Mapping of Chromosomal Imbalances in Pancreatic Carcinoma by Comparative Genomic Hybridization

Sabina Solinas-Toldo, Christine Wallrapp, Friedericke Müller-Pillasch, Martin Benz, Thomas Gress, and Peter Lichter


ABSTRACT

To identify recurrent chromosomal imbalances in pancreatic adenocarcinoma, 27 tumors were analyzed by using comparative genomic hybridization. In 23 cases chromosomal imbalances were found. Gains of chromosomal material were much more frequent than losses. The most common overrepresentations were observed on chromosomes 16p (eight cases), 20q (seven cases), 22q (six cases), and 17q (five cases) and underrepresentations on a subregion of chromosome 9p (eight cases). Distinct high-level amplifications were found on 1p32-p34, 6q24, 7q22, 12p13, and 22q. These data provide evidence for a number of new cytogenetically defined recurrent aberrations which are characteristic of pancreatic carcinoma. The overrepresented or underrepresented chromosomal regions represent candidate regions for potential oncogenes and tumor suppressor genes, respectively, possibly involved in pancreatic tumorogenesis.

INTRODUCTION

In Western countries, pancreatic cancer represents the fourth and fifth most common cause of cancer-related death in men and women, respectively, and the incidence appears to be increasing (1, 2). Prognosis of the disease is dismal, with a 5-year survival rate of <0.4% and a medial survival time following diagnosis of 4–6 months (2, 3). Early tumors are very difficult to detect; surgical resection is performed only when the tumor is localized, whereas advanced tumors extending further into other organs are not considered for resection. The development of new treatment modalities, as well as diagnostic and preventive approaches, require the understanding of the molecular mechanisms underlying the complex multistep process of tumorigenesis in the pancreas. In comparison to other tumors, the knowledge about the molecular biology of pancreatic cancer is very limited. Multiple cytogenetic aberrations of primary tumors and of cell lines derived from pancreatic carcinoma detected by using traditional cytogenetic techniques have been described (4–9). According to these studies, chromosomal aberrations of chromosome 1, loss of 6q, gain of chromosomes 7 and 20, and aberrations involving chromosomes 17 and 18 seem to be the most frequent rearrangements present in this tumor. However, it is difficult to obtain sufficient metaphase spreads of good quality from such tumor specimens for cytogenetic analysis. This could also result in the selection of analyzed clones which are not representative of the tumor. Moreover, many aberrant chromosomal regions may not have been identified due to the highly complex karyotypes of cultured cancer cells carrying both multiple numerical and structural chromosomal abnormalities. To contribute to the identification of the most common chromosomal abnormalities, we analyzed 27 cases of pancreatic adenocarcinoma by using CGH (10). This approach does not require metaphase preparation of the tumor sample and thus circumvents the limitations of karyotypic analysis possibly influenced by short-term culturing of tumor cells. CGH is based on the use of genomic DNA of tumor cells as a probe for fluorescence in situ hybridization to normal metaphase chromosomes (11, 12). The probe is cohybridized with genomic DNA, isolated from normal lymphocytes, and visualized with a different fluorochrome. Comparison of the signal intensities from tumor and control DNA probes allows the detection of chromosomal imbalances. Gains or losses of chromosomal material in the tumor are indicated by the increase and decrease of the ratio of the fluorescence signal intensities, respectively.

MATERIALS AND METHODS

Tumor Samples. Tumor tissue from patients with adenocarcinoma of the pancreas (n = 27; 18 males and 9 females) was provided by the Department of Visceral and Transplantation Surgery, University of Bern (Bern, Switzerland), Department of Surgery, University of Ulm (Ulm, Germany), Department of Pathology, University of Heidelberg (Heidelberg, Germany), and the Institute of Experimental Medicine (Budapest, Hungary) with written consent from each patient and after approval by the local Ethics Committees. The majority of samples derived from tumors of T1 or T2 [according to the UICC classification (13)], which are usually the only ones resected surgically (Table 1). Blocks of approximately 0.5 g of pancreatic cancer tissue were flash frozen in liquid nitrogen immediately after surgical removal. Tissue blocks were split into four pieces of the same size, and tissue fragments were trimmed to obtain frozen histological sections covering the complete tissue block. Sections of approximately 5 μm were fixed with acetone and stained with H&E according to standard procedures. Based on microscopic evaluation, only tissue fragments shown to contain more than 50% tumor cells were selected for DNA extraction. DNA preparation was performed according to a modified guanidinium thiocyanate extraction protocol, followed by centrifugation in a cesium chloride gradient (14).

