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Fhit down-regulation is an early event in pancreatic carcinogenesis

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Abstract

Aberrant Fhit expression characterizes a large proportion of primary pancreatic ductal adenocarcinomas (PDACs), but fragmentary information is available on Fhit expression during the phenotypic changes of pancreatic ductal epithelium during multistep transformation. We assessed Fhit expression by immunohistochemistry in two different multistep pancreatic carcinogenic processes: pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasia (IPMN). We considered 105 surgically treated PDACs/IPMNs and selected: 30 samples of non-neoplastic pancreatic parenchyma, 50 PanIN lesions, 30 IPMNs, 15 IPMNs with associated invasive carcinoma and 60 adenocarcinomas. Normal pancreatic ducts and surrounding acinar cells consistently showed moderate to strong Fhit immunoreactivity. Significant down-regulation of Fhit expression was observed in association with increasing severity of dysplasia/neoplasia in both carcinogenic processes. This was further confirmed by studying multiple lesions obtained from the same surgical specimen. Of 60 PDACs, only 14 showed Fhit expression comparable to

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Compliance with Ethical Standards: The materials used have been collected under Program 853 protocol 298CE 15/02/2002 and Program 1885 protocol 52438 on 23/11/2010. The protocols include informed consent of the patient and were approved by the local ethics committee of the Integrated University Hospital Trust of Verona. The first approval (prog. 853) regarded the collection of pancreas samples for use in molecular research studies. This was later updated (prog. 1885) for the creation of a coordinated biobank for the collection of samples from all cancer patients that included neoplastic and associated local and distant normal tissue. The approved programs include tissue processing and storage methods of FFPE tissues of both neoplastic and normal tissues.

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normal pancreatic ductal epithelium, while the remainder (77%) showed clearly negative or reduced Fhit expression. This study demonstrates that Fhit down-regulation is an early event in both multistep carcinogenic processes leading to PDAC.

Keywords

FHIT; pancreatic cancer; carcinogenesis; biomarkers

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth-leading cause of cancer-related deaths worldwide, with a 5 year survival time of less than 5% [1, 2]. Surgery still represents the only potentially curative treatment [1].

Accumulating evidence indicates that PDAC is characterized by marked genetic heterogeneity [3–5]. The study of this multifaceted molecular heterogeneity is further complicated by the existence of different carcinogenic cascades involved in normal ductal epithelium transformation.

In the last decade, three types of PDAC precursor lesions have been characterized: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary neoplasia (IPMN), and mucinous cystic neoplasia (MCN) [6]. They all follow multistep progression to invasive cancer characterized by an increasing degree of morphological and cytological atypia [7–10]. Several recent molecular studies investigated the molecular landscapes characterizing the progression of pancreatic cancer through its precursor lesions [11–15]. However, no biomarker is currently recommended for clinical use in the management of PDAC patients.

Chromosome fragile sites are preferential targets of replication stress, resulting in deletions involving encoded fragile genes [16–18]. One of the most commonly altered fragile sites is FRA3B/FHIT at chromosome band 3p14.2, which is among the chromosomal loci that show relatively frequent karyotypic anomaly and allelic loss in pancreatic cancer [19–23]. Consequently, it has been shown that expression of *FHIT* mRNA and Fhit protein is significantly down-regulated in primary PDACs [20, 24, 25]. *In vitro* and *in vivo* experiments demonstrated that Fhit overexpression in PDAC-derived cell lines leads to increased apoptosis, through activation of the caspase pathway [26].

Of note, early loss of Fhit has been shown to be an important step in the initiation of tumorigenesis in a variety of solid tumors, including among others of the lung, breast and colorectum [27–31]. However, excluding isolated case reports [32, 33], no study focused on evaluating Fhit expression in the early phases of pancreatic carcinogenesis.

The aim of this study was to explore down-regulation of Fhit expression by immunohistochemistry in the two main PDAC carcinogenic cascades: PanIN and IPMN.

