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Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the *BRCA2* region

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ABSTRACT **Homozygous deletions have been central to the discovery of several tumor-suppressor genes, but their finding has often been either serendipitous or the result of a directed search. A recently described technique [Lisitsyn, N., Lisitsyn, N. & Wigler, M. (1993) *Science* 259, 946–951] held out the potential to efficiently discover such events in an unbiased manner. Here we present the application of the representational difference analysis (RDA) to the study of cancer. We cloned two DNA fragments that identified a homozygous deletion in a human pancreatic adenocarcinoma, mapping to a 1-centimorgan region at chromosome 13q12.3 flanked by the markers *D13S171* and *D13S260*. Interestingly, this lies within the 6-centimorgan region recently identified as the *BRCA2* locus of heritable breast cancer susceptibility. This suggests that the same gene may be involved in multiple tumor types and that its function is that of a tumor suppressor rather than that of a dominant oncogene.**

Tumor-suppressor genes play a crucial role in the control of cell growth and differentiation. Loss of the function of tumor-suppressor genes is part of the cascade of genetic alterations which drive tumorigenesis (1). The biallelic inactivation of a tumor-suppressor gene typically involves an intragenic change (nucleotide substitution, small insertion, or microdeletion) within one allele, combined with inactivation of the other allele through the loss of a large chromosomal region. Although infrequent, sizable deletions involving both alleles have been observed. Such homozygous deletions have contributed to the discovery of several tumor-suppressor genes (*RB1, DCC*, and p16) (2–5).

Despite the fact that pancreatic adenocarcinoma is one of the more common human cancers (6), little is known of the genetic alterations in these tumors. One of the reasons is that the tumors generally are diagnosed at a late stage of tumorigenesis. This, together with the aggressive clinical course, severely limits the number of resected specimens available for research. Also, pancreatic adenocarcinomas characteristically exhibit an exuberant host desmoplastic response, resulting in a high admixture of nonneoplastic cells and hampering the molecular genetic analysis of primary tumor samples (7). Finally, familial patterns of pancreatic adenocarcinoma usually do not involve young ages of onset, high penetrance, or extensive pedigrees (8).

We have circumvented some of these problems by the development of a xenograft model of pancreatic adenocarcinoma that generates genetically stable cell expansions, free of infiltrating nonneoplastic human cells (9, 10). Molecular analysis of known oncogenes and tumor-suppressor genes has proven feasible; it is possible to identify both K-*ras* and p16 alterations in over 80% of pancreatic adenocarcinomas (7, 10, 11) and p53 mutations in at least 70% of the cases (12). However, a conventional search for novel loci of interest presented practical obstacles. Allelotyping had identified frequent loss of heterozygosity (LOH; deletion of only one allele), mainly at sites of known genes, such as 9p (p16), 17p (p53), and 18q (*DCC*) (7, 10). A limited number of xenografted specimens, and the typically large areas involved by LOH, precluded a standard search for smaller consensus areas of deletion. An alternative approach for the identification of tumor-suppressor genes preferably would allow high-resolution genome scanning without the need for a statistical analysis of numerous tumor specimens. The newly described technique of representational difference analysis (RDA) (13) suggested a promising approach.

RDA is a means for isolating DNA fragments that are present in only one of two nearly identical complex genomes. It utilizes a subtractive hybridization method but differs from conventional methods (14–16) by using "representations" of the genomes that have a reduction in complexity. Representations are generated by a PCR-based size selection applied to the restriction fragments of both genomes. Moreover, RDA takes advantage of both subtractive hybridization and DNA reassociation kinetics to favor the reiterated PCR amplification of the difference among the two genomes. It has been demonstrated that RDA can enrich difference products over a millionfold after three rounds of selection (13).

