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Identification of Two Common Regions of Allelic Loss in Chromosome Arm 12q in Human Pancreatic Cancer[1]

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ABSTRACT

Using the method of microsatellite analysis, we studied 40 tissues with pancreatic ductal adenocarcinoma and identified two commonly deleted regions on the long arm of chromosome 12. One (region A) was found between *D12S81* and *D12S1719* at 12q21 at a frequency of 67.5%, and the other (region B) was located between *D12S360* and *D12S78* at 12q22–q23.1 at a frequency of 60%; the latter was reported previously (M. Kimura, *et al.* Genes Chromosomes Cancer, *17:* 88–93, 1996). The results of microsatellite analyses were verified by fluorescence *in situ* hybridization. We further analyzed 19 pancreatic cancer cell lines by fluorescence *in situ* hybridization and found that 10 of them showed allelic loss at *D12S81* and 6 showed allelic loss at *D12S360*. Yeast artificial chromosome contigs were constructed to cover the deleted regions. Region B was completely covered by a 650-kb yeast artificial chromosome clone. The frequently deleted regions in chromosome 12q in pancreatic cancer that were identified here may provide new avenues for isolating novel tumor suppressor genes.

INTRODUCTION

Pancreatic cancer is one of the leading causes of cancer death in Japan, as well as in Western countries. The poor prognosis is due to difficulty in diagnosis and inefficiency in surgical and/or multidisciplinary treatment of the cancer (1). An understanding of the molecular mechanisms of pancreatic carcinogenesis would provide important molecular clues for the development of methods for early detection and efficient therapy of this very malignant disease. Recent cytogenetic studies using karyotype analyses, as well as microsatellite analyses, CGH,[4] and FISH, suggest that many genetic alterations occur in several chromosomal regions other than those including *MTS1*, *p53*, and *DPC4* (2–11). These results suggest possible involvement of unknown genes in pancreatic carcinogenesis. We previously performed an allelotype analysis to search for localization of putative tumor suppressor genes and identified a 1-cM region of common allelic loss in 12q22–q23.1 (12). However, our CGH analysis suggested the existence of a region of common allelic loss in 12q other than the one that we had identified by microsatellite analysis (13). Hence, we examined the entire region on the long arm of chromosome 12 with microsatellite analysis and FISH combined to construct a deletion map of the entire chromosome 12q arm in pancreatic ductal adenocarcinoma. Herein, we report identification of the two distinct regions of common allelic loss in 12q in human pancreatic cancer.

MATERIALS AND METHODS

Tissues and Cell Lines. The materials studied were from 40 cases of primary pancreatic ductal adenocarcinoma (23 men and 17 women, ages 31–81 years; mean age = 63.5 years). Thirty-three cases were surgically resected, and 7 were obtained at autopsy. The average survival period after diagnosis was 15.1 months. According to the clinical staging system of the Japan Pancreas Society (14), 2 cases were in stage II, 9 were in stage III, and 28 were in stage IV. A brief explanation of the clinical staging system of the Japan Pancreas Society was included in our previous report (13). In one case, no description of the clinical stage was available. Histological diagnoses of the tumors were as follows: 35 tubular adenocarcinomas, 2 papillary adenocarcinomas, 2 adenosquamous carcinomas, and 1 anaplastic carcinoma. Of the 40 tissues, 37 specimens were formalin-fixed, paraffin-embedded tissues, and 3 were frozen tissues. Tissues were cut to provide 13–15 20-μm-thick sections for microdissection. These sections were sandwiched between two 3-μm-thick sections that were stained with H&E for histopathological examination. Under a microscope, tumor cells were carefully dissected from the 20-μm-thick sections that had been deparaffinized, hydrated, and stained with hematoxylin. In each sample, normal cells were also collected from the adjacent normal tissue. DNAs were extracted from the dissected tissues, as described previously (12). Nineteen pancreatic cancer cell lines, PK-1, PK-8, PK-9, PK-59, PCI-6, PCI-10, PCI-19, PCI-35, PCI-43, PCI-64, PCI-66, PAN02JCK, PAN03JCK, PAN07JCK, PAN09JCK, Panc-1, MIA PaCa-2, SU.86.86, and BxPc-3, were cultured as described (15–24). The primary tumors from which these cell lines derived were not available.

