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## A framework for fibrolamellar carcinoma research and clinical trials

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**Cancer dependency Map:** <https://depmap.org/portal>

**ClinicalTrials.gov:** <https://clinicaltrials.gov/>

**European Union Drug Regulating Authorities Clinical Trials Database (EudraCT):** <https://eudract.ema.europa.eu/>

**Fibrolamellar Cancer Foundation Biobank:** <https://fibrofoundation.org/join-the-fight/donate-tissue/>

**FibroLamellar Omics (FLO):** [https://sethupathy-lab.shinyapps.io/flc\\_data/](https://sethupathy-lab.shinyapps.io/flc_data/)

**Fibrolamellar Registry:** <https://fibroregistry.org/>

**Fibrolamellar Tissue Repository:** <https://fibrolamellar.rockefeller.edu/repository>

**Kinase Chemogenomic Set (KCGS):** <https://www.sgc-unc.org/kinase-chemogenomics>

**MyPART:** <https://www.cancer.gov/pediatric-adult-rare-tumor>

**Natural History Study of Rare Solid Tumors:** <https://www.cancer.gov/pediatric-adult-rare-tumor/participate/natural-history>

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## Abstract

Fibrolamellar carcinoma (FLC), a rare, lethal hepatic cancer, occurs primarily in adolescents and young adults. Unlike hepatocellular carcinoma, FLC has no known association with viral, metabolic or chemical agents that cause cirrhosis. Currently, surgical resection is the only treatment demonstrated to achieve cure, and no standard of care exists for systemic therapy. Progress in FLC research illuminates a transition from an obscure cancer to one for which an interactive community seems poised to uncover fundamental mechanisms and initiate translation towards novel therapies. In this Roadmap, we review advances since the seminal discovery in 2014 that nearly all FLC tumours express a signature oncogene (*DNAJB1-PRKACA*) encoding a fusion protein (DNAJ-PKAc) in which the J-domain of a heat shock protein 40 (HSP40) co-chaperone replaces an amino-terminal segment of the catalytic subunit of the cyclic AMP-dependent protein kinase (PKA). Important gains include increased understanding of oncogenic pathways driven by DNAJ-PKAc; identification of potential therapeutic targets; development of research models; elucidation of immune mechanisms with potential for the development of immunotherapies; and completion of the first multicentre clinical trials of targeted therapy for FLC. In each of these key areas we propose a Roadmap for future progress.

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Fibrolamellar carcinoma (FLC; also known as fibrolamellar hepatocellular carcinoma (FL-HCC)) is a rare liver cancer that was defined originally by its distinctive histology, especially the abundant lamellar bands of intratumoural fibrosis that are reflected in the tumour's name<sup>1-5</sup> (Fig. 1). FLC primarily affects adolescents and young adults at a median age of approximately 22 years<sup>6,7</sup>. Cancers in individuals in this age group pose distinct challenges for the understanding of biology, the development of effective therapies, and the provision of care, insurance cover and psychosocial support<sup>8,9</sup>. Despite the significant advances in FLC research that we discuss in this Roadmap, predisposing factors have not been identified and the population at risk remains poorly defined. Moreover, effective systemic therapy remains elusive<sup>5</sup>.

While FLC cases have been reported around the world, the global incidence of this cancer remains uncertain. Systematic tracking is limited to the USA, where data from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute reveal only 0.2 new diagnoses per million person-years<sup>10,11</sup>. However, some reports have suggested that FLC comprises a higher fraction of primary liver cancers<sup>12-16</sup>. The absence of a unique designation for FLC in the International Code of Diseases 10th Revision (ICD-10) might lead to inadvertent under-counting of this cancer. Furthermore, analysis of liver tumours in The Cancer Genome Atlas (TCGA) collection revealed that half (three of six) of those that had both histological and molecular features diagnostic of FLC were inaccurately annotated as having conventional hepatocellular carcinoma (HCC)<sup>17</sup>. Thus, although FLC undoubtedly is a very rare cancer, its incidence might exceed that indicated by the SEER data.

FLC only infrequently responds to chemotherapy or targeted therapies currently approved for HCC, although these are often used by default without a clear rationale, and therefore have not improved the poor long-term survival rates<sup>5,7,18–20</sup>. Surgical resection remains the sole available treatment modality with curative intent. However, this option is viable for only a limited number of patients, and tumour recurrence and/or metastasis are common<sup>18–20</sup>. Orthotopic liver transplantation, utilized for selected patients, gives comparable overall survival (OS) to that reported for HCC<sup>21</sup>. Radiotherapy and interventional radiology also contribute to current management of FLC, although published data on the effect of these modalities on survival remain scarce<sup>5</sup>. There is no standard of care for systemic therapy<sup>10,11,20</sup>. Owing to the lack of specific early warning signs and diagnostic biomarkers, by the time patients present with classic symptoms such as abdominal pain, a palpable mass, anorexia and, rarely, gynaecomastia<sup>22</sup> or hyperammonaemia<sup>23–25</sup>, their disease is typically advanced and curative surgical resection is often not possible (FIG. 1).

Over the past 8 years significant progress has been made towards elucidating the molecular biology of FLC<sup>26</sup>. However, the challenge in translating knowledge into effective therapies remains unmet. An international group of researchers and physicians focused on FLC met in November 2019 at a Research Summit hosted by the Fibrolamellar Cancer Foundation, Greenwich, USA (for a list of relevant FLC databases, repositories and useful links, see BOX 1). The key goals were to share advances in laboratory and clinical studies, and to consider opportunities for future progress. Participants' areas of expertise included the clinical care of patients with FLC (both oncological and surgical), biology and genomics, immunotherapy, in vitro and in vivo modelling, and translational research. Patients and family advocates were also represented. All authors of this paper were present at the summit. In this Roadmap, we aim to provide a curated guide to ongoing initiatives. We review developments and unmet needs in four areas: molecular mechanisms, disease models, immunotherapy and clinical trials. For each, we provide a brief background, highlight new research findings and pose important unanswered questions. Finally, we offer a Roadmap for the future, prioritizing next steps towards the development of novel and effective therapeutics.