Here we apply RDA to the identification of DNA fragments that are deleted in neoplastic tissues. Normal tissue from the patient provides the "tester" sequences, and neoplastic cells provide the "driver" sequences in the hybridization reactions. RDA identifies a simple LOH, when a deletion involves a restriction fragment length polymorphism in such a way that the smaller fragment is deleted in the neoplasm and therefore is present only in the representation of the tester (normal) genome. Due to the PCR-based size exclusion, the larger allele is not present in either of the representations, and the 2:1 allele ratio seen upon comparison of the total genomic DNAs of normal and tumor is converted to a 1:0 ratio in the representations. Thus the existence of the larger allele in the driver will no longer prevent enrichment for the smaller allele in the tester (the "target," or deleted sequence in the tumor) (Fig. 1). In homozygously deleted regions, however, both alleles are absent from the driver genome and thus the target alleles do not

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Abbreviations: LOH, loss of heterozygosity; RDA, representational difference analysis; cM, centimorgan(s); STS, sequence-tagged site; YAC, yeast artificial chromosome; FISH, fluorescence *in situ* hybridization.

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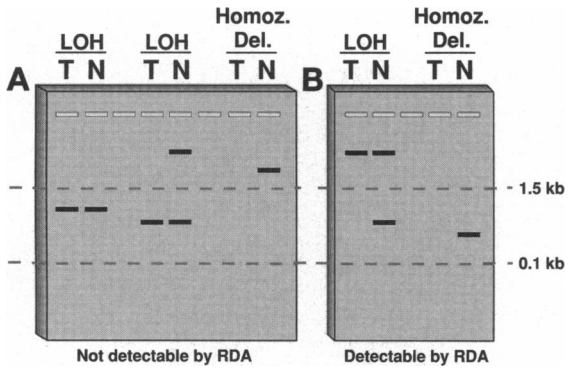

FIG. 1. Identification by RDA of DNA sequences deleted in tumors. The figure is a schematic representation of specific loci within electrophoretically separated restriction endonuclease-digested total genomic DNA from tumor (T) and corresponding normal tissue (N). The area between the broken lines depicts the PCR-based size selection, resulting in "representations" of the genomes. Homoz. Del., homozygous deletion. (*A*) RDA cannot identify losses of single alleles when the DNA fragments are nonpolymorphic in restriction fragment length (lanes 1 and 2), nor can it identify simple LOH wherein the remaining DNA fragment of the tumor lies within the boundaries of the size selection (lanes 4 and 5). Most DNA fragments in a region of homozygous deletion will lie outside the size selection area and therefore cannot be recovered by RDA (lanes 7 and 8). (*B*) RDA identifies DNA fragments at a site of simple LOH if the deletion involves the smaller fragment of a restriction fragment length polymorphism (lanes 1 and 2), and this technique detects a homozygously deleted DNA fragment provided that it lies within the representation (lanes 4 and 5).

need to be polymorphic in restriction fragment length in order to be detectable by RDA.

It can be reasoned that RDA would strongly favor the enrichment of homozygously deleted regions over areas of heterozygous loss in the tumor, allowing the identification of homozygous deletions even among the usually high background of LOH found in many malignancies. Assuming a polymorphism frequency in the human genome of 1 in 300 bp, and a necessity for the loss of the smaller of the two restriction fragments (half of the sites of LOH), the efficiency ratio for the identification by RDA of deleted fragments (comparing those within a homozygous deletion versus those within a site of simple LOH) will be 50:1 when a restriction endonuclease requiring a 6-bp recognition site at both ends of a fragment is used. That is, loss of a random DNA sequence should be detectable by RDA at least 50 times more often if the loss produces a homozygous deletion rather than simple LOH.

Here we describe the identification of a homozygous deletion in a pancreatic adenocarcinoma, using RDA. The homozygous deletion mapped to a 1-centimorgan (cM) region at chromosome 13q, flanked by the markers *D13S171* and *D13S260*. The premise that a tumor-suppressor gene might be located within the region of the homozygous deletion is strengthened by the localization of the recently identified *BRCA2* locus for heritable breast cancer susceptibility (17), which currently encompasses the entire region of the homozygous deletion.

