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

Running title: miR-21 expression in ampulla of Vater carcinogenesis

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ABSTRACT

Aims: Poor information is available on the molecular landscape characterizing the carcinogenetic process occurring in ampullary mucosa. MiR-21 is one of the most frequently up-regulated miRNAs in pancreatic adenocarcinoma, a tumor sharing similar molecular features with ampullary adenocarcinomas (AVCs).

Methods: We have profiled, by in situ hybridization (ISH), miR-21 expression in a series of 26 AVCs, 50 ampullary dysplastic lesions 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.

Results: 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 ($p= 0.0003$), HG-IEN ($p= 0.0001$), 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 ($p = 0.003$). 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.

Conclusions: 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.

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).

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.

MATERIALS AND METHODS

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 an epicenter 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 WHO 2010 classification (8) and immunohistochemical (CDX2 and MUC1) profiling (2). Samples were classified as: i) 46 intestinal-type adenoma (35 with low-grade dysplasia [low-grade intraepithelial neoplasia]; 11 with high-grade dysplasia [high-grade intraepithelial neoplasia]); 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 underwent to upper endoscopy for dysfunctional dyspepsia were collected. Patients with *Helicobacter pylori* infection, and gastrointestinal polyposis were excluded from the study.

Sample preparation and immunohistochemistry (IHC)

All samples were processed using the Galileo CK3500 Arrayer (www.isenet.it), a semiautomatic and computer-assisted Tissue Macro Array (TMA) platform, as previously described (10, 29).

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).

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 1h 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 H_2O_2 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.

Quantitative real-time polymerase chain reaction

FFPE samples (10 AVCs and their matched normal duodenal mucosa 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.

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).

RESULTS

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-regulation was observed during the carcinogenetic cascade ($p<0.001$), with 21/26 (80.8%) of cancer samples showing a miR-21 overexpression (**Figures 1A & 2**).

In comparison to control samples, a significant overexpression was found in samples of LG-IEN ($p= 0.0003$), HG-IEN ($p= 0.0001$), and AVCs ($p<0.0001$). No significant difference in miR-21 overexpression was observed between LG-IEN, HG-IEN and AVCs, even a higher miR-21 overexpression prevalence was observed in less differentiated lesions (LG-IEN 25/35 [71.4%] positive cases; HG-IEN 12/15 [80.0%] positive cases; AVCs 21/26 [80.8%] positive cases). This was even more evident for moderate to strong miR-21 overexpression (LG-IEN 6/35 [17.1%] positive cases; HG-IEN 5/15 [33.3%] positive cases; AVCs 11/26 [42.3%] positive cases).

To further confirm miR-21 overexpression in AVCs, we tested 10 samples with their normal matched mucosa by qRT-PCR analysis. AVCs showed a 1.7-fold increase over the controls ($p = 0.003$; **Figure 1B**).

Confirming the central role of p53 alterations in biliary tract cancers (9, 32-34), 44 out of 76 (57.9%) neoplastic samples showed p53 overexpression. Similar prevalence was observed among dysplastic and invasive samples; all control samples showed a regular p53 stain.

Thirteen neoplastic cases (17.1%) were characterized by a nuclear localization of β-catenin: the 20% (10/50) of dysplastic lesions and the 11% (3/26) of carcinomas. The 3 AVCs with aberrant expression of β-catenin were intestinal type.

Only two adenocarcinomas (7.7%; both intestinal-type) showed a HER2 overexpression (both 2+), which corresponded to a *HER2* gene amplification at CISH analysis.

No significant correlation was observed between miR-21 overexpression and p53, β-catenin or HER2 dysregulation.

DISCUSSION

The normal and neoplastic mucosa overlaying the ampulla of Vater are both characterized by a heterogeneous histological commitment, which results into a difficult and limited histopathological categorization of AVCs. In spite of our well-established understanding of the natural phenotypic history occurring in the progression from native epithelia to invasive carcinomas in the ampullary mucosa, little information is available on molecular pathogenesis. New molecular biomarkers are thus warranted to adequately stratify patients according to their risk of developing invasive cancer and to introduce targeted therapies based on a biological rationale.