## Molecular mechanisms

### **DNAJB1–PRKACA fusion: the molecular hallmark of FLC.**

FLC is a distinct disease from HCC<sup>26,27</sup>. As illustrated histologically, FLC is marked by the presence of thick lamellae of fibrous tissue surrounding clusters of large polygonal and eosinophilic tumour cells<sup>2,3,7</sup> (FIG. 1). By immunohistochemistry, FLC tumours consistently co-express cytokeratin 7 (CK7) and CD68 (REFS<sup>7,28–30</sup>). Global gene expression profiling also clearly distinguishes FLC from HCC<sup>17,31–34</sup>. Most crucially, FLC tumours characteristically express a fusion gene, *DNAJB1–PRKACA*, originally identified by Honeyman et al.<sup>35</sup> and since confirmed by multiple groups<sup>17,28,32,33,36–38</sup> (FIG. 2). The fusion results from a ~400 kb somatic heterozygous deletion on chromosome 19. This genetic alteration, and the lack of consistently recurrent mutations in classic tumour suppressor genes such as *PTEN* and oncogenes such as *CTNNB1*, helps distinguish FLC from conventional HCC and other malignancies<sup>26,27,32,39</sup>. Initially it seemed possible

that the gene fusion would be exclusive to FLC<sup>17,40</sup>. However, in 2020, two groups described some oncocytic neoplasms of the pancreas and bile duct that, like FLC, have eosinophilic cytoplasm due to the accumulation of altered mitochondria, and display recurrent rearrangements involving a *PRKAC* gene (*PRKACB* or *PRKACA*). The genetic alterations include *DNAJB1-PRKACA* fusions, indistinguishable from those observed in FLC<sup>41,42</sup>. Thus, the presence of this fusion alone does not uniquely diagnose FLC.

*DNAJB1* encodes a member of the HSP40 family of heat shock proteins<sup>43</sup>, while *PRKACA* encodes the  $\alpha$ -isoform of the catalytic subunit (C $\alpha$ ) of protein kinase A (PKA), the cyclic AMP-dependent protein kinase<sup>44-46</sup>. How the fusion of these genes might contribute to oncogenesis is discussed below, in the section 'Aberrant activation of PKA in FLC'.

Beyond the fusion gene, analyses of FLC have shown a low tumour mutational burden (TMB) and few other consistent genomic changes<sup>32,33,39,47</sup>. A genomic profiling study did identify activating promoter mutations for the telomerase reverse transcriptase (*TERT*) gene in 7 of 31 patients with FLC<sup>48</sup>. In addition, a whole-genome sequencing study in ten patients identified several genes that were mutated recurrently in tumours. For example, genetic lesions in *MUC4* were identified in four patients<sup>39</sup>. This observation is notable because mucin 4 (*MUC4*) can form a complex with HER2 (also known as ERBB2 or NEU2) to promote cell proliferation and migration<sup>49</sup>. A separate genome-wide study of 26 FLC samples identified focal deletions present in at least 25% of the patients, including at the *LKB1* (also known as *STK11*) and *TAF15* (also known as *FAM19A5*) loci<sup>32</sup>. These findings are concordant with observations that *LINC00473*, an *LKB1*-regulated gene, and *TAF15* are among the most aberrantly expressed genes in FLC, and that these loci are associated with FLC-specific chromatin changes<sup>50</sup>. Although mutations in *TP53* are uncommon in FLC, the activated, phosphorylated form of p53 seems to be excluded from its normal site of action in the cell nucleus, while MDM4, a negative regulator of p53, is present in the nucleus at elevated levels<sup>51</sup>. Dysregulation of the activity or stability of p53, a key tumour suppressor protein, could promote the growth and resistance to therapy of FLC<sup>38,51</sup>.

Some liver cancers have been reported to present with mixed histological features of FLC and conventional HCC<sup>52-54</sup>. Although these mixed tumours generally exhibit distinct genomic and transcriptomic profiles relative to canonical or 'pure' FLC<sup>31</sup>, a subset of them do harbour the *DNAJB1-PRKACA* oncogene<sup>37</sup>. A genomic study revealed the presence of this hallmark gene fusion in two of ten patients categorized as having mixed FLC-HCC<sup>55</sup>. Strikingly, six of the remaining eight fusion gene-negative mixed tumours were found to contain biallelic inactivating mutations in the tumour suppressor gene *BAP1* (which encodes BRCA1-associated protein 1). Conversely, 2 of 15 tumours classified histologically as pure FLC lacked the *DNAJB1-PRKACA* fusion and were *BAP1*-mutated. Most of the patients with fusion-positive pure FLC were relatively young (<40 years), whereas two patients with fusion-negative pure FLC tumours developed FLC later in life (ages 51 and 65 years)<sup>55</sup>. The mixed tumours bearing *BAP1* mutations arose in patients aged 27 to 54 years (median 39 years)<sup>55</sup>. These observations, along with misdiagnoses of HCC as FLC, might help account for the bimodal age distribution of FLC reported in some epidemiological studies<sup>10,11</sup>. These intriguing findings merit further validation in follow-up cohorts.

### Aberrant activation of PKA in FLC.

Tumours with the *DNAJB1-PRKACA* gene fusion and *BAP1*-mutated liver cancers seem to share a crucial functional feature, namely, aberrant activation of PKA. In the genomic study discussed previously, tumours with *BAP1*-inactivating mutations, regardless of histological classification, generally also displayed amplification of the *PRKACA* catalytic subunit gene (83%) and/or deletion of a regulatory subunit gene, *PRKAR2A* (94%)<sup>55</sup>. Both changes might be expected to deregulate PKA, rendering its activity less dependent on cAMP. When therapeutics are developed for canonical FLC, it will be important to determine whether they are also effective against tumours with a mixed FLC–HCC phenotype and those with *BAP1* mutations.

