift	Y	L3
77 K	8	272	gtg → atg	val → met	Missense	Y	
114 K	5+	141	tgc → cgc	cys → arg	Missense	Y	
	6	204	gag → tag	glu → STOP	Nonsense	N	
125 K	7	248	cgg → tgg	arg → trp	Missense	Y	L3
147 K	8	282	cgg → tgg	arg → trp	Missense	Y	LSH
195 K	7	244+	ggc → agc	gly → ser	Missense	Y	L3
		249	agg → ag-	arg →	Frameshift	Y	L3
338 K	7	244	ggc → agc	gly → ser	Missense	Y	L3
351 K	5	152	ccc → cccc	pro →	Frameshift	N	
398 K	6+	220	tat → tgt	tyr → cys	Missense	N	L3
	7	244+	ggc → agc	gly → ser	Missense	Y	L3
		249	agg → ag-	arg →	frameshif	Y	L3
406 K	5	174	agg → aag	arg → lys	Missense	Y	L2
421 K	5	177	ccc → cc-	pro →	Frameshift	Y	L2
452 K	7	248	cgg → tgg	arg → trp	Missense	Y	L3
463 K	8	273	cgt → cat	arg → his	Missense	Y	LSH
499 K	8	278	cct → tct	pro → ser	Missense	Y	LSH
627 K	5	151	ccc → acc	pro → thr	Missense	N	
678 K	7	244+	ggc → gcc	gly → ala	Missense	Y	L3
		249	agg → cgg	arg → arg	Silent	Y	L3
706 K	5	163	tac → ttc	tyr → phe	Missense	N	L2
757 K	5	141	tgc → tcc	cys → ser	Missense	Y	
817 K	7	244+	ggc → agc	gly → ser	Missense	Y	L3
		249	agg → ag-	arg →	Frameshift	Y	L3

specimen, when it is then possible to obtain a perfect vision of the morphology of the tissue and to make an accurate choice of the zone to be microdissected (Fig. 1). In some cases, it may happen that the oil used in this way stops the material isolated by the laser ray from reaching the cap even though it has been detached from the surface of the slide: in this case, the tissue appears too soft, and the fragments floating about in the oil are

difficult to hit with the tiny catapult, requiring a large number of shots before all of them are captured. These difficulties regarding transfer problems were solved by mounting the tissue on the supporting LPC-membrane, thus transferring even a wide selected zone with a single shot, in spite of the presence of the mineral oil (Fig. 1).

One of the most difficult problems arising during tumor research is that of their cell heterogeneity, which

