High-Density Screen of Human Tumor Cell Lines for Homozygous Deletions of Loci on Chromosome Arm 8p

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Tumor cell-specific homozygous deletions coinciding at a particular genetic location may indicate the inactivation of a nearby tumor suppressor gene. Forty-six human cancer cell lines of prostate, pancreatic, lung, liver, and colon origin were screened for homozygous deletions of 139 expressed sequence tag (EST) and sequence-tagged site (STS) loci spanning the entire short arm of chromosome 8. Only one Southern blot–verified homozygous deletion was detected in this set of cell lines. The deletion, in pancreatic tumor cell line MIA-PaCa-2, encompassed two screening loci, D8S549 and D8S1992, and overlapped another previously described homozygous deletion of band 8p22 in a metastatic prostate cancer specimen. Both deletions entirely removed the candidate tumor suppressor gene *N33*. These data define a consensus homozygous deletion region in chromosome band 8p22. *Genes Chromosomes Cancer 24:42–47, 1999.*

INTRODUCTION

Chromosome arm 8p has been identified as having high rates of allelic loss in many human cancer types, including prostate, colorectal, non-small cell lung, hepatocellular, bladder, laryngeal, and breast carcinomas (Emi et al., 1992; Knowles et al., 1992; Van der Bosch et al., 1992; MacGrogan et al., 1994; Yaremko et al., 1995; Sunwoo et al., 1996; Vocke et al., 1996). In many studies, the allelic losses of this chromosome arm appear to assort into two or more separate regions (e.g., 8p22 and 8p12–21), suggesting genetic or mechanistic complexity, or both. To date, no credible tumor suppressor candidates have been identified on the basis of 8p allelic loss.

The analysis of homozygous deletions, which are observed much less frequently than allelic losses, has been important in the discovery of several tumor suppressor genes such as *CDKN2A* (Kamb et al., 1994), *BRCA2* (Schutte et al., 1995; Wooster et al., 1995), *DPC4* (Hahn et al., 1996a), and *MMAC1/PTEN* (Li et al., 1997; Steck et al., 1997). In one study of 8p allelic loss in prostate cancer, Bova et al. (1993) described a node-metastatic prostate cancer with homozygous deletion of the macrophage scavenger receptor (*MSR*) locus. As the only known example of 8p homozygous deletion in any human cancer, this deletion and its genetic neighborhood in chromosome band 8p22 were physically mapped using radiation hybrids, yeast artificial chromosomes (YAC), and pulsed-field electrophoresis with numerous probes, expressed sequence tag (EST) and sequence-tagged site (STS) loci, and infrequent-cutting restriction sites as landmarks (Bookstein et al., 1994; Bova et al., 1996).

The deletion was found to extend from *MSR* 730 kb to 970 kb toward the telomere (Bova et al., 1996). Screening of the region for expressed sequences yielded *MSR* plus one novel gene, *N33*, spanning approximately 200 kb within the homozygous deletion (MacGrogan et al., 1996). N33 was homologous by amino acid sequence to an accessory subunit of oligosaccharyltransferase in *Saccharomyces cerevesiae* and to an open reading frame of unknown function in *Caenorhabditis elegans*. Interestingly, the upstream regulatory region of *N33* was found to be methylated, and gene expression silenced, in a number of human tumor cell lines (most frequently colorectal carcinomas). However, neither specific inactivating mutations nor tumor suppressor activity were detected by standard surveys or assays in prostate and colorectal cancer samples or cell lines, leaving the identification of *N33* as a tumor suppressor gene uncertain.

In this study, we sought to address the paucity of known 8p homozygous deletions by carrying out a systematic search for them in a panel of human tumor cell lines by using current EST/STS mapping resources and a high-throughput polymerase chain reaction (PCR) methodology. Of >400 such markers mapped to chromosome arm 8p, 180 were randomly chosen for screening of this ~50-Mb

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TABLE 1. Tumor Cell Line Screening Panel by Cancer Type[a]