Microsatellite Analysis. Allelic imbalances were detected by the microsatellite analysis method in 40 tissues, as described previously (12). Twenty-four microsatellite markers were used, as listed in Fig. 2, to cover the entire long arm of chromosome 12. Detailed information on the primers and PCR conditions used is available upon request. The primers were end-labeled with [^{32}P]ATP with polynucleotide kinase (New England Biolabs, Beverly, MA) and used for PCR. The amplified products were fractionated in a gel composed of 6% polyacrylamide, 8 M urea, and 32% formamide and then autoradiographed. Relative intensities of allele-specific bands were examined by quantitative densitometry using NIH Image software (details of this software are available at: http://cbel.dcrt.nih.gov/~mvivino/ImgEngrHTML/ImgEngr.html). LOH was defined as 50% or more reduction in relative intensity in one allele-specific band, as compared to the band of the normal counterpart. Homozygous alleles, microsatellite instabilities, and poor yield of PCR products for each marker were not scored in this study.

Construction of Contigs Using BAC and YAC. DNA superpools of YAC and BAC libraries (Research Genetics, Huntsville, AL) were screened by PCR with primers for the microsatellite markers, according to the manufacturer's instructions. BAC and YAC clones that were positive for microsatellite markers were purchased from Research Genetics. Agarose plugs of YACs and BACs were prepared and analyzed for their sizes using the CHEF Mapper pulsed-field gel electrophoresis system (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. Total yeast DNA, including YAC, was purified by the methods described (25). Cosmid libraries were generated from the DNAs of YACs and screened to select clones that harbor human sequences by colony hybridization with total human DNA as the probe (26). Cosmid clones harboring microsatellite markers were detected by hybridization with end-labeled oligonucleotide primers that were used for PCR amplifications. A

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[4] The abbreviations used are: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; LOH, loss of heterozygosity; BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome; STS, sequence-tagged site.

Fig. 1. Microsatellite analyses of tissues with pancreatic ductal adenocarcinoma. *Lanes T*, tumor; *Lanes N*, normal. *Arrowheads*, loss of allele-specific bands.

contig of YACs and BACs was constructed, based on PCR-mediated detection of the microsatellites and STSs in YAC and BAC DNAs. Sequences of STS markers WI-4478 and WI-4156 were obtained from the Whitehead/Massachusetts Institute of Technology STS-based map of the human genome (http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map).

Two-Color FISH Analysis. The two-color FISH method was used for the analysis of LOH in the 19 pancreatic cancer cell lines and 3 frozen tissues (cases 515, 550, and 551), according to methods described previously (27). A plasmid clone, pα12H8 (28), which harbored α-satellite DNA within the centromeric region of chromosome 12 (*D12Z3*/12cen), was obtained from American Type Culture Collection (Manassas, VA). Cosmid clones c872-3-E4, harboring *D12S81*, and c25, harboring *D12S360*, were cloned from the cosmid libraries generated from YAC DNAs, as described previously (27). The probe for 12cen (*D12Z3*) was labeled with digoxigenin-dUTP (Boehringer Mannheim, Mannheim, Germany). The probes for c872-3-E4 (*D12S81*) and c25 (*D12S360*) were labeled with biotin-dUTP (Boehringer Mannheim) by the nick translation method using the Nick Translation Kit (Boehringer Mannheim), according to supplier's recommendations. The probes were mixed with 150 ng of COT-1 DNA (Life Technologies, Inc., Rockville, MD) to suppress repetitive sequences. Hybridization was carried out at 37°C for 48 h. Development was carried out with rhodamine-antidigoxigenin antibody and avidin-FITC. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole. Signals were counted for at least 100 nuclei in each sample. Digital image analysis and display of the fluorescence image was done using the ISIS imaging system and Axioplan 2 microscope (Carl-Zeiss, Jena, Germany). The number of chromosomes was counted in the 4′,6-diamidino-2-phenylindole image of metaphase of the cell.

