DNAJB1-PRKACA is specific for fibrolamellar carcinoma

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Fibrolamellar carcinoma is a distinct subtype of hepatocellular carcinoma that predominantly affects young patients without underlying cirrhosis. A recurrent *DNAJB1-PRKACA* fusion has recently been reported in fibrolamellar carcinomas. To determine the specificity of this fusion and to develop routinely available clinical methods of detection, we developed an RT-PCR assay for paraffin-embedded tissues and a FISH probe for detection of the rearrangements of the *PRKACA* locus. We also developed an RNA *in situ* hybridization assay to assess expression levels of the total chimeric transcript and wild-type transcripts. A total of 106 primary liver tumors were studied by RT-PCR, including 26 fibrolamellar carcinomas (4 of which were metastases to the abdominal wall or lymph nodes), 25 conventional hepatocellular carcinomas, 25 cholangiocarcinomas, 25 hepatic adenomas, and 5 hepatoblastomas. RT-PCR was successful in 92% of tested fibrolamellar carcinoma cases (24/26) and the *DNAJB1-PRKACA* fusion transcript was found in all fibrolamellar carcinomas but not in other tumor types. FISH was tested in 19 fibrolamellar carcinomas and in 6 scirrhous hepatocellular carcinomas, which can closely mimic fibrolamellar carcinoma. Rearrangements of the *PRKACA* locus was seen in all 19 fibrolamellar carcinoma specimens, but in none of the scirrhous hepatocellular carcinomas. Finally, a RNA *in situ* hybridization strategy was positive in 7/7 successfully hybridized cases, and showed mRNA over-expression in all of the fibrolamellar carcinomas. In addition, the stromal cells embedded in the characteristic intratumoral fibrosis of fibrolamellar carcinomas and the background liver tissues were negative for the *DNAJB1-PRKACA* fusion by all tested methods. In conclusion, detection of *DNAJB1-PRKACA* is a very sensitive and specific finding in support of the diagnosis of fibrolamellar carcinoma.

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Fibrolamellar carcinoma is a morphologically distinctive type of hepatocellular carcinoma that makes up ~1% of all hepatocellular carcinomas. The neoplastic cells are monotonous with abundant, eosinophilic cytoplasm and prominent nucleoli. The tumor is also characterized by a distinctive intratumoral fibrosis that often has a lamellar pattern. Also there are distinctive clinical features, including young age of onset, relative to conventional hepatocellular carcinoma, and the lack of

background liver disease.¹ Interestingly, this general constellation of findings (young age, lack of underlying disease, and morphological monotony) is a common feature of translocation-associated tumors² and a recurrent chimeric gene was recently identified in the fibrolamellar carcinoma.³ However, the specificity of this fusion in the context of other primary hepatic neoplasms is unknown. To address this question and to develop clinically applicable test platforms, we developed an RT-PCR assay for use with paraffin-embedded tissues along with a FISH assay, both of which can detect the novel fusion at the transcriptional and genomic level, respectively. In addition, we developed a third independent method to semiquantitatively assess the total expression level of wild-type *PRKACA* and *DNAJB1-PRKACA* by using *in situ* hybridization.

