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MiR-21 up-regulation in ampullary adenocarcinoma and its pre-invasive lesions

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ABSTRACT

Poor information is available on the molecular landscape characterizing the carcinogenetic process leading to ampullary carcinoma. MiR-21 is one of the most frequently up-regulated miRNAs in pancreatic adenocarcinoma, a tumor sharing similar molecular features with ampullary adenocarcinomas (AVCs), above all with the pancreatic-biliary type. We profiled, by in situ hybridization (ISH), miR-21 expression in a series of 26 AVCs, 50 ampullary dysplastic lesions (35 low-grade [LG-IEN] and 15 high-grade [HG-IEN]) and 10 normal duodenal mucosa samples. The same series was investigated by immunohistochemistry for β-catenin, p53 and HER2 expression. HER2 gene amplification was evaluated by chromogenic in situ hybridization. To validate miR-21 ISH results we performed miR-21 qRT-PCR analysis in a series of 10 AVCs and their matched normal samples. All the normal control samples showed a negative or faint miR-21 expression, whereas a significant miR-21 up-regulation was observed during the carcinogenetic cascade ($p < 0.001$), with 21/26 (80.8%) of cancer samples showing a miR-21 overexpression. In comparison to control samples, a significant overexpression was found in samples of LG-IEN ($p = .0003$), HG-IEN ($p = .0001$), and AVCs ($p < 0.0001$). No significant difference in miR-21 overexpression was observed between LG-IEN, HG-IEN and AVCs. By qRT-PCR analysis, AVCs showed a 1.7-fold increase over the controls ($p = .003$). P53 was frequently dysregulated in both dysplastic and carcinoma samples (44 out of 76; 57.9%). A 20% (10/50) of dysplastic lesions and 11% (3/26) of carcinomas were characterized by a nuclear localization of β-catenin. Only 2 AVCs (7.7%; both intestinal-type) showed a HER2 overexpression (both 2+), which corresponded to a HER2 gene amplification at CISH analysis. This is the first study demonstrating a miRNA dysregulation in the whole spectrum of ampullary carcinogenesis. MiR-21 overexpression is an early molecular event during ampullary carcinogenesis and its levels increase with the neoplastic progression.

1. Introduction

Ampulla of Vater carcinomas (AVCs) represent a rare and heterogeneous group of cancers deriving from the ductal epithelium or intestinal mucosa of the papilla [1–4]. AVCs have been categorized into two main histological subtypes: intestinal and pancreatobiliary [2,5–8]. Histological classification based on morphological feature fails to have a prognostic utility and the molecular pathogenesis of AVCs and of their pre-invasive lesions is still not well understood [9,10].

MicroRNAs (miRNAs) are short non-coding RNAs which regulate gene expression post-transcriptionally and are known to play important roles in oncogenesis, angiogenesis and tissue differentiation [11,12]. Among them, microRNA-21 (*hsa-miR-21*) has been originally described to be up-regulated in pancreatic adenocarcinoma [13,14], and its overexpression has been related to decreased sensitivity to gemcitabine *in vitro* and with short survival in retrospective clinical studies [15–19]. Moreover, this miRNA has emerged as a major driver of carcinogenesis in many different gastrointestinal settings [12,20–27].

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Schultz and colleagues showed that microRNA expression profile in AVCs is very similar to pancreatic adenocarcinoma [28]. However, despite recent extensive investigations, no information is available on miRNAs (and on miR-21 as well) dysregulation occurring with each phenotypic change involved in AVC carcinogenesis.

In the present study, miR-21 expression was profiled by miRNA *in situ* hybridization (ISH) in a large series of formalin-fixed paraffin-embedded (FFPE) biopsy samples representing the whole spectrum of AVC oncogenesis. To validate obtained results we performed qRT-PCR in a series of 10 AVCs and their matched normal samples. Furthermore, the FFPE series was investigated for the expression of HER2, p53, and β–catenin in neoplastic epithelia.

2. Materials and methods

2.1. Cases

A consecutive cohort of 50 ampullary adenomas and 26 adenocarcinomas (all Caucasian; M/F 40/36; mean age 62.4 ± 15.4) were retrospectively selected from the electronic archives of the Surgical Pathology Unit at Padua University. All patients involved in this study gave their informed written consent. The Helsinki Declaration and the international and institute's ethical regulations on research on human tissues were followed. The same series was previously investigated for PD-L1 expression [10].

To be defined as AVC, a tumor should be characterized by its central part located: i) in the lumen or walls of the distal end of the common bile duct and/or pancreatic duct; ii) at the papilla of Vater; or iii) at the duodenal surface of the papilla [3,8].