MATERIALS AND METHODS

Case Report. An 84-year-old woman presented with painless obstructive jaundice and was found to have a mass in the head of the pancreas without evidence of metastases. Her medical history included a right-sided colon carcinoma curatively resected at the age of 61. Her family history included multiple incidents of adenocarcinoma, including her mother, who had an adenocarcinoma of the colon resected and who died of breast carcinoma at age 80, her mother's sister, who died of breast carcinoma at age 94, her mother's brother, who died of "stomach" cancer in his 80s, and the patient's brother, who died of colorectal carcinoma at the age of 52. The only siblings in these two generations unaffected by cancer were the patient's sister (alive, age 76) and her mother's sister, who died at the age of 29 from tuberculosis. Both children of the patient are unaffected to date.

Tissue Samples. Tissue specimens were obtained from the pancreas upon its resection at The Johns Hopkins Hospital. Histopathological examination revealed a moderately differentiated primary pancreatic ductal adenocarcinoma. The pancreas cancer was histologically distinct from her previous colorectal carcinoma, slides of which were reviewed. At the time of surgery, normal duodenal mucosa was fresh-frozen at −80°C and xenografts were generated by implantation of 2-mm^3 pieces of the primary tumor into athymic *nude* mice. Xenografts were harvested at a size of 1 cm^3, and DNA was prepared as described (10).

RDA. RDA was performed essentially as described by Lisitsyn *et al.* (13). The restriction endonuclease *Bam*HI and corresponding anchor primers were used for digestion of the DNA samples and subsequent PCR amplifications. For the xenograft-driven RDA, hybridization times were increased to 40 hr. A detailed protocol of the RDA procedure is available from the authors.

The RDA round 2 difference products were cloned by using the pBluescript II plasmid vector (Stratagene). Insert DNAs of individual clones were used as probes for Southern blots containing tester and driver amplicon DNA. These fragments were sequenced by the SequiTherm cycle sequencing method (Epicentre Technologies, Madison, WI) and 20-mer or 24-mer oligonucleotide pairs for sequence-tagged sites (STSs) were designed from these results.

PCR. STSs were amplified by using 40 ng of genomic DNA in 67 mM Tris·HCl, pH 8.8/4 mM $MgCl_2$/16 mM $(NH_4)_2SO_4$/10 mM 2-mercaptoethanol containing bovine serum albumin at 100 μg/ml, dATP, dCTP, dGTP, and dTTP at 200 μM each, each primer at 1 μM, and 2 units of *Taq* DNA polymerase (GIBCO/BRL) in a final reaction volume of 15 μl. The enzyme was added after a preheating step of 2 min at 94°C. For 20-mers, 35 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 1 min were followed by a final extension of 5 min at 72°C. For 24-mers, the annealing step was omitted and the extension step was increased to 2 min. Primer sequences for *DPC1* were 5′-CAGGTCTGAAACGTATAAAGG-3′ and 5′-GAGTCAAGGTAGGCTACTTC-3′, and for *DPC2*, 5′-CTTCCCCAGTGCTTCTAATG-3′ and 5′-CTCTCCTCATCTCTATTTCG-3′. Primer sequences for *DPC1′* were 5′-TTCTCCATCTTCCCACCTAACAGG-3′ and 5′-ATCAGCCATCTTGGCAGCAACTAG-3′, and for *DPC2′*, 5′-AAGCTTCCCCAGTGCTTCTAATGC-3′ and 5′-TTTCCACGTAGGCTGTTGGTGTAG-3′. Primer sequences for *LC01* were 5′-GCCTCCGGTAGGCTTTATTC-3′ and 5′-GAGCGAGACACAGGGATTTG-3′. Dinucleotide markers and the Généthon megaYAC library were purchased from Research Genetics (Huntsville, AL).