Pancreatic	Prostate	Colon	Liver	Lung
ASPC-1	ARCaP	CACO2	HEP3B	A549
BXPC-3	CA-HPV-10	COLO-205	HEPG2	NCI-H358
CAPAN-1	DU145	COLO-320	HLE	NCI-H596
CAPAN-2	LNCaP-FGC	DLD-1	SK-Hep-1	NCI-H661
COLO587	NCI-H660	EB		SK-LU-1
HPAC	PC-3	HCT 116		WI38
HPAF-11	PPC-1	HT-29		
HS700T	TSU-PR1	LS174T		
HS766T		RKO		
MIA-PaCa-2		SK-CO-1		
PANC-1		SW-1417		
		SW-1463		
		SW-403		
		SW-48		
		SW-480		
		SW-620		
		SW-837		
		WIDR		

[a]ARCaP DNA was substituted for SK-LU-1 DNA when it became available halfway through the screening procedure.

chromosome arm (Strachan and Reed, 1996). Although the extensive use of EST markers was expected to provide overrepresentation of gene-rich vs. gene-poor regions, this distribution was considered advantageous for detecting functionally significant deletions affecting expressed genes. However, the complete screening procedure yielded only a single new homozygous deletion, suggesting the relative rarity of this alteration, at least in the megabase-size range. Surprisingly, despite probe representation over the entire chromosome arm, the new homozygous deletion was located in 8p22 and overlapped the deletion previously characterized by Bova et al. (1996).

MATERIALS AND METHODS

Tumor Cell Lines and DNA Preparation

Tumor cell lines are listed in Table 1. Most were obtained from the American Type Culture Collection (Rockville, MD). TSU-PR1 was a gift from Dr. John Isaacs and ARCaP DNA was a gift from Dr. Leland Chung. The human/Chinese hamster somatic cell hybrid containing human chromosome 8, repository number GM10156C, was obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Total genomic DNA was extracted from cell lines by standard methods (Sambrook et al., 1989).

Mapping Resources and Primers for Homozygous Deletion Scan

A set of 160 STS/EST markers spanning 8p was identified at the Whitehead Institute/MIT Center for Genome Research Human Physical Mapping Project (Hudson et al., 1995) (http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map). Based on the associated cytogenetic, linkage and physical mapping data, marker D8S519 was selected to represent the centromeric boundary of the p arm. A set of 336 EST markers was identified at the National Center for Biotechnology Information (NCBI) Human Gene Map Project (Schuler et al., 1996) (http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96/msrch2), using 8pter and D8S519 as the interval endpoints. These EST markers have been ordered in "bins" defined by 82 radiation hybrid-mapped reference markers. The Whitehead and NCBI sets were merged based on their many shared markers. Redundant ESTs made from the same transcripts or cDNA clones were eliminated when detected. Of the ~400 resulting markers, 180 were chosen for PCR screening based on the availability of premade primers and random selection among binned ESTs (the final list of markers is available from the authors upon request). Premade oligonucleotide primers were supplied by Research Genetics (Huntsville, AL). One additional STS within an insertion-deletion polymorphism ("Del-118") (Wieland et al., 1992) was used to validate the detection of deletions by our PCR methods.

Polymerase Chain Reaction

We used a robotic workstation (Biomek 2000 Beckman Instruments, Inc., Fullerton, CA) to set up PCR reactions in 96-well plates (GeneAmp PCR System 9600, Perkin-Elmer, Norwalk, CT). For all primer pairs, 20 μl PCR amplifications with 100-ng DNA template (or TE buffer control) were performed under the following conditions: an initial denaturation step at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, followed by a final extension at 72°C for 5 min. Ten microliters of the PCR products were separated by electrophoresis in 2% agarose gels in 1 × TAE, visualized by ethidium bromide staining, and documented on an image analysis system (Alpha Imager 2000, Alpha Innotech Corp., San Leandro, CA).

DNA Clones

Plasmid clones containing selected EST sequences were obtained from the IMAGE collection (Genome Systems, St. Louis, MO). Genomic and cDNA clones in the 8p22 region were described previously (Bookstein et al., 1994; MacGrogan et al., 1996). Purified insert fragments were used as probes on Southern blots.

