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Special Article

The Structural Basis of Molecular Genetic Deletions

An Integration of Classical Cytogenetic and Molecular Analyses in Pancreatic Adenocarcinoma

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Molecular genetic alterations are known to be important in human carcinoma, but the structural basis of these changes is largely unknown. To examine the basis of these changes, we compared the karyotypic chromosomal abnormalities of primary pancreatic adenocarcinomas with the molecular changes identified in these same cancers. In 14 cancers with abnormal karyotypes, 65% (123 of 188) of the chromosomal arms with molecular loss of heterozygosity (LOH) were associated with karyotypic structural anomalies. Karyotypic changes accounting for these molecular allelic losses included 83 chromosome losses, 18 partial deletions, nine isochromosomes, eight additions, and five translocations. Eight homozygous deletions were also identified by molecular analyses. Of the three homozygous deletions identified at 9p21, the only karyotypic change was a single case in which one entire copy of chromosome 9 was deleted. Of the four homozygous deletions identified at 18q21.1, one showed a loss of both copies of chromosome 18, two showed a loss of one copy of chromosome 18, and the fourth had two structurally normal copies of chromosome 18. One homozygous deletion was identified at 13q12.3, and the karyotype revealed the loss of one entire copy of chromosome 13. The second copy of chromosome 13 in this carcinoma was structurally normal. These results indicate that chromosomal structural anomalies can account for two-thirds of the LOH in pancreatic adenocarcinomas and that most homozygous deletions are likely to be interstitial chromosomal deletions that are below the detection limit of conventional karyotypic analyses. Some of the molecular deletions detected as LOH on chromosomes with karyotypically normal structure can be explained by chromosomal loss with reduplication of the remaining chromosome. (Am J Pathol 1997, 150:383–391)

Pancreatic adenocarcinoma is the fifth most common cause of cancer death in the United States.[1–2] It is typically a very aggressive carcinoma and is usually diagnosed late in the course of the disease. Carcinoma of the pancreas carries a very poor prognosis; the overall 5-year survival is currently less than 5%.[2] An improved understanding of the molecular genetic changes responsible for the development of carcinoma of the pancreas may lead to novel strategies to diagnose and treat this disease at earlier stages.[3,4] For example, activating point mutations in codon 12 of the K-*ras* oncogene are present in more

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than 80% of pancreas cancers, and these mutations can be detected in duodenal juice and stool samples from patients with carcinoma of the pancreas.[5–7] Activating point mutations in K-*ras* and other known genetic changes are, however, not specific for pancreatic cancer, and the complex constellation of genetic alterations present in pancreatic cancer has not been fully explored. A more complete understanding of the molecular genetics of cancer of the pancreas is therefore needed.

Two general approaches are currently used to identify the tumor suppressor genes that are important in the development of a neoplasm. With classical cytogenetic techniques, individual chromosomal alterations can be visualized and examined. With molecular techniques, probes specific for each chromosome arm are used to identify specific genetic loci that have been lost. Although molecular studies can detect genetic alterations (losses of heterozygosity and homozygous deletions) in neoplasms, they do not provide information as to how these losses occurred. Correlating these molecular changes with structural changes may provide insight into the mechanisms responsible for these chromosomal events. We have previously analyzed a large number of pancreatic cancers using both classical cytogenetic and molecular techniques.[8–13] We report here an analysis of the correlation between cytogenetic abnormalities of pancreatic adenocarcinomas after short-term culture and the chromosomal losses as determined by molecular analyses of the same tumors expanded in a xenograft model.

Materials and Methods

Cytogenetic and molecular analyses were previously performed on a series of primary adenocarcinomas of the head of the pancreas.[8–10] In 17 tumors, complete data were available from both cytogenetic analysis and allelotyping. The 17 patients ranged in age from 48 to 84 years, and all diagnoses were confirmed histologically.

Methods for tumor recovery, karyotyping, and allelotyping and the results of these analyses have been reported previously.[8–13] In all cases, fresh carcinoma from surgically resected primary adenocarcinomas of the pancreas was harvested under sterile conditions in the Surgical Pathology Laboratory at The Johns Hopkins Hospital. Adjacent sections of carcinoma were submitted for cytogenetic and molecular genetic analyses.

