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Centrosome Abnormalities in Pancreatic Ductal Carcinoma

Norihiro Sato, Kazuhiro Mizumoto, Masafumi Nakamura, Kenjiro Nakamura, Masahiro Kusumoto, Hideaki Niiyama, Takahiro Ogawa, and Masao Tanaka

Department of Surgery I, Kyushu University Faculty of Medicine, Fukuoka 812-8582, Japan

Abstract

The centrosome plays an important role in microtubule nucleation and organization, ensuring the establishment of cell polarity and balanced chromosome segregation. Recent studies have suggested that the loss of cell polarity and/or chromosome missegregation (aneuploidy) in human malignant tumors could result from defects in centrosome function. Using immunofluorescence analysis with an antibody to γ-tubulin (a well-characterized centrosomal component), we examined surgically resected human pancreatic tissues for centrosome abnormalities. The tissues included ductal carcinomas (n = 13), adenomas (n = 3), endocrine tumors (n = 3), chronic pancreatitis (n = 5), and normal pancreatic tissues (n = 12). We found that most (85%) carcinomas and some adenomas displayed abnormal centrosome profiles, characterized by an increase in size and number of centrosomes, and by their irregular distribution. In contrast, none of normal ductal and stromal tissues showed these abnormalities. These findings suggest that centrosome abnormalities may develop at a relatively early stage of pancreatic ductal carcinogenesis.

Introduction

The centrosome, which consists of a pair of centrioles surrounded by electron-dense pericentriolar material, represents the microtubule organizing center of interphase and mitotic cells (1–3). The centrosome duplicates itself only once during each cell cycle with the duplication beginning near the G1-S transition and completing during the G2 phase (2, 4). Before mitosis, duplicated centrosomes separate to produce two mitotic spindle poles that organize the mitotic apparatus (4–6). In addition, during interphase the centrosome nucleates and organizes the cytoplasmic microtubules, which leads to redistribution of cellular organelles and the establishment of cell polarity (3, 7, 8).

Because the centrosome plays an important role in the maintenance of cellular polarity and chromosome segregation during mitosis, the characteristic loss of cell polarity and abnormal chromosome number (aneuploidy) commonly seen in human malignant tumors could result from defects in centrosome function (9–11). Support for this idea comes from studies describing abnormal centrosome number during carcinogenesis induced by abrogation of the p53 tumor suppressor gene (12, 13) and by a tumor-amplified kinase STK15 (14). These studies provide a direct link between centrosomes and tumorigenesis. In fact, several recent studies documented structural and functional abnormalities of centrosomes in human malignant tumors originating from a variety of tissues including the breast, prostate, brain, lung, and colon (10, 11).

Pancreatic cancer is one of the most aggressive malignant tumors and has an extremely poor prognosis (15, 16). In patients with pancreatic cancer, early diagnosis and treatment would have a great impact on survival time. We reported previously that telomerase, a ribonucleoprotein enzyme responsible for cell immortality and carcinogenesis, was highly activated in the pancreatic juice obtained from patients with pancreatic ductal carcinoma, indicating that telomerase activity has an important diagnostic value (17).

Despite the recent diagnostic improvements using molecular techniques, there are still difficulties in diagnosing pancreatic cancer, suggesting a definite need for innovative diagnostic modalities (18). Therefore, we examined centrosomes in human pancreatic tissue sections obtained from patients with ductal carcinoma and other pathological states to evaluate whether centrosome abnormalities are specific for cancer cells in situ, and thus the presence of such abnormalities could be a sensitive diagnostic marker. The results demonstrated that almost all pancreatic ductal carcinomas and some adenomas exhibited a striking defect in centrosome profiles, whereas normal pancreatic tissues and ductal cells of chronic pancreatitis tissues did not. These results suggest that centrosome abnormalities can occur early in the multistep process of pancreatic ductal carcinogenesis and that detection of these abnormalities may be of value for assessing the underlying malignant potential in pancreatic lesions.