Statistical Analysis. Clinicopathological features, including age, sex, histological type, clinical stage, and prognosis, were collected from clinical

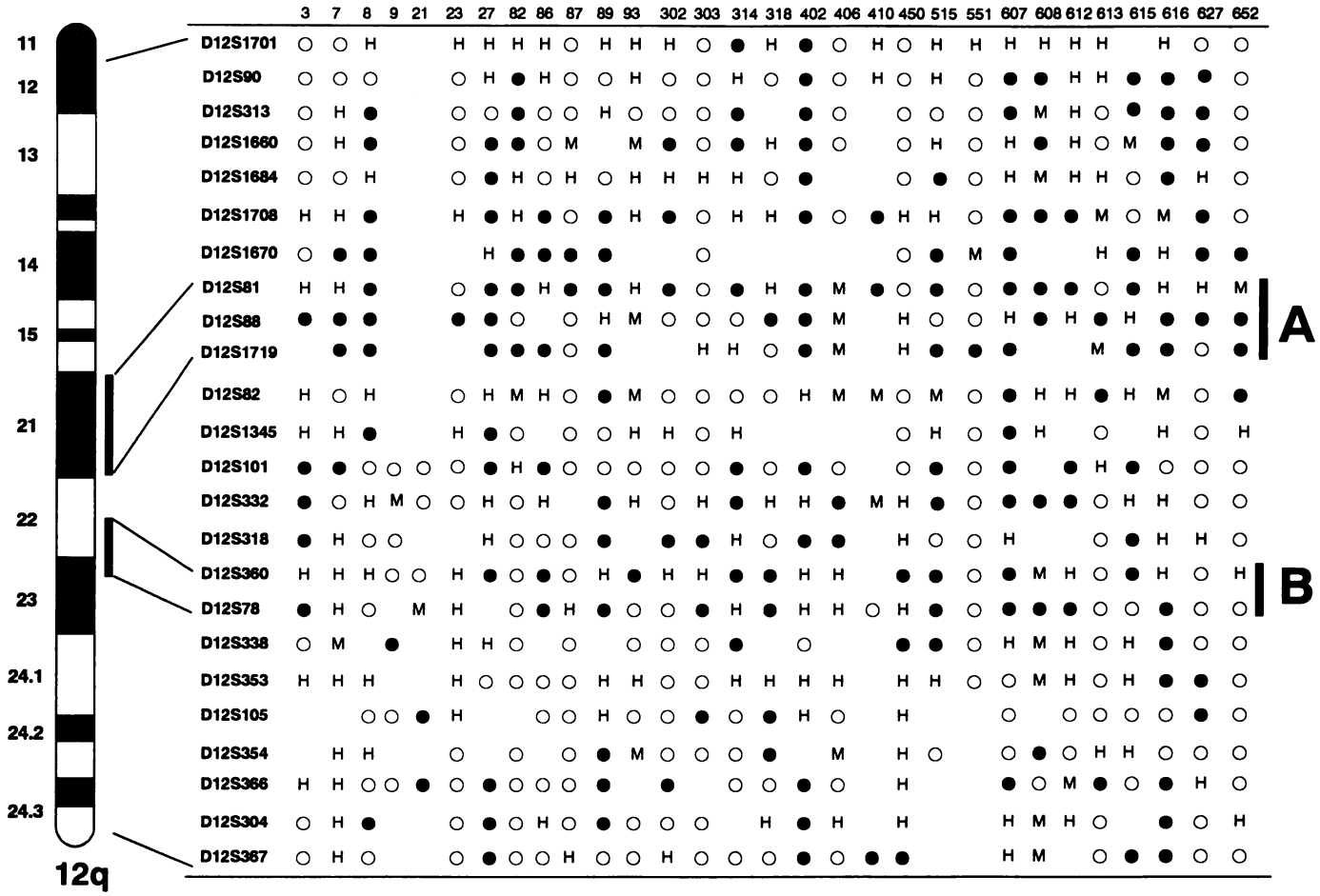

Fig. 2. A deletion map on chromosome 12q. ○, retention of heterozygosity; ●, LOH. *H*, homozygosity; *M*, microsatellite instability; *blanks*, no information due to the limited amount of samples or poor yield of PCR products. Only tumors that exhibited at least one locus of interstitial allelic loss are shown. *Right*, common regions of deletion (*A*, region A; *B*, region B).