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Materials and methods

A total of 26 fibrolamellar carcinomas were studied, including 19 cases from the Mayo Clinic and 7 cases from the University Hospital of Heidelberg, which were obtained from the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the local regulations and the approval of the ethics committee. Additional cases were also selected from Mayo Clinic files of conventional hepatocellular carcinomas ($N=25$), scirrhous hepatocellular carcinomas ($N=6$), intrahepatic cholangiocarcinomas ($N=25$), hepatoblastomas ($N=5$), and hepatic adenomas ($N=25$). Each case was histologically confirmed by two pathologists (Mayo cases: RPG and MST; Heidelberg cases: TL and PS).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Using hematoxylin and eosin (H&E)-stained sections as guides, tumor sections with at least 50% tumor cellularity were macrodissected from unstained 5- μ m thick sections of paraffin-embedded tissue. Total RNA was extracted with the TRIzol Reagent kit (Invitrogen). After RNA concentration was measured by spectrophotometry (Nanodrop Technologies, DE), 0.5–1 μ g of RNA from each case was added to separate 20 μ l reactions and converted into cDNA by using the iScript Select cDNA Synthesis kit with the supplier's provided instructions (Bio-Rad Laboratories, CA). The *DNAJB1-PRKACA* fusion transcript (Genbank accession numbers: *DNAJB1*: NM_006145.1; *PRKACA*: NM_002730.3) was amplified by using a PCR primer set with the sequences as follows: *DNAJB1* exon 1: 5'-GGAGAAGTTCAAGGAGATCGCT (forward) and *PRKACA* exon 2: 5'-CAAGTGGGCTGTGTTCTGAG (reverse) with PCR product size 163 bp. The housekeeping gene phosphoglycerokinase (*PGK1*) was amplified along with *DNAJB1-PRKACA* fusion transcript to check the RNA quality, using these primer sets: 5'-CAGTTTGAGCTCCTGGAAG (forward) and 5'-TGGAGATGAGAAAATGCTAA G (reverse), which result in a predicted PCR amplicon size of 126 bp (Genbank accession number: NM_000291.2).

PCR was carried out in a 25 μ l final reaction volume containing 2 μ l of cDNA template DNA, 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 0.2 mM of each deoxynucleotide, 0.3 μ M of forward and reverse primers, and 1.0 U of Taq DNA polymerase (Invitrogen). Thermal cycling was conducted by using the following parameters: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR product for each case was visualized under ultraviolet wavelength after 3% agarose gel electrophoresis and staining with ethidium bromide. As a quality control, reactions were

repeated in 16 cases to confirm the accuracy of the results. In addition, the PCR products from 8 cases of fibrolamellar carcinoma were sequenced by the Sanger method. The sequence chromatograms were compared with the fusion sequence of *DNAJB1* exon 1 to *PRKACA* exon 2.

Fluorescence In Situ Hybridization (FISH)

PRKACA rearrangements were analyzed with a break-apart FISH strategy. Human bacterial artificial chromosomes flanking the *PRKACA* gene locus were identified by using the University of California Santa Cruz February 2009 Assembly hg19. The telomeric clones (RP11-63F22, CTD-2003D17, and CTC-708A18) were labeled by nick translation with Spectrum Green and the centromeric clone (CTC-548K16) was labeled with Spectrum Orange (Abbott Molecular, IL). Labeled clones were combined to create a dual-color, single fusion probe set. Paraffin sections from fibrolamellar carcinomas ($N=19$) and scirrhous hepatocellular carcinomas ($N=6$) were cut 5 μ m thick and mounted on silanized slides, baked for 15 min at 90 °C, and then deparaffinized in xylene. Slides were dehydrated in 100% ETOH and allowed to air dry. Pre-treatment in 10-mM citric acid was followed by a NaCl protease treatment to remove proteins and non-DNA cellular components. Slides were dehydrated in an ethanol series and air dried. Next, 5–15 μ l of DNA probe working solution was applied to the hybridization area, an 18 $\times$ 18 or 22 $\times$ 22 mm coverslip was placed over the top, and the edges of the coverslip were sealed with a continuous bead of rubber cement. The slide and probe were co-denatured and hybridized overnight. Slides were then washed and DAPI counterstain was applied as well as a glass coverslip. Visualization of the FISH signals was accomplished by using fluorescence microscope and pictures were captured by using a FISH imaging system (CytoVision, Leica Biosystems). Two experienced FISH technologists (DK and SKN) independently scored 50 tumor nuclei for each case.

RNA In Situ Hybridization (RNA-ISH)

We semiquantitatively assessed the expression levels of the combined total expression of *DNAJB1-PRKACA* and native *PRKACA*. A custom set of RNAscope double Z probe pairs was designed to span *DNAJB1* exon 1 and *PRKACA* exon 2, which are fused together in the novel chimera. A separate set of probe pairs spanned portions of the normal *PRKACA* exon 2.