Materials and Methods

Cell Culture and Preparation. The human pancreatic cancer cell line MIA PaCa-2 was donated by the Japanese Cancer Resource Bank (Tokyo, Japan). Pancreatic fibroblasts were initially grown from surgical specimens from a 33-year-old male patient with chronic pancreatitis and were used at five to six passages. Cells were routinely cultured in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C in a humidified atmosphere containing 10% CO2.

Exponentially growing cells were plated at a density of 5 × 104 cells/well on 12-mm glass coverslips in 24-well plates with 1 ml of DMEM containing 10% fetal bovine serum. After overnight incubation, the cells were washed with PBS, fixed in cold methanol, and incubated with permeabilization buffer [0.1

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1 To whom requests for reprints should be addressed, at Department of Surgery I, Kyushu University Faculty of Medicine, Fukuoka 812-8582, Japan. Phone: 81-92-642-5444; Fax: 81-92-642-5458; E-mail: mizumoto@mailserver.med.kyushu-u.ac.jp.
m PIPES (pH 6.9), 1 mM EGTA, 4 M glycerol, 0.5% Triton X-100, and 1 mM GTP] for 1 min. The coverslips were placed on glass slides and subjected to centrosome immunofluorescence.

Tissue Samples and Preparation. Formalin-fixed, paraffin-embedded blocks of pancreatic tissues were prepared after surgical resection performed at Department of Surgery I, Kyushu University Hospital, from January 1995 to August 1998. The specimens included 13 primary pancreatic ductal carcinomas, 3 adenomas (1 intraductal papillary adenoma, 1 mucinous cystadenoma, and 1 serous cystadenoma), 3 malignant endocrine tumors, 5 chronic pancreatitis tissues, and 12 normal pancreatic tissues. Normal pancreatic tissues were obtained from a peripheral soft part of the pancreas away from the tumor in noncancer patients. These normal pancreatic tissues were proved to include no neoplastic or dysplastic lesion by careful histological analyses. Five-μm-thick sections of the pancreatic tissues were mounted on coated slides, deparaffinized in xylene, and placed in 100% ethanol. The sections were rehydrated in a descending gradient of ethanol-water to 70% ethanol, transferred to PBS, and subjected to centrosome staining.

Centrosome Staining and Image Analysis. The sections or cells on slides were incubated with a blocking solution (10% normal goat serum, 3% BSA, and 0.5% gelatin in PBS) for 1 h and subjected to a monoclonal antibody to γ-tubulin (GTU-88; Sigma) at a dilution of 1:300 for 1 h. The antibody-antigen complexes were detected by a FITC-conjugated goat antibody to mouse IgG (1:200; Biosource International, Camarillo, CA) by incubation for 1 h at room temperature. Some cells or tissue sections were also stained using a polyclonal antibody to pericentrin (BAbCO, Richmond, CA) or a monoclonal antibody to α-tubulin (Amersham International, Buckinghamshire, United Kingdom). The slides were washed extensively with PBS-T (PBS containing 0.1% Tween 20) after each incubation. Finally, the sections or cells were counterstained with propidium iodide (20 μg/ml; Sigma) for nuclear DNA, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and visualized using a laser scanning microscope, Olympus LSM-GB200 system (Tokyo, Japan). To determine centrosome abnormalities in each tissue section, at least 100 cells were carefully examined with regard to the diameter, number, and distribution of γ-tubulin staining spots. Centrosomes were considered abnormal if they

Fig. 1  Immunofluorescence staining of human pancreatic fibroblasts and MIA PaCa-2 carcinoma cells. Cells were immunostained with an antibody to γ-tubulin, followed by an FITC-conjugated secondary antibody (for γ-tubulin, green) and propidium iodide (for nuclear DNA, red). A, fibroblast at interphase; B, MIA PaCa-2 cell containing a large γ-tubulin staining spot (arrow); C, MIA PaCa-2 cell containing three γ-tubulin staining spots. Bars, 10 μm.