MiRNAs' dysregulation play a leading role since early phases of (gastrointestinal) oncogenesis; furthermore they may influence tumor responsiveness to chemotherapy (35). Their relative stability in FFPE samples pinpointed this class of molecules as reliable biomarkers with potential diagnostic/prognostic implications to be introduced into clinical practice.

Several studies have shown that miR-21 is one of the most frequently up-regulated miRNAs in several type of cancers including pancreatic adenocarcinomas (15, 16, 18-21, 26, 27, 36-40). The oncogenic properties of miR-21 are further supported by functional studies showing that inhibition of miR-21 expression reduces proliferation and generates a pro-apoptotic and anti-angiogenesis response of several cancer cells lines (16, 17, 19). From a clinical point of view, its overexpression has been correlated with a poor clinical outcome and resistance to chemotherapy (15, 16,

18, 37).

To our knowledge, our study is the first that demonstrates dysregulation of miR-21 in the whole spectrum of ampullary carcinogenesis. We were able to demonstrate that miR-21 overexpression is an early event in ampullary carcinogenesis and its levels increase with the neoplastic progression.

Beside miRNAs' dysregulation, two recent whole-exome sequencing studies (32, 41) confirmed previous literature findings on the most molecular alterations observed among AVCs, supporting a central role for five main molecular pathways: i) RAS/PI3K (*HER2*, *KRAS*, *PIK3CA*); ii) TGFβ (*ELF3*, *SMAD4*); iii) WNT (*APC*, *CTNNB1*); iv) p53 (*CDKN2A*, *ATM*, *TP53*); v) Chromatin remodeling (*ARID2*, *ARID1A*). Intestinal-type carcinomas are characterized by a molecular signature similar to what observed in colorectal cancer, whereas pancreatobiliary-type to that of pancreatic ductal adenocarcinoma (28, 32). However, a significant overlap with common mutations (*KRAS*, *TP53*, *CTNNB1*, *SMAD4*) were observed in both subtypes (42).

We missed to find any relationship between miR-21 overexpression and p53, β-catenin or HER2 dysregulation. This could be explained by the relative low number of analyzed cases. However, our results are in line with the current Literature.

The p53 oncosuppressor role is at the center of many cellular pathways that respond to DNA damage, improper mitogen stimulation, and cellular stress. The importance of p53 in carcinogenesis is indicated by the presence of mutations in the p53 pathway in nearly all cancers (43). In AVCs, p53 dysregulation occurs during malignant transformation from the adenoma and continues during the tumor progression in carcinoma. Moreover clinical prognosis of de novo carcinomas with p53 overexpression was worse than that of the remaining patients (44). It has been shown that miR-21 suppresses p53-mediated apoptosis contributing to chemotherapy tumor resistance (43).

β-catenin plays a critical role in cell-to-cell adhesion by linking cadherins to the actin cytoskeleton and regulate transcription in the WNT signaling pathway. Indeed, upon WNT activation, β-catenin is translocated from the membrane to the cytoplasm and nucleus, where it interacts with transcriptional activators to modulate a number of target genes associated with increased growth, invasion and cellular transformation, such as c-MYC or cyclin D1. Recently has been shown that miR-21 upregulated the protein expression level of β-catenin in glioma (45) and lung cancer cells (46) and

increased CyclinD1 gene expression (46). Therefore β-catenin may be an important downstream mediator of miR-21 that allow to regulate proliferation, migration, invasion and resistance to chemotherapy of cancer cells. In fact APC/β-catenin is the most frequent genetic pathway underlying colon carcinogenesis and is associated with the classic adenoma-carcinoma sequence; this can confirm our finding that the 3 AVCs with aberrant expression of β-catenin were intestinal type. In addition we can postulate that also dysregulation of WNT/β-catenin signaling pathway is an early event in ampullary carcinogenesis.