The *DNAJB1-PRKACA* mRNA transcript found in the vast majority of patients with FLC is translated to a chimeric protein of ~46 kDa (REF.<sup>35</sup>), designated here as DNAJ–PKAc, in which the J-domain of HSP40 replaces a small segment at the amino terminus of the catalytic subunit of PKA (FIG. 2). This mutant protein retains activity as a serine–threonine-specific protein kinase<sup>27,33,35,56</sup>. The introduction of *DNAJB1-PRKACA* into mouse liver via CRISPR–Cas9 or transposon-mediated gene transfer suffices to induce tumorigenesis, implying that DNAJ–PKAc can act as an oncoprotein<sup>57,58</sup>. Although it has been shown that equally robust expression of wild-type *PRKACA* fails to drive tumour formation, the kinase activity of DNAJ–PKAc is required for tumorigenesis<sup>58</sup>. A recent preprint reports data from the preclinical evaluation of a potent, selective active site inhibitor of PKAc that slowed the growth of a human FLC tumour line in a patient-derived xenograft (PDX) model<sup>59</sup>. However, clinical development of such an inhibitor seems to be highly problematic because of on-target toxicity attributable to inhibition of normal PKAc, in line with the enzyme's important physiological roles in multiple tissues<sup>46,60</sup>.

The mechanism by which DNAJ–PKAc transforms cells remains poorly understood and will probably be critical for the development of effective targeted molecular therapies, a central aim of FLC research. The factors that control the level of cAMP in FLC tumour cells are also not well known. As with certain other cancers (for example, lung, colorectal, pancreatic, prostatic and breast cancers)<sup>61,62</sup>, the peptide neurotensin (NTS) could have a role in oncogenesis by binding to its receptor NTSR1 (NTS receptor type 1) and driving increased production of intracellular cAMP<sup>63</sup>. Nonetheless, genetic, structural, biochemical and cell biological studies have illuminated key differences in PKA activity between FLC and normal cells.

Wild-type PKA is a heterotetrameric holoenzyme (R<sub>2</sub>C<sub>2</sub>) comprising four subunits, namely, two catalytic (C) subunits and a regulatory (R) subunit homodimer that inhibits enzymatic activity<sup>45</sup>. Cooperative binding of cAMP to the R subunits liberates the kinase function of the C subunits. There are three C isoforms (C $\alpha$ , C $\beta$  and C $\gamma$ ), and four functionally non-redundant R isoforms (RI $\alpha$ , RI $\beta$ , RII $\alpha$  and RII $\beta$ ). While RII $\beta$  is the predominant isoform in the liver, RI $\alpha$ , a 'master regulator' of PKA<sup>64</sup>, is the only regulatory subunit isoform reported to be upregulated in human FLC tumour cells<sup>34,65</sup>.

Some relevant insights have come from a heritable condition called Carney complex, which accounts for a small minority (~1%) of FLC cases. Carney complex is marked by

multiple, mainly benign tumours of the heart, skin and endocrine system, as well as altered pigmentation<sup>66</sup>. The disorder is caused by activation of PKA via deleterious mutations in *PRKARIA*, which encodes RI $\alpha$ . A report from 2018 describes three individuals with Carney complex who developed histologically typical FLC, but without a *DNAJB1-PRKACA* gene fusion in their tumours<sup>67</sup>. In these patients, sequence analysis confirmed a heterozygous mutation in *PRKARIA* in germline DNA, although intact RI $\alpha$  was still expressed from the unaffected allele in normal cells. However, the cancer cells lacked detectable RI $\alpha$  protein, consistent with loss of the remaining wild-type *PRKARIA* allele as a key event in tumorigenesis. Thus, the absence of this regulatory subunit seems sufficient to deregulate kinase activity and drive elevated phosphorylation of PKA substrates associated with neoplastic growth.

A corresponding mechanism for deregulation of the chimeric catalytic subunit found in the great majority of FLC tumours is less apparent. Enzymological and structural studies indicate that the catalytic core is essentially unaffected in DNAJ-PKAc<sup>56,68-70</sup>. Similarly, endogenous protein kinase inhibitor peptide (PKI) blocks the activity of the fusion protein and the wild-type kinase with equal efficiency<sup>56</sup>. Moreover, like wild-type C subunits, the oncogenic DNAJ-PKAc C subunits interact with R subunits to form functional holoenzymes, and PKA activity in FLC cells remains responsive to cAMP<sup>65,68,69</sup>. Nevertheless, the addition of the J-domain of HSP40 to the C subunit can change biochemical properties when paired with certain regulatory subunits. For example, the holoenzyme formed by the mutant DNAJ-PKAc subunit with RII $\beta$  subunits is activated more readily by cAMP than the corresponding wild-type holoenzyme<sup>71</sup>.

In 2021, Olivieri and colleagues identified changes in allosteric properties of the hallmark chimeric catalytic subunit that could contribute to deregulating signal transduction<sup>72</sup>. They noted that normal C subunits (PKAc) exhibit positive binding cooperativity between ATP and the substrate to be phosphorylated. After transfer of a phosphate group, negative binding cooperativity of ADP facilitates release of the phosphoprotein product. However, addition of the flexible J-domain in DNAJ-PKAc strongly attenuates the allosteric binding cooperativity between nucleotide and PKI (a pseudo-substrate). Exactly how this affects the pattern of substrate phosphorylation in FLC cells remains to be determined. Along with other structural studies<sup>70</sup>, it does offer some hope that subtle differences between DNAJ-PKAc and wild-type PKAc could facilitate the discovery of selective small-molecule inhibitors of the oncogenic chimaera.

