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Comparative genomic analysis of tumors: Detection of DNA losses and amplification

(cancer/oncogenes/difference analysis/polymerase chain reaction)

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ABSTRACT **We demonstrate the use of representational difference analysis for cloning probes that detect DNA loss and amplification in tumors. Using DNA isolated from human tumor cell lines to drive hybridization against matched normal DNA, we were able to identify six genomic regions that are homozygously deleted in cultured cancer cells. When this method was applied in the reverse way, using normal DNA to drive hybridization against tumor cell DNA, we readily isolated probes detecting amplification. Representational difference analysis was also performed on DNAs derived from tumor biopsies, and we thereby discovered a probe detecting very frequent homozygous loss in colon cancer cell lines and located on chromosome 3p.**

A variety of genetic lesions are found in tumors, including rearrangement, gene amplification, point mutation, deletion, and acquisition of viral genomes (1, 2). Delineating the genes involved has often led to important new insights into the pathophysiology of cancer. Therefore, efficient methods for the discovery of new genetic lesions in tumors may accelerate our management of that disease. Recently we described a method, called representational difference analysis (RDA), for analyzing the differences between complex but highly related genomes (3).

RDA combines three elements: representation, subtractive enrichment, and kinetic enrichment. The procedure is carried out in two stages. The first comprises the preparation of representations for driver and tester DNAs, during which small restriction endonuclease fragments (called ARFs) are ligated to oligonucleotide adapters and amplified by the polymerase chain reaction (PCR). The second stage is comprised of the reiterative hybridization/selection steps. Prior to the hybridization/selection step, only tester molecules are fitted with a new pair of defined oligonucleotides at their 5′ ends. After reannealing tester and driver, the mixture of molecules is treated with DNA polymerase. This adds the complement of the defined oligonucleotides to both 3′ ends of only self reannealed tester DNA fragments. When the defined oligonucleotide is used as primer in PCR of the mixture, such molecules can participate in exponential amplification. This serves three purposes. (*i*) Molecules of tester that reanneal to the excess of driver are "subtracted out" since the heteroduplexes they form with driver have the primer complement on one end only, thus leading to inefficient, linear amplification of only one strand. (*ii*) Abundant sequences of tester will reanneal faster than less abundant ones (kinetic enrichment) so that sequences that are enriched or amplified become even more enriched. (*iii*) PCR amplification increases the yield so that the hybridization/selection step can be reiterated or the product can be cloned and analyzed. Two of three rounds of hybridization/selection are employed to achieve full purification of the difference products.

RDA may be used in two ways. (*i*) With tumor DNA taken as driver, and matched normal DNA as tester, one may identify the allelic loss of polymorphic loci and hemizygous or homozygous deletions leading to the loss of restriction endonuclease fragments in the tumor. (*ii*) With tumor DNA taken as tester, and matched normal DNA as driver, one may detect small restriction endonuclease fragments present only in the tumor genome arising following viral infection, genetic rearrangement, or point mutation (3). In the same manner, but by a different mechanism, one can identify restriction endonuclease fragments that have increased in copy number due to gene amplification in the tumor. Using these two approaches we have identified seven genomic loci that appear to be homozygously lost in different tumor cell lines and two loci that have undergone gene amplification. The probes marking these loci may prove to be valuable tools in the search for recessive and dominant oncogenes.