RESULTS

RDA. We performed RDA on a human pancreatic adenocarcinoma, essentially as described (13). The strategy is schematically represented in Fig. 2. Tumor DNA was used to drive the subtractions, whereas corresponding normal DNA was used as the tester. Tissue from primary tumors, typically infiltrated with nonneoplastic cells, should not effectively drive the subtractions. We therefore used a carcinoma that had been propagated in an athymic *nude* mouse. Such xenografted tumors are genetically stable and do not contain detectable nonneoplastic human cells (9, 10). As these xenografts contain

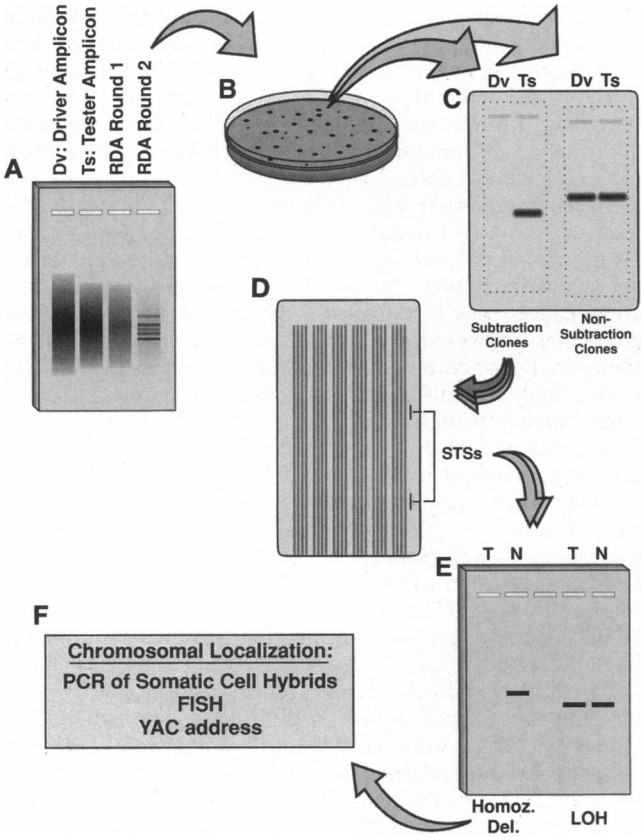

FIG. 2. Schematic strategy for analysis of RDA-generated clones. (*A*) RDA is performed. (*B*) Difference products are cloned by using a plasmid vector, and individual clones are picked. (*C*) Clones are evaluated by using them as probes in multiple Southern blots containing driver (Dv) and tester (Ts) amplicons. Subtraction clones are those present in the tester but absent from the driver; the nonsubtraction clones represent the background nontarget sequences which escape RDA enrichment. (*D*) Subtraction clones are sequenced and STS primer pairs are designed from separate positions within each sequence. (*E*) STS primers are used in PCR to evaluate the original total genomic DNA samples of tumor (T) and normal tissue (N) to exclude those clones representing simple LOH. (*F*) STSs that identify sites of homozygous deletion are used in chromosomal localization techniques and yeast artificial chromosome (YAC) contig generation. FISH, fluorescent *in situ* hybridization.

up to 50% murine cells, we modified the RDA protocol of Lisitsyn *et al.* by increasing the time of DNA annealing to 40 hr.

Genomic representations of the xenograft and normal DNA were generated by using the restriction endonuclease *Bam*HI. After two rounds of RDA, a distinct pattern of DNA fragments was visible upon electrophoretic separation of the difference product (Figs. 2*A* and 3*A*). The round 2 difference product was cloned by using a plasmid vector (Fig. 2*B*). True subtraction fragments were detected by using Southern blots of the tester and driver representations (Fig. 2*C*). This analysis revealed that >80% of 60 randomly selected fragments were subtraction products—i.e., they were absent from driver and present in tester.