Test tube biochemistry over many years suggested that the release of C subunits from R subunits is required for the activation of PKA by cAMP<sup>44</sup>. In the past 5 years, high-resolution studies of intracellular PKA have revealed, instead, that catalytically active holoenzymes remain intact in the cytoplasm, with some R-C subassemblies constrained to within 150 to 250 Å of target substrates<sup>73</sup>. This finding implies that a potential mechanism for the oncogenic activity of DNAJ-PKAc could be mis-localization and consequent dysregulation of holoenzymes. PKA binds via the R subunits to A-kinase anchoring proteins (AKAPs), which regulate PKA localization and substrate specificity<sup>73-75</sup>. Structural analyses support the notion that the J-domain (from HSP40) in DNAJ-PKAc can perturb interaction with AKAPs by altering the position of the dimerization or docking domain of

PKA holoenzyme<sup>71</sup>. Furthermore, Turnham et al. demonstrated that DNAJ–PKAc interacts with heat shock protein 70 (HSP70) via the J-domain<sup>76</sup> (FIG. 2). They infer that this interaction creates a pathological AKAP signalling island. Compound screening in a murine hepatocyte cell line engineered to express DNAJ–PKAc suggested that this complex could be targeted pharmacologically by a combination of inhibitors of mitogen-activated protein kinase (MAPK) signalling and HSP70. Clinical development of such drug combinations will first require confirmation of their utility in human FLC cells.

Another aspect of intracellular compartmentation also seems to contribute to oncogenesis in FLC. Cytoplasmic phase separation, the creation of distinct spatial compartments within a solution, has important roles in the formation of membrane-less organelles<sup>77,78</sup>. In 2020, Zhang et al. discovered that the ubiquitously expressed PKA regulatory subunit RI $\alpha$  normally undergoes liquid–liquid phase separation, thereby spatially constraining cAMP to microdomains within the cytoplasm<sup>79</sup>. This phase separation leads to the formation of RI $\alpha$  bodies, described by these authors as “biomolecular condensates [that] act as a dynamic ‘sponge’ in recruiting and retaining cAMP and [catalytically] active PKA<sub>cat</sub>”. Remarkably, they found that the DNAJ–PKAc fusion protein abolishes RI $\alpha$  phase separation, resulting in aberrant cAMP–PKA signalling that promotes cellular transformation. Both the loss of RI $\alpha$  phase separation and the transforming activity depend, at least in part, on the recruitment of HSP70 by DNAJ–PKAc, and also the absence from the fusion protein of a myristoylation site located near the N terminus of the normal C subunit of PKA<sup>79</sup>.

### Additional therapeutic targets?

Research efforts continue to identify specific substrates and downstream signalling pathways activated by DNAJ–PKAc that are most critical to oncogenesis (FIG. 2). Global analyses of the transcriptomes and focused studies of selected proteins in FLC models and primary tumours have shown elevated copy numbers, expression or activity of genes in several pathways that could contribute to transformation by the fusion kinase. These include the following: the MAPK pathway<sup>32,50,76,80</sup>; components of the epidermal growth factor receptor (EGFR) pathway and the related receptor tyrosine kinase ERBB2<sup>31,34,37,38</sup>; the mechanistic target of rapamycin (mTOR)<sup>81,82</sup>; fibroblast growth factor receptor 1 (FGFR1)<sup>82,83</sup>; the WNT– $\beta$ -catenin pathway<sup>34,58,84</sup>; and aurora kinase A (AURKA), a mitotic regulator<sup>34,38</sup>. However, while multiple reports agree on activation of certain of these pathways (for example, MAPK), the available data on others are more limited and sometimes contradictory. Substantial further work is required to determine which, if any, represent valid targets for FLC therapy. In initial multicentre clinical studies in patients with FLC, neither a pan-HER inhibitor of EGFR and ERBB2 kinase activity, nor an mTOR inhibitor, nor an AURKA inhibitor showed significant benefit as a single agent (see section ‘Clinical trials’). Sporadic responses to other targeted therapies have been observed in various small case series, reported in both meeting abstracts and peer-reviewed publications, but prospective clinical studies are urgently needed<sup>85–87</sup>.

Genome-scale studies are beginning to reveal additional candidate targets for new FLC treatments. Cataloguing non-coding RNAs in FLC has uncovered RNA species and the pathways they regulate that might have therapeutic significance<sup>17,88</sup>. One of the most

dysregulated small RNAs in FLC is microRNA-375 (miR-375), which is dramatically suppressed in primary human tumours and in several models that express the DNAJ–PKAc chimaera. Restoration of miR-375 in a human FLC cell line suppresses growth and migration of the cancer cells, possibly through the inhibition of expression of proteins in the Hippo signalling pathway, including yes-associated protein 1 (YAP1) and connective tissue growth factor (CTGF)<sup>89</sup>. The potential therapeutic benefit of this and other miRNAs of interest in FLC, including miR-10b<sup>89</sup> and miR-548p<sup>88</sup>, merits further investigation. Long non-coding RNAs (lncRNAs) have also emerged as key regulators of oncogenesis and metastasis in a growing number of cancer types<sup>90–93</sup>. The lncRNA LINC00473 is part of a reported gene signature of FLC<sup>17</sup>. Moreover, the FLC-specific active regulatory elements near LINC00473 are highly enriched in binding motifs for cAMP response element binding protein (CREB)<sup>50</sup>, an important downstream mediator of PKA signalling that has been implicated as a key transcription factor in FLC<sup>33</sup>. However, the molecular functions of LINC00473, and other lncRNAs<sup>88</sup>, in initiation and/or maintenance of this cancer are currently unknown.

Other systematic approaches to target identification follow from the recognition that regulatory alterations might underlie the ‘addiction’ of cancers to specific oncogenic signals<sup>94</sup>. One study utilized a novel technique called chromatin run-on sequencing (ChRO-seq), which helps define tumour-specific super-enhancers that mark nearby genes that are critical for tumour behaviour. This method also points to aberrant MAPK signalling in FLC tumours. In addition, it reveals several previously unsuspected genes, including *CA12* (encoding carbonic anhydrase 12) and *SLC16A14* (encoding the orphan transporter known as monocarboxylic acid transporter 14), for proteins that seem to have important roles in the survival of FLC tumour cells<sup>50</sup>. Strikingly, *SLC16A14* is more highly expressed in FLC than in any other tumour type or normal tissue for which data are publicly available<sup>50</sup>. Studies in FLC cell models suggest that combination therapy with inhibitors of these genes as well as MAPK pathway inhibitors might offer a new therapeutic approach for this cancer<sup>50</sup>.