MATERIALS AND METHODS

Cell Lines and DNA Samples. Renal cell carcinoma (RCC) cell lines UOK112, UOK114, UOK124, UOK132, UOK108, UOK111, UOK127, UOK146, and UOK154 and normal DNAs from the same patients were obtained as described (4). Colorectal cancer cell lines VACO 429, VACO 441, VACO 432, VACO 456, VACO 476, and RBX and matched normal DNAs were established according to ref. 5. Cell line NCI H1770 (small cell lung carcinoma) and Epstein–Barr virus (EBV)-immortalized lymphocytes from the same patient were supplied by J. D. Minna (Southwestern Medical School, Dallas). DNAs from the melanoma tumor cell lines AH-Mel, FF-Mel, BD-Mel, and DX-Mel and matched EBV-immortalized cells were the gift of A. Houghton (Memorial Sloan–Kettering Cancer Center). Cell lines A382 (astrocytoma), VM-CUB-2 (bladder cancer), SK-LC-6, SK-LC-13, SK-LC-14, SK-LC-17, and SHP-77 (lung cancers), and WILTU-1 (Wilms tumor) were from the J. Fogh collection (Memorial Sloan–Kettering Cancer Center). All other tumor cell lines were obtained from the American Type Culture Collection. DNAs NA04844 and NA11102 and human/rodent somatic cell hybrid mapping panel no. 2 were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). The "standard blotting panel" included *Bgl* II digests of DNAs from tumor cell lines BD-Mel and AH-Mel (melanomas); T24 and VM-CUB-2 (bladder cancers); SK-BR-3 and MCF7 (breast cancers); HT-29, SW480, and SW620 (colon cancers); A-172 and U-118 MG (glioblastomas); A-382 (astrocytoma); NCI H1770, SK-LU-1, SK-LC-6, SK-LC-13, SK-LC-14, SK-LC-17, and

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Abbreviations: RDA, representational difference analysis; RCC, renal cell carcinoma; EBV, Epstein–Barr virus; FISH, fluorescence *in situ* hybridization; LOH, loss of heterozygosity.

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SHP-77 (lung cancers); SK-N-MC, SK-N-SH, and IMR-5, (neuroblastomas); G-401 (Wilms tumor); and normal control DNA NA04844. Allele frequency blots were prepared using *Bgl* II digests of human DNAs from various races (allele frequency kit, BIOS Laboratories, New Haven, CT). The standard PCR panel included DNAs from tumor cell lines BT-20, MCF7, SK-BR-3, T-47D, BT-549, MDA-MBA-435S, MDA-MB-436, MDA-MB-231, MDA-MB-453, and MDA-MB-468 (breast cancers); UOK124, UOK161, UOK114, UOK112, UOK132, UOK154, UOK127, UOK111, UOK146, and UOK108 (RCCs); LS180, SW403, SW480, HT-29, LoVo, DLD-1, Caco-2, HCT-15, VACO 429, and VACO 441 (colon cancers); FF-Mel, BD-Mel, AH-Mel, DX-Mel, HT-144, SK-Mel-2, SK-Mel-3, G-361, WM266-4, and Malme-3M (melanomas); T24, VM-CUB-2, UM-UC-3, J82, SCaBER, HT-1376, RT-4, and HT-1197 (bladder cancers); and normal control DNA NA04844. Tumor and normal cells were grown as recommended and DNAs were purified using cell culture DNA Maxi kit (Qiagen, Chatsworth, CA). Diploid and aneuploid nuclei were separated by flow cytometry from a biopsy of a patient with Barrett esophagus (6), and 100 ng of DNA from each diploid and aneuploid fraction (10^5 nuclei each) was purified after lysis in SDS/proteinase K buffer, phenol/chloroform extraction, and ethanol precipitation.

RDA. The RDA procedure was performed as described (3, 7) using *Bgl* II restriction endonuclease (New England Biolabs). A more complete protocol is available upon request (8). When DNAs from flow-sorted material were used, 100 ng of each driver and tester was digested with *Bgl* II and ligated to adaptors in a volume 30 μl as described (3). After ligation, 10 μg of tRNA (5 mg/ml), 90 μl of TE buffer, 30 μl of 10 M ammoninum acetate, and 380 μl of ethanol were added. The DNA pellet was recovered by centrifugation and dissolved in 10 μl of TE buffer. Forty microliters of the DNA ligate was PCR amplified for 20 cycles in a volume of 400 μl as described (3) taking two tubes for preparation of driver and two tubes for preparation of tester representation. To get sufficient quantity of DNA, 40 μl of the product of the first PCR was directly added to each of 12 tubes used for preparation of driver representation and reamplified for 5 cycles in a volume of 400 μl under standard conditions (7). The subsequent PCR amplification of tester representation was made in the same way, taking 2 tubes. All subsequent steps were performed as described (3). RDA difference products were digested with *Bgl* II, ligated to *Bam*HI-digested and dephosphorylated pBluescript SK(−) (Stratagene), and transformed into *Escherichia coli* XL-Blue competent cells according to the supplier's recommendations.