CGH. Probe preparation, hybridization, and image acquisition were performed as described previously (15). Briefly, metaphase chromosomes were prepared from female peripheral blood leukocytes according to standard procedures. Control DNA was isolated from peripheral blood lymphocytes of a healthy male individual. One μg of tumor DNA and 1 μg of control DNA were labeled using nick translation with biotin and digoxigenin, respectively. The two labeled DNAs were combined with 80 μg of human Cot-1 DNA and coprecipitated in ethanol. The DNA was then resuspended in 12 μl of hybridization mixture (10% dextran sulfate, 50% formamide, and 2× SSC) and denatured at 75°C for 5 min. Probe and Cot-1 DNA were then allowed to reanneal at 37°C for 25 min and were hybridized to denatured normal female chromosomes. Hybridization was allowed to proceed for 3 days in a moist chamber at 37°C. Posthybridization washing in 50% formamide/2× SSC at 42°C and in 0.1× SSC at 60°C, biotinylated tumor DNA was detected with avidin-FITC and digoxigenin-labeled control DNA with mouse antidigoxigenin antibody conjugated to TRITC. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole and embedded in antifade solution (Vectashield). 4

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3 To whom requests for reprints should be addressed. Phone: 49-6221-424619; Fax: 49-6221-424639.

4 The abbreviations used are: CGH, comparative genomic hybridization; UICC, International Union Against Cancer; TRITC, tetramethyl rhodamine.

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Table 1 UICC classification of the analyzed tumor samples

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Digital Image Analysis. Gray level images of 15–20 metaphases were taken separately for each fluorochrome using a fluorescence microscope (Zeiss) equipped with selective single-bandpass filters and with a cooled charged coupled device camera (Photometries). The ratio of FITC:TRITC fluorescence intensities along each individual chromosome was calculated using dedicated software described in more detail elsewhere (16). For each chromosome the ratio values obtained from 10 to 12 metaphase cells were averaged, and the resulting profile was plotted next to chromosomal ideograms. Chromosomal imbalances were detected on the basis of the ratio profile averaged, and the resulting profile was plotted next to chromosomal ideograms. Chromosomal imbalances were detected on the basis of the ratio profile deviation from the balanced value (FITC/TRITC = 1). The values 1.25 and 0.75 were used as diagnostic cutoff levels indicating overrepresentations and underrepresentations, respectively. These thresholds for overrepresentation and underrepresentation correspond to the values expected for a trisomy or monosomy in 50% of the diploid cells. Chromosomal regions were scored as imbalanced regions when the ratio profile either reached or exceeded the diagnostic thresholds. The thresholds were thoroughly tested as reported previously (16–18) and successfully applied in numerous CGH studies by a number of laboratories (e.g., see Refs. 19–21). Overrepresentations were considered high-level amplifications when the ratio of the fluorescence exceeded the value of 2.0, or when the FITC fluorescence showed a strong distinct signal detected by visual inspection and the corresponding ratio profile was diagnostic for overrepresentation.

The use of female metaphase cells for hybridization did not allow us to obtain CGH profiles for the Y chromosome. However, most of the Y chromosome consists of a heterochromatin block containing clustered tandem repeats, a feature common to the heterochromatic and centromeric regions. These areas often show very low signal intensity due to the high suppression by Cot-1 DNA. This results in gross ratio variations from only small variations of the fluorescence intensity (15, 16, 22). Accordingly, these regions were not considered for CGH analysis.

