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ORIGINAL ARTICLE

Correlation between different p53 expression patterns and chromosome 17 imbalances in pancreatic ductal adenocarcinoma based on tissue microarray analysis

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Summary

Purpose: *p53 (gene location: 17p13.1) overexpression is a common event in pancreatic ductal adenocarcinoma (PDAC), a highly aggressive malignant neoplasm. Although specific mechanisms of p53 gene deregulation have been identified, correlation between p53 expression and chromosome 17 gross numerical imbalances (aneuploidy) are under investigation.*

Methods: *Using tissue microarray technology, 60 paraffin-embedded tissue samples of histologically confirmed primary PDACs were cored and re-embedded to the final recipient block. Immunohistochemistry (IHC) for p53 expression and chromogenic in situ hybridization (CISH) for chromosome 17 numerical alterations were performed. Digital image analysis was applied for p53 expression levels evaluation (Nuclear Labelling Index-NLIs).*

Results: *p53 overexpression was detected in 38/60 (63.3%), whereas chromosome 17 aneuploidy was observed in 21/60 (35%) cases, respectively. Polysomy was identified in 19 cases, whereas monosomy in 2 of them. p53 overall expression was strongly correlated to the stage of the examined tumors (p=0.02). Chromosome aneuploidy was not associated to tumors' stage and grade (p=0.42, p=0.71, respectively). Although overall chromosome 17 centromeric imbalances were not correlated with p53 overexpression (p=0.32), both cases with monosomy demonstrated high expression levels.*

Conclusion: *p53 overexpression combined with chromosome 17 numerical imbalances characterizes a significant proportion of PDACs. Because commercially available anti-p53 antibodies detect mutant and also wild-type protein expression levels, chromosome 17 monosomy maybe is a gross genetic criterion for discriminating them due to point mutation that frequently affects the remaining allele.*

Key words: chromogenic *in situ* hybridization, chromosome, p53, pancreatic ductal adenocarcinoma, tissue microarrays

Introduction

PDAC is the most malignant and lethal neoplasm among those that derive from exocrine pancreas [1]. Extensive cytogenetic analyses have shown that pancreatic ductal carcinogenesis is a progressive process including chromosome and specific gene imbalances [2]. Malignant transformation of ductal epithelia demonstrates "early" genetic events such as k-ras oncogene mutations, "intermediate" such as p16 suppressor gene inactivation, and finally "late" events such as p53 and DPC4 inactivation or BRCA2 mutations [3].

Without dispute, p53 is a key regulator of the genome stability and function [4]. The corresponding gene (17p13.1) encodes a nuclear phosphoprotein with a molecular mass of 53 kDa acting as a transcription factor that negatively regulates cell proliferation [5]. It is also involved in a significant number of cell-signaling pathways, including cell cycle, programmed cell death, and DNA repair. The protein is expressed at a low level in

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normal cells and at a high level in a variety of neoplastic and malignant epithelia [6]. p53 overexpression due to point mutations is frequently detected by immuno-histo-cytochemistry assays in about 50% of malignancies derived from epithelia of different origin [7]. Concerning PDACs, p53 overexpression ranges between 40 and 80% based on several studies [8,9]. Furthermore, p53 gene aberrations occur not only as somatic mutations in PDAC, but also as germline mutations in some cancer-prone families with Li-Fraumeni syndrome [10,11].

In this study, we analyzed p53 at the protein expression level by IHC and we also determined chromosome 17 numerical imbalances by applying CISH on tissue microarray cancerous cell sub-populations. Our aim was to identify potential correlation between p53 protein expression and chromosome 17 aneuploidy.

Methods

Study and control groups

For the purposes of our study we obtained sixty (n=60) paraffin-embedded tissue samples of histologically confirmed primary and sporadic PDACs derived from patients who had undergone radical (Whipple) or partial pancreatic resection between 1998 and 2005. Thirty-nine cases were referred to male patients with a median age of 64 years (range 50-78) and 21 to female patients with a median age of 66.8 years (range 54-79). 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 at the Department of Pathology (417 VA General 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 the Digestive System Tumors Classification criteria of the World Health Organization (WHO 2000) [12]. Clinicopathological data are demonstrated on Table 1.