Characterization and Mapping of RDA Probes. Plasmid inserts were PCR amplified and those with distinct sizes were selected, purified, and hybridized to Southern blots containing *Bgl* II representations of driver, tester, one normal male, and one normal female DNA prepared as described (7). Sequences present in tester but not in driver representations were hybridized to Southern blots containing *Bgl* II-digested DNAs from the standard blotting panel and to allele frequency blots. These blots were washed two times, 30 min each, in 0.1× SSC/0.5% SDS at 68°C. Selected plasmid inserts were sequenced on both strands, using Sequenase T7 DNA polymerase reagent kit (United States Biochemical) as recommended by the supplier. Oligonucleotides derived from the sequences were synthesized and used for screening the standard PCR panel of DNAs. Two hundred fifty nanograms of template was taken per each 100-μl PCR containing 1 μM primers. Amplification was made for 32 cycles as described (7). Negative reactions were independently repeated two times.

Mapping of probes on human chromosomes was performed by PCR using 250 ng of DNAs from National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel no. 2 as templates under the same conditions (7). To sublocalize probes on chromosome 3, DNA from hybrid clone GM 11102 retaining the der(3) t(3;16) (q13.2;q13) chromosome was used (NIGMS Human Genetic Mutant Cell Repository). Fluorescence *in situ* hybridization (FISH) was performed as described (9).

RESULTS

Tumor DNA as Driver. We performed RDA on 16 individual pairs of tumor DNAs (used as driver) and matched normal DNAs (used as tester) derived from the same patient, as otherwise cloning of polymorphic differences between different individuals predominates. In all cases, we used *Bgl* II as the restriction endonuclease to prepare representations. Pure tumor DNAs were isolated from 15 tumor cell lines (including 9 RCCs and 6 colon cancer cell lines), and normal DNA was derived from unaffected blood or tissue but not from EBV-immortalized cell lines lest viral DNA fragments be cloned. In one case we used a fluorescence-activated cell sorter to fractionate nuclei from an esophageal cancer biopsy into aneuploid and diploid fractions that were used for preparation of driver and tester DNA, respectively.

In each application of RDA, 2–13 difference products were observed and cloned into plasmids. Plasmid clones were picked at random and inserts of different sizes were analyzed by hybridization to blots containing representations from the normal (tester) and tumor (driver) DNAs, as well as *Bgl* II

Table 1. Analysis of RDA probes derived using tumor DNA as driver

Cell line	Selected for initial characterization	Found to be informative*
RCC		
UOK112 (♂)	13†	13 (0/13/0)
UOK114 (♀)	12†	4 (3/0/1)
UOK124 (♀)	12†	4 (4/0/0)
UOK132 (♂)	10†	9 (3/6/0)
UOK108 (♀)	2	2 (2/0/0)
UOK111 (♀)	5	5 (5/0/0)
UOK127 (♂)	3	3 (2/1‡/0)
UOK146 (♀)	3	3 (1/1‡/1)
UOK154 (♀)	5	1 (1/0/0)
Colon cancer		
VACO 429 (♂)	2	1 (0/0/1)
VACO 441 (♀)	3	3 (1/0/2)
VACO 432 (♂)	2	1 (1/0/0)
VACO 456 (♀)	2	1 (1/0/0)
VACO 576 (♀)	2	2 (2/0/0)
RBX (♂)	2	1 (1/0/0)
Barrett esophagus		
BE 758 (FACS-sorted nuclei) (♂)	5	5 (0/4/1§)
Total	83	58 (27/25/6)

FACS, fluorescence-activated cell sorter.

*Entries are a (b/c/d), where a is the total number of probes detecting DNA loss in tumors, judged to be: b, LOH; c, hemizygous loss; d, presumably homozygous loss (see *Discussion*). All but two probes judged to detect hemizygous loss were derived from the Y chromosome. The difference between quantities of initially selected probes (83) and informative probes (58) was due to the presence of the repeat sequences (9 cases), non-human DNA contaminating tester (5 cases), and single copy sequences present in tester and driver DNAs (11 cases).