Several other chromosomal regions have been recognized as being difficult for CGH analyses (15, 16, 22). Careful testing of our CGH protocol by multiple hybridization experiments using normal test DNA versus normal control DNA revealed that two of these regions occasionally show ratio values which would be diagnostic for imbalances: chromosomal region 1p32-pter and chromosome 1q. In contrast, none of the other regions that has been considered difficult for CGH analyses showed a significant deviation of the ratio values. To avoid the presentation of data with a small potential of being false positive, we did not score the CGH results regarding these two regions, except for the high copy number amplifications (Fig. 1).

When CGH is performed with tumor DNA from a female patient and control DNA from a male donor, the ratio value for the balanced state of the X chromosome is different (value 2). Since in this study the control DNA was always from a male donor and test and control DNA were not always matched for sex, scoring of X chromosomal imbalances had to take the sex of the tumor patient into account.

RESULTS

Of a total of 27 cases of pancreatic carcinoma analyzed with CGH, 23 cases showed chromosomal imbalances. With the exception of chromosomes 2 and 10, all chromosomes were involved in imbalances. A schematic representation of the genetic imbalances obtained from the analysis of 10–12 metaphases for each tumor are schematically summarized in Fig. 1 and represent the aberrations observed for each chromosome in this study. The most frequently identified imbalances were gains of chromosomal material on chromosome 16p in eight cases, with the commonly gained region of distal 16p; chromosome 20 in seven cases, five of which were on 20q only; chromosome 22q (six cases); chromosome 17q (five cases); and chromosomes 18p and 16q (four cases each). In two tumors the presence of an isochromosome i(18p) was suggested by the overrepresentation of 18p and deletion of 18q. Significant loss of chromosomal material was found in the telomeric part of 9p in eight cases and in an interstitial portion of 13q and 6q in four cases each. For the chromosomal regions 1p32-pter and 19, the ratio profiles indicated overrepresentation in 8 and 11 tumors, respectively. These were, however, not scored (*, Fig. 1), since the data in these regions are potentially false positive (for detailed description, see “Materials and Methods”). Profiles of aberrant chromosomes involved in at least five tumors, i.e., chromosomes 9, 16, 17, and 20, are shown in Fig. 2a. In three cases for each of the chromosomes 9, 16, and 20, the profile deviated from the balanced value in the same direction, but did not reach the diagnostic threshold. These instances are shown separately in Fig. 2. Distinct high copy number amplifications were observed in the following regions: 1p32-p34, 6q24, 7q22, 12p13, each in one case, and 22q in two cases (Figs. 1 and 2b). Chromosomal regions 7q22 and 12p13 were also involved in the gain of chromosomal material in two other tumors and 22q in four other tumors. In four cases, no aberration could be detected, suggesting the presence of a balanced karyotype. Although a requirement for the tumor samples used was the presence of at least 50% cancer cells, there is the possibility that this fraction was too low for CGH detection.

DISCUSSION

Although cytogenetic analyses of pancreatic adenocarcinoma have revealed complex karyotypic changes, only a few recurrent aberrations have emerged from these studies (4–9). We analyzed 27 cases of pancreatic adenocarcinoma using CGH, a very efficient and valuable tool to detect chromosomal imbalances as demonstrated for a variety of different tumors (e.g., Refs. 17–22). In the present study, a number of characteristic genetic changes were identified which were previously not recognized as recurrent aberration using cytogenetic techniques. The newly described overrepresented or underrepresented areas define regions which contain putative proto-oncogenes and tumor suppressor genes, respectively, presumably involved in the pathogenesis of pancreatic tumors. In the following text, we emphasize those candidate genes which reside in the highly ampliﬁed regions.

The most frequently found imbalance, gain of the distal region of the short arm of chromosome 16, has not been described before as a recurrent genetic change in this tumor type. In contrast, gain of 20q, the second most frequent genetic imbalance detected using CGH, confirms previous reports using chromosome banding analysis (5, 7). Chromosome 22q was found to be overrepresented in six tumors of
CGH IN PANCREATIC CARCINOMA

Fig. 1. Summary of genetic imbalances detected in 27 pancreatic adenocarcinomas. Vertical lines on the left, chromosome ideograms indicate loss of genetic material; vertical lines on the right, gain of genetic material. Bold lines, high-level amplifications. Numbers above each line, case analyzed. The distal part of chromosome 1p and chromosome 19 are labeled (*), since in these two regions only high copy number amplifications were scored. In this illustration, low copy number imbalances of these regions were neglected for reasons which are outlined in detail in “Materials and Methods.”