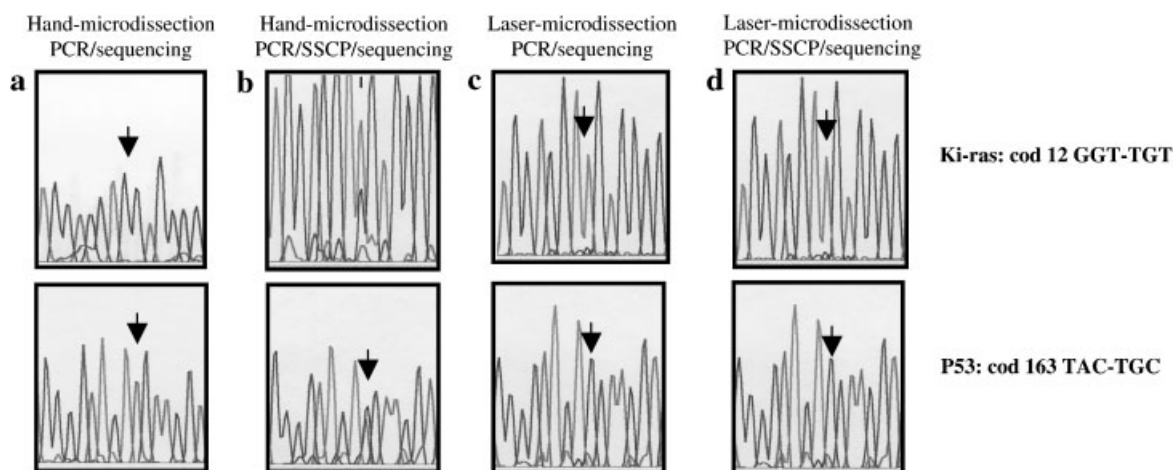


Fig. 4. Comparison between mutational analysis of genes *k-ras* and *p53* by means of hand-microdissection and with the use of the LPC system. With the former technique, direct sequencing showed no mutations (a), which were picked up only by means of sequencing following the SSCP technique (b); LPC-microdissection made it possible to identify mutations with both techniques (direct sequencing or SSCP/sequencing) (c–d).

TABLE 2. Distribution of 29 mutations identified in *k-ras* gene exon 1 in 67 CRCs

Mutated codon	Type of mutation	Mutated sequence (aminoacid)	No. of mutations (%)
12	Transitions (1st pos.) (2nd pos.)	GGT-AGT (Gly-Ser)	2 (14)
		GGT-GAT (Gly-Asp)	5 (36)
	Transversions (1st pos.) (2nd pos.)	GGT-TGT (Gly-Cys)	3 (21)
		GGT-GTT (Gly-Val)	4 (29)
	Total mutations		14
13	Transitions (2nd pos.)	GGC-GAC (Gly-Asp)	15 (100)
Total mutations			15

depends on their clear molecular heterogeneity (Moyret et al., 1994; Keohavong et al., 2004). The multi-step model has revealed that a neoplasia develops progressively as a result of the accumulation of various alterations of at least five or six different genes implicated in cell cycle control, differentiation and apoptosis (programmed cell death) (Kinzler and Vogelstein, 2002). This fact not only explains why the tumor mass often presents with cell sub-clones which may be extremely different one from the other, both with regard to phenotype and genotype, but is also why the routine clinical use of biomolecular analysis is so enormously complicated. Furthermore, in order to be really accurate, tumor genotyping requires a series of costly and time-consuming steps.

Before the technique of laser microdissection was developed, tumor cells would be isolated onto a slide by means of manual microdissection; this meant that the selected zone often contained a significant proportion of other cell types which weaken the tumor-specific signals. Mutational screening requires clear discrimination between tumor-correlated genic alterations and wild-type genotypes. Unfortunately, mutational analysis performed with the use of direct sequencing permitting PCR is impeded by the presence of wild-type phenotype, and therefore genotype, cells. Thus throughout the years, several other, alternative mutational assays which by-pass this problem have been preferred; of these, the one with the highest sensitivity and specificity has proved to be the SSCP (Hongyo et al., 1993; Moyret et al., 1994; Soong and Iacopetta, 1997; Kutach et al., 1999; Keohavong et al., 2004). This analysis permits PCR and, in fact, discriminates by means of aberrant electrophoresis migration bands, mutated alleles which may represent as little as 15–20% of all the alleles. Nevertheless, in order to identify by sequencing the type of alteration revealed by this technique, only the mutated allele must be isolated, which means that the band with altered electrophoretic migration must be cut, and its DNA eluted, purified, and reamplified, in order to obtain a sufficient quantity for sequencing. All of this obviously takes a great deal of time and money and may also lead to loss of the sample.

The advent of laser microdissection is a procedure which easily solves these problems of accuracy, costs, and time.

In a group of 67 cases of CRC, we compared the results obtained with the technique of hand-microdissection for the mutational analysis of *k-ras* and *p53* genes with those obtained with the use of the LPC system. We analyzed the DNA obtained with both techniques, one

part by means of direct sequencing and one part with the SSCP analysis, followed by sequencing performed in various preparatory steps. Of the 67 cases analyzed, 40 proved to be wild-type when mutational analysis was performed by sequencing or by SSCP/sequencing and preceded by hand microdissection or by LPC-microdissection.