†The difference products after two rounds of hybridization/selection were cloned; in all of the rest of the experiments cloning was performed after three rounds.

‡Probes 127-1 and 146-1 were found to be deletion polymorphisms, absent on both autosomes of 7 of 35 and 3 of 35 normal humans, respectively.

§This result is presumed but was not confirmed because of the small amount of sorted tumor nuclei available.

Table 2. Homozygous losses detected in tumor cell lines

Probe	Chromosome location	Homozygous loss*†	Sequences of primers used for PCR	PCR product, bp
UOK114-18	3p	1/74	CATTTCTTTAGGGTTCATTGTTGGAGC GAGCCCAGCCAGCAGTCCCACC	293
UOK146-4	11	1/113	CCATGCTGCCTCCGTTGACACTCA TGGCAACAATATCCATCCCTTTCCTG	283
UOK124-6‡	2	2/113	GTCTTCTCTCCCTCTTTCCCTCCC TGGCAGTAGAAGAGGAAAGATGTGTG	319
UOK146-8‡	9	13/113	TGTGCTCCCAGTCCTGCAGTCATC AGGGAACTCTGATGGTAGACTGGTC	261
UOK132-12‡	9	6/86	GCCCCTCTAAAAGATAAGGTCTTGGT GATCTGAGCCCCTGGAAGAAGTTAG	272
VACO 429-6	20	1/86	GGGAACAGTTCTCTTACAGCCACAC ACAGAGGTGACAACAAGGTCAGTGG	351
VACO 441-1	18	1/86	CCAGCTGTGTCCTCTCAGCAACAG ACATGATGCTGGCCTAGGTGAACTG	268
VACO 441-9	18	1/86	TCTAGGAACTGCCAGTGAGTGCTTG GTACTAACCAAGGAGCTGGTGACAC	244
BE758-6	3p	6/86	GCTAAGCCTGGGGGAGTTGCTGAC GATTACTAAGGCTTTGAAAGCTGGCC	315

*The numbers show the ratio of the number of cell lines with apparent homozygous loss (see *Discussion*) to the total number of analyzed cell lines. The primary determination was by PCR. The losses were detected in the following cell lines: probe UOK114-18 in UOK114;† probe UOK146-4 in UOK146; probe UOK124-6 in UOK141 and VM-CUB-2; probe UOK146-8 in UOK108, UOK122LN, UOK162, AH-Mel, Malme-3M, UM-UC-3, RT-4, MDA-MB-231, A-382, U-118 MG, A-172, SK-LU-1, and SK-LC-14; probe 132-12 in AH-Mel,† FF-Mel,† MDA-MB-231, A-382, U-118 MG, and A-172; probe VACO 429-6 in VACO 429; probes VACO 441-1 and VACO 441-9 in VACO 441; probe BE758-6 in LS180,† SW480,† HT-29,† LoVo,† MDA-MB-436,† and VM-CUB-2.† See text for origins of cell lines.
†PCR data were additionally confirmed by genomic Southern blotting for the indicated cell lines.
‡The probe was found to detect LOH in the initial normal/tumor DNA pair.

representations of normal male and female DNAs. The "informative" probes, which were hybridizing to one band on a blot, and were absent in the driver representation, were taken for further analysis, except for those that derived from the Y chromosome (loss of the Y chromosome information was frequently observed in RCCs). In search for clones detecting single copy sequences that are frequently lost in tumors, informative probes were hybridized to blots containing *Bgl* II-digested DNAs from a standard blotting panel of human tumor cell lines. Those probes that were commonly polymorphic at *Bgl* II sites were presumed to have arisen by loss of heterozygosity (LOH) and were not further studied unless they did not detect any bands in at least one tumor DNA on a blot. Probes of this type, as well as the remaining nonpolymorphic single copy probes, were sequenced, and oligonucleotides derived from the sequence were synthesized to be used for PCR screening of total genomic DNA from tester, driver, and panels of human tumor cell lines. When we could not detect probe sequences in the genomic DNA that was used as driver, we generally assumed that the probe detected homozygous deletion. All probes absent in two or more DNA samples from standard PCR panel were hybridized to allele frequency blots containing *Bgl* II digests of human DNAs from various races. This way we were able to find two probes that did not hybridize to any sequences in several normal human DNAs. We thus presume that these two probes actually detect hemizygous loss of a deletion polymorphism (see Table 1, footnote ‡). Tables 1 and 2 summarize all of our results obtained using tumor DNA as driver.