For molecular studies of allelic loss, carcinomas were implanted subcutaneously in athymic nude mice. Carcinomas xenografted in this manner are enriched for neoplastic cells and have been shown to be genetically stable.[10,12] The xenografted carcinomas were harvested when the tumors reached about 1 cm in size. Allelotype analyses were performed on DNA from the xenografted carcinomas using 283 commercially available microsatellite markers (Research Genetics, Huntsville, AL) for polymorphic loci on all 39 nonacrocentric chromosomal arms (a complete list of the microsatellite markers used in this study is available on the World Wide Web at http://www.path.jhu.edu/pancreas_markers). All markers listed in the web site were used in this study; however, not all markers were informative in all of the carcinomas examined. Gross statistical details on the informativeness of the markers are given in a previous publication.[10] Several cancers were examined using all of the markers, and after an initial screen only the markers that were found to be adequately informative were used on the remaining cancers. Polymerase chain reaction was performed as has been described,[10] the products were separated on a 6% polyacrylamide-8 mol/L urea gel, and autoradiography was performed. Analyses of homozygous deletions at 9p21, 13q12, and 18q21.1 were performed as previously described.[11–13]

For cytogenetic analyses, primary carcinomas obtained from surgical specimens were harvested as primary cultures (1 to 12 days). Slides were prepared and examined as previously described.[8,9] Clonal abnormalities were defined and described according to International System for Human Cytogenetic Nomenclature.[14]

Karyotypes and allelotypes from the same cancers were directly compared to determine whether specific allelic losses identified at the molecular level could be accounted for by chromosomal losses, structural abnormalities, or breakpoints identified by classical cytogenetics. The approximate chromosomal band locations of the polymorphic markers used in the allelotyping analyses are available on the World Wide Web (http://www.path.jhu.edu/pancreas_markers), and these locations were taken from published genetic maps[15–18] and from the Research Genetics web site (http://www.resgen.com). A correspondence between a karyotypic abnormality and an allelic loss was defined as a chromosomal structural anomaly overlying the map location of the genetic marker.

Results

We compared the allelotypes, homozygous deletion analyses, and karyotypes of 17 adenocarcinomas of

Table 1. *Sites of Allelic Loss and Metaphase Karyotypes for 14 Pancreatic Adenocarcinomas*