Fig. 2  The number of centrosomes per cell in pancreatic fibroblasts and MIA PaCa-2 cells. At least 200 cells were counted for each cell type.
measured >2 \mu m in diameter and/or if they were present in three or more per cell. Raw digital fluorescence images were recorded electronically and were printed with a Fujix Pictography 3000 color printer (Tokyo, Japan) using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**Statistical Analysis.** Data were expressed as mean ± SD. Statistical significance was analyzed using the Fisher’s exact probability test or the Mann-Whitney U nonparametric test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Centrosomes in Human Pancreatic Cancer-derived Cells and Normal Fibroblasts.** We first examined centrosomes in MIA PaCa-2 human pancreatic cancer cells and pancreatic fibroblasts by immunostaining with an antibody to \( \gamma \)-tubulin, a well-characterized component of the centrosomes (19–22). In pancreatic fibroblasts during interphase, a single \( \gamma \)-tubulin-stained spot of uniform size was observed (Fig. 1A), and in mitotic cells, a pair of spots was detected, one at each pole of the spindle. Nearly all fibroblasts displayed normal centrosome numbers, with only 2% of cells containing three centrosomes (Fig. 2). In contrast, a significant proportion of MIA PaCa-2 cells showed multiple (≥3) \( \gamma \)-tubulin-stained spots of various sizes and shapes (Fig. 1, B and C). The percentage of MIA PaCa-2 cells containing three or more centrosomes was 12%, which was significantly higher than that of fibroblasts (\( P = 0.01 \)). When stained with another well-characterized centrosome protein, pericentrin (22, 23), each cell type showed similar staining pattern to that of \( \gamma \)-tubulin. Double-staining of MIA PaCa-2 cells with \( \gamma \)-tubulin antibody and pericentrin antibody revealed colocalization of the \( \gamma \)-tubulin and pericentrin signals (Fig. 3A), implying the general accumulation of centrosomal proteins in tumor cells. To evaluate whether the observed staining structures really represent abnormal centrosomes with a functional consequence, we used \( \alpha \)-tubulin antibody for microtubule staining. Double-labeling of tumor cells by using antibodies to pericentrin and \( \alpha \)-tubulin demonstrated that the abnormal pericentrin spots were localized at the central parts of multiple microtubule asters (Fig. 3B). These findings suggest that the striking defects in centrosome structure are involved in the assembly of multipolar spindles in pancreatic cancer cells.

**Centrosomes in Pancreatic Tissues.** Next, we examined centrosomes in a series of 36 pancreatic tissue sections of ductal carcinomas, adenomas, endocrine tumors, chronic pancreatitis, and normal pancreatic tissues. Table 1 shows the clinical profiles and centrosome abnormality status of each case.

In normal pancreatic tissues, \( \gamma \)-tubulin staining was very weak or almost undetectable at low magnification (Fig. 4A). In all cell types of normal pancreatic tissue (including ductal cells, acinar cells, and islet cells), \( \gamma \)-tubulin staining was detected as a

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**Fig. 3** Coimmunostaining of MIA PaCa-2 cells with pericentrin antibody and \( \gamma \)-tubulin or \( \alpha \)-tubulin antibody. A, cells were double-stained with \( \gamma \)-tubulin antibody (green) and pericentrin antibody (red). Both staining patterns were superimposed by electronic image processing (superimposition). The colocalized signals of \( \gamma \)-tubulin and pericentrin are yellow. B, cells were double-stained with \( \alpha \)-tubulin antibody (green) and pericentrin antibody (red). Superimposed image of \( \alpha \)-tubulin and pericentrin signals show localization of pericentrin spots at each center of multiple microtubule asters (yellow). Bars, 10 \mu m.
single discrete spot of uniform size (about 1 μm in diameter) located in a perinuclear area, which indicated no abnormality (Fig. 4B). All of the 12 normal pancreatic tissues showed similar centrosome staining patterns. A low level of diffuse staining was sometimes detected in normal tissues, which most likely represented the cytoplasmic γ-tubulin known to be present in normal cells.