Typical results and analysis are shown in Fig. 1. In this particular case, RDA was performed using DNA from the RCC cell line UOK146 as driver. One of the probes (UOK146-8) cloned from the third round of hybridization/selection (Fig. 1*A*, lane c) was found to be absent in the *Bgl* II representation of the tumor DNA. It was further analyzed by Southern blotting and PCR (Fig. 1 *B* and *C*, respectively), indicating its frequent homozygous loss in many tumor cell lines. Subsequent PCR analysis of driver and tester DNAs indicated that UOK146-8 in fact detected loss of a small allele of a rare *Bgl* II polymorphism in the cell line UOK146 (data not shown) and was present in the difference product due to LOH rather than homozygous loss in the original tumor. Probe UOK146-8 represents one of three probes detecting apparent homozygous loss in at least one tumor source but isolated by virtue of LOH in the original tumor (see Table 2).

All probes that detected homozygous loss in at least one tumor cell line were mapped to human chromosomes using a panel of monochromosomal human/rodent somatic cell hy-

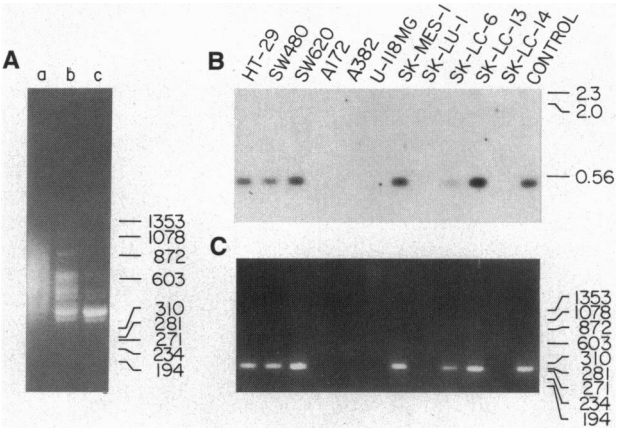

FIG. 1. Cloning of probes detecting DNA losses in RCC cell line UOK146 (tumor DNA used as driver). (*A*) Agarose gel electrophoresis of difference products obtained after the first (lane a), second (lane b), and third (lane c) hybridization/selection steps. Sizes are indicated in bp. (*B*) Autoradiogram obtained after hybridization of probe 146-8 (350 bp in length) to Southern blot containing *Bgl* II-digested DNAs from indicated tumor cell lines. Sizes are indicated in kb. (*C*) Agarose gel electrophoresis of PCR products amplified from indicated tumor cell line DNAs using primers derived from the 146-8 sequence (expected fragment length, 261 bp). Sizes are indicated in bp.

brids (see *Materials and Methods*). In two cases, an additional human/rodent hybrid was used to resolve location to 3p or 3q (see Table 2).

Tumor DNA as Tester. We also used RDA by taking DNA from tumor cell lines as tester and DNA from matched normals as driver. The cell lines used were melanoma (AH-Mel), small cell carcinoma of the lung (NCI H1770, gift of John Minna), and two RCC cell lines (UOK161 and UOK124). In two cases (AH-Mel and NCI H1770) difference products were observed and were discernible even after the first round of hybridization/selection. In each of these cases individual products cloned from the second round of hybridization/selection detected high-level amplifications (30- to 100-fold) in the tumor DNA used as tester. Additionally, RDA products from NCI H1770 were found to be amplified in a neuroblastoma cell line, IMR-5. The sequences from NCI H1770 were mapped to chromosome 2, and those from AH-Mel were mapped to chromosome 3. The entirety of the RDA product from the second round of hybridization/selection of the melanoma tumor cell line was used as a probe for FISH to metaphase preparations from the AH-Mel cell line. Two and in some cases three homogeneously staining regions were readily observed in tumor cells with this probe (see Fig. 2).