Case	Allelic loss	Karyotype
PX9	2p,6p,6q,7p,7q,17p,18q,22q	42-165, XY,del(2)(p23),del(6)(q13),tas(7:11)(p22:q24) idic(11)(q24),−13,−14,−17,−18,−21[cp9]
PX16	10q,12p,17p,18p,18q21q,22q	40-43,XY,−Y,−10,−12,add(12)(?p11), add17(?p11),−18x2,−21x2,−22,+3mar,+dms[cp10]
PX17	1q,1p,3p,6p,6q,9p,11p,13q, 17p,18p,18q,20p,21q	46,XY[20]/69-71<3n>XY,i(1q)X2,del(1)(q12),+3,+5,−6X2 add(11)(p13),−13,−15,−20,−21,inc[cp4]
PX20	1p,2q,3p,3q,7q,8p,9p, 11p,12p,12q,17p,22q	44-45,XY,del(7)(q31),−17[2]/77-82<3n>, XXY,idem,+7,+19[2]
PX23	3p,6p,6q,8p,9p,10q,11p,12p, 12q,17p,17q,18q,20q,21q,22q	46,XX[10]/42-43,XX,+1,+3,−6,−10,−12,−13 t(14q15q),−15,−17,−18,−21,−22,+5mar[cp5]
PX24	1q,2q,3p,4p,7q,8p,8q,9q,10q, 12q,17p,18q,19p,20q,21q	46XX[16]/64-70,XX,−X,i(1q),+4,−4,+5,i(6p),−7 add(7)(p22),−9,−10,del(11)(q14),+13,−14, i(17q),−18X2,+19,+20X2,add(21)(p11),−21[cp6]
PX27	1p,2p,5q,6p,6q,8p,11p,12q, 13q,14q,17p,17q,18q,19p	32-43,X-X,del(1)(p34),del(2)(p?21),add(3)(p11),−4,−5,−6 add(7)(p?13),i(8q),−12,−13,−14,−17,−18,−19,−21, add(22)(p11),+4mar,+dm[cp11]
PX28	1p,2p,3q,4p,5q,9p,10q,11p,11q, 13q,14q,17p,18p,18q,21q,22q	64-66<3n>,XXY,−1,−2,−4,−8,−9,−13,+i(17q)X2 −18,−20,−21,−22,+mar1X2,+mar2,+mar3 +mar4X2,+rX2[cp8]
PX30	1p,2q,3q,6p,6q,8p,9p, 11p,12q,13q,17p,17q, 18p,18q,19p,19q,21q	46XY[20]/36-39,X,−Y,del(1)(p13),del(3)(q21) −4,−6,dic(8:20)(p12:q1?3.3),del(9)(p13),−10, del(11)(p15.1),−12,−13,t(14q15q),−16,−17,−18,−19, der(19)t(13;19)(q12;q13),−20,−21,2mar+r,+dms[cp8]
PX53	1p,1q,2p,2q,5q,6p,6q,8p,10q, 11p,12q,15q,17p,17q,19p,22q	42-45,XX,del(1)(p3?5),+del(1)(q42),−2,del(2)(p11), del(4)(p11),der(5)t(5;5)(p14;q?14),i(?p5),−6, del(6)(q1?3),inv(7)(p22q32),del(8)(p22),del(9)(q21) −10,der(11)t(11;12)(p14;q13),−12,−13,dup14(q32q31) −15,−17,add(17)(q24),add(19)(p13.3),add(21)(p11) ?del(21)(q22),−22,+dms[cp11]
PX56	5p,6p,6q,8p,9p,9q,11q,12p,12q, 13q,15q,17p,18p,18q,19q,20p,22q	46,XY[11]/62-65,XY,+2,+del(3)(p12),−4X2,−5,−6 del(6)(q22)X2,del(8)(p12)X2,−9,add(9)(?p21)X2,−10X2 −13X3,add(17)(p11)X2,−18X2,−19,−20,−21,i(?21q), −22X2[cp4]
PX64	1p,3p,4p,6q,8p,9p,9q,13q 17p,18p,18q,19p,22q	46,XX[4]/68-89,<4n>,XXXX,+der5?t(5;8)(p11;p11) −7,−12,−13,+?dm,inc[cp4]
PX65	1p,3p,3q,8p,8q,9p,9q,11q,12q 13q,14q,15q17p,19p	46,XX[8]/63-68,XX,−X,i(3q)X2,+add(3)(q12),−8, i(8q)X2,−9,i(10q),−13,i(q13)X2,−14,i(14q)X2, der(17)t(17;22)(p11;q11)X2,−22X3 +4mar[cp3]/84-127,idemX2[cp]
PX67	1p,3p,3q,4p,4q,5p,5q, 9p,10q,17p,18q	41-45,XY,i(1q),der(3)t(3q14q) −4,−18,+2mar[cp7]

For each tumor, only clonal losses are indicated, and shared sites that illustrate the mechanisms of loss have been underlined. In the allelic loss column, all losses are lumped together by chromosome arm.

the pancreas. Fourteen of these 17 pancreatic tumors had abnormal karyotypes. The three carcinomas with normal karyotypes were from early in our cytogenetic experience and likely represent the characterization of nonneoplastic cells. These three cases were not used in further analyses. The karyotypes and allelic losses for the 14 remaining tumors are summarized in Table 1. In this table, allelic losses that could be accounted for by karyotypic changes are underlined.