## Disease models

Model systems are important for the definition of biological processes and vital to the discovery of effective therapeutics. Over the past 6 years, several systems genetically engineered to express a DNAJ–PKAc fusion protein have been described, including a murine cell line (AML12)<sup>76</sup>, and various transformed human cell lines<sup>33,95</sup> as well as in vivo mouse<sup>57,58</sup> and zebrafish<sup>96</sup> models. In addition, human PDX tumour lines have been generated<sup>36,97</sup>, and cells from one PDX line have been utilized for in vitro studies<sup>50,89</sup>. Although the currently available models provide biological insights and facilitate molecular dissection of FLC, they also have important limitations (TABLE 1). Primary human FLC biospecimens are also available from biobanks at Massachusetts General Hospital, Boston, USA, and Rockefeller University, New York, USA (BOX 1).

Two independent groups described the use of CRISPR–Cas9-mediated deletion, via guide sequences introduced by hydrodynamic injection, to engineer a murine homologue of the *DNAJB1–PRKACA* fusion gene in hepatocytes of young adult mice<sup>57,58</sup>. Both groups observed that the chimaera sufficed to induce liver tumorigenesis, albeit slowly and with



incomplete penetrance. The tumours in this model resembled those in human FLC, although they lacked fibrolamellar bands. Addition of an activating mutation in the  $\beta$ -catenin gene (*CTNGB1*), as observed in a small minority of human FLC cases, accelerated tumour initiation and growth<sup>58</sup>. Exposure to the hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine further enhanced tumorigenesis<sup>58</sup>.

A similar approach in zebrafish has not yet yielded frank malignant tumours. However, the generation of a DNAJ–PKAc chimeric protein from an engineered fusion of the homologous genes in hepatocytes induced hepatomegaly and increased hepatocyte size. Early characterization of this model indicated non-resolving inflammation<sup>96</sup>. This observation might reflect a species difference in terms of the effects of the fusion protein, because, despite altered expression of inflammatory genes and enhanced cytokine production in a subset of human tumours<sup>32</sup>, overt inflammation has not been reported as a characteristic feature of FLC.

The first published PDX model sheds light on the potential lineage derivation of human FLC. This tumour line, designated FLC-TD-2010 (listed as PDX-1 in TABLE 1), was developed from cells present in ascitic fluid of a patient at an advanced stage of disease<sup>36</sup>. Prior to injection into immune-deficient mice, the cells were first cultured briefly in serum-free medium and on a hyaluronan gel substrate, which are conditions known to promote the survival of normal endodermal stem or progenitor cells. The resulting line is strongly enriched for tumour-initiating cells and displays striking transcriptomic and phenotypic similarities to a normal adult population of primitive biliary tree stem cells, which can give rise to hepatic, cholangiocytic and pancreatic cell lineages<sup>98,99</sup>. These data indicate that the biliary tree stem cell is a potential cell type of origin for FLC<sup>30–33,36</sup>. The detection of *DNAJB1–PRKACA* in human intraductal oncocytic papillary neoplasms of the pancreas and bile duct supports the concept that this fusion gene can drive transformation of multipotent endodermal stem or progenitor cells to yield primary cancers outside the liver, in addition to FLC<sup>41,42</sup>. However, the de-differentiation of more mature liver or pancreatic or biliary ductal cells remains possible<sup>100</sup>, as suggested by the murine models of FLC in which the fusion gene is inserted into cells of the adult liver. No stable cell lines of human FLC have yet been reported in the literature. However, one line has been established from the original PDX-1 model that is slow-growing and retains the *DNAJB1–PRKACA* fusion across numerous passages (N. Bardeesy, personal communication; see FLX-1 in TABLE 1).

Lalazar et al. described six new PDX lines derived by direct implantation of tumour specimens from both the liver (one primary and one recurrent tumour) and metastases<sup>97</sup> (PDX-2 to PDX-7 in TABLE 1). The new PDXs facilitated the first reported large-scale screening of compounds for the ability to kill dissociated FLC tumour cells in short-term culture (72 h). Active compounds were also assessed on cells obtained directly from patients, taken from lymph node metastases or ascitic fluid, and in vivo in mice bearing the PDX. A library of >5,000 compounds, selected as suitable for drug repurposing, included clinically approved drugs, drugs in preclinical development and other relevant bio-active species. The results revealed promising activity of several classes of inhibitors, including those for topoisomerase I (TOPOI) and histone deacetylase (HDAC). Napabucasin exhibited the most potent activity of the compounds screened. This drug has been touted as a potential

blocker of the stem cell activity of cancers via targeting the signal transducer and activator of transcription 3 (STAT3) pathway<sup>101,102</sup>. Indeed, reports indicate that napabucasin reduces cancer stem cell properties of human HCC and biliary tract cancer cells<sup>103,104</sup>. The activity against FLC of a drug thought to target cancer stem cells seems to be consistent with both the high proportion of tumour-initiating cells in the PDX-1 model<sup>36</sup> and the strong resemblance of the transcriptomes of that line and of multiple primary FLC tumours to normal biliary tree stem cells<sup>17,36</sup>. However, napabucasin's anticancer activity does not depend exclusively on the attenuation of STAT3 phosphorylation and signalling. The compound was shown to induce pleiotropic effects through the generation of reactive oxygen species, induction of DNA damage and decreased synthesis of certain proteins via inhibition of eukaryotic initiation factor 4A<sup>97,105</sup>. Another important observation from the compound screen is that inhibitors of the anti-apoptotic protein BCL-X<sub>L</sub>, but not BCL-2, synergized with drugs such as the TOPOI inhibitor topotecan and the HDAC inhibitor panobinostat<sup>97</sup>. Overall, the study may represent a major step towards novel clinical trials of individual drugs and/or combinations possessing documented activity against FLC cells.