The sequences of the cloned fragments were used to design primers to amplify STSs (Fig. 2*D*). Fourteen of 16 STSs derived from unique subtraction fragments were present in normal and xenograft total genomic DNA, consistent with sites of simple LOH in the carcinoma (Fig. 2*E*). Two STSs, designated *DPC1* and *DPC2*, were present in normal but absent from xenograft DNA, indicating that they were homozygously deleted in the pancreatic carcinoma. As a control for DNA quality, duplex PCR was performed for both *DPC1* (Fig. 3*B*) and DPC2 with concurrent use of STS primers for an irrelevant locus (*LC01*),

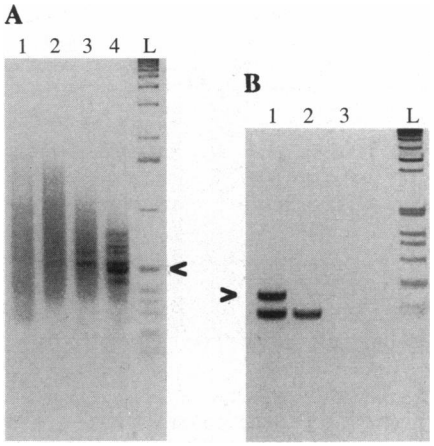

FIG. 3. (*A*) RDA of the pancreatic carcinoma xenograft. Lane 1, PCR-generated amplicon of the xenograft (driver); lane 2, amplicon of normal DNA (tester); lanes 3 and 4, difference product after first and second round of hybridization–amplification, respectively; lane L, 1-kb DNA ladder (GIBCO/BRL). The arrowhead marks 510 bp. (*B*) Duplex PCR analysis with the concurrent use of the STS primer pairs for *DPC1* and for an irrelevant locus (*LC01*) which serves as a positive control for PCR. Lane 1, normal DNA as template; lane 2, xenograft DNA as template; lane 3, template-negative control; lane L, 1-kb DNA ladder. Arrowhead indicates the amplification product of the STS *DPC1*.

which localized to chromosome 14. To exclude simple insertion/deletion polymorphisms, an adjacent sequence of each cloned fragment was amplified with additional STS primers, designated *DPC1′* and *DPC2′*.

As a control, we performed a parallel RDA in which the driver DNA was provided by a cell line derived from the same pancreatic carcinoma. Seven of 8 unique subtraction fragments from this RDA had been identified in the xenograft-driven RDA. These fragments included *DPC1*.

Localization and YAC Contig. The STSs *DPC1* and *DPC2* both localized to chromosome 13 upon PCR analysis of monochromosomal somatic cell hybrid DNAs of NIGMS mapping panel 2 (Coriell Cell Repositories, Camden, NJ) (18). Both subtraction fragments, *DPC1* and *DPC2*, were used to screen a chromosome 13 phage library (American Type Culture Collection). Two-color FISH, using the whole phage DNAs as probes, localized *DPC1* and *DPC2* as distinct nonoverlapping nearby sites on a metaphase preparation, below the centromere of chromosome 13 (Fig. 2*F*).

PCR screening of the Généthon megaYAC library (19) resulted in a YAC contig, encompassing the *BRCA2* region at 13q12–13. YAC y886d8 contained both *DPC1* and *DPC2* and the marker *D13S171*. YAC y951a3 contained *DPC1* and the markers *D13S171* and *D13S267*, whereas y931f4 contained *DPC2*, *D13S260*, and *D13S290*. Five additional YACs confirmed the contig (Fig. 4). Analysis with the markers *D13S289*, *S290*, *S260*, *S171*, *S267*, *S219*, and *S220* did not reveal interstitial deletions within these YACs. YACs suspected to be chimeric, on the basis of Généthon data and our own data, were excluded from the contig.

Dinucleotide markers *D13S289*, *S290*, *S260*, *S171*, *S267*, *S219*, and *S220* in this region were all found to be present in the xenograft DNA, exhibiting a pattern of simple LOH upon comparison with normal DNA. Thus the entire homozygous deletion in the carcinoma mapped between the markers *D13S171* and *D13S260* at band 13q12.3 (Fig. 4). PCR analysis for the candidate tumor-suppressor genes *Brush-1* (21) and *RFC3* (22) revealed the expected PCR products in xenograft DNA. None of the eight YACs in the contig contained the *Brush-1* sequence. Microsatellite instability was not identified at any locus in the carcinoma.