MATERIALS AND METHODS

Tissues

A series of formalin-fixed and paraffin-embedded (FFPE) tissue samples obtained from 105 pancreatectomies (male:female=66:39; mean age 74.3 ± 12.1) for PDACs ($n=60$; G2=38, G3=22; pTNM Stage II=36, III=16, IV=8), IPMNs ($n=30$), and IPMNs with an associated invasive carcinoma ($n=15$) were retrospectively retrieved from the ARC-Net biobank at Verona University Hospital (www.arc-net.it). Original slides were jointly re-evaluated by three GI pathologists (MF, BR and CL) according to WHO 2010 criteria [6], the Baltimore Consensus Meeting [10], and the Verona Consensus Meeting [9].

From the 60 PDAC-pancreatectomies, 50 PDAC-associated PanIN lesions were further selected and classified as low-grade PanIN ($n=30$), or high-grade PanIN ($n=20$) [10].

The 30 IPMNs were further subclassified according to (i) their immunohistochemical profile [34] as intestinal ($n=17$), pancreatobiliary ($n=9$), oncocytic ($n=2$), or gastric ($n=2$) type; (ii) their highest-grade focus of dysplasia [9] as low ($n=12$), or high grade ($n=18$). The two gastric-type and 10 of the intestinal-type IPMNs were characterized by low-grade dysplasia. Of the IPMNs, seven of the intestinal-type, the two of oncocytic-type and the nine of pancreatobiliary-type were characterized by high-grade dysplasia.

Eight of 15 IPMNs with an associated invasive carcinoma were intestinal type, the other seven were pancreatobiliary. All the 15 IPMNs featured high-grade dysplasia. The invasive component was classified as tubular in seven and colloid in eight.

A series of 30 non-neoplastic and non-atrophic pancreatic parenchyma samples adjacent to IPMN were selected, and pancreatic ducts were considered as controls and retained for Fhit score.

Immunohistochemistry (IHC)

Immunostaining was performed on 4 μ m-thick FFPE sections using anti-Fhit antibody (clone ZR 44; Zymed Laboratories by ThermoFisher, South San Francisco, CA; dilution 1:200) [35], with an automatic stainer (Bond instrument, Vision Biosystem Leica, Milan, Italy). Immunostaining was performed after antigen retrieval with 10 mM citrate buffer, pH 6.0, as previously described [36, 37]. Detection was with streptavidin-biotin complex using the LSAB2 system (DAKO, Carpinteria, CA) with diaminobenzidine as chromogen. Appropriate positive and negative controls were selected from a previous series of gastric samples already characterized for Fhit expression [38] and run concurrently.

Fhit immunostaining was considered positive when cells showed unambiguous cytoplasmic staining. In positive cases, also some nuclear staining was observed. Fhit staining intensity (jointly considering the cytoplasmic and nuclear immunoreactivity) was evaluated using a semi-quantitative H-score, defined as the aggregate of the total percentage of tumor cells expressing Fhit at three intensity levels from 0, +1 (weak intensity), +2 (moderate intensity) or +3 (strong intensity).

In brief, the H score was defined as: (Percent of Fhit 1+ tumor cells multiplied by intensity of 1) + (Percent of Fhit 2+ tumor cells multiplied by intensity of 2) + (Percent of Fhit 3+ tumor cells multiplied by intensity of 3). This composite score ranges from 0 (a tumor which is completely negative) to a maximum of 300 (a tumor in which all the cells feature 3+ staining). Starting from its original description in 1995 [39], H-score has been successfully implemented in biomarker expression evaluation. A great advantage of this method is its comprehensive evaluation of the specimen: protein expression observed among all tumor/lesional cells in the slide are included in the analysis, avoiding possible bias due to tumor heterogeneity.

Statistical analysis

Differences in Fhit expression between pathology subgroups were evaluated by (paired)*t*-test and ANOVA, as appropriate. The statistical analysis was performed using STATA software (Stata Corporation, College Station, Texas, USA).

RESULTS

Fhit expression in non-neoplastic pancreatic parenchyma

Normal pancreatic ducts and surrounding acinar cells consistently showed moderate to strong Fhit immunoreactivity (average H-score of 270.7 ± 24.9 ; Figure 1A) mainly observed in the cytoplasm (Figure 2A). Also ducts in chronic pancreatitis showed moderate to strong Fhit immunoreactivity (Figure 2B).

Fhit is significantly down-regulated in PanIN lesions and PDACs

Significantly down-regulated Fhit expression was observed in both PanINs (average H-score of 188.4 ± 90.4) and PDACs (average H-score of 89.5 ± 75.1) in comparison to normal pancreatic ducts (both $p < 0.001$; Figure 1A).