TMA construction

Areas of interest were identified on H&E-stained slides by a conventional microscope (Olympus BX-50, Menvile, NY, USA). The corresponding paraffin blocks were obtained for construction of one TMA block. Using TMArrayer-100 (Chemicon International, USA), all of the source blocks were cored and cylindrical cores (diam 1.5 mm) were transferred from each conventional donor block to the recipient block. After 3 mm microtome sectioning and H&E staining, we observed microscopically that each case was adequately represented (Figure 1).

Table 1. Clinicopathological data and combined IHC & CISH results

	p53			*Chromosome 17*		
Cases (n=60)	*L/M n=22*	*H n=38*		*Normal n=39*	*Aneuploidy n=21 (Polysomy 19, Monosomy 2)*	
	%	*%*	*x^2, p*	*%*	*%*	*x^2, p*
Gender			NS			NS
Male (n=39)	43.5	56.5		63.5	36.5	
Female (n=21)	41	59		71	29	
Grade			NS			NS
I (n=14)	49.5	50.5		43.5	56.5	
II (n=29)	62	38		59.5	40.5	
III (n=17)	52.5	47.5		63.5	36.5	
TNM stage			0.02			NS
I (n=11)	49	51		58	42	
II (n=24)	41.5	58.5		67.5	32.5	
III (n=17)	39	61		58.5	41.5	
IV (n=8)	43.5	56.5		62.5	37.5	
Location			NS			NS
Head (n=44)	49.5	50.5		57.5	42.5	
Body (n=10)	48	52		59.5	40.5	
Tail (n=6)	53.5	46.5		61.5	39.5	

L/M: low/moderate p53 expression, H: high p53 (over) expression, p-value derived from x^2 test, NS: statistically not significant
Interpretation of NLI (image analysis evaluation): High >10 stained nuclei per opt. field, Moderate 5-10 stained nuclei per opt. field, Low/negative 0-4 stained nuclei per opt. field

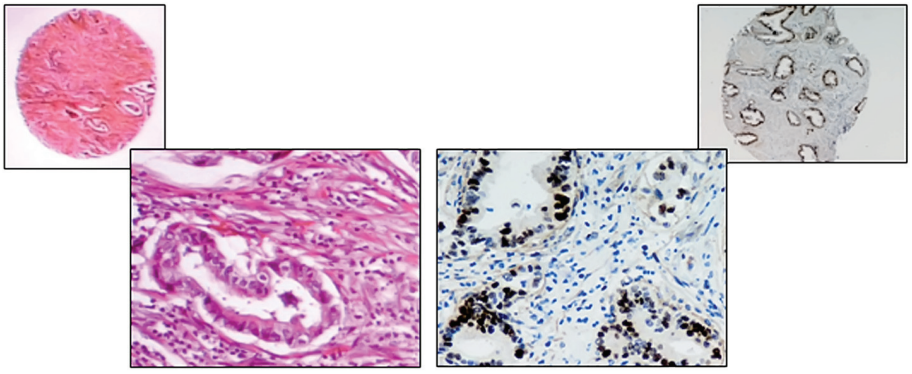

Figure 1. p53 expression based on tissue microarray cores. A tissue core with cancerous pancreatic epithelia in glandular formation (H&E stain, left). p53 high expression level in a PDAC tissue core. Note multiple overexpressed nuclei (p53 monoclonal antibody stain, right). Original magnification 10×, inside tissue cores 40×.