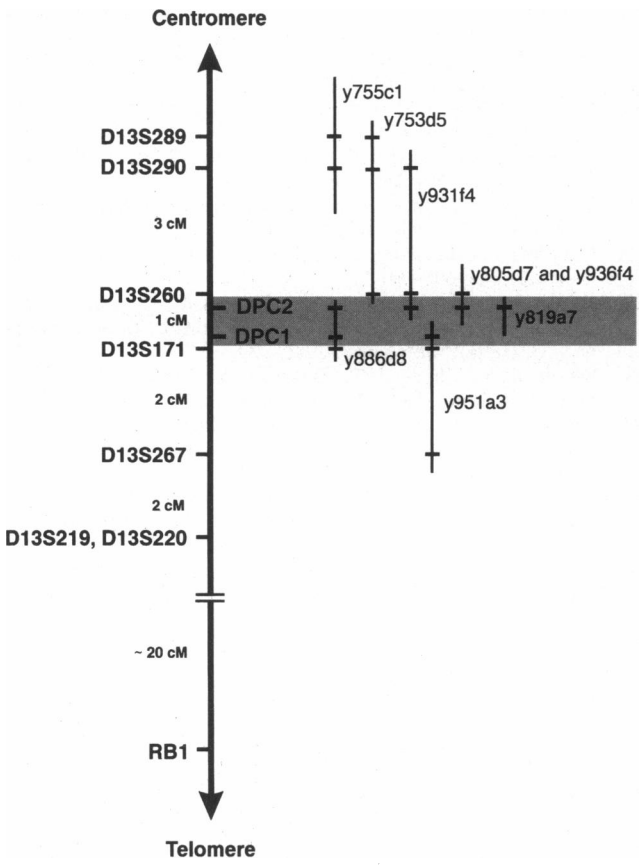

FIG. 4. Schematic map of the region 13q12–13 and flanking markers. The gray area represents the *DPC* region homozygously deleted in the pancreatic carcinoma. The positions of *D13S219, S220, S267, S171, S260, S289*, and *S290* markers and their genetic distances in cM (labeled on the heavy line) were adapted from the 1993–94 Généthon human linkage map (20). The positions of STSs *DPC1* and *DPC2*, which lie within the homozygous deletion of the pancreatic carcinoma, and the order of *D13S289* and *S290*, are based on studies of the YAC contig (light lines) and on the demonstrated presence of one remaining allele of the *D13S219, S220, S267, S171, S260, S289*, and *S290* markers in the xenograft DNA. The positions of the endpoints of the YACs are drawn arbitrarily.

PCR analysis for *DPC1* and *DPC2* revealed that these STSs were present in all of 45 additional pancreatic adenocarcinoma xenografts and in 10 cell lines derived from pancreatic carcinoma (ATCC; ref. 10). On the basis of the localization of the homozygous deletion, an analysis using the polymorphic dinucleotide markers from chromosome 13q was performed (20). This revealed that 7 of 29 pancreatic adenocarcinoma xenografts had LOH of chromosome 13q that spanned 13q12.3. One tumor, reported to have the cytogenetically identified translocation t(13;19)(q12;q13) (23), did not have LOH detectable by using the available markers.

DISCUSSION

RDA has been described by Lisitsyn *et al.* (13) as a means to isolate single-copy sequences that are present in only one of two otherwise nearly identical complex genomes. These investigators showed that RDA can identify binary polymorphisms and polymorphisms linked to a trait of interest (13, 24). Recently, it also has been shown that RDA can identify DNA losses and amplifications in tumors (25), as well as DNA sequences from unknown pathogens in infected tissues (26). Here we have applied RDA for the identification of DNA sequences that are deleted in tumors.

Pancreatic carcinomas, as well as other carcinomas, can exhibit an average fractional allelic loss at least as high as 20% (7, 27). Overwhelmingly, the detected deletions are LOHs; that is, only one of the two alleles is deleted. Although complete data are not available, the occurrence of deletions involving both alleles is considered to be infrequent. Owing to the total loss of particular genetic information, the cellular effect of most homozygous deletions is assumed to be deleterious. Indeed, the homozygous deletions reported to date are relatively small. The significance of the identification of a homozygous deletion is best illustrated by their contribution to the discovery of several tumor-suppressor genes (*RB1, DCC*, and p16) (2–5). The potential for identifying homozygous deletions among a high background of heterozygous losses suggests RDA as a powerful approach for the identification of novel tumor-suppressor genes.