RNA *in situ* hybridization was performed manually using RNAscope 2.0 HD Brown FFPE Reagent Kit (Advanced Cell Diagnostics, Hayward, CA) according to the manufacturer's instructions. In brief, 5- μ m paraffin-embedded tissue sections from seven randomly selected cases of fibrolamellar carcinoma

were pretreated with heat and protease prior to hybridization with the target oligonucleotide probes. Preamplifier, amplifier, and HRP-labeled oligos were then hybridized sequentially, followed by chromogenic precipitate development. Each sample was quality controlled for RNA integrity with an RNAscope probe specific to Hs-PPIB RNA and for background with a probe specific to bacterial dapB RNA. Specific RNA staining signals were identified as brown, punctate dots. Samples were counterstained with Gill's Hematoxylin. The slides were then reviewed and semiquantitatively scored by two pathologists (RPG and MST) by using the manufacturer-provided guidelines on a scale of 0–4.

Results

The characteristics of the patients with fibrolamellar carcinoma are summarized in Table 1. Histologically, all cases of fibrolamellar carcinoma revealed the classic histological features (Figure 1a). In a single case, the classic features of fibrolamellar carcinoma in one area gave way to a growth pattern that resembled a more typical hepatocellular carcinoma, with a sheet-like growth of tumor cells with increased nuclear:cytoplasmic ratio and a loss of the prominent cytoplasmic eosinophilia (Figures 2a

and b). This case was included because some prior studies have considered this histological pattern as a separate entity, using terms such as 'combined hepatocellular carcinoma–fibrolamellar carcinoma'.

The fusion transcript *DNAJB1-PRKACA* was detected by RT-PCR in 24 of 24 (100%) successfully amplified cases of fibrolamellar carcinoma. The fusion was present in the tumor but not in the background liver. In 16 cases where duplicate RT-PCR was done, all reactions were repeatedly positive for the fusion transcript. Amplicon sizes were confirmed by gel electrophoresis (Figure 1b). Sanger sequencing of the amplicon in 8 cases revealed *DNAJB1* exon1 fused to *PRKACA* exon 2 in all cases (Figure 1c). All cases had the same breakpoint. RT-PCR failed in 2 (8%) cases of fibrolamellar carcinoma, likely because of RNA degradation. RT-PCR for the *DNAJB1-PRKACA* fusion transcript was negative in all other tested primary liver tumors where testing was successful: 18 conventional hepatocellular carcinomas, 21 hepatic adenomas, 21 intrahepatic cholangiocarcinomas, and 4 hepatoblastomas. RT-PCR failures were all because of RNA degradation. Thus, RT-PCR was successful in 92% of fibrolamellar carcinoma specimens and 80% of tested primary epithelial liver tumor cases and was specific and sensitive for fibrolamellar carcinoma.

Table 1 Characteristics of the patients with fibrolamellar carcinoma

Case number	Specimen	Age at diagnosis (yrs)	Tumor size (cm)	RT-PCR result	Direct sequencing confirmed <i>DNAJB1</i> exon1 fused to <i>PRKACA</i> exon 2	<i>PRKACA</i> rearrangement by FISH
1 ^a	Liver	29	10.5	+	ND	+
2	Liver	31	19	+	+	+
3 ^a	Liver	25	11	Failed	ND	+
4	Liver	Recurrence of case 3	NA	Failed	ND	+
5	Liver	Recurrence of case 3	NA	+	ND	+
6 ^a	Liver	21	16	+	+	+
7	Lymph node	47	17.5	+	ND	+
8	Liver	19	7.5	+	ND	+
9	Liver	19	10	+	ND	+
10	Liver	44	8 ^b	+	ND	+
11 ^a	Liver	19	13.3	+	ND	+
12	Liver	15	14	+	+	+
13 ^a	Lymph Node	16	3.5 ^b	+	+	+
14	Liver	29	11.7	+	ND	+
15 ^a	Liver	25	10.2	+	ND	+
16	Liver	19	14.6	+	+	+
17	Liver	21	11.2	+	+	+
18	Liver	32	7.4	+	+	+
19 ^a	Liver	19	13.2	+	ND	+
20	Abdominal wall	27	0.7	+	ND	ND
21	Liver	19	9	+	+	ND
22	Liver	Recurrence of case 21	NA	+	ND	ND
23	Abdominal wall	Recurrence of case 21	NA	+	ND	ND
24	Liver	26	13	+	ND	ND
25	Liver	24	10.2	+	ND	ND
26	Liver	32	3.5	+	ND	ND

Abbreviation: ND, not done.