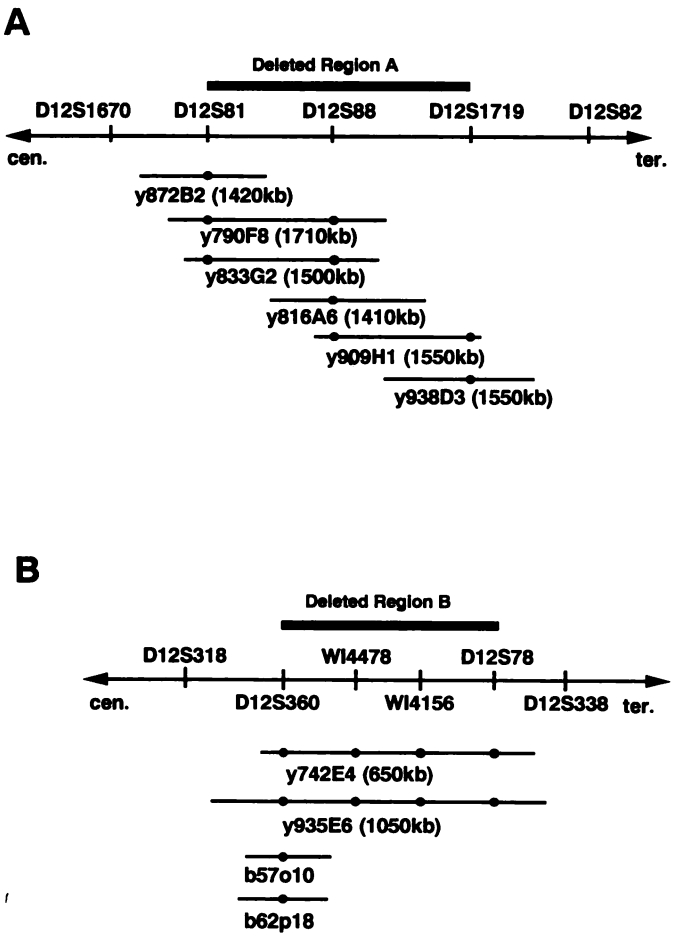

Fig. 3. YAC and BAC contigs covering the commonly deleted regions. *Numbers in parentheses*, actual size of each YAC (in kb). *A*, a total of six YACs covered the region between *D12S81* and *D12S1719*. *B*, a total of two YACs and two BACs covered the region between *D12S360* and *D12S78*.

charts. Correlations between LOH and clinicopathological features were statistically analyzed using Statview (Abacus Concepts Inc., Berkeley, CA) software.

RESULTS

We analyzed LOH on the entire 12q arm in 40 microdissected tissues taken from pancreatic ductal adenocarcinomas. LOH was determined by detection of an allelic imbalance between normal and tumor tissues in PCR-mediated amplified products of microsatellite markers. Typical examples of the microsatellite analyses are shown in Fig. 1. In case 551, one allele-specific amplified band for *D12S1719* in the tumor tissue (Fig. 1, *top*, *arrowhead*) was significantly reduced in intensity, compared with a corresponding band of the normal tissue. At the flanking markers *D12S88* and *D12S82*, no imbalance was observed. In case 627 (Fig. 1, *middle*), loss at *D12S88* and retention at *D12S1719* were observed; in case 515 (Fig. 1, *bottom*), retention of *D12S88*, with loss at flanking markers of *D12S81* and *D12S1719*, was observed.

Results of the microsatellite analyses are summarized in Fig. 2. Two common regions of allelic loss were found: one was between *D12S81* and *D12S1719* in 12q21 (Fig. 2, |*A*; region A), and the other was between *D12S360* and *D12S78* in 12q22–q23.1 (Fig. 2, |*B*; region B). In some of the tumors (cases 82, 87, 302, 515, and 551), results did not clearly indicate that region A was the common region of allelic loss (see "Discussion"). The frequency of allelic loss in region A was 67.5% (27 of 40), and that in region B was 60% (24 of 40). According to Dib *et al.* (29), both regions A and B are less than 1 cM. Regions of common allelic loss other than regions A and B were also detected: a region centromeric from *D12S1684* (see case 615), a region between *D12S82* and *D12S332* (see case 7), a region between *D12S353* and *D12S354* (see cases 303 and 627), and a region between *D12S366* and *D12S304* (see cases 8, 27, 302, 402, 410, 607, 613, 615, and 616).