The presence of amplified sequences from tumors in the RDA product even after one round of hybridization/selection suggested to us that such difference products would dominate over single copy sequence differences and hence that the detection of gene amplification might not require matched tumor and normal DNAs. To test this idea, we took AH-Mel DNA as tester and either a single or pooled DNA from 10 unrelated humans as driver. In either case, RDA products were observed even after one round of hybridization/selection, and difference products obtained after the second round were found to map to the same amplified region in AH-Mel as the RDA products found using the matched normal DNA as driver.

DISCUSSION

The RDA methodology may be successfully applied to the analysis of genetic lesions in tumors in two formats. In the first format, the use of tumor DNA as driver and normal DNA as tester leads to the discovery of probes that detect "loss" of information in tumors: in the present cases, LOH, hemizygous loss, and homozygous loss. Such losses may indicate the loss of function of a "tumor suppressor" gene, or a set of such genes, that in some way contributes to the evolution of the neoplastic population in its host. When tumor DNA was used as driver, 58 of 83 RDA products were informative—that is, detected loss of genetic information in the tumor source under analysis—and all 16 RDA comparisons of tumor and normal pairs yielded informative probes. In the second format, the use of tumor DNA as tester and normal DNA as driver leads to the discovery of probes that detect "gain" of information in tumors: in the present cases, DNA amplification. Such amplification may result in the increased production of certain proteins, encoded by dominant oncogenes, that contribute to the growth advantage of the neoplastic population in its host. Gene amplification was observed in two of four RDA comparisons of matched normal and tumor cell line DNAs.

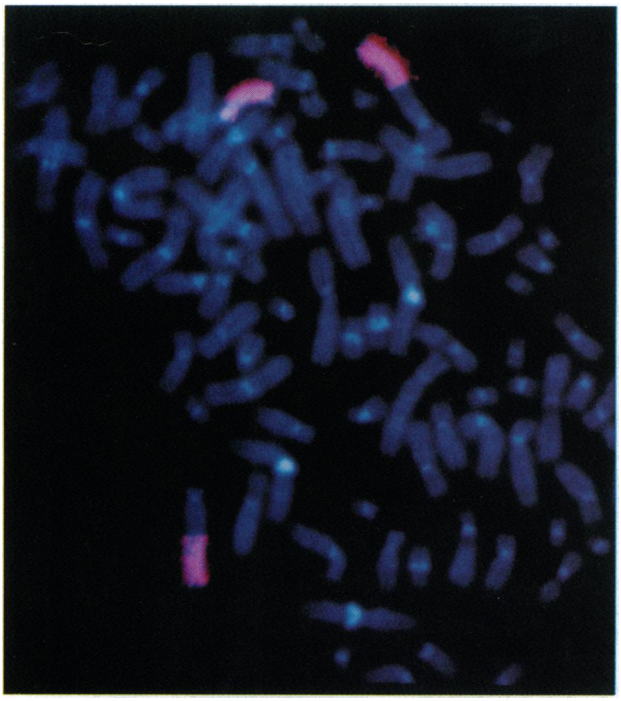

FIG. 2. Homogeneously staining regions detected by FISH of metaphase chromosomes from the AH-Mel cell line with nick-translated total difference product obtained after two rounds of hybridization/selection (tumor DNA used as tester).

LOH is detected by RDA at polymorphic loci where only one allele contains a small amplifiable restriction endonuclease fragment (ARF), present in the representation of the normal DNA, but lost in the tumor DNA. We estimate that after digestion with a restriction endonuclease such as *Bgl* II, polymorphic ARFs (or PARFs) occur at a frequency of about one per 1–3 megabases in the human genome. We did not further study the probes that detected LOH in tumor/normal pairs, except for 3 of a total of 27 probes of this type, which detected homozygous loss in at least one other tumor source.

Hemizygous loss can occur in tumors from males if part or all of the Y chromosome is lost. We observed this type of lesion frequently in RCC, as have others previously (10). Hemizygous loss can also occur if individuals have inherited only one copy of certain autosomal sequences and the remaining copy is lost by any of the same mechanisms that underlie LOH at polymorphic markers. We believe that two instances of loss we observed were of this type because the probes in question detected "deletion polymorphisms" in the human population—i.e., sequences that are not present in all humans (see Table 1, footnote ‡).