Losses of Heterozygosity

In the 14 pancreatic adenocarcinomas, we detected a total of 188 allelic losses, defined as a loss of heterozygosity (LOH) of at least one microsatellite marker on a chromosomal arm. One hundred twenty-three (65%) of these 188 allelic losses could be accounted for by abnormalities identified in corresponding karyotypes. Whole chromosome losses accounted for the largest number of these allelic losses (83 of 123). The chromosomes most frequently lost in this series of tumors were chromosomes 13 (10 of 14 carcinomas) and 18 (9 of 14 carcinomas). The remaining 40 sites of allelic loss that could be accounted for by karyotypic changes were caused by structural abnormalities. In these instances, it was necessary to compare the location of the breakpoint involved in the structural abnormality with the genetic map location of the microsatellite markers defining the allelic loss (Figures 1 to 3). For example, Figure 3 shows the LOH and cytogenetic changes for a representative carcinoma (case PX27). Chromosomes 1p and 2p

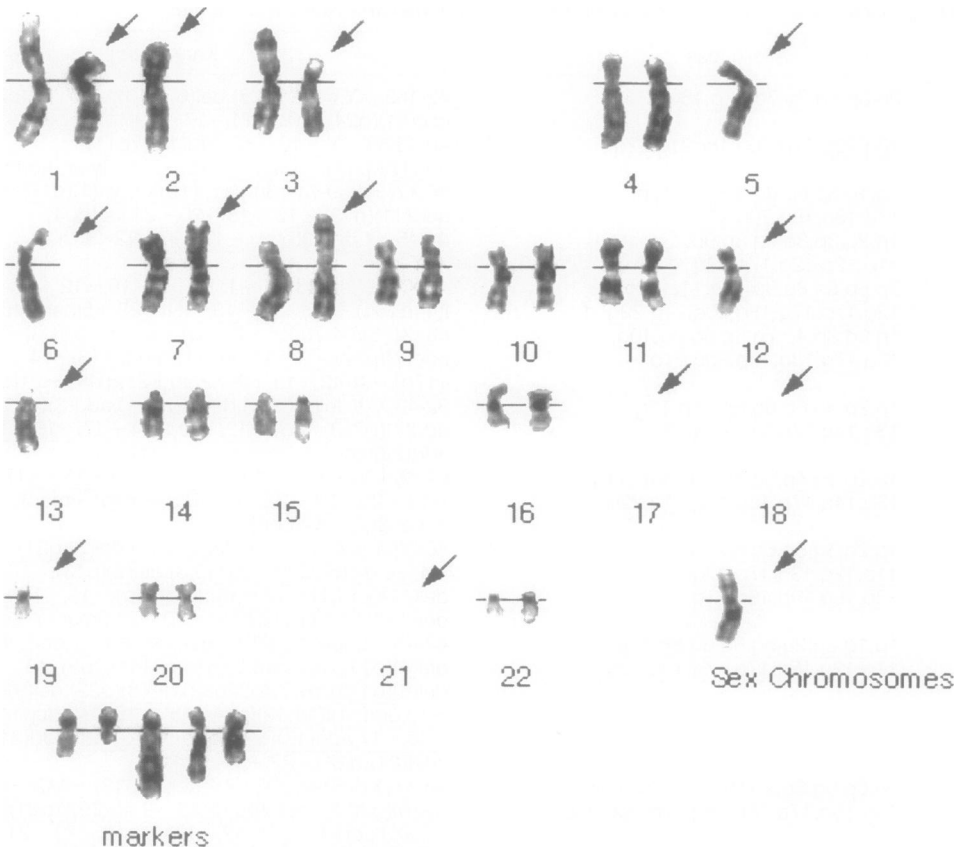

Figure 1. *Metaphase karyotype of a representative adenocarcinoma (case PX27).* **Arrows** *indicate the clonal cytogenetic abnormalities.*

have LOH for microsatellite markers that correspond to deletions identified in the Karyotype of the carcinoma. Also present in this example are extensive regions of LOH on chromosomes 6, 12 to 14, 17, and 18, which correspond to whole chromosomal losses in the karyotype. The LOH on 8p represents the molecular correlate of the isochromosome 8q noted in the karyotype.

Deletions of only a portion of a chromosome accounted for 18 of the allelic losses identified at the molecular level and were generally associated with genetic markers lost at the structural breakpoint and at more distal (telomeric) regions. Other structural abnormalities that corresponded to allelic losses included nine isochromosomes, eight additions of unidentifiable material, and five translocations. Chromosomal arms with the highest frequency of structural abnormalities in this series included 1p (six tumors), 8p (six tumors), and 17p (five tumors).