Focusing on grade of dysplastic PanIN lesions, significant down-regulation in Fhit expression was observed in high-grade PanINs (average H-score of 147.1 ± 101.9) in comparison to low-grade lesions (average H-score of 216.0 ± 71.1 ; $p = 0.007$; Figure 1B), defined according to the Baltimore Consensus Meeting [10]. Twenty-four of 30 (80%) low-grade PanINs showed moderate to strong Fhit expression (H-score > 150 ; for example, Figure 2A).

Of the 60 PDACs, 14 (23%) showed Fhit expression comparable to their normal counterparts (H-score > 150) and 16 (27%) presented negative or focally faintly positive Fhit expression (Figure 2B). No significant association was observed between Fhit immunoreactivity and PDAC grade and stage. Comparing PanINs to the corresponding invasive carcinoma from the same surgical specimen, significant down-regulation was observed in all ($p < 0.001$; Figure 1C), except for 4/50 cases (8%), which showed slight Fhit overexpression in the PDAC counterpart, and 8/50 cases (16%), which showed a similar downregulation of Fhit expression in dysplastic and invasive neoplastic epithelia (Figure 2C).

IPMN dysplastic epithelia are characterized by significant loss of Fhit expression

Fhit was significantly down-regulated in IPMN lesions (average H-score of 130.3 ± 55.6) in comparison to non-neoplastic ducts ($p < 0.001$; Figures 1A and 3), but significantly higher in comparison to PDACs ($p = 0.010$) and to IPMN with associated PDACs (average H-score of 86.0 ± 69.1 ; $p = 0.0249$). Significant differences were observed in Fhit H-score according to histotype, showing that oncocytic- and gastric-type IPMNs exhibit higher Fhit expression than intestinal- and pancreatobiliary-type (oncocytic = 225.0 ± 21.2 ; gastric = 200.0 ± 56.6 ; intestinal = 124.1 ± 47.4 ; pancreatobiliary = 105.6 ± 45.0 , Figure 1D).

As observed in the PanIN setting, a significant steady decrease in Fhit expression was associated with increasing grade of dysplasia of the lesions, with a significant Fhit down-regulation in high-grade *versus* low-grade lesions ($p = 0.032$; average H-score 112.8 ± 51.9 vs 156.7 ± 52.3 ; Figure 1E).

In IPMNs with an associated invasive carcinoma, Fhit expression was significantly lower in the invasive carcinoma than in the matched dysplastic component ($p = 0.008$; Figure 1F) in all but one of 15 cases (7%), this case showing slight Fhit overexpression in the PDAC part, and 4/15 cases (27%) which showed similar Fhit down-regulation in IPMN and associated PDAC.

DISCUSSION

FHIT gene alterations have been shown to drive events during pancreatic carcinogenesis. We have previously shown that aberrant transcripts of the *FHIT* gene can be detected in 50% of primary human PDACs, which may result in loss of Fhit protein expression [20]. Simon et al. reported *FHIT* gene alterations in 70% of PDAC-derived cell lines [25]. Furthermore, Tsujiuchi et al. observed *FHIT* cDNA alterations in 73% of nitrosamine-induced Syrian golden hamster PDACs [40].

FHIT locus deletions are among the earliest gene alterations detected in human pre-invasive neoplastic lesions [41, 42], and Fhit-deficient cells are more likely to acquire cancer-promoting mutations in oncogenes/tumor suppressor genes, expediting the cellular transformation process [43]. In lung adenocarcinomas, *FHIT* gene alterations are associated with initiation of the neoplastic process rather than with progression to an invasive tumor and distant metastasis [28, 43, 44], further supporting early involvement of *FHIT* in multistep carcinogenic processes. However, no study systematically investigated *FHIT* status in the early phases of pancreatic ductal carcinogenesis.

We studied Fhit expression by immunohistochemistry in PanIN and IPMN, which constitute the most important multistep cascades of pancreatic carcinogenesis. These lesions are characterized by different molecular landscapes. PanINs show frequent early activating point mutations in the *KRAS* gene while high grade PanINs show frequent alterations in *CDKN2A* and *TP53* [45]. IPMNs are characterized by recurrent mutations in *GNAS* and *RNF43* [34].

