ROS1 fusions are absent in a series of 109 pancreatic ductal adenocarcinomas

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Abstract:

Introduction:
Pancreatic ductal adenocarcinoma (PDAC) accounts for up to 95% of all malignant pancreatic neoplasms and constitutes the most frequent histopathologic entity among pancreatic neoplasms. Prognosis is mainly dependent on the stage of disease at diagnosis and overall outcomes are poor. KRAS-mutants are frequently observed in PDAC. There is, however, a percentage of PDAC cases with wild-type KRAS. ROS1 is a tyrosine kinase that has been recognized as a promising therapeutic target, when rearranged. Rearrangements have rarely been reported to be present in malignancies of the pancreato-biliary system, such as cholangiocarcinoma.

Methods:
We analyzed a series of 109 patients diagnosed with PDAC for KRAS-mutations and ROS1 expression. KRAS wildtype cases were also screened for NRAS-mutations. In cases of ROS1 expression, a fluorescence in-situ hybridization (FISH) was performed to check for ROS1 rearrangement.

Results:
On immunohistochemistry 5/109 cases (4.6%) of PDAC showed mild cytoplasmatic ROS1 expression. None of the 5 cases was found to be rearranged by FISH. 95/109 (87.1%) PDAC cases harbored a KRAS mutation. The 14 KRAS-wildtype cases had no NRAS mutations. Furthermore, a combination of ROS1 expression and KRAS mutation has been observed in three cases.

Conclusions:
We conclude that despite its significance as a therapeutic target in many other malignancies, ROS1 does not play a central role in the pathogenesis of PDAC. Furthermore, we confirm that KRAS mutations are a dominant factor promoting PDAC, while no NRAS mutations were found.

Introduction:
Pancreatic ductal adenocarcinoma (PDAC) accounts for up to 95% of all malignant pancreatic neoplasms and is the fourth most cause of cancer related death worldwide.12 Patients do generally not present with any or only with non-specific symptoms in the early disease process. Standard PDAC treatment consists of radical surgical resection followed by adjuvant chemotherapy and a close follow-up. Major problems are diagnosis at late stages of disease as well as peak occurrence in elderly and often multimorbid patients, both making the resection technically more challenging for the surgeon and overall treatment more stressful for the patient.1 In many cases, curative resection is hindered by locally advanced tumor growth.4 The above stated circumstances result in stage dependent five-year survival rates between 1 and 14% and a mean five-year survival rate below 8%, which has not significantly increased over the past two decades.14

From a histopathologic point of view, PDAC is an entity with several different morphological features conforming to the rising complexity of the disease on a genomic level.1 Using genome-wide array-based comparative genomic hybridization analysis, a subset of mutational patterns has been identified. A subclassification of PDAC governed by these patterns has been suggested in an attempt to correlate the diverse biologic behavior of PDAC with the new genomic findings.4,10 However, KRAS mutations on codons 12, 13 and 61, respectively, have been identified as the major driving force in PDAC development.

KRAS is, like NRAS and HRAS, a member of the RAS family of GTP-binding proteins involved in cellular proliferation. Mutations at codon 12 are frequently observed and desensitize the binding protein to hydrolytic cleavage at the nucleotide binding site, thus leaving it in a constitutively active state.11 KRAS mutations have been recognized as a hallmark of PDAC occurring early on in cancer development in 88 to 100% of cases.11,12,13 These mutations are found alongside with HER2/neu overexpression already in early precursor lesions of PDAC, while other aberrations such as inactivation of p16, loss p53, SMAD4/DPC4 and BRCA2 mutations appear in later stages of PDAC development.16 In contrast little detail is known about the underlying molecular profile of the small percentage of KRAS wild-type cases in PDAC and its possible connection to cancer progression. NRAS mutations occur on the same codons as KRAS mutations but are scarce in PDAC.17

ROS1 is a receptor tyrosine kinase encoded by the ROS1 gene and has first been found to be rearranged in both glioblastoma cell lines and tumor samples.18 It has subsequently been recognized as a proto-oncogene over-expressed in many other malignancies.

