Chromogenic in situ hybridization analysis of chromosomes 7, 9, and 17 in pancreatic ductal adenocarcinoma based on tissue microarrays

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Summary

Purpose: Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive neoplasm. Many different chromosomal alterations have been identified including structural or numerical changes. In this study we performed a molecular analysis of chromosomes 7, 9, and 17 based on tissue microarrays (TMA).

Materials and methods: Using TMA technology, 50 paraffin-embedded tissue samples of histologically confirmed primary PDACs were cored twice and re-embedded to the final recipient block. Chromogenic in situ hybridization (CISH) was performed using centromeric probes of the corresponding chromosomes. SPSS (chi square test and interrater kappa) was performed for statistical analysis.

Results: Chromosome 17 analysis detected aneuploidy in 19 (38%) cases. Similarly, aneuploidy regarding chromosome 9 was identified in 9 (18%) cases, whereas 14 (28%) cases were aneuploid, concerning chromosome 7. Statistical significance was assessed, correlating chromosome 7 with grade and stage (p=0.016 and p=0.027, respectively) and chromosome 9 to grade (p=0.023). Similarly, analyzing normal-appearing ductal epithelia adjacent to cancer cell populations, 2 cases were found with alterations regarding chromosome 9 and 17.

Conclusion: Molecular analysis for chromosomes 7, 9, and 17 in PDAC confirmed that there is a variety of numerical alterations, and some of them represent very early genetic events in the progression of carcinogenic process. Performance of CISH, also, provides an easy, accurate approach for their detection, even in a small tissue sample, such as TMA cylindrical cores.

Key words: chromogenic in situ hybridization, chromosome instability, pancreatic ductal adenocarcinoma, tissue microarrays

Introduction

Carcinogenesis represents a multi-step progressive process in human solid tumors [1,2]. According to extensive cytogenetic analyses, it is guided by chromosomal alterations, including structural or numerical changes (aneuploidy) and specific gene deregulations, such as methylation, deletions, intragenic mutations and amplifications [3-5]. Response to DNA damage, control of cell cycle and especially DNA replication are critical genetic events in normal cells mediated by genes such as p53 (suppressor gene, G1 to S phase checkpoint and apoptosis regulator) or DNA mismatch repair system factors (ie MLH1, MSH2, MSH6) [6-8]. Inactivation of those genes leads to chromosome aberrations (gains, losses or centromere amplification) and accumulation of those genome abnormalities, including telomere dysfunction, are responsible for the neoplastic and finally malignant transformation of normal epithelia [9-11].

PDAC - which is characterized by the term “silent killer”- is a highly aggressive type of cancer and
the most common pancreatic malignancy, comprising 85-90% of all exocrine pancreatic tumors [12,13]. Although chronic pancreatitis appears to be the main risk factor and substrate for the carcinogenetic process, a small proportion of pancreatic cancers seem to derive de novo via an unexplored mechanism [14,15]. Genetic instability is detected in a variety of chronic inflammation-dependent cancers such as Barrett’s oesophagus, ulcerative colitis or chronic gastritis [16,17]. All of these pre-neoplastic lesions appear to share a similar pattern of progressive malignant transformation process. In many examined cases, chromosomal instability has been detected in normal-appearing sporadic epithelia adjacent to cancer cell populations, evidence of an early genetic damage probably related to oxidative stress [18,19].

In this study, we performed a CISH protocol using TMA technology, for the identification of chromosomes 7, 9 and 17 numerical alterations in PDAC. These chromosomes contain many critical genes, which are frequently deregulated in PDAC and also in premalignant lesions, termed Pancreatic Intraepithelial Neoplasia (PanIN I-III) (Table 1).

Materials and methods

Study group

For the purposes of our study we obtained 50 paraffin-embedded tissue samples of histologically confirmed primary PDACs derived from patients who underwent radical (Whipple) or partial pancreatic resection between 1998 and 2004. Twenty-nine cases were from male patients with a median age of 62.5 years and 21 from female patients with a median age of 66.8 years. Tissue sections from benign-appearing epithelia (n=10), adjacent to malignant tissue were used as normal control group. The local Ethical Committee consented to the use of these tissues in the Department of Pathology (417 VA Hospital-NIMTS, Athens, Greece) for research purposes. The archival samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnosis. All adenocarcinomas were graded and staged according to Digestive System Tumours Classification criteria of the World Health Organization (WHO 2000). Clinicopathological data are demonstrated in Table 2.

TMA construction

Areas of interest were identified on H&E-stained slides by a conventional microscope (Olympus BX-50). The corresponding paraffin blocks were obtained for the construction of one TMA block. Using TMArrayer-100 (Chemicon International, USA), all of the source blocks were cored twice in order to increase the reliability of TMA method and 1-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient block. The final constructed TMA block contained approximately 100 cores (50 pairs) of tissue cylindrical specimens. After 3 mm microtome sectioning and H&E staining, we observed microscopically that each case was represented by at least one or two tissue spots, confirming the adequacy of the corresponding cases (tissue cylinders) (Figure 1).