^aRNA *in situ* hybridization using a probe to *DNAJB1* exon 1-*PRKACA* exon 2 was positive in each of these tested cases.

^bMultiple tumor nodules identified, the largest of which is included in this Table.

Figure 1 (a) Photomicrograph of a fibrolamellar carcinoma (FLC) characterized by neoplastic cells with an abundant granular eosinophilic cytoplasm, a round nuclei, and a prominent nucleoli arranged in trabecula and separated by bands of fibrosis ($\times 200$ original magnification). (b) Representative gel image showing RT-PCR for *DNAJB1-PRKACA* fusion transcripts (top) and a housekeeping gene *PGK1* (bottom) in four FLC (lanes 1–4), one conventional HCC (lane 5) and one cholangiocarcinoma (lane 6). Lane 7 is a positive control and no reverse transcriptase in RT reaction (no RT) is used as negative control (lane 8). The *DNAJB1-PRKACA* fusion transcript is detected in all four FLC, but not in the other two tumors. A 25-bp molecular maker (M) is used. (c) Sequencing chromatogram displaying the sequence of the transcript which corresponds to a fusion of *DNAJB1* exon1 and *PRKACA* exon 2. (d) FISH demonstrated the rearrangement of *PRKACA* in $>90\%$ of the tumor cells (separated green and red signals) often with loss of red signal probes which correspond to the 5' end of *PRKACA* in keeping with the fusion event.

FISH identified translocations of *PRKACA* locus in 19 of 19 (100%) fibrolamellar carcinomas (Figure 1d), including the single case with diffuse areas that resembled typical hepatocellular carcinoma (Figures 2c and d). In all cases, the abnormal signal patterns, indicating translocation, were diffuse and seen in $>90\%$ of tumor cells. The

translocation was not associated with homozygous deletion of the other chromosome, as tumor cells demonstrated heterozygous loss of the 5' probe, consistent with retention of a normal *PRKACA* allele in addition to the translocation. Furthermore, because many of the tumor cells were tetraploid or aneuploid, many tumor cells had more than one

Figure 2 (a) In this case, there were areas of typical FLC as shown here including oncocytic tumor cells separated by a dense fibrous stroma. The tumor cells were also remarkable for brown granular pigment ($\times 200$ original magnification). (b) Elsewhere, the tumor displayed a diffuse sheet-like growth with a higher nuclear:cytoplasmic ratio and pink secretions distending bile canaliculi, features which are atypical for FLC ($\times 200$ original magnification). (c) *PRKACA* was rearranged in the tumor cells from the typical areas of FLC as shown by FISH. (d) Similarly in the atypical areas with diffuse growth, *PRKACA* was rearranged supporting the clonal relationship of the two areas.

copy of both the normal and the abnormal *PRKACA* gene. In addition, the nuclei of the spindle shaped stromal cells revealed two intact signals for *PRKACA* (not shown). Normal intact signals were also observed in the nuclei of background hepatocytes. All six cases of scirrhous hepatocellular carcinoma were negative for *PRKACA* rearrangement.

By *in situ* hybridization, the total levels of *PRKACA* and *DNAJB1-PRKACA* were strongly expressed (3+ or greater; >10 dots per cell at $\times 200$ original magnification) diffusely in the cytoplasm of neoplastic cells of each of the 7 tested cases of fibrolamellar carcinoma (3+ in 4 cases; and 4+ in 3 cases) by RNA-ISH using the RNAScope method (Figure 3). The nonneoplastic liver was consistently negative. Lymphocytes and endothelial cells showed only 1+ staining (1 dot per cell at

$\times 200$ original magnification) consistent with expression of normal native *PRKACA* mRNA.