Of the 67 cases examined, 36% (24/67) showed mutations in *p53* and 40% (27/67) in *k-ras* (Fig. 4).

Following hand-microdissection, the mutations were identified only by means of SSCP/sequencing (Fig. 4b); direct sequencing did not show up any mutations (Fig. 4a). On the contrary, LPC-microdissection made it possible to identify the mutations with either technique (direct sequencing or SSCP/sequencing) (Fig. 3c,d), confirming the validity of the LPC system to reduce the time required for mutational analysis. As Figure 3, in fact, shows, when the specimen is hand-microdissected, there is a strong likelihood of scraping normal cells, that is, wild-type alleles, together with the tumor cells, and therefore mutated alleles, which means that it is impossible to establish their molecular type by means of simple direct sequencing. The selection of tumoral cells by means of LPC, however, results in a number of tumoral versus wild-type alleles which it is possible to identify by means of direct sequencing.

CONCLUSIONS

Laser microdissection systems have now taken the place of needle manipulation for scraping off cells from the sample, leading to great improvement in the technique; the LPC-system is particularly quick and efficient, and its use makes it possible to isolate a smaller number of homogeneous cells; this method is less dependent on the operator's skill and not as subject to possible contaminations as other techniques, since it entirely eliminates contact between the operator and the prepared sample (Schutze et al., 1998). LPC-microdissection makes it possible to perform extremely high-precision microcuts, thus improving the quality of the material obtained and guaranteeing a more accurate biomolecular characterization of samples. Moreover, the use of the LPC-system leads to a considerable reduction in the time and costs required for biomolecular profile analysis.

This technique, used together with other modern methods of biomolecular analysis, guarantees a more efficient and accurate genotyping of tumor samples, and improves the stratification of patients with CRC, thus permitting a more personalized therapeutic approach. If in the future our results are confirmed by further clinical

studies involving a larger number of cases, the LPC technique could be used in future routine clinical procedures.

ACKNOWLEDGMENTS

We would like to thank Mrs. Pamela Gardner for help in the preparation of the text.