In a recent NGS analysis of 32 AVCs the most frequently amplified gene was *ERBB2* and approximately 13% of AVCs exhibit *ERBB2* amplification, without predilection for subtype (42). On the other hand, in contrast to what observed in the gastroesophageal setting (47), no HER2 overexpression was observed in pre-invasive lesions. According to previous studies, we provide data correlating *ERBB2* in situ hybridization and IHC results in AVCs. Obtained results confirm that the IHC scoring criteria used for gastric carcinoma work well for AVCs for predicting gene amplification. Furthermore a similar molecular profile to *ERBB2*-amplified gastric carcinoma, can represent a hypothetical benefit from similar targeted therapy but the significance of the *ERBB2* amplification detected in this study is yet to be determined.

In conclusion, we demonstrated an early involvement of miR-21, p53 and β-catenin in ampullary carcinogenesis. Since miR-21 exerts its oncogenic function through a multi-pathways targeting, further larger and multi-centric studies should investigated the altered molecular cascades resulted from miR-21 early dysregulation to find novel reliable early biomarkers of ampullary mucosa transformation.

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TABLE 1. Main pathological features of the considered series.

Class	#	Histotype (Intestinal/ PB)	miR-21			p53 (*n*; %)	HER2 (*n*; %)	β-catenin (*n*; %)
			0 (*n*; %)	1^+ (*n*; %)	2^+ (*n*; %)			
Normal	10	-	10/10 (100.0%)	0/10 (0.0%)	0/10 (0.0%)	0/10 (0.0%)	0/10 (0.0%)	0/10 (0.0%)
LG-IEN	35	35/0	10/35 (28.57%)	19/35 (54.28%)	6/35 (17.14%)	20/35 (57.1%)	0/35 (0.0%)	7/35 (20.0%)
HG-IEN	15	11/4	2/15 (13.33%)	8/15 (53.33 %)	5/15 (33.33 %)	7/15 (46.7%)	0/15 (0.0%)	3/15 (20.0%)
AVC	26	19/7	5/26 (19.23 %)	10/26 (38.46 %)	11/26 (42.30%)	17/26 (65.4%)	2/26 (7.7%)	3/26 (11.5%)

Notes: PB= pancreatobiliary; LG-IEN= low-grade intraepithelial neoplasia; HG-IEN= high-grade intraepithelial neoplasia; AVC= Ampulla of Vater carcinoma;