CISH probes

Chromosome status (numerical alterations) was determined by the ready-to-use biotin-labeled chromo-

Table 1. Genes related with PDAC*

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Cytogenetic band</th>
<th>Gene</th>
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<tbody>
<tr>
<td>7</td>
<td>7p12-13</td>
<td>EGFR</td>
</tr>
<tr>
<td>9</td>
<td>9p21-22</td>
<td>p16ink4a</td>
</tr>
<tr>
<td>17</td>
<td>17p13.1-3</td>
<td>p53</td>
</tr>
<tr>
<td></td>
<td>17q12-23</td>
<td>HER2/neu</td>
</tr>
<tr>
<td></td>
<td>17q21-22</td>
<td>Topoisomerase IIa</td>
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<tr>
<td></td>
<td>17q21</td>
<td>BRCA1</td>
</tr>
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</table>

*pancreatic ductal adenocarcinoma

Table 2. Clinicopathological data of patients with PDAC*

<table>
<thead>
<tr>
<th></th>
<th>n=50</th>
<th>%</th>
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<tr>
<td>Sex</td>
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<td></td>
</tr>
<tr>
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<td>29</td>
<td>58</td>
</tr>
<tr>
<td>female</td>
<td>21</td>
<td>42</td>
</tr>
<tr>
<td>Tumor origin</td>
<td></td>
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</tr>
<tr>
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<tr>
<td>I</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>III-IV</td>
<td>36</td>
<td>72</td>
</tr>
</tbody>
</table>

*pancreatic ductal adenocarcinoma
somes 7, 9, and 17 double-stranded centromeric probes (Zymed/InVitrogen SPOT-Light Centromere Detection kit), which recognize the specific repetitive centromeric DNA sequences known as α-satellite DNA.

**CISH Assay**

CISH SPOT-Light (Chromogenic ISH Detection Kit-Zymed) was applied. The CISH Polymer and the HRP Detection Kit (Zymed) include steps similar to IHC. CISH assay for the detection of chromosomes 7, 9, and 17 status was performed on 5 µm-thick serial paraffin sections of the TMA block, adjacent to those used for IHC. Three slides were incubated at 37°C overnight followed by 2 h incubation at 60°C. Then, they were deparaffinized in xylene 2 times, 5 min each and in ethanol 3 times, 3 min each. The slides were incubated in Tris-EDTA buffer within a microwave oven at 700 W, and at 95°C for 10 min. Those sections were treated enzymatically by pepsin digestion at 37°C for 5 min, washed in PBS, dehydrated in graded series of ethanol, and air-dried. Twenty µl of ready-to-use biotin-labeled centromeric probes were applied to each TMA section. The tissue sections containing the added probe were denatured by placing the slides in a PCR machine equipped with a slide block at 95°C for 5 min. The slides were then placed in a moist slide box and incubated at 37°C for overnight hybridization. After the hybridization process, the sections were stringently washed in 0.5× standard saline citrate at 75°C for 5 min. TMA sections were placed in 3% H2O2 and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas Block™, was applied and incubated for 10 min. The biotin-labeled centromere probes were detected by incubation with HRP conjugated streptavidin for 30 min, followed by DAB development (CISH Centromere Detection Kit, Zymed) for 30 min. Finally, TMA sections were lightly counterstained with hematoxylin and coverslipped with Histomount™. At the end of the process, CISH centromeric signals were easily visualized as dark brown or blue scattered dots, using a conventional bright-field microscope at low magnification (×10, ×20, ×40) (Figure 2). Interpretation of centromeric signals was based on Zymed’s Chromosome CISH™ Test Interpretation Guide. According to this, in cases characterized as normal (diploid), regarding chromosomes 7, 9 or 17 status, 2 centromeric signals per nucleus in >50% of the examined cancer cells were identified. Similarly, 3 to 5 or only one signal per nucleus in >50% of the examined cancer cells were considered as aneuploidy (polysomy and monosomy, respectively). In some cases characterized by borderline levels of aneuploidy (30-40% of the examined cancer cells) this genetic event was considered to be the final result of the CISH interpretation. The results of molecular analysis are described on Table 3.