The homozygous deletion identified here by RDA maps to chromosome 13q12.3. Allelic loss at 13q is found in pancreatic carcinoma and in a wide variety of other tumor types. The tumor-suppressor gene *RB1*, located at 13q14, is a candidate target gene within these areas of deletion. However, mutations or other evidence of inactivation of *RB1* have been found in only a subset of tumors (28–30). As for pancreatic adenocarcinoma, previous immunohistochemical analyses of Rb protein expression found no evidence of *RB1* inactivation (7). The identification of a homozygous deletion at 13q12.3 in a pancreatic adenocarcinoma strengthens the suspicion that, besides *RB1*, at least one additional tumor-suppressor gene is located on chromosome 13q. Recently, a syndrome of familial breast cancer susceptibility (*BRCA2*) was linked to a 6-cM region at 13q12–13, between the markers *D13S267* and *D13S289* (17). Although the *BRCA2* candidate region encompasses the deletion we describe here, it as yet is not established whether the same genetic target is involved in pancreatic and breast carcinomas. If the target loci were postulated to be identical, the finding of a homozygous deletion would narrow the region for a gene search to the 1-cM region bounded by *D13S171* and *D13S260*. It would also indicate that *BRCA2* susceptibility is not due to a dominant oncogene (31) but could be attributed to a tumor-suppressor gene along the model proposed by Knudson, wherein both alleles must be inactivated to achieve the full tumorigenic phenotype (1).

One of our carcinomas under study contains a translocation of 13q, with the breakpoint observed at or near the *DPC* locus (23). However, our analysis with dinucleotide markers did not reveal LOH at any flanking site of 13q in this particular carcinoma. LOH analysis might underestimate the fraction of cases with genomic alterations in a particular gene. It is also possible that additional cases of pancreatic carcinoma harboring a homozygous deletion would have gone undetected, since the markers flanking the *DPC* region are located 1 cM apart. We reported a possibly analogous situation with the p16 tumor-suppressor gene, wherein we detected two pancreatic carcinomas as having a homozygous deletion upon the use of two flanking markers, and yet an additional eight homozygous deletions were identified only upon analysis of the p16 gene itself (10). Similarly, the majority of homozygous deletions involving *RB1* are intragenic. Conversely, other tumor-suppressor genes, like p53, rarely are inactivated by homozygous deletion. Additional evidence for the involvement of a tumor-suppressor gene of general importance for pancreatic carcinoma includes our finding of LOH that spans 13q12.3 in nearly a quarter of the cases. This frequency of LOH at 13q is comparable with that found for breast carcinoma (32–34) and may be significant even though measurably less than frequencies found at loci of some other tumor-suppressor genes. We postulate that a 1-cM region at 13q12.3, flanked by markers *D13S171* and *D13S260*, contains a tumor-suppressor gene that is involved in pancreatic carcinoma.

The patient in the present study was a member of a familial clustering of adenocarcinomas of various organ sites (see *Case Report*). Two related points can be elaborated. First, the age of onset in this familial cluster is rather late. Indeed, an onset at older age is the pattern found for most familial pancreatic carcinoma pedigrees (8). Many, if not most, familial clusters of carcinoma in the general population do not reproducibly involve onset at young age. A comprehensive understanding of monogenic and polygenic influences on cancer susceptibility will have to include studies of these less distinctive phenotypic patterns of susceptibility (35). Second, it will be of interest to determine whether the individuals of the presently reported familial cluster are hemizygous in the region, which would suggest that the putative tumor-suppressor gene at 13q12.3 might be involved in a variety of malignancies. This would be consistent with the frequent occurrence of allelic loss at 13q in multiple tumor types that is not readily attributable to inactivation of the *RB1* tumor-suppressor gene (7, 36–38).

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