Our study. Its commonly overrepresented region is characterized in part by two high copy number amplifications, defining 22q12-q13 as the critical region. This region harbors three proto-oncogenes (23): PDGFB, ECGF1, and EWSR1.

The distal part of chromosome 9p confined to the region 9p21—>9pter was found frequently lost (30% of the cases). Notably, in three additional cases the ratio profiles of this region were clearly shifted toward the threshold for underrepresentation (Fig. 2a). Although they do not reach the diagnostic value, they are suggestive for the existence of a loss of the corresponding chromosomal material in a smaller portion of tumor cells. Whereas previous karyotypic analyses of pancreatic tumors did not reveal 9p deletions, two tumor suppressor genes, CDKN2A (p16) and CDKN2B (p15), located in this region are known to be frequently involved in allelic loss in pancreatic cell lines and xenografts (24). Caldas et al. (24) proposed the CDKN2A gene as a functional target for the frequent loss of chromosome 9p. Recently, this observation has been confirmed (25, 26), the latter reference reporting the occurrence of mutations in the CDKN2A gene in 34% of primary pancreatic tumors. Thus, our CGH results are in agreement with the current hypothesis of an important role for CDKN2A or CDKN2B in the development or progression of pancreatic carcinoma.

Chromosome arm 6q appears to be an area recurrently lost in cancer of the pancreas (5, 9). In the present study, in only four cases could loss of 6q be diagnosed with CGH (Fig. 3a), whereas in 12 cases the CGH profile suggested loss of chromosomal material in this region,
but the ratio values did not exceed the diagnostic threshold (Fig. 3b). The high frequency of ratio values below the diagnostic threshold might be due to the loss of material in a smaller portion of cells. Since in the corresponding 12 cases many other chromosomal imbalances could be diagnosed using CGH, this might indicate that loss of 6q is a late event.

A remarkable finding of the present CGH study is the occurrence of six high copy number amplifications in 4 of the 27 tumors. Previous cytogenetic studies have not revealed double minute chromosomes or homogeneously staining regions, which both are chromosomal equivalents of such amplifications. Since high copy number amplicons are usually confined to DNA segments ≤1 MB, they provide a good basis for the identification of candidate proto-oncogenes. In the amplified region 1p32-p34, such candidate genes are the malignant TFS1 gene (1pter-p22.1) and RAB3B, a member of the RAS oncogene family (1p32-p31). The high copy number amplification on 6q24 coincides with the localization of the MYB and MAS1 oncogenes, the latter known to be associated with tumor-specific rearrangements. The high-level amplification on 7q22 is close to the proto-oncogenes WNT2 and MET; the latter encodes the receptor for hepatocyte growth factor scatter factor and is located on 7q31. MET has recently been found to be up-regulated in the majority of pancreatic ductal adenocarcinoma and overexpressed in some human pancreatic cancer cell lines, suggesting a possible role for this receptor in the growth and behavior of pancreatic cancer (27). The overrepresentation of 12p coincides with the localization of the proto-oncogene RAS (12p12.1) and of the cyclin CCND2 gene (12p13; Ref. 23). About 90% of the pancreatic carcinoma cases contain a point mutation at the 12th codon of the RAS oncogene (28, 29). Overrepresentation of 12p in two more cases of our study might be considered as additional support for a role of the RAS gene in pancreatic tumorigenesis.

Surgical resection of pancreatic adenocarcinomas is usually performed only when tumors are localized and restricted to a confined area (see above). Accordingly, tumor samples available for genetic studies are derived from similar tumor stages greatly impeding correlation analyses of tumor stages or clinical course of the disease with
Fig. 3. Average ratio profiles of chromosome 6 (16 tumor cases) exhibiting conspicuous deviation from the value representing the balanced state of the chromosome copy number. a, four cases showing significant loss of chromosomal material on the long arm of chromosome 6. b, 12 cases suggesting loss of chromosomal material in this region but with a ratio value that did not reach the diagnostic threshold.

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