The inclusion condition for this study was a concordant diagnosis among two gastrointestinal pathologists (MF and CL) based on morphological criteria of the World Health Organization (WHO) 2010 classification [8] and immunohistochemical profiling [2]. Samples were classified as: i) 46 intestinal-type adenoma (35 with low-grade dysplasia [low-grade intraepithelial neoplasia; LG-IEN]; 11 with high-grade dysplasia [high-grade intraepithelial neoplasia; HG-IEN]); ii) 4 non-invasive papillary neoplasm, pancreatobiliary type (all characterized by high-grade dysplasia); iii) 19 intestinal-type adenocarcinomas; 7 pancreatobiliary-type adenocarcinoma. The intestinal-type adenomas were further categorized according their phenotype in tubular (n = 35), tubulovillous (n = 9), and villous (n = 2).

As normal control, 10 normal peri-papillary biopsy samples from patients subjected to upper endoscopy for dysfunctional dyspepsia were collected. Patients with *Helicobacter pylori* infection, and gastrointestinal polyposis were excluded from the study.

2.2. Sample preparation and immunohistochemistry (IHC)

All surgical samples were processed using the Galileo CK3500 Arrayer (www.isenet.it), a semiautomatic and computer-assisted Tissue microarray (TMA) platform, as previously described [10,29]. Two tissue cores (1 mm in diameter) were obtained from each considered lesion.

IHC stainings were automatically performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon Tyne, UK) in the BOND-MAX system (Leica Biosystems) on 4 μm-thick FFPE sections with the primary antibodies for HER2 (CB11; Leica; ready to use), β-catenin (17C2; Leica; 1:10), and p53 (EP9, Cell Marque, Rocklin, California; 1:50).

IHC reaction for p53 was evaluated in percentage of nuclear staining in neoplastic cells; the results were dichotomized as negative (< 50% of positive cells) and positive (≥50%) [30]. β-catenin was considered positive in the presence of a nuclear staining in neoplastic epithelia.

HER2 expression was evaluated with the score used for the characterization of gastric adenocarcinoma. To test *HER2* gene amplification in IHC 2+ cases, an *HER2* chromogenic *in situ* hybridization (CISH) was performed according to the manufacturer's protocol (DuoCISH kit, DakoCytomation, Glostrup, DK) [29,31].

2.3. miR-21 in situ hybridization

In situ hybridization (ISH) was performed using the GenPoint™ Catalyzed Signal Amplification System (DakoCytomation) according to the manufacturer's protocol. Briefly, slides were incubated at 60 °C for 30 min and deparaffinized [26,29]. Sections were treated with Proteinase K (DakoCytomation) for 30 min at room temperature, rinsed several times with dH_2O, and immersed in 95% ethanol for 10 s before air-drying. The slides were prehybridized at 49–56 °C for 1 h with mRNA ISH buffer (Ambion) before overnight incubation at 49–56 °C in buffer containing the 5′-biotin-labeled miR-21 miRCURY™ LNA detection probe (Exiqon, Woburn, MA, USA) or the scrambled negative control probe (U6, Exiqon) at a final concentration of 200 nM. The slides were washed in both Tris-buffered saline with Tween (TBST) and GenPoint™ stringent wash solution (54 °C for 30 min), then exposed to H2O2 blocking solution (DakoCytomation) for 20 min, and then further blocked in a blocking buffer (DakoCytomation) for 30 min before they were exposed to primary streptavidin–horseradish peroxidase (HRP) antibody, biotinyl tyramide, secondary streptavidin–HRP antibody, and DAB chromogen solutions, following the manufacturer's protocol. The slides were then briefly counterstained in hematoxylin and rinsed with TBST and water before mounting.

Only cytoplasmic miR-21 intensity in epithelial cells was retained for scoring purposes and cases were classified as: 0 = negative or faint expression in most cells; 1^+ = low expression in most cells or moderate expression in < 50% of the cells; 2^+ = moderate to strong expression in most cells.

2.4. Quantitative real-time polymerase chain reaction

FFPE samples (10 AVCs and their matched normal duodenal mucosa samples, 7 LG-IEN and 5 HG-IEN samples) were deparaffinized with xylene at 50 °C for 3 min. Total RNA extraction was done using the RecoverAll kit (Ambion Inc, Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using the GeneAmp PCR 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), and gene expression levels were quantified using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) [26].

The NCode™ miRNA qRT-PCR method (Invitrogen, Carlsbad, CA, USA) was used to detect and quantify mature miR-21 (primer sequence: 5′-CGGTAGCTTATCAGACTGATGTTGA-3′) according to the manufacturer's instructions. Normalization was done with the small nuclear RNA U6B (Invitrogen). PCR reactions were run in triplicate, including no-template controls. The data were analyzed using the comparative CT method.

2.5. Statistical analysis

Differences between groups were tested by applying the paired *t*-test and chi-square test. To verify a hypothetical linear trend of miR-21 expression in neoplastic progression we performed a Cochran–Armitage test for trend. *P* values < 0.05 were considered significant. The statistical analysis was performed using STATA software (Stata Corporation, College Station, TX).

3. Results

The main histopathological and immunophenotypical features of the considered series are summarized in Table 1.

All the 10 normal control samples showed a negative or faint miR-21 expression at ISH analysis, whereas a significant miR-21 up-