IHC assay and antibodies

Protein expression levels were determined by the application of a ready-to-use monoclonal antibody: anti-p53 (clone DO7-DAKO, UK; dilution at 1:40). IHC assay was carried out on 3 μm serial paraffin sections of the TMA block described above. Two slides were deparaffinized and rehydrated. En Vision IHC protocol (DAKO Corp, Denmark) was performed using an automated IHC staining system (I 6000-Biogenex, USA). This IHC protocol is based on a water-soluble dextran polymer system, preventing the endogenous biotin reaction, which is responsible for the background in the stained slides. Briefly, the sections were incubated with the primary antibody for 30 min at room temperature and then incubated with the polymer for 30 min. The antigen-antibody reaction was visualized using 3-3,diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate. Finally, the corresponding TMA sections were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control slides, the primary antibodies were omitted. According to the manufacturer's guidelines, breast cancer tissue samples previously reported to strongly express p53 were used as positive controls. Nuclear staining pattern was considered acceptable for the evaluation of IHC specificity (Figure 1).

Computerized image analysis assay (CIA)

In order to evaluate the IHC results not in a qualitative way but in a more accurate and fast way, we performed CIA by using a semi-automated system with the following hardware features: Intel Pentium Dual core 23-bit, bright-field microscope Nikon Eclipse TE300 & Nikon digital camera DS, Nikon Corp, Tokyo, Japan and the following software: Windows XP/ NIS-Elements AR v3.0, Nikon Corp, Tokyo, Japan. NLI for all examined cases was evaluated. Measurements were performed in 5 optical fields per case and at magnification of 400 (40×10). A digital library was constructed including the previous referred fields. In a rectangular active window on the computer screen each pixel contained a 24-bit value, called RGB "TRIPLE". This RGB-triple is made up of 3 separate 8-bit samples. Each sample represents the level of brightness of its respective color channel: red, blue or green. Finally, these brightness values represent levels within a 256-level scale (0-255). A macro was implemented. According to this, all stained nuclei (DAB stained- dark or more light brown oval or circular objects) per case in the corresponding optical fields were measured and the final number was filed in Excel sheets (Figure 2). Interpretation of NLI is described on Table 1.

CISH probes

Chromosome 17 numerical alterations were determined by the ready-to-use biotin-labeled chromosome 17 double-stranded centromeric probe (Zymed/ InVitrogen SPOT-Light Centromere Detection kit, USA), which recognizes the specific repetitive centromeric DNA sequences known as α-satellite DNA.

CISH assay

CISH SPOT-Light (Chromogenic ISH Detection Kit-Zymed/InVitrogen, USA) was applied. The CISH Polymer and the HRP Detection Kit (Zymed/InVitrogen, USA) include steps similar to IHC. CISH assay for the detection of chromosome 17 signals was performed on 5 μm-thick serial paraffin sections of the TMA block,

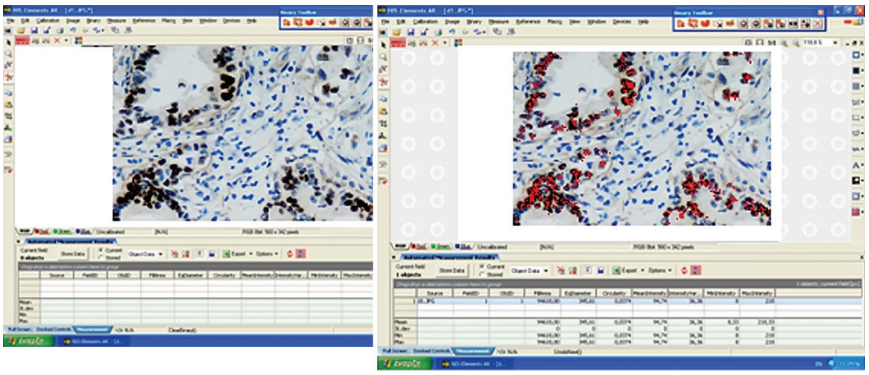

Figure 2. p53 protein expression evaluated by computerized image analysis. Red areas represent stained nuclei. Original magnification 40×.

adjacent to those used for IHC. Two 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 in 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-labelled centromeric probe 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% H_2O_2 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-labelled centromeric 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 cover slipped with HistomountTM. 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 3). Interpretation of centromeric signals was based on Zymed's Chromosome CISH™ Test Interpretation Guide. According to this, in cases characterized as normal (diploid) 2 centromeric signals per nucleus in >50% of the examined cancer cells were identified. Similarly, 3 -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. CISH results are described on Table 1.