Particular attention was paid to abnormalities on chromosome 9p, the site of the *p16* (*MTS1*) gene; 17p, the site of the *p53* gene; 13q, the site of the *BRCA2* gene[13,19]; and 18q, the location of the deleted in pancreatic carcinoma locus 4 (*DPC4*)[11,20] and the deleted in colorectal carcinoma genes.[21] Nine of the 14 carcinomas had LOH on 9p, and four of the nine had corresponding chromosomal structural abnormalities (three complete deletions and one partial deletion). All 14 of the carcinomas demonstrated LOH of 17p, and 10 of these had karyotypic anomalies accounting for the allelic loss (six deletions, two isochromosomes, and two additions). Eleven of the 14 carcinomas had LOH of 18q, and nine of these had chromosomal abnormalities (all deletions). Six carcinomas had allelic loss of 13q, and all six had karyotypic deletion of 13q.

An alternate way of looking at the same data sets would be to examine each site of LOH (rather than chromosome arms) for comparison with the karyotypes. A total of 587 sites of LOH were identified in this series of carcinomas, and 355 (60.5%) of these could be accounted for by chromosomal abnormalities detected in the karyotype.

Homozygous Deletions

Eight homozygous deletions were identified by molecular analyses.[11–13] Three homozygous deletions

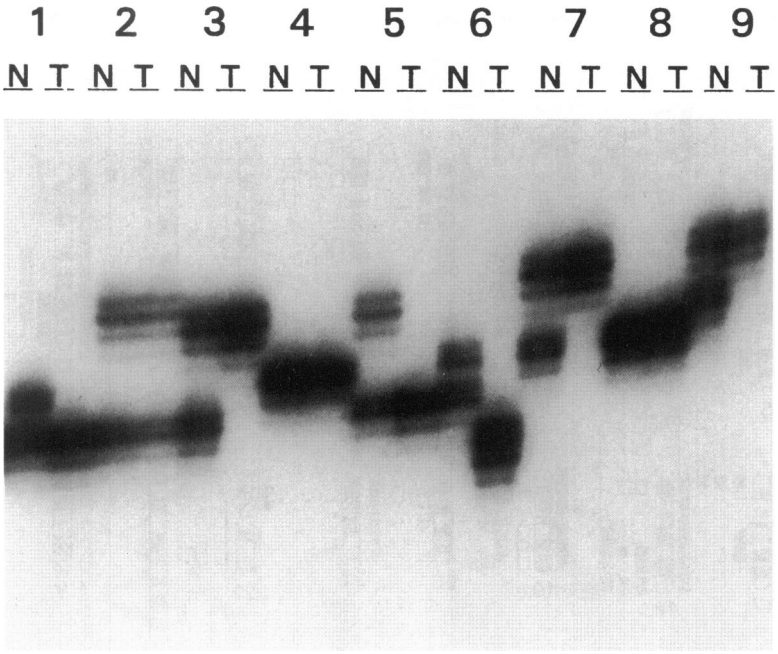

Figure 2. *Molecular analysis of LOH. Representative electrophoretic gel lanes of pancreatic adenocarcinoma* (T) *and corresponding normal tissue* (N) *for nine cases. LOH is demonstrated in cases 1, 3, 5, 7, and 9. Case 2 shows retained heterozygous alleles in the tumor. Cases 4 and 8 are uninformative, and case 6 has the mutator phenotype from a mismatch repair defect.*

were identified at 9p21 (cases PX23, PX28, and PX64).[12] However, in only one of these cases was a corresponding karyotypic change found, consisting of loss of a copy of chromosome 9. Four homozygous deletions were identified at 18q21.1 (cases PX16, PX27, PX30, and PX64);[11] one of these cases had a cytogenetic loss of both copies of chromosome 18, two showed a loss of one copy of chromosome 18, and in the fourth carcinoma both copies of 18 seemed structurally normal. One homozygous deletion was identified at 13q12 (case PX27),[13] and karyotyping of that carcinoma revealed the loss of an entire copy of chromosome 13. The second copy of chromosome 13 appeared structurally normal. Thus, seven of eight homozygous deletions involved a small and karyotypically invisible interstitial deletion within an otherwise structurally normal chromosome copy.