In contrast to the low levels of staining in normal tissues, pancreatic ductal carcinoma displayed a high level of γ-tubulin staining when analyzed at low magnification (Fig. 4, C and E). Higher magnification revealed that γ-tubulin-stained spots were abnormal in size and number, and they were located randomly throughout the cytoplasm (Fig. 4, D and F). Cancer cells often had an enlarged focus of γ-tubulin that was greater in diameter than centrosomes in normal cells (3–5-fold greater). The number of centrosomes seen in an individual section of normal tissues ranged from 0 to 2 per cell, whereas the range seen in individual cancer sections was 0 to 10 per cell. According to the criteria defined above, the presence of centrosome abnormalities was found in 11 of 13 specimens (85%) of pancreatic adenocarcinoma; the incidence was higher than in normal pancreatic tissues (P < 0.0001). Quantitative analysis demonstrated that the mean number of centrosomes per nucleus in pancreatic cancer sections was significantly larger than that in normal pancreatic tissue sections (3.9 ± 1.3 versus 1.4 ± 0.3, P < 0.001). Although the number of staining spots per cell (nucleus) in a 5-μm-thick section would be estimated smaller than the total number of centrosomes within a given cell, we demonstrate that even such a thin section can reveal a significant difference in centrosome number between normal and cancer cells. Similar results were obtained with centrosome staining by using the pericentrin antibody. Furthermore, immunostaining of tumor centrosomes with antibodies to γ-tubulin and pericentrin revealed coincidence of signals (Fig. 5), thus confirming that the observed staining structures really reflected abnormal centrosomes. Two of the three pancreatic adenoma sections (one intraductal papillary adenoma and one mucinous cystadenoma) also demonstrated abnormal centrosome structures (Fig. 6A).

None of the endocrine tumor sections exhibited abnormal centrosomes, although these tumors were malignant (Fig. 6B). On pathological examination, the tumor cells resembled normal islet cells without atypical features. The diagnosis of malignancy was made based on the findings of local infiltration and distant metastases.

In chronic pancreatitis tissues, ductal cells showed normal centrosome profiles in all five specimens (Fig. 6C), whereas some acinar cells showed positive γ-tubulin staining. However, the staining pattern was not spot-like and seemed to be different from that found in pancreatic ductal carcinomas. Moreover, staining of the same sections with pericentrin antibody did not demonstrate colocalized signals. Therefore, it is unlikely that the observed γ-tubulin staining in chronic pancreatitis tissues represented centrosome abnormalities.

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a Tumor stage was assessed according to Union International Contre Cancer-Tumor-Node-Metastasis staging.

b Centrosomes were considered abnormal if they measured >2 μm in diameter and/or if they were present in three or more per cell.

c Tumor cells showed moderate to severe atypia.
Fig. 4  Immunofluorescence staining of normal pancreatic tissue and pancreatic ductal carcinoma. Formalin-fixed, paraffin-embedded human pancreatic tissues were stained using γ-tubulin antibody, followed by an FITC-conjugated secondary antibody (green) and propidium iodide (red). A and B, normal pancreatic tissue; C and D, well-differentiated pancreatic ductal adenocarcinoma; E and F, poorly differentiated pancreatic ductal adenocarcinoma. Bars: A, C, and E, 50 μm; B, D, and F, 5 μm.
In the present study, we found that the centrosomes in nearly all pancreatic ductal carcinomas displayed structural abnormalities, such as an increase in their number and size, and an irregular distribution. Quantitative analysis demonstrated a significant difference in centrosome number between normal and cancer cells. In addition, double-labeled immunofluorescence analysis of MIA PaCa-2 pancreatic cancer cells suggest that these aberrant centrosomes contribute to the assembly of multipolar spindles, which may result in the improper segregation of chromosomes during mitosis. These results are consistent with previous studies describing centrosome abnormalities in human malignant tumors of the breast, prostate, brain, lung, and colon (10, 11). To our knowledge, however, this is the first report to demonstrate centrosome abnormalities in pancreatic carcinoma.