TABLE 2. PCR-Negative Cell Lines and Loci, With Methods and Results of Confirmation

Cell line	Locus	Method	Result
NCI-H660	WI-13936	Nested PCR	Present
BXPC-3	D8S504	Nested PCR	Present
BXPC-3	SGC33989	Southern blot	Present
MIA-PaCa-2	D8S1992	Southern blot	Deleted
MIA-PaCa-2	D8S549	Southern blot	Deleted

Preparation of Southern Blots and Hybridization

Ten-microgram aliquots of high-molecular-weight DNA were digested overnight with *Eco*RI restriction enzyme. Digests were run on 1% agarose gels and blotted onto nylon membrane filters as described (Sambrook et al., 1989). Probes were radiolabeled by random priming. Hybridizations were performed in RapidHyb (Amersham, Arlington Heights, IL) for 1 hour at 68°C. Final washes were in 0.1 × SSC, 0.5% SDS at 68°C.

RESULTS

First-Pass and Clean-Up PCR Screening

DNA from 46 cancer cell lines (Table 1) and a human chromosome 8 somatic cell hybrid (as a control for correct chromosomal mapping) were screened with 180 PCR markers (a total of 8,640 reactions, including negative controls) in two 48-well groups per 96-well plate. Forty-one markers (23%) failed to amplify in most or all templates with our standardized PCR conditions, and these loci were dropped from further consideration. The remaining 139 loci amplified with the chromosome 8 control and the great majority of tumor cell templates. The DNA samples that failed to amplify with any of the "working" loci were retested in individual reaction tubes. Most of the repeated reactions were positive for the expected PCR product, suggesting a technical basis for the negative initial result. Ultimately, only five loci were repeatedly negative in three different cell lines (Table 2). Interestingly, in one pancreatic cancer cell line, MIA-PaCa-2, the two tentatively deleted loci were adjacent to one other and located in the previously mapped region of 8p22 (Bova et al., 1996).

Confirmation of Homozygous Deletion

To distinguish homozygous deletion from rare polymorphisms of primer binding sites, we performed Southern blot analysis with hybridization probes derived from original IMAGE collection EST clones, or, if these were not available, nested primer sets designed from the EST sequence (Table 2). By these methods, the putative deletions of three loci (D8S504, SGC33989, and WI-13936) were refuted (data not shown). This left only the two adjacent loci, D8S549 and D8S1992, for further consideration. D8S549 is a CEPH/Genethon $(CA)_n$ microsatellite polymorphism without an available hybridization probe; D8S1992 is a $(CA)_n$ polymorphism located near exon 3 of *N33* as described by Bova et al. (1996). Because both of these loci were located within our previously mapped genetic region, we turned to the numerous precisely mapped PCR and hybridization probes that already existed. Homozygous deletion in MIA-PaCa-2 cells was confirmed in Southern blotting analysis with sev-

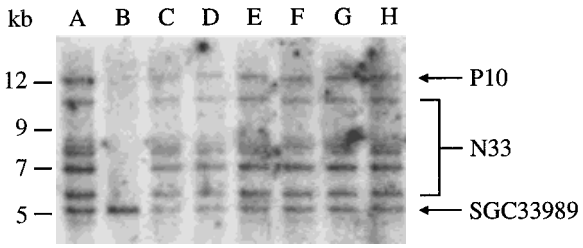

Figure 1. Homozygous deletion of *N33* in MIA-PaCa-2 DNA. Southern blots were prepared as described with DNA samples as follows: A, BXPC-3; B, MIA-PaCa-2 (homozygous deletion of *N33* and *P10*); C, HT29; D, COLO587; E, DU145; F, CAPAN-2; G, HCT116; H, HS766T). Probes N33C (MacGrogan et al., 1996), P10 (Bova et al., 1996), and SGC33989 (http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96/msrch2) were applied sequentially without stripping between hybridizations. Autoradiography was performed with phosphor storage screens (Molecular Dynamics, Inc., Sunnyvale, CA)