Discrepancies between Molecular and Karyotypic Analyses

Although the correlations between the allelotypes and karyotypes were often striking, many of the retained alleles and allelic losses identified at the molecular level were not detected in the karyotype of the corresponding cancer. For example, in case PX27 (Figure 3), molecular analyses identified retained alleles on 1p, 3p, 4p, 5p, 5q, 6p, 7p, 12p, 17p, 18p, 19p, 19q, and 21q, which were not detected in the karyotype of that cancer. Some of this retained genetic material is presumably present in the four marker chromosomes, which were identified in the karyotype of case PX27. Marker chromosomes represent clonal unclassifiable genetic material in karyotypes.

A number of allelic losses identified at the molecular level were also not detected in the karyotype of the corresponding cancer. Thirty-five percent of the chromosomal arms with LOH could not be accounted for by karyotypic changes, and there were 13 chromosomes that had extensive regions of LOH yet appeared normal on karyotypic analysis. Possible explanations for these discrepancies are detailed below.

Discussion

Information gained by the allelotyping and karyotyping of malignancies has led to a greater understanding of the genetic events underlying tumor development. Specifically, loci harboring tumor suppressor genes can be presumptively identified by a high prevalence of allelic losses and by recurrent chromosomal abnormalities. Reports of allelic losses and karyotypic abnormalities for specific human malignancies are usually reported separately, largely because molecular genetic and cytogenetic laboratories operate independently. When these analyses are indeed performed on the same neoplasms, most comparisons of cytogenetic changes with allelic losses have been restricted to descriptions of single