The significance of ROS1 rearrangements has been discussed prominently in adenocarcinoma of the lung, but also in gastrointestinal neoplasms such as colorectal adenocarcinoma, gastric adenocarcinoma and cholangiocarcinoma.19-25 Generally, ROS1 rearrangements represent driver mutations occurring mutually exclusive to other driver mutations involving EGRF, KRAS or RET.26,27

As a tyrosine kinase, ROS1 has been found to be a potential target for a range of tyrosine kinase inhibitors (TKI) such as Lortatibin or Crizotinib. Although only up to 2% of non-small cell lung carcinomas (NSCLC) harbor the rearrangement, testing for ROS1 rearrangements is recommended to facilitate targeted therapy in positive cases, which has been shown to grant a significant advantage in terms of response and overall survival compared to conventional chemotherapy alone.28,29 For reasons of cost efficacy, a screening method involving immunohistochemistry (IHC) followed by fluorescence-in-situ-hybridization (FISH) has been suggested.30,31

As stated above, ROS1 rearrangements have been reported in malignancies of the gastrointestinal tract and the pancreato-biliary system.16-22 However, there is no published data assessing the potential role of ROS1 rearrangements or ROS1 overexpression in PDAC.
Therefore, we sought to elucidate the frequency of both ROS1 expression and ROS1 rearrangement in a series of ductal adenocarcinomas of the pancreas.

Material and Methods:

Patients and case characteristics

Embracing a time frame from 2005 to 2015, we retrospectively subjected a cohort of 109 consecutive cases of ductal adenocarcinoma of the pancreas to this study. All patients had the tumor resection performed in the department of general and visceral surgery at the Klinikum Lippe in Detmold, Germany. All patient information was handled anonymized and according to the declaration of Helsinki. Verbal informed consent of each patient included was obtained individually beforehand.

Inclusion and exclusion criteria

All consenting patients with resectable pancreatic ductal adenocarcinoma (PDAC) were included in the giving time frame. Exclusion criteria were histological tumor entities other than PDAC and cases with mixed histology, respectively.

Statistical analysis

Patient age ranged from 39 to 84 years with a mean age of 69.17 years. Sex distribution was almost equal with 52 patients being female and 57 being male. The male to female ratio was 1.096. T stage according to the TNM guidelines of the International Union Against Cancer (UICC, 7th edition) ranged from T1 to T4. The most frequent tumor stage was T3 (n = 100), followed by T2 (n = 6), T4 (n = 2) and T1 (n = 1). Nodal positivity was detected in 74 cases (68%) and a primary R0 situation was achieved in 87 cases (80%).

Most of the specimen were resected from the head and body of the pancreas, 8 cases of our cohort underwent surgery with a lesion in the tail of the pancreas.

Immunohistochemistry

Firstly, immunohistochemical analysis of ROS1 was performed on 4 μm formalin-fixed and paraffin embedded (FFPE) tissue slides. Following dewaxing and heat-induced antigen retrieval, each case was stained with an antibody dilution of 1:300 on a Ventana Benchmark (Ventana Medical Systems, Tucson, AZ, USA) immunostainer platform using standardized operating procedures.32 The antibody clone used (clone D4D6, Cell Signaling, Cambridge, UK) has been validated for this purpose on lung carcinomas and successfully used on tumors of the pancreato-biliary system before.19,33

Semiquantitative evaluation of immunohistochemical staining was performed according to the H-score scoring system suggested by Yoshida et al. and previously used by Cha et al., on tissue of a ROS1 positive non-small cell lung cancer case as an on slide positive control.34,35

Fluorescence in-situ-hybridization (FISH)

ROS1 FISH analysis was performed using the SPEC ROS1 dual color break apart probe (ZytoVision, Bremerhaven, Germany) in combination with the ZytoLight FISH-Tissue Implementation Kit by Zytovision (product number Z-2082-20, ZytoVision, Bremerhaven, Germany) on formalin fixed paraffin embedded tumor-slides.