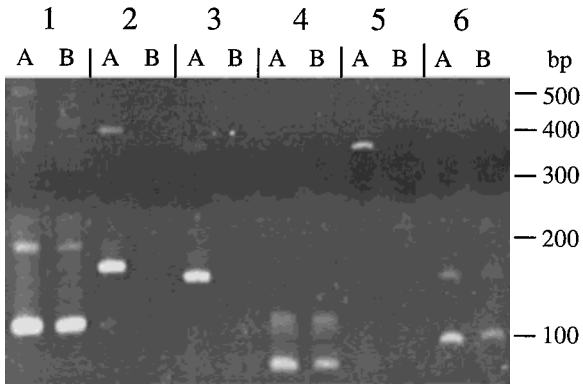

Figure 2. Additional PCR-based deletion analysis of MIA-PaCa-2 DNA. PCR was performed as described with primers specific for the following 8p22 loci (Bova et al., 1996; MacGrogan et al., 1996): 1, E17 (present); 2, C18–2644 (deleted); 3, J12 (deleted); 4, E3 (present); 5, N33 exon 5 (deleted); 6, E2d (present). Templates: A, NCI-H596 (control); B, MIA-PaCa-2. Primer sequences for E3 and E17 were previously published (Bookstein et al., 1994); the others were: C18–2644 (5′-CCTCTAAGTCCCTGTGTCAG and 5′-AAGCGCTAATGGGTCTCT); J12 (5′-AGATCTGAAGAAGATGATCAC and 5′-TGTCTTGACTTCCCTTATG); N33 exon 5 (5′-GGTGGCATGTTTCTGAGT and 5′-CTTCTCATTCAGACTTAAGTTC); E2d (5′-TGAAGCCATCTGTGGG and 5′-GTAAGAACTGTTAAGTGGAAAG).

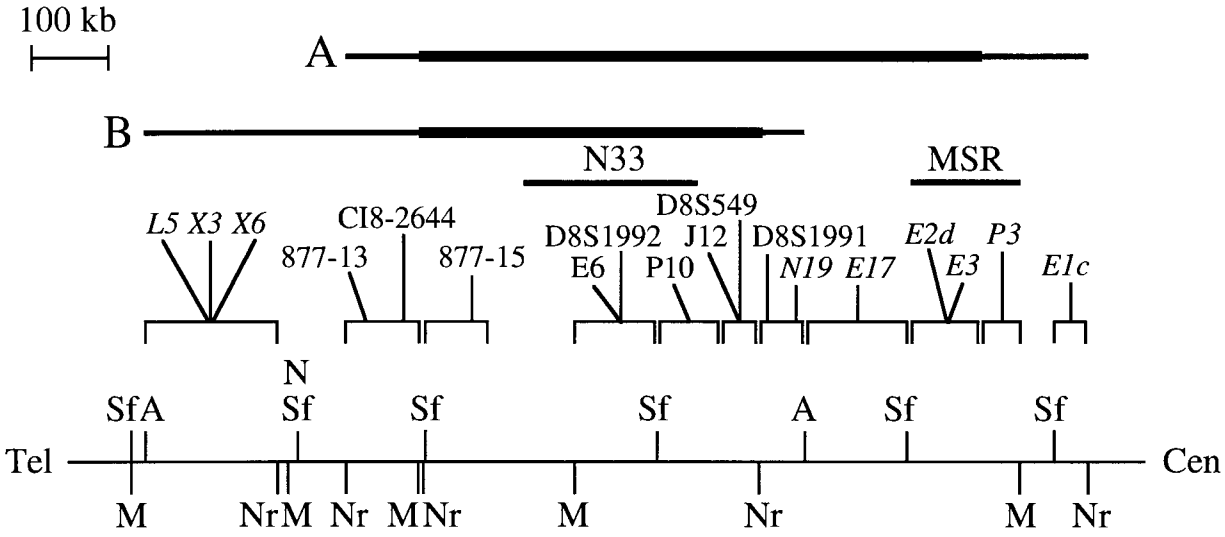

Figure 3. Restriction map of markers in chromosome 8p22 and their deletion in two cancers. Deletion "A" was previously described by Bova et al. (1996) in a node-metastatic prostate cancer sample; deletion "B" is the subject of the present report. The minimal and maximal sizes of deletions are shown as thick and thin lines, respectively. The displayed markers are those tested in tumor "B"; the retained loci are shown in italics. Brackets show the minimum interval to which each marker was mapped, as in Bova et al. (1996). Intervals were defined by infrequently cutting restriction sites (A: *Asc*I; M, *Mlu*I; N, *Not*I; Nr, *Nru*I; Sf, *Sfi*I) or by YAC ends (Bova et al., 1996). The locations of two known genes, *N33* and *MSR*, are shown. Tel: telomeric; Cen: centromeric.

eral different single-copy probes, including *N33* cDNA, which showed deletion of all exons (Fig. 1).