The centrosome plays a key role in the organization of cytoplasmic microtubules, in the determination of cell polarity, and in the establishment of the bipolar mitotic spindle (7, 8, 24). It is not difficult to envision that the characteristic loss of cell polarity and/or abnormal chromosome segregation in human malignant tumors could arise from defects in centrosome functioning. Recently, there has been increasing evidence suggesting a direct link between centrosome dysfunction and chromosome missegregation during tumorigenesis. For example, inactivation of the p53 tumor suppressor protein induced abnormal centrosome amplification, resulting in unequal segregation of chromosomes (12). Furthermore, ectopic expression of a human serine/threonine kinase STK15/BTAK, known to be overexpressed in multiple human cancers, caused abnormal centrosome amplification, aneuploidy, and transformation (14). These studies suggest that centrosome duplication, which may be disrupted by certain oncogenic molecules, is crucial for maintaining the genomic stability. Our present results may provide further evidence suggesting a possible connection between centrosomes and pancreatic tumorigenesis, although the exact mechanism by which the centrosome defects directly contribute to the development of pancreatic cancer remains to be determined.

An unexpected observation was that of high levels of \(\gamma\)-tubulin staining in pancreatic adenomas (intraductal papillary adenoma and mucinous cystadenoma). In pancreatic carcinogenesis, the concept of an adenoma-carcinoma sequence is applicable (25). Clinically, intraductal papillary adenoma and mucinous cystadenoma are considered to have malignant potential. Indeed, tumor cells in both of the \(\gamma\)-tubulin-positive specimens showed foci of papillary prolifer-
ation with moderate to severe atypia by histology. Therefore, the centrosome abnormalities detected in some adenomas would reflect these pathological features and the underlying malignant potential. Furthermore, a recent study suggested that abnormal centrosome amplification occurred at a very early stage in skin tumorigenesis, even in benign papillomas of transgenic mice expressing a p53 mutation (13). On the basis on these observations, we hypothesize that centrosome abnormalities might develop at an early stage, probably in adenomas, of pancreatic tumorigenesis. We are presently examining centrosome abnormalities in a larger series of pancreatic adenomas and early-stage carcinomas to test this hypothesis.

It is interesting that the pancreatic endocrine tumor specimens in this study showed normal centrosome staining patterns despite their histological grade of malignancy. Malignant endocrine tumors are often well differentiated with few or no areas of marked atypia or mitotic activity. Thus, the pathogenesis of endocrine tumors is not likely to be characterized by the loss of cellular polarity that is commonly found in carcinomas. This pathological feature could explain the absence of the centrosome abnormalities in malignant endocrine tumors. It is also possible that endocrine tumors can occur through different genetic instability pathways that are not associated with the centrosome defects.

The differential diagnosis between chronic pancreatitis and pancreatic cancer remains a clinical challenge because an inflammatory mass resulting from chronic pancreatitis often masquerades as pancreatic cancer (18, 26). Using a sensitive PCR-based semiquantitative assay, we found previously that telomerase, a ribonucleoprotein associated with cell immortality and a malignant phenotype, was activated exclusively in surgically resected specimens of pancreatic cancer and in the pancreatic juice obtained from patients with ductal carcinomma (17, 27, 28). We concluded that telomerase activity in the pancreatic juice could serve as a useful diagnostic tool to distinguish carcinoma from adenoma and pancreatitis. Our present results document that centrosome abnormalities were found in almost all pancreatic carcinomas and some adenomas but not in ductal cells of chronic pancreatitis tissues. These findings herald a possible clinical application of the presence of centrosome abnormalities in cytological specimens as an aid in assessing malignant potential in pancreatic lesions. Furthermore, this method may be able to detect these abnormalities in samples containing a very small number of cells, as indicated by the immunofluorescence study on cultured cells. A careful analysis of the centrosome abnormalities in cell samples in pancreatic juice obtained preoperatively is presently under way.

In summary, centrosome abnormalities were found in nearly all pancreatic ductal carcinoma specimens but not in normal pancreatic tissues. This study provides, for the first time, evidence that centrosome abnormality may occur at a relatively early stage of pancreatic ductal carcinogenesis. The precise mechanism by which these centrosome defects lead to genetic instability and how they contribute to tumor progression has yet to be studied.

**Acknowledgments**

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