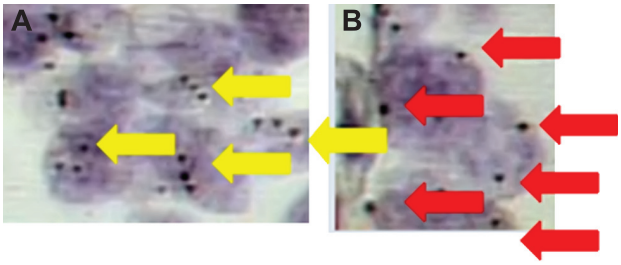

Figure 3. Chromogenic *in situ* hybridization analysis in PDAC tissue cores. **A:** a case with chromosome 17 polysomy. Note 3-4 centromeric signals per nucleus (yellow arrows) **B:** a case with chromosome 17 monosomy. Note only one centromeric signal per nucleus (red arrows) Original magnification 40×.

Statistical analysis

Associations between variables including protein expression levels, chromosome 17 signals and clinicopathological parameters such as sex, tumor grade, stage and anatomical origin were performed using chi square test (SPSS Inc Chicago IL v.11.0). Two tailed p-values <0.05 were considered statistically significant.

Results

All of the examined tissue cores – including the ones of the control group – expressed different levels of p53 protein. Concerning cancerous tissues, 38 out of 60 cases (63.3%) overexpressed the marker (high levels), whereas the rest of them (n=22) demonstrated moderate (n=20) and also low (n=2) expression NLIs. Addi-

tionally, normal pancreatic epithelia showed moderate (n=5) or low marker expression (n=5). Overall, p53 protein expression was strongly correlated only to the stage of the examined malignancies (p=0.02).

CISH analysis identified chromosome 17 aneuploidy in 21/60 (35%) cases. Polysomy (3-5 centromeric signals per nucleus) was identified in 19 cases, whereas monosomy (only one centromeric copy per nucleus) in 2 of them. Chromosome aneuploidy was not associated to the stage and grade of the cases (p=0.42, p=0.71, respectively). Interestingly, although overall chromosome 17 centromeric imbalances were not correlated with the overall p53 overexpression (p=0.32), the 2 cases with monosomy demonstrated high levels of expression.

Discussion

Experimental studies based on mouse model analysis have introduced mutant Tp53-mediated chromosomal instability as a crucial genetic event in the carcinogenetic process of pancreatic ductal epithelia [13]. Furthermore, aberrant expression of the protein seems to correlate with an aggressive phenotype in patients with PDAC, affecting even the progression of the carcinogenetic process through pancreatic intraepithelial neoplasia (PanIns 1-3), grade or the stage of disease [14,15]. Although p53 expression levels obtained by IHC are used generally as a potential prognostic marker for a variety of carcinomas, including breast and gastrointestinal malignancies, their association with the corresponding molecular mechanisms of p53 gene deregulation in PDAC is still a matter of investigation [16,17].