We next screened YAC and BAC libraries, followed by construction of contigs, guided by microsatellite and STS markers. To minimize erroneous interpretation by deleted and/or rearranged YAC clones, we made efforts to isolate as many YAC clones as possible to cover the deleted regions. To cover region A, six YAC clones were isolated and used for the construction of a contig, as shown in Fig. 3*A*. For region B, two YACs and two BACs were isolated and used for the construction of a contig, as shown in Fig. 3*B*. A YAC clone, y742E4, which was estimated to be 650 kb by pulsed-field gel electrophoresis, was found to completely cover region B.

We further constructed cosmid libraries from the YAC clones, and a cosmid contig was partially constructed by previously described methods (27). Using the cosmid clones derived from these libraries, two-color FISH analyses in tissues were performed with a probe for the centromere of chromosome 12 (pα12H8; *D12Z3*) and cosmid clones c872-3-E4 (*D12S81*) and c25 (*D12S360*). Typical examples are shown in Fig. 4. Cell nuclei from case 515 showed three signals for *D12Z3*/12cen, whereas only one signal for *D12S360* was observed (Fig. 4*A*). The reduced number of signals for each locus, compared with those for the centromere, indicated allelic loss of the locus. This was consistent with the LOH data from the microsatellite analysis.

We also analyzed 19 pancreatic cancer cell lines by FISH. Allelic loss was detected in some cell lines: a decreased number of signals for

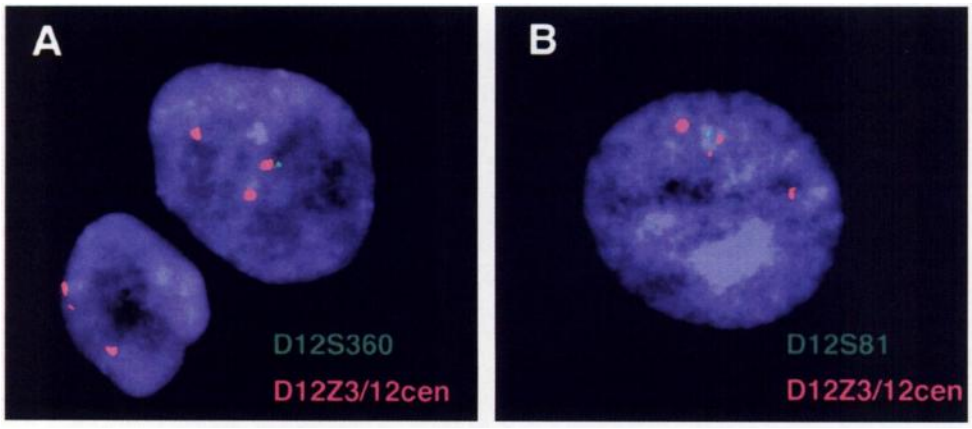

Fig. 4. Typical examples of FISH analyses. *A*, clones for *D12Z3*/12cen (*red*) and *D12S360* (*green*) were used. Three red signals and one green signal in case 515 indicated an allelic loss at *D12S360*. *B*, clones for *D12Z3*/12cen (*red*) and *D12S81* (*green*) were used. Four red signals and one green signal in the interphase nucleus of pancreatic cancer cell line PAN09JCK indicated an allelic loss at *D12S81*.

Table 1 *Alterations in copy number, as detected by FISH*

Cell line	Probe (locus) pα12H8 (*D12Z3*/12 cen)	c872-3-E4 (*D12S81*)	c25 (*D12S360*)	No. of chromosomes[a]
PK-1	4	2[b]	4	69
PK-8	4	4	4	82
PK-9	4	2[b]	4	63
PK-59	4	4	4	82
PCI-6	4	4	4	52
PCI-10	3	2[b]	3	60
PCI-19	4	4	4	62
PCI-35	4	4	4	80
PCI-43	4	2[b]	2[b]	56
PCI-64	3	3	3	54
PCI-66	3	3	3	58
PAN02JCK	8	4[b]	4[b]	60
PAN03JCK	8	4[b]	4[b]	82
PAN07JCK	4	4	4	70
PAN09JCK	3	1[b]	3	72
Panc-1	4	3[b]	3[b]	62
MIA PaCa-2	3	2[b]	2[b]	46
SU.86.86	4	4	4	56
BxPc-3	3	2[b]	2[b]	58
No. of samples with loss/total no. of samples		10/19	6/19	

[a] Total number of chromosomes (mode), as determined by counting 15 or more nuclei.
[b] Allelic losses.

microsatellite loci was observed, as compared to signals for the centromere (Fig. 4*B*). The results of FISH for each cell are listed in Table 1. The numbers of chromosomes observed in the great majority of the cells are listed as well.