Discussion

Fibrolamellar carcinoma is rare, with ~ 50 new cases diagnosed per year in the United States.⁴ The etiology of fibrolamellar carcinoma is unclear and there is no histological or clinical evidence for chronic liver disease in the background liver. The genetic changes underlying fibrolamellar carcinoma are clearly different than the typical hepatocellular carcinoma, but significant driver mutations were unknown, with fibrolamellar carcinomas lacking the typical driver mutations found in hepatocellular carcinoma. However, a recent study of 10

Figure 3 (a) High-magnification representative photomicrograph of a FLC ($\times 400$ original magnification). (b) RNA *In situ* hybridization using a probe to *DNAJB1-PRKACA* is positive in the neoplastic cells ($\times 400$ original magnification).

fibrolamellar carcinoma cases identified a recurrent somatic intrachromosomal deletion on chromosome 19, leading to a *DNAJB1-PRKACA* fusion transcript.³ Our study confirms the presence of the *DNAJB1-PRKACA* fusion in a further 26 fibrolamellar carcinomas. Together, these data are an independent confirmation of 100% sensitivity of this fusion for fibrolamellar carcinoma. Also of note, in the fibrolamellar carcinoma case with foci that resemble more typical hepatocellular carcinoma, *PRKACA* rearrangements by FISH were found in both components.

The survey of other primary liver carcinomas in this study revealed the absence of the fusion transcript in typical hepatocellular carcinomas, scirrhous hepatocellular carcinomas, hepatic adenomas, hepatoblastomas, and cholangiocarcinomas, supporting the hypothesis that the *DNAJB1-PRKACA* fusion transcript is very specific for fibrolamellar carcinoma. Scirrhous hepatocellular carcinoma is a known morphological mimic of fibrolamellar carcinoma, but the absence of the rearrangement of *PRKACA* highlights the point that they are fundamentally different tumors.

Fibrolamellar carcinoma has been shown to over-express the protein known as anterior gradient-2,⁵ which is normally expressed in the bile ducts, as well as other markers of biliary differentiation, and may even show glandular differentiation.⁶ These observations suggest the possibility of some shared genetic changes with cholangiocarcinomas. In addition, subsets of cholangiocarcinomas have recently been shown to have recurrent translocations.^{7,8} However, none of the cholangiocarcinomas in this studied revealed a *DNAJB1-PRKACA* translocation.

Rare reports of fibrolamellar carcinomas arising in livers with metachronous or adjacent hepatic adenomas are present in the literature.^{9,10} These

observations suggest that some hepatic adenomas may also share similar key genetic events with fibrolamellar carcinoma. However, no hepatic adenomas in our study revealed the *DNAJB1-PRKACA* fusion. Lastly, we tested a small number of the rare childhood tumor, hepatoblastoma, in order to more fully determine the specificity of the fusion across the spectrum of primary epithelial liver tumors. Hepatoblastomas were also negative for the fusion gene.

The presence of a distinct defining genetic event in fibrolamellar carcinoma consolidates it as a unique variant of hepatocellular carcinoma. The correlation of a distinct morphology and a distinct genetic event is interesting and raises the consideration of whether other morphological variants of hepatocellular carcinoma harbor distinct genetic changes. In support of this, the recently described chromophobe variant of hepatocellular carcinoma is characterized by an altered lengthening of telomere phenotype together with its distinctive morphology.¹¹ Other morphologically unique variants of hepatocellular carcinoma, such as clear cell hepatocellular carcinoma, steatohepatic hepatocellular carcinoma, and scirrhous hepatocellular carcinoma, may be associated with unique genetic changes, allowing for a morphology based subclassification that reflects specific genetic driver events, which in turn may lead to a better understanding of tumor biology and implementation of precisely targeted therapies.