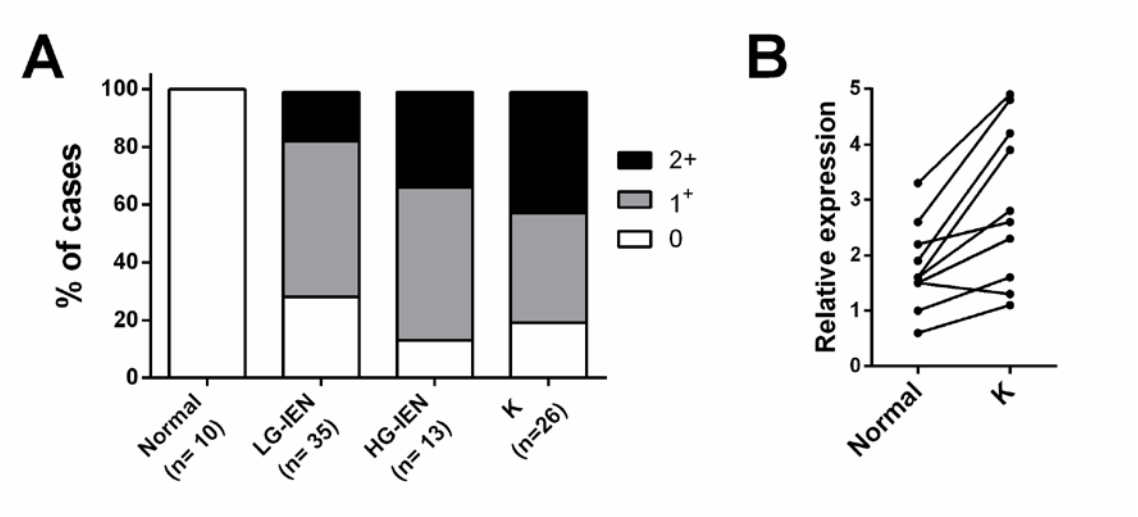

Figure 1. (**A**) miR-21 expression distribution at *in situ* hybridization (ISH) analysis. (**B**) Consistent with the ISH results, miR-21 was significantly overexpressed by 1.7-fold in AVC tissues in comparison to matched normal mucosa by qRT-PCR.

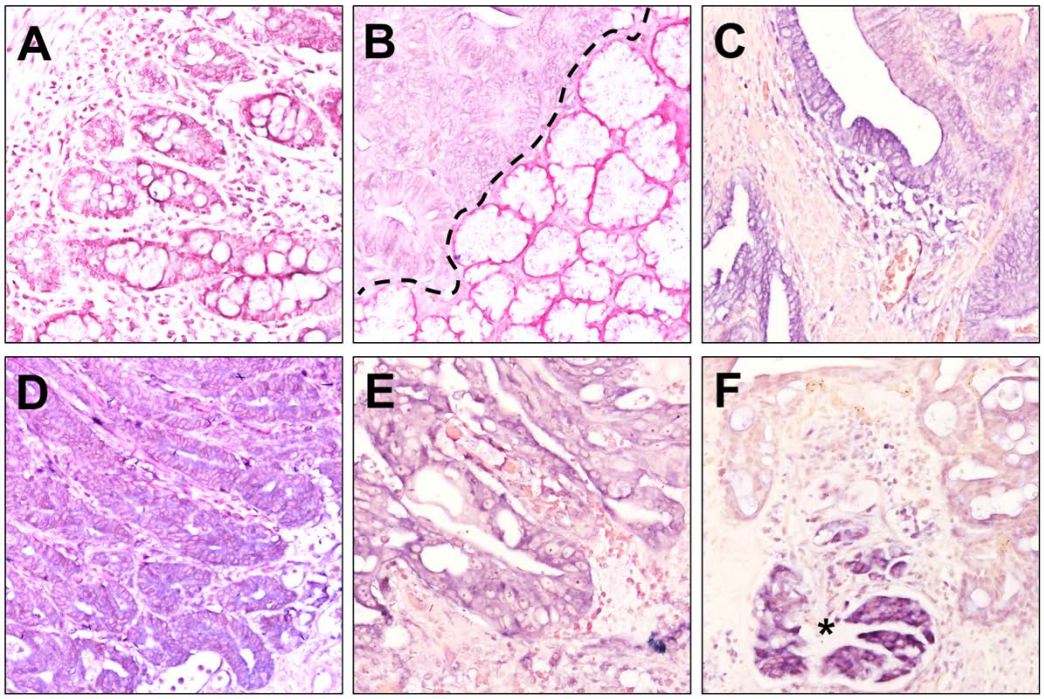

Figure 2. Representative miR-21 ISH stainings during ampullary carcinogenesis. Normal duodenal mucosa showing none or weak miR-21 expression (**A**). LG-IEN (upper left over the dotted line) showing weak miR-21 expression, but higher expression in comparison with normal surrounding mucosa (lower right) (**B**). Two cases of HG-IEN showing moderate (1^+) to strong (2^+) miR-21 expression (**C-D**). Intestinal type AVC showing moderate miR-21 expression (**E**). The invasive front of an AVC (asterisk) showing strong expression in comparison to normal surrounding mucosa (**F**). Original magnifications 20x and 40x. LG-IEN= low-grade intraepithelial neoplasia; HG-IEN= high-grade intraepithelial neoplasia; AVC= Ampulla of Vater carcinoma.