## Immunotherapy

Immunotherapies have revolutionized cancer treatment and, in some cases, provide robust and lasting protection to patients. Favourable results in clinical trials have led regulators in the USA and Europe to approve immune checkpoint blockade for patients with diseases including, among others, melanoma, renal cell carcinoma, non-small-cell lung cancer and HCC<sup>106–108</sup>. Can such therapies be developed for FLC? A significant challenge is that the responsiveness of cancers to various immune modulations might correlate positively with TMB, as has been observed with checkpoint blockade immunotherapy<sup>109–111</sup>. This finding is often attributed to the random generation of large numbers of neoantigens that could be targeted by the immune system<sup>110</sup>. Indeed, in 2020, the FDA approved the use of the PD1 immune checkpoint inhibitor (ICI) pembrolizumab to treat adult and paediatric patients with TMB-high solid tumours, agnostic of the tissue of origin<sup>112,113</sup>.

Genomic studies indicate that FLC falls at the low end of the TMB spectrum, suggesting that this cancer would be immunologically 'cold'<sup>32,33,38,39,48</sup>. However, the demonstration that some FLC tumours robustly express markers of multiple immune-suppressive mechanisms, including PDL1 (also known as CD274), PD1 (also known as CD279), B7-H3 (also known as CD276) and indoleamine 2,3-dioxygenase, in a pattern consistent with adaptive immune resistance, challenges the assumption that checkpoint blockade therapy necessarily would be ineffective<sup>114</sup>. No systematic reports have appeared on the efficacy of individual ICIs against FLC, and only a few anecdotal accounts have been published<sup>26,86,115</sup>. Increasingly, ICIs are being employed in combination, and sometimes also in conjunction with other therapeutic modalities, to expand the range of patients in whom they are effective<sup>116</sup>. A clinical trial led to the approval by the EC (European Commission) and the FDA of combined ICIs against PD1 and CTLA4 (also known as CD152), nivolumab and ipilimumab, for the treatment of HCC previously treated with sorafenib<sup>117</sup>. While FLC was excluded from that study, a case report documents a long-lasting, near-complete response to the nivolumab–ipilimumab combination in a patient with advanced, metastatic FLC and low TMB<sup>118</sup>.

Even for TMB-low tumours, it is possible that the immune system could effectively target a specific antigen common to a particular type of cancer. What neoantigen(s) might be accessible for immune attack in FLC? One obvious candidate is the DNAJ–PKAc oncogenic driver protein itself, in which the fusion junction could comprise a unique epitope not found in normal cells<sup>26,119,120</sup>. A precedent exists in the robust immune recognition of a comparable junction in the ETV6–RUNX1 driver fusion protein of paediatric acute lymphoblastic leukaemia<sup>121</sup>. In addition to the use of checkpoint blockade to enhance the immune response to tumours bearing such a neoantigen, therapeutic routes that could be explored include vaccination with a peptide corresponding to the junction (discussed later), or adoptive cell therapy with a cloned T cell receptor specific for a junctional epitope.

## Clinical trials

Surgical intervention, when possible, remains the mainstay of FLC treatment, given that there are no effective, standard or agreed-upon systemic therapies for advanced disease<sup>5,11</sup>. Patients with oligometastatic disease have been shown to benefit from repeated local control interventions with the goal of life prolongation, including resection of the primary tumour, metastasectomy, radiation and embolization with or without the addition of chemotherapy. Advanced surgical approaches include parenchyma-sparing hepatectomies and the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure<sup>122,123</sup>. While these methods attempt to radically resect all visible sites of disease, data regarding their effect on OS in patients with FLC are lacking. Therefore, in patients with metastatic disease, systemic therapies remain an important aspect of care, and there is an urgent need for clinical studies to advance new concepts.

Current chemotherapeutic regimens utilized for FLC are often based upon retrospective data analysis and vary among institutions<sup>5</sup>. Treatment regimens currently in regular use include gemcitabine and oxaliplatin (GEMOX), 5-fluorouracil (5-FU) and oxaliplatin, and 5-FU and interferon<sup>124,125</sup>. Paediatric patients with FLC have historically been treated with hepatoblastoma-specific regimens, yielding a modest response<sup>126</sup>. However, assessment of regimens for FLC in clinical trials has been limited, and many clinicians consider this cancer to be generally resistant to chemotherapy<sup>6,11,19,48</sup>.

Patients with FLC are eligible for a current international, prospective clinical trial led by the Children's Oncology Group (COG), which is enrolling patients with HCC (with FLC considered as an HCC 'variant') to receive cisplatin and doxorubicin postoperatively, in the completely resected context, or cisplatin–doxorubicin–sorafenib versus cisplatin–doxorubicin–sorafenib alternating with GEMOX and sorafenib in the metastatic or unresectable context (Paediatric Hepatic Malignancy International Therapeutic Trial [NCT03533582](#); European Union Drug Regulating Authorities Clinical Trials Database (EudraCT) number 2016-002828-85). The inclusion of sorafenib in this trial is noteworthy. This compound has activity against several kinase activities that conceivably could be relevant in FLC, including RAF serine–threonine kinase phosphorylation in the RAS–RAF–MAPK pathway, and the vascular endothelial growth factor receptor tyrosine kinases (VEGFR2 and VEGFR3)<sup>127,128</sup>. Sorafenib was the first molecularly targeted drug to receive FDA approval for treatment of HCC, achieving that status in 2007 on the basis of a

nearly 3-month improvement in OS in the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) study, from which FLC was excluded<sup>129</sup>. While FLC and HCC are clearly distinct entities, single-centre and individual case reports indicate that a number of oncologists have utilized sorafenib in patients with FLC, both paediatric and adult<sup>18,85</sup>. For example, in one single-institution experience, four of nine patients with advanced FLC who received sorafenib with palliative intent (median duration 8 months) showed temporary disease stabilization, but progression-free survival (PFS) did not improve<sup>86</sup>. Newer kinase inhibitors, in particular lenvatinib, have been approved in Europe and in the USA for the treatment of HCC, against which the drug has superior efficacy to sorafenib<sup>130</sup>. Preliminary data reported in a 2021 meeting abstract show encouraging initial results in patients with FLC treated with lenvatinib in combination with GEMOX or nivolumab, although not yet in the context of a formal, prospective research study<sup>131</sup>.