In the current study we analyzed p53 at the protein expression level in archival surgical specimens of PDACs in order to correlate them with chromosome 17 centromeric numerical imbalances. In a significant subset of the examined cancerous tissue cores we observed overexpression of the marker. Additionally, chromosome 17 aneuploidy (polysomy or monosomy) was identified in a relatively high percentage (21%) of them. Overall, p53 protein expression was associated with the stage of the tumors. The majority of the overexpressed cases was characterized by advanced disease (increased stage including liver and distant node metastasis). Although chromosome 17 instability was not correlated with the grade and stage of the analysed malignancies and also with overall p53 expression, cases with monosomy were strongly correlated with high levels of overexpression. Concerning p53 protein expression in PDAC, many studies based on IHC analysis have shown different results. In some of them, p53 overexpression was correlated with the expression of molecules that affect directly or indirectly pathways in which p53 acts as a regulator. Functional interactions between p53 and COX-2 have been already identified [18]. Thus, the p53-dependent upregulation of COX-2 was proposed to be another mechanism by which p53 could abate its own growth-inhibitory and apoptotic effects. Furthermore, the same study showed that accumulation of p53 was associated with COX-2 overexpression in premalignant and malignant ductal lesions, an evidence of the interactive role of COX-2 and p53 in pancreatic cancer carcinogenesis. Similarly, another study explored the role of the combined retinoblastoma (RB) and p53 deregulation [19]. It was well established that the RB protein is an important cell cycle regulator. RB also plays an important role in the regulation of apoptosis, which is mediated by interaction of p53 signalling mediators. Co-analyzing the protein expression levels of those molecules, the authors concluded that evaluating RB expression combined with the mediators of the p53 pathway p21WAF1/CIP1 and Bax may provide more accurate information regarding clinical outcome beyond the one provided by RB expression alone. Focused on the same analytical process, another group of investigators showed that p53 overexpression increased progressively according to the severity of the lesion and seemed to be a later event in the development of PanIN if compared to p21WAF1/CIP1 expression. The authors confirmed also the possible p53 independent p21WAF1/CIP1 expression in some PanIN2, PanIN3 lesions and invasive carcinomas [20].

Different p53 protein expression patterns provided by IHC characterize PDACs but they can not elucidate specific mechanisms of gene alteration. Furthermore, p53 IHC-based analysis is dependent on different protocols, and antibody clones that detect aberrant but also wild-type form of the molecule provided limited and controversial data regarding clinical significance as screening test in patients with PDAC [21]. Furthermore, there are differences in evaluating the results under bright-field microscopy due to subjectivity in screening by different pathologists. Owing to these reasons, molecular analysis provides a rational and accurate explanation of p53 overexpression in PDAC [22]. It is suggested that loss of heterozygosity (LOH) (p53 allele deletion) happens frequently in PDAC, and is correlated also with malignant transformation and also the metastatic process [23]. Many studies have already identified point and frame-shift mutations that affect the biological behavior of the neoplasm [24,25]. Additionally, concerning the role of p53 gene in pancreatic carcinogenesis, a study showed that allelic loss is often the first "genetic hit" in p53 gene's biallelic inactivation [26]. The same deregu-

lation mechanism appears to be obvious, analysing also the DPC4 suppressor gene deregulation.

Limited data exist concerning the role of simultaneous p53 overexpression and chromosome 17 numerical imbalances in PDAC. Similarly to our current molecular study, other authors based on fluorescence *in situ* hybridisation (FISH) or CISH analysis, detected chromosome 17 imbalances (polysomy and monosomy) that might affect p53 gene numerical stability [27-29]. Additionally, based on our findings, chromosome 17 monosomy seems to be a reliable gross genetic event in order to identify accurate p53 overexpession based on the biallelic deregulation (deletion or absence of the normal allele/mutation in the remaining allele) mechanism that has been previously reported [26]. Additionally, chromosome 17 monosomy is associated with an aggressive phenotype in PDAC and also in breast cancer [30-33]. Extended investigation of the mechanisms that lead to p53 overexpression in PDAC correlates with the development of targeted anti-TP53 agents due to increased expression of p53 regulators, such as tumor protein 53-induced nuclear protein 1 (TP53INP1). This molecule is a pro-apoptotic stress-induced p53 target gene, which dramatically decreases in PDAC [34]. Furthermore, protein analysis regarding molecules that regulate p53 metabolism, such as MDM-2, is a crucial process for an accurate evaluation of p53 protein overexpression [35].

In conclusion, p53 overexpression correlates to advanced disease (increased stage of malignancy) in PDAC. Furthermore, although chromosome 17 numerical imbalances are not associated with overall p53 protein expression, cases with monosomy may provide the proper molecular substrate for mutant protein expression due to a mechanism of deletion/mutation that happens frequently in PDACs.

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