Mapping of Chromosomal Imbalances in Pancreatic Carcinoma by Comparative Genomic Hybridization

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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 cyogenetically defined recurrent aberrations which are characteristic of pancreatic carcinoma. The overrepresented or underrepresented chromosomal regions represent candidate regions for potential oncopgenes and tumor suppressor genes, respectively, possibly involved in pancreatic tumorigenesis.

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 median 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 CGH4 (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 I). 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 anneal at 37°C for 25 min and were hybridized to denatured normal female chromosome. Hybridization was allowed to proceed for 3 days in a moist chamber at 37°C. After posthybridization washes 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).

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

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

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 (Photometrics). The ratio of FITC:TRITC using dedicated software described in more detail elsewhere (16). For each fluorescence intensities along each individual chromosome was calculated (Zeiss) equipped with selective single-bandpass filters and with a cooled charged coupled device camera (Photometrics). The ratio of FITC:TRITC using dedicated software described in more detail elsewhere (16).

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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,
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Fig. 2. a, average ratio profiles of the chromosomes (Chrom.) most frequently affected by loss (Chrom. 9) and by gain (Chrom. 16, 17, and 20) of chromosomal material. The ratios of FITC/TRITC fluorescence are plotted along the chromosome ideograms. The intensity ratio of a balanced copy number calculated from the mode of the intensity ratio (value of 1.0, central line), and the threshold for overrepresentations (value of 1.25, right line) and underrepresentations (value of 0.75, left line) are shown (see Ref. 16). Centromeric regions are known to be difficult in CGH analyses (see "Materials and Methods"). Accordingly, these regions were not considered and appear as shaded boxes. Ratio profiles suggesting a copy number change of the respective chromosomal region but not reaching the diagnostic threshold are listed separately on the right (under bold lines). b, average ratio profiles of six high copy number amplifications in five genomic regions found in four different tumor samples. Below each profile a representative example is shown.

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 $\leq$ 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 TPS1 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...
genetic data. Since it is very difficult to obtain a sufficient number of tumors from different stages, an analysis of steps and/or sequences of genetic alterations occurring during pancreatic tumor progression can hardly be performed by using only clinical specimens. In the present study, the number and nature of genetic imbalances identified in the lower (cases 2, 10, 11, 17, and 19) and higher stage tumors (cases 3, 15, 22, and 23) are not distinguishable (Table 1 and Fig. 1). Despite such shortcomings regarding the relationship of genetic changes and tumor progression, the identification of candidate genes relevant for pancreatic adenocarcinoma will allow the study of pathogenesis of pancreatic cancer by alternative routes, e.g., by using animal models. The present CGH analysis provides new entry points for studies aimed at the isolation of such candidate genes: several chromosomal regions were identified which are likely to harbor oncogenes and tumor suppressor genes relevant for the development or progression of pancreatic adenocarcinoma; some of these have not yet been associated with pancreatic cancer, such as the ones on chromosomes 1p, 13q, and 22q, suggesting a set of new candidate genes for the study of genetic changes involved in pancreatic adenocarcinoma.

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