Despite the rarity of FLC, studies of several targeted therapies have shown that multicentre clinical trials specific for this cancer can meet recruitment goals. The choices of drugs were based primarily on certain clinical observations, together with results of the initial systematic analyses of gene expression in FLC tumours<sup>31,32,34,36</sup>. The first example of such a study tested everolimus as a single agent or in combination with an anti-oestrogenic regimen. Everolimus inhibits the serine–threonine protein kinase activity of mTOR complex 1 (mTORC1), which selectively regulates translation of mRNAs encoding key elements of the cell's protein synthesis machinery<sup>132</sup>. The anticancer activity of everolimus correlates with its ability to inhibit the phosphorylation of substrates such as ribosomal protein S6 kinase (S6K). The active, phosphorylated forms of mTORC1 and S6K have been observed in biopsy specimens from patients with FLC<sup>81</sup>. A study in 2015 detected a high level of the phosphorylated form of the key downstream substrate ribosomal protein S6 (P-S6) in 96% of FLC specimens ( $n = 23$ ), while P-S6 was observed only rarely in other carcinomas of the liver (13% of HCCs and 0% of intrahepatic cholangiocarcinomas) or normal liver (3%)<sup>82</sup>. Aromatase expression in FLC and its association with the occasional incidence of gynaecomastia in male patients suggested the combined use of everolimus with the aromatase inhibitor letrozole, plus the luteinizing hormone-releasing hormone agonist leuprolide<sup>22,133,134</sup>. A randomized phase II trial (NCT01642186) evaluated everolimus alone, anti-oestrogen therapy (letrozole plus leuprolide) or the three agents together in FLC; upon progression, patients in the everolimus or anti-oestrogen arms could receive the complete combination. Of 28 patients enrolled, none achieved the primary end point of PFS at 6 months (median PFS was 2.7 months; median OS was 12.4 months), and the study was halted after an interim analysis indicated lack of clinical activity<sup>135</sup>.

A separate phase II multicentre study (NCT02234986) targeted the serine–threonine kinase AURKA, a cell-cycle regulator for the G2/M transition believed to promote centrosome maturation and mitotic spindle assembly<sup>136</sup>. Transcriptome and kinome studies point to activation of AURKA as a downstream consequence of both the expression and kinase activity of the DNAJ–PKAc fusion protein<sup>34</sup>. An open-label study of the oral AURKA inhibitor ENMD-2076, which also inhibits several kinases associated with angiogenesis, enrolled 35 patients with metastatic, advanced FLC<sup>137</sup>. One patient showed a partial response, and 20 had stable disease. However, the OS (19 months) and PFS (3.9 months) did not meet the study's primary or secondary end points. The investigators concluded that the

results did not support further testing of ENMD-2076 as a single agent for the treatment of FLC<sup>138</sup>.

Some RNA-based studies and/or immunohistochemical analyses of human FLC tumours have indicated at least modestly elevated expression of one or both of the EGFR (encoded by *EGFR* (also known as *ERBB1*)), a tyrosine kinase, and its closest relative (encoded by *ERBB2* (also known as *HER2*)), a receptor for which a specific ligand remains to be identified<sup>31,32,34,139,140</sup>. However, FLC typically does not feature either activating mutations of *EGFR*, which frequently occur in lung and certain other cancers, or high levels of amplification of *HER2*, as seen in a subset of breast cancers. Nevertheless, patients with FLC were included in a current clinical trial (NCT01953926) evaluating neratinib, an irreversible pan-HER receptor tyrosine kinase inhibitor which targets both the EGFR and ERBB2 proteins<sup>141</sup>. A 2021 meeting abstract states that initial results did not show substantial benefit for single-agent neratinib in 15 patients with advanced FLC who had failed other systemic regimens. However, several patients with FLC received neratinib plus pembrolizumab (with or without everolimus) under expanded access regulations, leading to an objective partial response in one and extended disease stabilization in another<sup>142</sup>. The investigators recommend continued exploration of combinations of neratinib with other anticancer agents.

As FLC PDX lines and other disease models, including cell lines and 3D organoids, become more widely accessible, large-scale screening could reveal compounds and drug combinations with unanticipated potential to revolutionize systemic therapy for this cancer<sup>97</sup>. In parallel, future efforts at targeted therapy will take advantage of the growing knowledge of FLC mechanisms discussed above. In addition, ‘genetic dependency mapping’, the systematic elucidation of genes and pathways that tumour cells require to survive and grow, has potential to identify an expanded, unbiased set of therapeutic targets and to help elucidate drug mechanisms<sup>143–146</sup>. These strategies are key components of the proposed Roadmap for future research aimed at translation to novel clinical trials and improved therapy (TABLE 2). At the level of individual patients, assays on cells taken directly from tumour biopsy samples might enable the selection of optimal therapeutic regimens<sup>147,148</sup>. This could be particularly important for drugs such as neratinib, for which in vitro responses of FLC cells taken from patients have been observed to vary substantially from tumour to tumour<sup>97</sup>.

With respect to immunotherapy, an investigator-initiated study of pembrolizumab at four sites in the USA is currently open to enrolment for patients up to age 30 years with HCC or FLC. This trial might shed more light on whether a single-agent PD1 ICI has efficacy against FLC (NCT04134559). A study of another PD1 inhibitor, nivolumab, in conjunction with local yttrium-90 resin microsphere radioembolization therapy<sup>149</sup> for liver tumours, in which patients with FLC were eligible, led by investigators at the Clinical University of Navarra, Spain (EudraCT number 2017-000232-34; NCT03380130), was presented at a 2020 meeting<sup>150</sup>. However, the report did not specifically address whether any patients with FLC were enrolled or responded to therapy.

A clinical trial opened in late 2020 at the MD Anderson Cancer Center, Houston, TX, which combines the frequently used combination of 5-FU and recombinant interferon<sup>125</sup> with nivolumab (NCT04380545). A key hypothesis is that the chemotherapy agent and interferon will potentiate the effect of the PD1 ICI. A peer-reviewed paper of initial observations with this triple drug therapy in 14 patients with FLC at Rush University Medical Center (Chicago, IL) was published in 2021 (REF.<sup>151</sup>). The investigators report that the regimen was well tolerated and suggest the potential for clinical benefit<sup>151</sup>.

