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Detection of Genetic Loss in Tumors by Representational Difference Analysis

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A variety of genetic lesions are found in tumors, including DNA losses, point mutations, gene amplifications, and rearrangements (Lasko et al. 1991; Salomon et al. 1991). Frequent losses of both alleles at a given locus or losses of one allele with functional inactivation of the other have been detected in many tumor types. These genetic lesions, manifesting themselves as loss of heterozygosity (LOH) and hemizygous and homozygous deletions, have been found to be the hallmarks of the presence of tumor suppressor genes. Many approaches have been taken in the past to identify these genes, but recently we have developed a new method that is both general and efficient (Lisitsyn et al. 1993, 1995). The method, called representational difference analysis, or RDA, is designed for analyzing the differences between complex but highly related genomes and combines three elements: representation, subtractive enrichment, and kinetic enrichment. The first stage of the procedure comprises the preparation of representations from the genomes, during which DNAs are cut with restriction endonuclease, ligated to oligonucleotide adapters, and amplified by the polymerase chain reaction (PCR). Since only small fragments (<1 kb in length), called ARFs, are efficiently amplified by standard PCR procedures, representations have at least tenfold lower complexity than initial DNAs. This enormously increases the efficiency of the second stage, comprising the reiterative hybridization/selection steps during which ARFs present in one sample, the tester, but not in the other, the driver, are selectively enriched. We describe here the application of RDA to discover sequences that are lost in tumors.

CLONING SEQUENCES LOST IN TUMORS

We performed RDA on 16 individual pairs of tumor DNA (used to derive driver) and matched normal DNA (used to derive tester) from the same patient. We isolated 15 DNAs from tumor cell lines (including 9 renal cell and 6 colon cancer cell lines) with normal DNAs derived from unaffected blood or tissue. In one case, we used a fluorescent activated cell sorter to fractionate nuclei from an esophageal cancer biopsy into aneuploid and diploid fractions used for preparation of driver and tester DNA, respectively.

In each application of RDA, difference products were cloned and analyzed by blot hybridization. The "informative" probes hybridized to DNA from the normal representation but not the tumor representation. Some of these probes mapped to the Y chromosome. Loss of Y chromosome information is frequently observed in renal cell carcinomas (Presti et al. 1991). Other probes detected binary polymorphisms at *Bgl*II sites and were presumed to reflect loss of heterozygosity in tumor. Finally, some probes did not hybridize at all to total genomic DNA from the tumor. Probes of this type were sequenced, and oligonucleotides were derived for use in PCR screening of genomic DNA from the tester and driver sources, and from panels of normal human and tumor cell lines. Occasionally, we found probes that did not hybridize to several normal human DNAs. We presume that these probes reflect hemizygous loss in the tumor of a deletion polymorphism common in the human population (see Table 1, footnote c). Table 1 summarizes the types of probes we obtained (for further details, see Lisitsyn et al. 1995).

HOMOZYGOUS LOSS ON CHROMOSOME 3p

From 16 comparisons, 6 pairs yielded probes that appear to detect homozygous loss in the tumor used as driver. Three probes were found to be homozygously deleted in other DNAs isolated from a collection of 100 tumor cell lines established from different types of cancer but not from normals (N. Lisitsyn and R. Lucito, unpubl.). Probe 758-6, derived from a patient with Barrett's esophagus, detected frequent losses in cancers of the digestive tract. Loss detected with this probe has also been observed in breast, bladder, and lung tumors. This probe has been analyzed in greatest detail.

758-6 was mapped to chromosomal region 3p by PCR analysis of monochromosomal human/rodent cell hybrids. The probe was used for screening a chromosome-3 cosmid library, and a cosmid contig was built by chromosome walking (see Fig. 1). Single-copy probes were derived from this contig and used to screen DNAs from a collection of human colon cancer cell lines and xenografts. Of 175 tumor DNAs, 20 (11%) lacked sequences from at least one of the probes from this region. In contrast, losses were observed in 1 of 122 lung cancer cell lines (S. Bader and J. Minna, unpubl.). Figure 1 shows the different patterns of loss that were observed in colorectal cancer cell lines and xenografts.

Additional probes derived from a cosmid contig were used in hybridizations to Southern blots containing DNAs harboring deletions. Several hybridizing restric-

Table 1. Analysis of RDA Probes Derived Using Tumor DNA as Driver

	Selected for initial characterization	Found to be informative[a]
Renal cell carcinoma cell lines		
UOK 112 (male)	13[b]	13 (0/13/0)
UOK 114 (female)	12[b]	4 (3/0/1)
UOK 124 (female)	12[b]	4 (4/0/0)
UOK 132 (male)	10[b]	9 (3/6/0)
UOK 108 (female)	2	2 (2/0/0)
UOK 111 (female)	5	5 (5/0/0)
UOK 127 (male)	3	3 (2/1[c]/0)
UOK 146 (female)	3	3 (1/1[c]/1)
UOK 154 (female)	5	1 (1/0/0)
Colon cancer cell lines		
VACO 429 (male)	2	1 (0/0/1)
VACO 441 (female)	3	3 (1/0/2)
VACO 432 (male)	2	1 (1/0/0)
VACO 456 (female)	2	1 (1/0/0)
VACO 576 (female)	2	2 (2/0/0)
RBX (male)	2	1 (1/0/0)
Barrett's esophagus		
BE 758[d] (male)	5	5 (0/4/1[e])
Total:	83	58 (27/25/6)

[a] Entries are a(b, c, d), where a is the total number of probes detecting DNA loss in tumors, judged to be: b, loss-of-heterozygosity; 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), nonhuman DNA contaminating tester (5 cases), and single-copy sequences present in both tester and driver DNAs (11 cases).