In spite of the different genetic background, both dysplastic lesion types featured significant loss in Fhit expression in comparison to normal ductal epithelia. Moreover, a faint to negative Fhit expression consistently characterized more advanced lesions (i.e. high-grade dysplasia and PDAC). Overall, these data further support a central role for *FHIT* down-regulation in the initiation of neoplasia in duct epithelia.

In contrast to previous findings [20], we observed moderate to strong cytoplasmic Fhit immunoreactivity in normal pancreatic acinar cells. This might be related to the applied antisera, which were different from earlier studies. However, in duct epithelia consistent down-regulation of Fhit expression has been demonstrated in PDAC samples [20, 24].

The clinical impact of Fhit evaluation as a novel diagnostic tool is limited by several factors, especially in small biopsies or in cytology specimens. Firstly, detection of Fhit expression cannot differentiate between invasive and intraepithelial neoplasms. Secondly, normal surrounding tissue is needed as positive control for staining performance. Finally, Fhit expression may also be lost early in low-grade lesions, which can lead to over-diagnosis.

We did not investigate molecular mechanisms leading to this early loss. A recent matched pair sequencing study on genomic DNA from 14 PDAC and associated PanIN lesions showed that *FHIT* is deleted in 50% of tumors, with associated loss in expression [23]. In this series, *FHIT* rearrangements observed in PDACs were only in part validated in adjacent PanINs [23]. However, this might be related to molecular heterogeneity observed in PDAC (and therefore in PDAC-associated pre-invasive lesions) [46]. Of note, PanIN lesions are characterized by chromosomal instability [47–50], and therefore the down-regulation of Fhit expression we observed in our series may be associated with *FHIT* gene loss rather than with epigenetic mechanisms. On the other hand IPMNs are characterized by chromosomal stability and presence of activating mutations [32], which support an epigenetic mechanism rather than gene deletion to explain significant down-regulation of Fhit expression observed in these tumors.

Our study shows that Fhit down-regulation is an early event in multistep carcinogenic processes leading to PDAC. These data further support Fhit as a key tumor suppressor in pancreatic duct epithelia.

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Figure 1. Fhit expression is down-regulated early in pancreatic carcinogenesis

Immunohistochemical expression is represented as H-score values. PanIN= Pancreatic intraepithelial neoplasia; LG= low grade; HG= high grade. (A) Fhit expression distribution in the series of samples examined. (B) Fhit expression according to dysplasia grade of PanIN. (C) Matched PanIN-PDAC. (D&E) Distribution of Fhit H-score according to histotype and grade of IPMNs, respectively. (F) Fhit expression in matched dysplastic and invasive epithelia in 15 IPMNs with an associated invasive carcinoma. Boxplots are with whiskers from minimum to maximum of all data.

Figure 2. Fhit expression is significantly down-regulated in pancreatic ductal adenocarcinoma (PDAC) and in PDAC-associated pancreatic intraepithelial neoplastic (PanIN) lesions
(A) A low-grade PanIN lesion (asterisk) showing Fhit expression comparable to expression in normal surrounding pancreatic parenchyma. (B) A representative Fhit-negative adenocarcinoma intermingled with Fhit-positive pancreatic ductal epithelia. (C) A high-grade PanIN lesion (arrow heads) and coexisting PDAC, both showing negative/low Fhit expression. (Original magnifications, 4 $\times$, 10 $\times$, and 20 $\times$).

Figure 3. Down-regulation of Fhit expression in IPMNs

(A and B) IPMNs (asterisks) with low-grade dysplasia showing negative/low Fhit expression in comparison to Fhit-positive adjacent pancreatic parenchyma. (C) A Fhit positive IPMN (arrow heads), showing down-regulation of Fhit expression in higher grade epithelia. (D) IPMN with low-grade dysplasia showing negative (arrow heads)/low Fhit expression in comparison to Fhit positive adjacent pancreatic parenchyma (asterisk). (E) Fhit-negative IPMN with high-grade dysplasia. (F) Mild to moderate Fhit expression in an IPMN with high-grade dysplasia and a coexisting Fhit-negative colloid adenocarcinoma (arrow heads). (Original magnifications, 4 $\times$, 10 $\times$, and 20 $\times$).