The results of FISH in fibrolamellar carcinoma indicate that essentially all neoplastic cells harbor the gene fusion, further underscoring its biological importance and suggesting the gene fusion is an early event in tumor genesis. FISH also provided an opportunity to assess the stromal cells within the lamellar fibrosis of the tumor. The spindle stromal cells revealed intact *PRKACA* signal patterns,

indicating that they are not clonally related to the tumor and instead are most likely induced via a tumor cell–stromal interaction. FISH studies, RT-PCR results, and *in situ* hybridization were all negative in the background liver, suggesting that the fusion is not a common preneoplastic event in the background liver, but instead is an early step occurring in, or leading to, clonally expanding cells that ultimately give rise to fibrolamellar carcinomas.

RNA *in situ* hybridization demonstrated overexpression of total *PRKACA* and chimeric transcripts in fibrolamellar carcinoma. The validity of the RNAScope system for semiquantitative assessment of mRNA in formalin fixed paraffin-embedded tissues has been shown previously by other groups.^{12–14} In the tested samples, nonneoplastic liver was consistently negative suggesting very low or absent expression of *PRKACA* in normal liver. In endothelial cells and lymphocytes, ISH staining was limited, with only 1 + expression (1 dot per cell) in lymphocytes and endothelial cells. These data also suggest that RNA *in situ* hybridization strategies may be designed to detect fusion transcripts and could potentially be employed to detect a wide variety of fusions in various tumor types in formalin fixed paraffin-embedded tissues. However, our probe design detects total expression, and not exclusively the chimeric transcript, so this would require an additional validation.

DNAJB1, located at 19p13.2, encodes a heat shock protein, Hsp40 which is involved in protein folding within cells.¹⁵ In times of stress, the protein translocates from its usual cytoplasmic location to the nucleus and nucleolus of the cell.¹⁶ *PRKACA*, located at 19p13.1, encodes the catalytic subunit of the serine/threonine protein kinase A.¹⁷ Under physiological conditions, protein kinase A is responsible for the phosphorylation of numerous target proteins within the cell. Cellular processes affected by protein kinase A activity include glucose and lipid metabolism,¹⁸ MYC activity,¹⁹ Wnt signaling pathway activation,²⁰ and mitochondrial biogenesis.²¹ The connection of *PRKACA* to mitochondrial biogenesis is interesting because of the well-known increased mitochondrial mass in fibrolamellar carcinoma,^{1,22} which is unexplained by mitochondrial genome mutations and was hypothesized to be related to a defect in the mitochondrial biogenesis.²³ Further study is warranted to elucidate the relationship between the fusion gene and mitochondrial biogenesis, as this may lead to improve understanding of tumor cell energy metabolism and survival. Honeyman *et al*³ proposed the chimera formed by *DNAJB1* and *PRKACA* leads to upregulation of *PRKACA* activity by a promoter switch mechanism. Interestingly, point mutations^{24–26} in *PRKACA* and copy number gain²⁴ of *PRKACA* have been shown as recurrent genetic abnormalities underlying functional adrenal adenomas and adrenal hyperplasia. The discovery of a translocation involving *PRKACA* is a third

mechanism of activation of the *PRKACA* gene in tumors.

Fibrolamellar carcinomas remain challenging to diagnose for both clinical care and research studies. The H&E findings are very sensitive but a variety of histological mimics reduces the specificity. This problem can be mitigated by the use of CK7 and CD68 immunostains, which are widely available, easy to perform, and easy to interpret: all fibrolamellar carcinomas should be both CK7 and CD68 positive. The new methods reported in this study have the advantage of identifying a key molecular lesion and, when combined with morphology, we believe will approach 100% sensitivity and 100% specificity. The use of immunostains or molecular assays to confirm the diagnosis of fibrolamellar carcinoma should be the foundation of future clinical and translational studies on this tumor.

In conclusion, the *DNAJB1-PRKACA* fusion gene was 100% sensitive for fibrolamellar carcinomas in this study and, based on a survey of an additional 105 primary liver tumors, appears to be highly specific, suggesting it will be a useful diagnostic assay. The fusion is present in essentially all tumor cells, indicating it is an early mutational event in the development of fibrolamellar carcinoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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