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

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

[d] Nuclei from a biopsy were sorted by flow cytometry into aneuploid (tumor) and diploid (normal) fractions.

[e] This result is presumed, but was not confirmed because of the small amount of sorted tumor nuclei available.

tion fragments were observed in tumors that were absent in normals, presumably as a result of rearrangements occurring at the ends of some of the deletions. This observation rules out the possibility that probe 758-6 detects a deletion polymorphism and that the loss of sequences is caused by the same types of mechanisms that underlie loss of heterozygosity at polymorphic markers. Work is in progress to identify transcribed sequences from this region.

DISCUSSION

The RDA methodology may be successfully applied to detection of DNA losses in tumors, readily providing probes that detect homozygous deletions. As we were able to demonstrate, some of these deletions are relatively small (< 50 kb) and, thus, positional cloning of genes that must be inactivated in tumors becomes much more efficient, as compared to other techniques used for this purpose (e.g., allelotyping, linkage analysis of predispositions in families, and cytogenetic studies followed by microdissection). Since some of these probes are deleted in more than one DNA isolated from tumor cells, it is possible that the deleted locus contains a gene that is commonly inactivated in tumors.

It is well documented that some genes known to be disrupted by homozygous deletions in tumors regulate cellular growth, differentiation, and genomic stability. Some of these genes have strong tumor suppressor phenotypes after transfection into tumor cell lines. Our screening technique based on RDA methodology holds promise for the identification of new genes participating in these or other processes. We have taken a similar approach for the cloning of dominant oncogenes. These are frequently amplified in tumors, and probes detecting amplifications can be efficiently cloned by RDA when tumor DNA is used to derive tester (Lisitsyn et al. 1995). Although amplified regions are usually rather large, one can map candidate oncogenes more precisely by finding the minimal region common to all amplifications at a given locus. The use of RDA for the analysis of cancers thus opens up new avenues for understanding the etiology of the disease and for the development of prognostic and diagnostic markers.

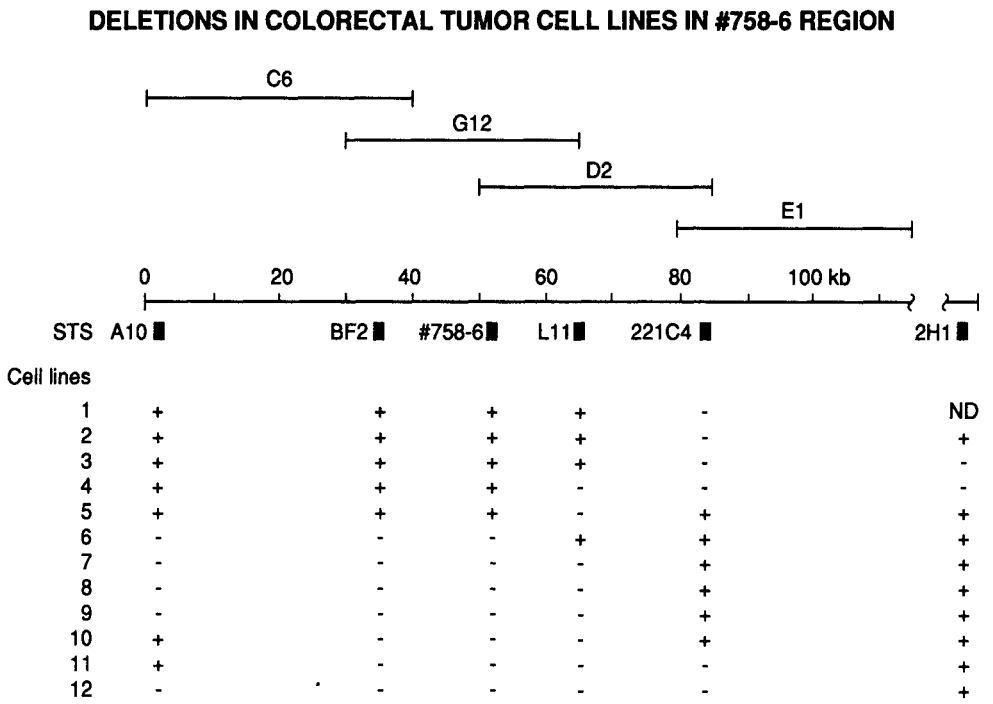

Figure 1. Physical map of a chromosome 3p region. On top are shown cosmids from a region isolated by chromosomal walking. Patterns of the homozygous loss of STSs (thick bars) detected by PCR analysis are depicted on the bottom. Pluses and minuses indicate presence or absence of the probe in DNAs from colorectal cancer cells.

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