LITERATURE CITED

- Albanese I, Rinaudo CD, Alberti M, Bazan V, Russo A, Migliavacca M, Bazan P, Dardanoni G, Tomasino RM, La Farina M. 1997. Ras and myc analysis in primary and metastatic colorectal carcinomas: Specific ras mutations are associated with Dukes'D stage and mucinous histotype. *Int J Oncol* 10:293–300.
- Bazan V, Migliavacca M, Zanna I, Tubiolo C, Grassi N, Latteri MA, La Farina M, Albanese I, Dardanoni G, Salerno S, Tomasino RM, Labianca R, Gebbia N, Russo A. 2002. Specific codon 13 K-ras mutations are predictive of clinical outcome in colorectal cancer patients, whereas codon 12 K-ras mutations are associated with mucinous histotype. *Ann Oncol* 13:1438–1446.
- Bernsen MR, Dijkman HB, de Vries E, Figdor CG, Ruiter DJ, Adema GJ, van Muijen GN. 1998. Identification of multiple mRNA and DNA sequences from small tissue samples isolated by laser-assisted microdissection. *Lab Invest* 78:1267–1273.
- Dillon D, Zheng K, Costa J. 2001. Rapid, efficient genotyping of clinical tumor samples by laser-capture microdissection\PCR\SSCP. *Exp Mol Pathol* 70:195–2001.
- Ellsworth DL, Shriver CD, Ellsworth RE, Deyarmin B, Somiari RI. 2003. Laser capture microdissection of paraffin-embedded tissues. *Biotechniques* 34:42–44.
- Grant K, Jerome WG. 2002. Laser capture microdissection as an aid to ultrastructural analysis. *Microsc Microanal* 8:170–175.
- Greene S, Stockton P, Kozyreva OG, Gaston KE, Flake GP, Mohler JL. 2003. Mutational analysis of the androgen receptor using laser capture microdissection and direct sequencing. *Methods Mol Biol* 218:287–302.
- Hongyo T, Buzard GS, Calvert RJ, Weghorst CM. 1993. 'Cold SSCP': A simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. *Nucleic Acids Res* 21:3637–3642.
- Hunt JL, Swalsky P, Sasatomi E, Niehouse L, Bakker A, Finkelstein SD. 2003. A microdissection and molecular genotyping assay to confirm the identity of tissue floaters in paraffin-embedded tissue blocks. *Arch Pathol Lab Med* 127:213–217.
- Judex M, Neumann E, Lechner S, Dietmaier W, Ballhorn W, Grifka J, Gay S, Scholmerich J, Kullmann F, Muller-Ladner U. 2003. Laser-mediated microdissection facilitates analysis of area-specific gene expression in rheumatoid synovium. *Arthritis Rheum* 48:97–102.
- Keohavong P, Gao WM, Zheng KC, Mady H, Lan Q, Melhem M, Mumford J. 2004. Detection of K-ras and p53 mutations in sputum samples of lung cancer patients using laser capture microdissection microscope and mutation analysis. *Anal Biochem* 324:92–99.
- Kinzler KW, Vogelstein B. 2002. Basic concepts in cancer genetics. In: *The genetic basis of human cancer*. 2nd edn. New York: McGraw-Hill Publishing Division. pp 3–131.
- Kutach LS, Bolshakov S, Ananthaswamy HN. 1999. Detection of mutations and polymorphisms in the p53 tumor suppressor gene by single-strand conformation polymorphism analysis. *Electrophoresis* 20:1204–1210.
- Moskaluk CA, Kern SE. 1997. Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. *Am J Pathol* 150:1547–1552.
- Moyret C, Theillet C, Puig PL, Moles JP, Thomas G, Hamelin R. 1994. Relative efficiency of denaturing gradient gel electrophoresis and single strand conformation polymorphism in the detection of mutations in exons 5 to 8 of the p53 gene. *Oncogene* 9:1739–1743.
- Nagasawa Y, Takenaka M, Matsuoka Y, Imai E, Hori M. 2000. Quantitation of mRNA expression in glomeruli using laser-manipulated microdissection and laser pressure catapulting. *Kidney Int* 57:717–723.
- Paweletz CP, Liotta LA, Petricoin EF III. 2001. New technologies for biomarker analysis of prostate cancer progression: Laser capture microdissection and tissue proteomics. *Urology* 57(Suppl 4A):160–163.
- Russo A, Migliavacca M, Zanna I, Valerio MR, Latteri MA, Grassi N, Pantuso G, Salerno S, Dardanoni G, Albanese I, La Farina M, Tomasino RM, Gebbia N, Bazan V. 2002. p53 mutations in L3-loop zinc-binding domain, DNA-ploidy, and S phase fraction are independent prognostic indicators in colorectal cancer: A prospective study with a five-year follow-up. *Cancer Epidemiol Biomarkers Prev* 11:1322–1331.
- Schindler M. 1998. Select, microdissect, and eject. *Nat Biotechnol* 16:719–720.
- Schutze K, Posl H, Lahr G. 1998. Laser micromanipulation systems as universal tools in cellular and molecular biology and in medicine. *Cell Mol Biol* 44:735–746.
- Sirivatanauksorn Y, Drury R, Crnogorac-Jurcevic T, Sirivatanauksorn V, Lemoine NR. 1999. Laser-assisted microdissection: Applications in molecular pathology. *J Pathol* 189:150–154.
- Soong R, Iacopetta BJ. 1997. A rapid and nonisotopic method for the screening and sequencing of p53 gene mutations in formalin-fixed, paraffin-embedded tumors. *Mod Pathol* 10:252–258.