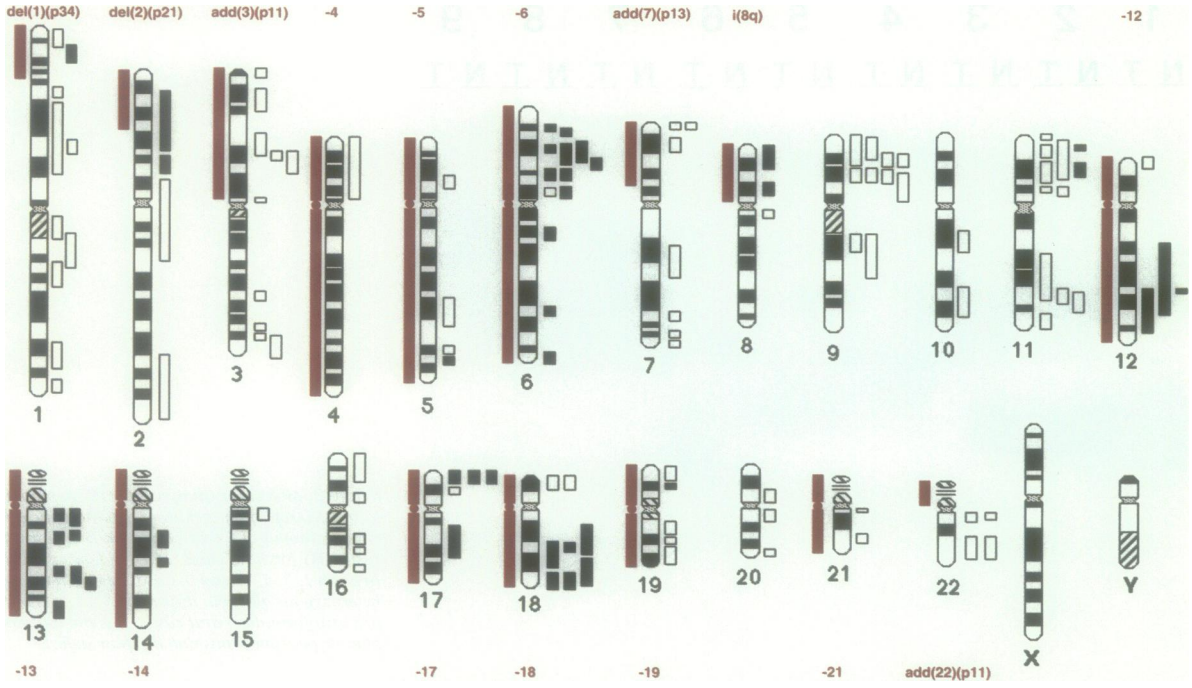

Figure 3. *Ideogram of the 22 human autosomes with the approximate chromosomal locations of microsatellite markers used in determining allelic losses for one case of pancreatic adenocarcinoma (case PX27). Each bar represents one marker used in the LOH study. LOH is indicated by a* **solid black bar,** *and retention of heterozygous alleles is indicated by an* **open bar** *on the right side of the chromosome. Chromosomal losses found by karyotyping are shown in* **red** *on the left of each chromosome, and the cytogenetic abnormalities for each chromosome are indicated in* **red**. *Note the single allelic losses corresponding to the small deletions on chromosomes 1 and 2, the multiple allelic losses corresponding to losses of chromosomes 6, 12 to 14, 17, and 18, and the loss of 8p corresponding to an isochromosome 8q. In this carcinoma, homozygous deletions attributable to interstitial deletions were identified at the loci of* DPC4 *(18q21) and* BRCA2 *(13q12) genes.*

chromosomes.[22–24] Each technique is labor intensive, and rarely are both complete allelotypes and detailed karyotypes obtained on the same tumor specimens. Nonetheless, comparisons of cytogenetic and molecular genetic analyses may be instructive for two reasons. First, they may illuminate the limitations of each technique. Second, classical metaphase cytogenetic analyses can supplement molecular analyses by providing a structural basis for genetic losses identified at the molecular level. We have been able to combine and analyze the cytogenetic and molecular genetic data from 14 pancreatic adenocarcinomas.

In the present study, a substantial percentage (65%) of the chromosomal arms with LOH in the 14 pancreatic carcinomas with abnormal karyotypes could be accounted for by structural changes identified by classical cytogenetic techniques. The most common karyotypic changes associated with LOH were complete loss of one copy of a chromosome or partial deletion of that chromosome. Interestingly, the most consistent correlation between cytogenetic and allelotypic changes were found in analyzing chromosomal regions at sites of known tumor suppressor genes. For example, *p53* mutations have been reported in 50 to 70% of pancreatic carcinomas.[25,26] All 14 cases examined in the present study had an LOH of 17p at markers near the *p53* locus. Ten (71%) of these 14 carcinomas had karyotypic anomalies that could account for the allelic loss (six chromosomal losses, two isochromosomes, and two additions). Similarly, the deleted in colorectal carcinoma and the recently described *DPC4* genes on 18q form an important region of allelic loss in many human malignancies,[27] and homozygous deletions and point mutations of *DPC4* have been identified in pancreatic cancers.[11,20,27] Eleven of the 14 pancreatic carcinomas included in the present study had LOH of 18q, and nine (82%) of these 11 had chromosomal abnormalities (all chromosomal losses), which could account for this LOH. The second familial breast cancer gene, *BRCA2*, has recently been identified on chromosome 13q12.[19] On this chromosomal arm, six the 14 tumors showed LOH, and karyotypic analysis of these carcinomas showed loss of one entire copy of this chromosome in all six (100%) cases. Genetic alterations of the *p16* gene, including point mutations, have been described in more than 80% of pancreatic adenocarcinomas.[12] In this series of 14 tumors, nine had LOH involving

9p21, presumably targeting the *p16* gene. Three of these nine tumors showed loss of an entire copy of chromosome 9, and one had a partial deletion that included the locus of the *p16* gene. These combined results demonstrate that the known tumor suppressor genes in pancreatic adenocarcinomas are commonly inactivated by loss of one allele through the deletion of an entire chromosome or a large portion of the chromosome and by inactivation of the second allele by a mutation not detectable cytogenetically.