Statistical analyses, seeking any relationship between clinicopathological features, including age, sex, survival period, clinical stage, and histological type, were also performed, but we could not find any significant correlation between allelic loss and any of these features (data not shown).

DISCUSSION

In this study of microsatellite analysis for LOH in the entire long arm of chromosome 12 in pancreatic ductal adenocarcinoma, we identified two independent commonly deleted regions. One was within a 1-cM region between *D12S81* and *D12S1719* at 12q21 (region A), and the other was also within a 1-cM region between *D12S360* and *D12S78* at 12q22–q23.1 (region B). The latter was completely covered by a single 650-kb YAC clone. Most of the samples with LOH showed interstitial deletions in either one or both of the two regions; three cases had lost 12q completely. An interesting pattern was observed in cases 82 and 515: they showed allelic retention at *D12S88*, with allelic losses on flanking markers of both sides. This result implied that one allele lost a region distal from *D12S88*, and the other allele lost a region proximal from *D12S88*. If the putative tumor suppressor gene extends over *D12S88*, then it is possible to explain the results of this LOH study, particularly in tumors from cases 82, 87, 302, 314, 515, and 551. A similar situation has been reported in colorectal tumor at the *MCC* and *APC* loci at 5q21 (30). Some regions of common allelic loss other than regions A and B were also predicted by several tumors. Frequencies of allelic loss in these regions were relatively low: roles of inactivations of genes in these regions may not be very important in pancreatic carcinogenesis. However, we cannot totally exclude the possibility that some tumor suppressor genes exist in these regions.

Allelic imbalances that were detected in microsatellite analysis were confirmed as allelic loss in two-color FISH analysis (Fig. 4*A*). Results of FISH in cell lines indicated frequent allelic loss at this locus. Some cells showed an apparent increase in the copy number of *D12Z3*/12cen, as compared with the number of total chromosomes. Even in those cells, copy numbers for the chromosome regions detected by the probes were decreased. These results suggested that LOH would result in inactivation of the putative tumor suppressor gene. Allelic loss at *D12S81* on 12q21, detected in PK-1, PK-9, and PK-59 cells, and retention, detected in PK-8 cells, were consistent with our previous results examining CGH (13).

Some karyotype analysis reports failed to detect alterations in chromosome 12 in pancreatic cancer (6, 8, 10); however, LOH was reported in frequencies as high as 56% in xenografted tissues by microsatellite analysis (9). Brat *et al.* (31) reported that the discrepancies between karyotype analysis and microsatellite analysis are accounted for by the different sensitivities of these methods. Our results may support this idea: many tumors analyzed in this study showed small interstitial deletions.

LOH in chromosome 12q was observed in several malignant tumors. Frequent LOH in 12q has been observed in gastric cancers by means of analyses using microsatellite markers and RFLP markers (32–34). CGH analysis of head and neck squamous cell carcinoma showed an interstitial deletion involving 12q21 (35). Although only a few reports of detailed investigations of 12q in tumor tissues have been published, a high frequency of LOH in 12q22 has been observed in the male germ cell tumors. However, the common region of allelic loss in this tumor type is between *D12S101* and *D12S346* (36), a site located outside of the commonly deleted regions detected in this study.

Contigs consisting of YAC and BAC clones give us information on the actual physical sizes of the deleted regions. Region A in 12q21 was covered by two overlapping YAC clones, y833G2 (1500 kb) and y909H1 (1550 kb). Thus, the distance between *D12S81* and *D12S1719* was 3 Mb or less. Region B in 12q22–q23.1 was covered by a single YAC clone, y742E4. The actual physical size of this region was estimated to be no larger than 650 kb. The YAC contigs constructed were in good agreement with those mapped by the Whitehead Institute.

Our results strongly suggest the existence of putative tumor suppressor genes on 12q21 and 12q22–q23.1. Further studies are necessary to clone and characterize putative tumor suppressor genes in these regions.

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