After hybridization slides were analyzed with an Olympus BX51 fluorescence microscope equipped with filters for simultaneous detection of ZyOrange and Rhodamin Signals of the SPEC ROS1 Dual color break apart probe. Split-signal events were counted per 50 tumor cell nuclei. Positive events were defined as a signal distance of at least one signal diameter. A cut-off of 15% tumor nuclei was used for detection of positive cases.

KRAS and NRAS analysis

Each case was additionally analyzed for the presence of KRAS mutations and, in case of KRAS wildtype, NRAS mutations using the strip assay method (Medipro, Hockenheim, Germany) modified according to Ausch et al. The KRAS strip assay used (Medipro, Hockenheim, Germany) covered 29 mutational genotypes in codons 12, 13, 59, 60, 61, 117 and 146 on the KRAS gene, namely Ala12 (c.35G>C), Arg12(c.34G>C), Asp12 (c.35G>A), Cys12 (c.34G>T), Ile12 (c.34_35delGGinsAT), Leu12 (c.34_35delGGinsCT), Ser12 (c.34G>A), Val12 (c.35G>T), Ala13 (c.38G>C), Arg13 (c.37G>C), Asp13 (c.38G>A), Cys13 (c.37G>T), Ser13 (c.37G>A), Val13 (c.38G>T), Gly59 (c.176C>G), Thr59 (c.175G>A), Val60(c.179G>T), Arg61 (c.182A>G), His61 (c.183A>C; c.183A>T), Leu61 (c.182A>T), Lys61 (c.181C>A), Asn117 (c.351A>C; c.351A>T), Gly117 (c.349G>A), Pro146 (c.436G>C), Thr146 (c.436G>A), Val146 (c.437C>A). For NRAS mutation analysis a strip assay with a coverage of 22 mutations in codons12, 13, 59, 60, 61 and 146 respectively was used to identify the mutations Ala12 (c.35G>C), Arg12 (c.34G>C), Asp12 (c.35G>A), Cys12 (c.34G>T), Ser12 (c.34G>A), Val12 (c.35G>T), Arg13 (c.37G>C), Asp13 (c.38G>A), Cys13 (c.37G>T), Val13 (c.38G>T), Asp59 (c.176C>A), Thr59 (c.175G>A), Arg60 (c.179G>C), Gly60 (c.179G>A), Arg61 (c.182A>G), Glu61 (c.181C>G), His61 (c.183A>C; c.183A>T), Lys61 (c.181C>A), Asn117 (c.351A>C; c.351A>T), Gly117 (c.349G>A), Pro146 (c.436G>C), Thr146 (c.436G>A), Val146 (c.437C>A).

Results:

Histological findings and mutational status

In our series of 109 ductal adenocarcinomas of the pancreas we found that five cases showed mild cytoplasmatic granular expression of ROS1 (Figure 1). The on slide controls used validated the staining results. Two of the five cases harbored a KRAS G12D mutation, while one had a G12R mutation and two were found to be KRAS wildtype. On fluorescence in-situ-hybridization no split signal was detectable in either of the five cases (Figure 2). NRAS mutations were not detectable in our collection of 109 cases. Overall, Gly12Asp was the KRAS mutation most frequently observed in our cohort (n = 47; 43%), followed by Gly12Val (n = 26; 12%) and Gly12Arg (n = 17; 16%). Other mutations at codon 12, namely Gly12Cys (n = 1; 1%) and Gly12Ser (n = 1; 1%), were less frequent. At codon 61 we found the mutation Gln61His in three cases (3%). 14 cases were found to be KRAS wild type (13%; Figure 3).

Discussion:

Pancreatic ductal adenocarcinoma (PDAC) is among the leading causes of cancer related deaths worldwide. KRAS mutations have been identified as a major factor in PDAC development and progression, occurring in around 90 percent of cases. Even though the remaining proportion of KRAS wild-type cases is clustered into a subset of different genotypes, the significance of other potential oncogene aberrations or driver mutations in these cases and in progressed PDAC remains unclear.37

In other cancers such as colorectal carcinoma, adenocarcinoma of the lung and gastric cancer, ROS1 rearrangements are therapeutic targets providing an advantage in terms of progression free survival, if present.22,27,28,29 In intrahepatic cholangiocarcinoma ROS1 overexpression in absence of rearrangements was found to be associated with better survival.19 To date, neither the role of ROS1 rearrangements nor the presence of its overexpression have been examined in PDAC.