**Statistical analysis**

Associations between chromosome status and clinicopathological parameters such as grade and stage

<table>
<thead>
<tr>
<th>Table 3. CISH results in PDACs³ and correlations</th>
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<tbody>
<tr>
<td>Numerical alterations n=50</td>
</tr>
<tr>
<td>Chromosome 7</td>
</tr>
<tr>
<td>normal (diploid)</td>
</tr>
<tr>
<td>polysomy</td>
</tr>
<tr>
<td>monosomy</td>
</tr>
<tr>
<td>Chromosome 9</td>
</tr>
<tr>
<td>normal (diploid)</td>
</tr>
<tr>
<td>polysomy</td>
</tr>
<tr>
<td>monosomy</td>
</tr>
<tr>
<td>Chromosome 17</td>
</tr>
<tr>
<td>normal (diploid)</td>
</tr>
<tr>
<td>polysomy</td>
</tr>
</tbody>
</table>

* α-satellite DNA

³pancreatic ductal adenocarcinoma

Kappa analysis: Chromosome 7 vs. Chromosome 9 0.416
Chromosome 9 vs. Chromosome 17 0.261
Chromosome 17 vs. Chromosome 7 0.687
were performed using the chi square test (SPSS Inc Chi
cago IL v.11.0). Cohen’s interrater kappa was also es-
timated along with its 95% CI to evaluate concordance
between the 3 chromosomal markers. By its de
inition, a kappa value of 1 denotes complete agreement, values
more than 0.75 are characterized as excellent agree-
ment, values between 0.4-0.75 show fair to good agree-
ment, values more than 0 but less than 0.4 poor agree-
ment, and a kappa value of 0 indicates that the observed
agreement is equal to chance. Two tailed p-values <0.05
were considered to be statistically significant.

Results

CISH results were successfully obtained from all
50 PDAC cases. According to CISH Test Interpretation
Guide, chromosome 17 analysis detected aneuploidy
(polysony, 3-5 centromeric signals per nucleus) in 19
(38%) cases. Similarly, aneuploidy regarding chro-
mosome 9 was identified in 9 (18%) cases (2 demon-
strated polyosomy and 7 monosomy), whereas 14 (28%)
cases concerning chromosome 7 were characterized
as aneuploid (13 cases demonstrated polysony and
1 monosomy). No statistical significance correlating
chromosome 17 status and clinicopathological pa-
rameters was found (p=0.37 for stage, and p=0.682 for
grade). In contrast, chromosome 7 status was strongly
associated to grade and stage of the examined tumors
(p=0.016 and p=0.027, respectively), whereas chro-
mosome 9 status was significantly correlated with grade
but not with stage (p=0.023 and p=0.354, respectively).
Correlations between chromosome status and sex or
tumor origin were not established. Analyzing the chro-
mosomal status in the control group (normal-appear-
ing ductal epithelia adjacent to cancer cell populations) we
identified 2 cases demonstrating aneuploidy (one with
chromosome 9 monosomy and one with chromosome
17 polysony). Kappa analysis showed a high degree
of concordance between chromosome 7 and 17 status
(k=0.687), whereas lower levels of agreement regard-
ing chromosome instability were observed between
chromosome 9 and 7 (k=0.416) and between chro-
mosome 17 and 9 (k=0.261). In 7 cases, combined chro-
mosome 7, 17, and 9 instability was confirmed (Figure
3). Finally, overall chromosomal instability was cor-
related to grade (p=0.043), but not to stage (p=0.254)
in the examined PDAC cases.

Discussion

This study was designed for the identification
and evaluation of chromosomal aberrations in PDACs

![Figure 2. CISH analysis in PDAC: A: a case of chromosome 9 monosomy (1 centromeric signal per nucleus); B: a case of chromosome 17 aneuploidy (polysony, 3-4 centromeric signals per nucleus); C: normal (diploid) pattern of chromosome 17 in a cancerous duct (2 centromeric signals per nucleus). Original magnification×40.](image1)

![Figure 3. Histogram of the combined chromosome analysis in PDACs.](image2)
regarding chromosomes 7, 9, and 17. Using a CISH protocol, we detected numerical alterations based on the measurements of centromeric signals per nucleus in cancer cell subpopulations of the corresponding TMA. Polysomy of chromosome 7 and 17 (centromeric gain) and also monosomy of chromosome 9 (centromeric loss/absence) were the main identified genetic events. Although a variety of numerical alterations was confirmed by analysing those cases, a small proportion of them was characterized by combined chromosomal instability. In other words, all of the examined chromosome markers demonstrated simultaneous numerical alterations in those cases correlated to grade of differentiation. Similarly, chromosome 7 and 17 co-instability showed a higher level of concordance, compared to the other associations. Another previous published study showed a strong concordance regarding numerical alterations of those two chromosomes [20]. It is known that chromosome 7 and also 8 are among the most frequently altered chromosomes in PDAC [21]. Similarly, loss of the entire chromosome 9 has been confirmed by the application of fluorescence in situ hybridization (FISH) [22]. Comparative genomic hybridization (CGH) has also shown that chromosomes 7, 9 and 17 are common targets for chromosomal aberrations (gains or losses) in early stages of pancreatic cancerogenesis [23]. Molecular analyses of precursor lesions with varying degree of histological atypia (PanINs I-III) identified a variety of chromosomal and also specific gene copy number changes [24].