Additional hybridization and PCR loci were used to define further the extent of the deletion in this cell line (Fig. 2). The centromeric boundary was confined to a ~50-kb *Nru* I-*Asc*I restriction fragment based on a breakpoint between D8S1991 and N19, which both map to this fragment (Fig. 3). This boundary was thus located within the previously described deletion ("A" in Fig. 3), and *MSR* was excluded from the new deletion ("B" in Fig. 3).

Mapping of the telomeric boundary of "B" was less refined. It was telomeric to the breakpoint in "A," based on results with locus 877–13, which was retained in "A" but deleted in "B." Probes L5, X3, and X6, located within a common ~170-kb interval, were all retained in "B." The intervals with retained and deleted markers were further separated by an 80-kb predicted segment without any available probes; thus, the telomeric deletion boundary was located somewhere within these three segments.

Our attempts to measure the deletion size by pulsed-field gel electrophoresis of agarose-embedded MIA-PaCa-2 DNA failed because of its resistance to digestion at relevant restriction sites (e.g., *Asc*I) found in YAC DNA (data not shown). Because of the uncertainty about the telomeric boundary of deletion "B," its size is deduced to be between 440 kb and 860 kb (Fig. 3). Deletions "A" and "B" thus overlap by at least 440 kb, with the common deletion region containing the entire *N33* gene (MacGrogan et al., 1996).

DISCUSSION

Homozygous Deletions of Chromosome Arm 8p Are Uncommon

The complexity of allelic losses, the paucity of homozygous deletions, and the recent availability of hundreds of physically mapped EST markers led us to screen a panel of tumor cell lines blindly for additional homozygous deletions on 8p. Only one homozygous deletion was detected despite a reasonably high average marker density (~3 per Mb) and an intrinsic bias toward gene-rich regions. Although no direct comparison can be made to other studies, these results stand in contrast to, at another extreme, a 30% rate of homozygous deletion of markers on 18q in pancreatic cancer xenografts (Hahn et al., 1996a). Of course, more deletions might be found if marker densities and numbers of cell lines were increased, up to a practical limit. It is of interest that the single new deletion was found in a tumor cell line of pancreatic origin, a cancer type also host to a unique homozygous deletion of the *BRCA2* gene (Schutte et al., 1995). Taken together with the high overall frequency of deletions detected in pancreatic tumor cell lines (Hahn et al., 1996b), pancreatic cancers may have an unusual predilection for suffering large homozygous deletions.

A Second, Overlapping Homozygous Deletion in 8p22

In light of the apparent rarity of large 8p homozygous deletions, it is striking that the deletion in MIA-PaCa-*2* cells coincided with a previously described tumor-specific deletion despite a search across the entire chromosomal arm. Three alternative explanations may be considered. First, deletion of *N33*, although rare, is a selective event in oncogenesis, i.e., *N33* is a tumor suppressor gene. Second, the deletions actually target another nearby gene, so far unknown. Third, this region of 8p*22* is intrinsically predisposed to deletion without any biological selection for the event in cancer cells. The last explanation may also be formulated as a lack of selection against homozygous deletions in this region compared to deletions elsewhere on 8p. The data at hand are insufficient to enable us to distinguish among these possibilities. Although *N33* appears to lack tumor suppression activity by standard assays, perhaps its loss could play a role at a specific oncogenic stage that is not detected by these tests. On the other hand, the Giemsa-dark staining properties of 8p*22* (Strachan and Reed, 1996) and the limited numbers of expressed sequences assigned to this region suggest that it is relatively gene-poor. It has been theorized that the strong biological selection against homozygous deletion of essential genes is not operative in gene-poor regions. Finally, there are many examples of innately unstable genomic sequences, especially in cancer cells, e.g., *FHIT* (Ohta et al., 1996). Additional genetic analysis will be necessary for a full understanding of the significance of these findings in 8p*22*.

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