Here, we sought to elucidate the potential role of ROS1 overexpression and ROS1 rearrangements in KRAS wild-type cases of PDAC by means of immunohistochemistry and fluorescence-in-situ-hybridization,
Taking into consideration tumor localization, sex and age distribution, TNM stages and histological grading, the cohort analyzed here is representative for pancreatic ductal adenocarcinoma (PDAC). It must be stated though, that all patients analyzed came from a relatively confined region, increasing the risk of confounding and underrepresentation because of a restricted gene pool.

Based on our findings, there was no statistical evidence for a contribution of ROS1 rearrangements or ROS1 overexpression in the development of this entity. The antibody clone used for immunohistochemical analysis has been well evaluated in several other studies before. Samples were analyzed conventionally as whole tissue sections, avoiding the use of tissue microarrays in order to obtain more representative samples. Hence, intra-tumoral heterogeneity of ROS1 expression, which has been seen in KRAS mutated and ALK rearranged cases of colorectal carcinoma, could be ruled out as a confounding factor in our study. Moreover, out of five cases with mild cytoplasmatic ROS1 expression, three cases carried a KRAS mutation in absence of a ROS1 rearrangement. NRAS mutations, either alone or co-occurring with KRAS mutations, were not found. Our study does again confirm the high prevalence of KRAS mutations in PDAC with Gly12Asp (n = 47; 43%) being the most frequent mutation in our study. This is in accordance to the reported data, that the majority of KRAS mutations is located on exon 2 and arises on codons 12 and 13.

While our methods covered the majority of KRAS and NRAS mutations reported to be present in PDAC, other studies have found different aberrations in even smaller collections of KRAS wild-type cases. Thus, there are few case studies reporting on patients presenting with ALK rearrangement, BRAF V600E mutation in single KRAS wild type PDAC. These findings suggest that there might indeed be a set of gene aberrations accounting for the up to 5% of KRAS wild-type cases in PDAC.

In a small study, oncogenic ALK fusions and a rare RAS mutation have been found in separate KRAS negative cases of PDAC, indicating that development of a small subset of pancreatic cancers could be driven by oncogenic aberrations other than the KRAS mutation. However, ALK expression has been found to be extremely rare in PDAC.

In case reports of adenocarcinoma of the lung, concurrent KRAS mutations and ROS1 rearrangements have been detected as well as EGFR mutations occurring together with ROS1 rearrangements, respectively. However, ROS1 rearrangements and KRAS mutations usually occur mutually exclusive, when detected in malignancies.

The presence of NRAS mutations in PDAC is rarely reported. Yet, one very recent study showed a significant positive correlation between high NRAS expression by means of immunohistochemistry and progression free and overall survival in a cohort of resectable PDAC. How these new insights fit into the general view of RAS signaling as a hallmark of PDAC remains to be elucidated.

**Conclusion**

Based on our series of 109 cases, we conclude that there is no statistical evidence for either ROS1 rearrangements or NRAS mutations to play a crucial role in the pathogenesis of PDAC. Before this background, the significance of the small subset of cases with immunohistochemical positivity for ROS1 shown here must be viewed doultfully, even in the presence of successful on slide controls. On the other hand, our findings contribute to the general view of the dominant role of KRAS mutations in PDAC.

**Conflict of Interest and Source of Funding:**

There is no conflict of interest for any of the authors. The authors have no financial support or funding sources to declare.

**References**


Figures

IHC Panel Figure 1
Results of KRAS mutation analysis (n = 109)

- G12D: 43%
- G12V: 15%
- G12R: 13%
- Q61H: 24%
- G12C: 1%
- G12S: 3%
- WT: 1%

Mutation Distribution Figure 3