Stereotypic gene alterations provide progression of tumorigenesis but the exact mechanism and sequence of those alterations remain under investigation [25]. A main question raised by the molecular analysis of premalignant lesions is the timing of specific gene deregulations. Concerning chromosome 17, one of the most important genes is the p53 suppressor gene. According to the results of some studies, loss of heterozygosity (LOH), meaning the deletion of one allele, at 17p occurs before the inactivation of the gene due to intragenic mutation (about 75% of PDAC cases) [26]. Using immunohistochemistry for the evaluation of p53 overexpression and molecular analysis for the detection of gene instability, Wiletz et al. concluded that LOH occurred before mutated protein expression [27]. This idea has also been supported strongly by the observation that analyzing p53 gene in inherited syndromes or familial cancers, such as Li-Fraumeni syndrome, which are characterized by germline mutations, intragenic alteration comes first [28]. But in sporadic cases of PDAC the mechanism seems to be different [29]. In the current study, chromosome 17 imbalances were detected even in normal-appearing ductal epithelia adjacent to cancerous tissue. This observation, combined with the identification of those chromosomal numerical alterations in all of the degrees of differentiation (Grade I-IV), is an evidence that PDAC is characterized by the presence of numerous complex structural aberrations and copy number changes in the entire range of its histological atypia.

Similarly, monosomy of chromosome 9, which contains the p16INK4A suppressor gene, acting as a negative regulator of cell cycle, appears to be an early and common genetic event. Loss of the entire chromosome or of the specific gene locus combined with intragenic mutation of the remaining allele or even homozygous gene deletion leads to deregulation of cell cycle, especially in the cases of cyclin D1 (positive cell cycle regulator mediating G1 to S transition) overexpression [30,31]. Concerning also chromosome 7 instability, we observed that polysomy is the main genetic alteration. Because epidermal growth factor receptor (EGFR) deregulation is correlated with aggressiveness in many solid tumors, such as colon, hepatocellular and pancreatic, and additionally represents a substrate for novel targeted therapeutic strategies via monoclonal antibodies or intracellular tyrosine kinase inhibitors, mechanisms of gene alteration maybe include predominantly deletion combined with intragenic point mutations rather than gene amplification, as it happens in other types of cancer (colon, non-small cell carcinoma) [32,33].

In the current study we performed a CISH protocol in order to evaluate chromosome 7, 9 and 17 status. CISH has drawn more attention, since it is capable of evaluating gene amplification/deletion, chromosome aneuploidy or chromosomal translocations simultaneously with tissue morphology on the same slide, using routine light microscopy. This way, large regions of each tissue section can be scanned rapidly with hematoxylin counterstain. Several studies have shown that CISH is a reliable alternative to FISH method and both of them demonstrate a high level of concordance, comparing their results (92-98%) [34,35]. Also, TMA technology decreased cost and time for the molecular analysis of the examined cases. This technology enables multiple studies of tumors.

The range of TMA applications appears to be very broad [36]. The equipment needed to make TMAs is not complicated and mainly is friendly to the user. Commercially available TMA hardware consists of a two-needle system with a slightly different diameter combined with a mechanism allowing the introduction of holes at defined areas in a recipient block. A multitude of studies utilized progression TMAs to
find associations between gene or chromosome alterations and protein overexpression, as in our study [37]. TMAs are an excellent substrate of multi-tumor combined research but the main question is the level of representativity of an entire potentially heterogeneous tumor by the use of only one or two cylindrical cores with a diameter of 0.6 or 1 mm. In fact, there are controversial data. Rubin et al. analysed the ki 67 immunohistochemical NLI in 10 separate cores of 88 cases of prostate cancer. They observed that more than 4 cores of each case did not add significant information about the ki 67 overexpression [38]. Similarly Camp et al. studied the expression of oestrogen and progesterone receptors and HER2/neu in 2-10 tissue cores obtained from the same donor blocks in a set of 38 invasive breast carcinomas. A concordance of 95-99% was reached by the analysis of 2-4 cores of each case [39]. In our study we used 2 cores of each case in order to increase the sensitivity in the final interpretation of the results.

In conclusion, molecular analysis for chromosomes 7, 9, and 17 in PDAC showed that this highly aggressive neoplasm is characterized by a variety of numerical alterations, and some of them represent very early genetic events in the progression of the carcinogenetic process. Performance of CISH also provides an easy, accurate approach for their detection even in a small tissue sample, such as TMA cylindrical cores.

Acknowledgements

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