Although the correlations between the karyotypes and the allelotypes were often dramatic, a number of discrepancies were found. For example, 35% of the chromosomal arms with LOH could not be accounted for by karyotypic changes. It seems likely that the genetic changes leading to these losses are below the detection limit of conventional cytogenetic analyses or involve complex rearrangements that obscure chromosomal identities. Although these explanations may account for some of the discrepancies, they do not account for discrepancies that involve large amounts of genetic material. In this series of carcinomas, there were a total of 13 chromosomes that had extensive regions of LOH yet appeared normal on karyotypic analysis. This finding is best explained by the mechanism of nondisjunctional chromosomal loss with reduplication of the remaining chromosome, as described by Cavenee et al[28] in retinoblastoma. Chromosomal loss with reduplication would lead to LOH at all loci of a particular chromosome, yet the genetic changes would not be apparent in the karyotypes because of the presence of two structurally normal chromosomes. Other more complex mitotic recombination and duplication events could also fail to give cytogenetically abnormal chromosomes, but would involve loss of heterozygous genetic material and would cause apparent discrepancies between karyotypic and molecular analyses.[28,29] It should therefore be reemphasized that determinations of LOH and copy number are not synonymous.

Some discrepancies may also arise from the criteria used in cytogenetic analyses. For example, the karyotyping of one of the carcinomas in this study revealed loss of one copy of chromosome 18 in only two cells. However, the International System for Cytogenetic Nomenclature guidelines require that at least three cells show the genetic change; therefore this loss of 18, detected in the molecular analyses, is formally absent in the reporting of the karyotype of this tumor.

Alternatively, if the predominant clone analyzed by cytogenetic techniques were to differ from the clone analyzed by molecular techniques, then discrepancies would occur. It is thus also possible that different clones within the primary tumor were analyzed by the two techniques, despite attempts to analyze adjacent tumor foci. This seems unlikely, because different sites of these tumors have been shown to harbor identical allelic loss patterns.[10] It is also possible that genetic changes introduced in the xenografted tumors could explain the differences. This possibility also is improbable in the present set of xenografted pancreatic tumors, because allelic and sequence stability have previously been demonstrated in xenografts that were established in parallel and serially propagated.[10]

Tumor suppressor genes can also be inactivated by loss of both copies of the gene. Indeed, it is known that 64% of pancreatic cancers harbor at least one homozygous deletion, and 19% harbor two or more.[12,20] Eight homozygous deletions at sites of known tumor suppressor genes were identified by molecular analyses in the current series. In only one of these eight cases could the loss of both copies of the affected gene be accounted for by structural changes identified by classical cytogenetics. In four of the cases structural deletions could account for loss of only one copy of the gene, whereas no structural changes were identified in three other cases. In general, homozygous deletions are thought to be only 1 to 2 megabases in size,[11,20] and the present study confirms that homozygous deletions are usually caused by small interstitial deletions not detectable by classical cytogenetics.

Although most of the LOH identified at the molecular level was accounted for by numerical and structural changes in the karyotypes, the converse was not necessarily true. Many of the karyotypic changes identified in these carcinomas did not have corresponding molecular allelic losses. In the case of structural abnormalities, such as stable translocations, allelic loss would only be expected if the structural breakpoint involved the region encompassed by the microsatellite marker. Importantly, "marker chromosomes" were present in 8 of the 14 tumors. These marker chromosomes represent unclassifiable genetic material in the karyotypes, and they may explain how genetic material preserved in the allelotype studies can seem to be deleted in the karyotypic studies. The recent application of spectral karyotyping to tumor cytogenetics may eliminate this problem of unidentifiable marker chromosomes. Another potential source of discrepancy could come from the growth of minor tumor subclones in the short-term primary cultures used for karyotyping.

In summary, approximately two-thirds of chromosomal arms with allelic losses in pancreatic carcinomas have corresponding chromosomal structural abnormalities. Most striking were the correlations of allelic losses and karyotypic changes in chromosomal regions known to have specific tumor suppressor genes. In contrast, homozygous deletions are often small and beyond the limits of detection of classical cytogenetics. Marker chromosomes and chromosomal or subchromosomal loss with reduplication of the remaining chromosome can account for some of the apparent discrepancies between karyotypic studies and molecular analyses.

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