Homozygous loss occurs when both alleles of an autosome are lost in the tumor cell. In general, LOH should occur in bigger blocks than homozygous loss. Thus probes that detect homozygous loss are more likely to be closely linked to a tumor suppressor gene. Happily, such probes are readily found by RDA. In our experiments, the ratio of the number of probes detecting apparent homozygous loss to the number detecting LOH was 6:27. Since a 10-kbp homozygously deleted region would have about a 50% chance of containing an ARF, while a 1-megabase loss on one of two autosomes would generate, on average, only a single PARF, we can estimate that in the tumors we have sampled the ratio of genome length that has been homozygously deleted to the length that has lost heterozygosity is $6 \times 10 \times 10^3$ to 27×10^6, or about $1–4.5 \times 10^2$. We must take these numbers with caution because we have not proven that the six probes that detect complete loss in the tumor genome truly arose by homozygous loss. Some of these might represent hemizygous loss at undetected rare deletion polymorphisms. In fact, we expect that some deletion polymorphisms, being recessive organismic lethals, would not be found in the human population in the homozygous state but could occur in tumors by LOH if they were not cell lethals.

Of the total nine probes that detect homozygous loss, probes UOK132-12 and UOK146-8 probably detect the region on chromosome 9 previously known to undergo frequent homozygous loss; and the two probes from VACO 441 both derive from the vicinity of the *DCC* locus on chromosome 18 (unpublished data), a region previously known to undergo LOH. One probe, derived from the Barrett esophagus biopsy (BE758-6), that detects frequent homozygous loss in colon

cancer and other cell lines, does not yet appear to map to a known tumor suppressor locus (unpublished data). This probe maps to 3p, and intensive study of the surrounding genomic region is necessary. The other probes detect homozygous deletion in at most two cell lines and do not yet appear to coincide with known loci.

When tumor DNA is used as tester, RDA has the potential to detect several types of genetic alterations, including the presence of viral genomes, genomic rearrangements, and a very small proportion of point mutations. Such genetic changes can give rise to restriction endonuclease fragments present in tumor that are not present in the matched normal DNA. In the four tumor cell lines investigated here, no probes for lesions of these types were encountered. Rather, RDA yielded probes that detected highly amplified single copy sequences. These probes do not arise because of absolute differences between normal and tumor DNA, but because their relative abundance in tumor has led to their kinetic enrichment during the RDA procedure. For the isolation of highly amplified sequences in tumor DNA, any normal human DNA can be used as driver, providing a means to clone such sequences from any tumor cell. The two regions we found amplified map to chromosomes 2 (from small cell lung cancer) and 3 (from melanoma). The region on chromosome 2 is also found amplified in a neuroblastoma cell line and hence may contain N-*myc* (11). Amplification on chromosome 3 has not been previously described.

For the application of RDA to cancer, the source of driver must be relatively free of the unique tester source (5%). While this is not a problem when normal DNA is used as driver, the tumor source for driver preparation should not be derived from unprocessed biopsies because of the presence of contaminating normal stromal elements. Established tumor cell lines are a convenient source of pure DNA, but the tester DNA must come from the normal host, and this source is often no longer available. Moreover, established tumor cell lines may have accumulated genetic alterations that were not present in the original tumor. We have experimented successfully with flow-sorted nuclei from biopsies. This method of separating tumor DNA from normal DNA is based on differences in ploidy, but other methods (e.g., short-term culture and/or separation of tumor cells from normal cells by surface antigens or other properties) might be employed.

RDA will not detect all differences between tumor and normal DNAs. First, only differences in the respective representations can be found. Since a representation is of lower complexity than the genomic DNA from which it derives, not all differences will be represented. Second, since RDA is a PCR-based methodology and multiple cycles of PCR are used (in excess of 80 cycles for the representation and three rounds of hybridization/selection), only the most efficiently amplifiable differences are readily detected. This probably explains the relatively narrow size range (250–350 bp) of the probes we isolated that detect homozygous deletions. Thus cloning the differences between two samples by RDA using a single restriction endonuclease will hardly exhaust all of their abundant differences.

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