An innovative immunotherapy clinical trial specific to FLC began recruiting patients in March 2020 at Johns Hopkins University, Baltimore, MD. It is designed to focus on the potential attack on cancer cells by T cells with receptors for the signature DNAJ–PKAc driver protein of FLC. The study tests a synthetic peptide vaccine spanning the junction sequence linking the two components of the chimeric protein, corresponding at the mRNA level to the fusion of exon 1 of *DNAJB1* to exon 2 of *PKACA*. The amino acid sequence at the junction should be unique to DNAJ–PKAc and identical in all patients<sup>35</sup>. The tumour in a few patients also contains a second form of the fusion protein due to a minor RNA splice variant between a donor site within exon 2 of the *DNAJB1* sequence and the start of exon 2 of *PRKACA*. Patients also receive nivolumab and ipilimumab (NCT04248569). Thus, the trial combines a precision immunotherapy directly targeting the FLC oncoprotein with broad immune checkpoint blockade. Observations to date indicate that the regimen has clinical activity (M. Yarchoan, personal communication), but the results have not yet been published.

Additional concepts in immunotherapy remain to be evaluated. For example, a proposed trial at the US National Cancer Institute was designed to test whether oral vancomycin can enhance the immune response against FLC by altering the gut microbiome (NCT04025567). However, the trial was closed prematurely because of slow accrual of patients. Preclinical observations in mice indicate that depletion of commensal bacteria from the gut increases levels of CXCL16, leading to the recruitment of natural killer T cells and a subsequent effect on liver tumours, but this has not yet been tested in an FLC model<sup>152</sup>.

Looking ahead, emphasis should be placed on multi-agent as opposed to single-agent trials, aimed at targeting multiple protein and pathway alterations driven by the *DNAJB1*–*PRKACA* fusion oncogene. Also, it will be important to focus on broad enrolment of clinical trial patients, with specific inclusion of patients at age 12 years and above, per regulatory agency allowances, and consistent with the prevalence of FLC in the adolescent and young adult population.

## Roadmap

Many scientists and clinicians working on FLC have forged strong cross-disciplinary and multi-institutional collaborations. These interactions facilitate progress, from understanding of molecular mechanisms to translational findings and clinical trials, all ultimately in the service of developing new treatments and improving outcomes for a cancer that presently lacks a standard of care. This is one of many examples of the role of collaborative networks that include patient-led groups in advancing research in rare and neglected diseases<sup>153–155</sup>.

Ongoing discussions among the authors of this article and other colleagues for more than 2 years have contributed to a Roadmap for FLC research, which can be categorized into four key goals (TABLE 2):

- Define the FLC patient base
- Develop targeted molecular therapies
- Develop immunotherapies
- Devise effective clinical regimens

We appreciate that others in the field might also be working towards the same ends, pursuing similar or superior strategies. While the specific suggestions in TABLE 2 are conditioned by the biology of FLC and the needs of the affected adolescent and young adult population, the overall goals mirror those recognized as essential to improve treatments and discover cures for other rare cancers<sup>155–157</sup>.

## Conclusions

FLC epitomizes both the strengths and challenges of research focused on a very rare cancer. The discovery that nearly all FLC tumours share a unique oncogenic driver, the DNAJ–PKAc fusion protein, shifted the landscape from an obscure variant of HCC to a distinct entity with a unique molecular mechanism centred on a regulatory protein kinase, PKA, from which much of our current understanding of signal transduction evolved. Translation to effective therapies now seems within reach, both to attack the underlying biochemistry and cell biology and to enhance the immune response to a cancer with low mutational burden but an obligate oncogenic driver that might serve as an ideal neoantigen. Yet, as the proposed Roadmap emphasizes, research remains constrained by limitations in basic tools such as cell lines, human tumour xenografts and animal models, along with resources and sufficient patients to support clinical trials. Advances in our fundamental understanding of FLC are likely to bring novel insights to more common cancers that are harder to study because of greater phenotypic and genomic heterogeneity and genetic instability. Progress will continue to depend heavily on active cooperation within the research community, in partnership with patients and caregivers.

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**Box 1 |****Fibrolamellar carcinoma databases and repositories**

- FibroLamellar Omics (FLO): [https://sethupathy-lab.shinyapps.io/flc\\_data/](https://sethupathy-lab.shinyapps.io/flc_data/). A browser for genome-scale fibrolamellar carcinoma (FLC) data, including small RNA sequencing, RNA sequencing and chromatin run-on sequencing data.
- Cancer Dependency Map: <https://depmap.org/portal>. A systematic effort to catalogue as completely as possible the genetic and pharmacological vulnerabilities in a wide array of established cell lines and 3D organoid cultures from a comprehensive collection of common and rare human cancers.
- Kinase Chemogenomic Set (KCGS): <https://www.sgc-unc.org/kinase-chemogenomics>. A collection of specific small-molecule kinase inhibitors<sup>165</sup>. At the time of writing, the KCGS includes 645 inhibitors and covers 250 enzymes across all kinase families and includes compounds active against many orphan kinases.
- Fibrolamellar Cancer Foundation Biobank (Massachusetts General Hospital): <https://fibrofoundation.org/join-the-fight/donate-tissue/>. A centralized repository of tumour tissue and blood samples contributed by patients with FLC to help advance research, available to qualified researchers interested in studying FLC.
- Fibrolamellar Tissue Repository (Rockefeller university): <https://fibrolamellar.rockefeller.edu/repository>. A collection of tissue and blood specimens contributed by patients with FLC. Specimens will be distributed only to scientists at Rockefeller University and other approved institutions who have received authorization from their respective Institutional Review Boards. Because tissue samples are rare, investigators must submit a request that justifies their proposed research and use of tissue from the Repository.
- The Fibrolamellar Registry: <https://fibroregistry.org/>. A collection of health data about patients with FLC, stored in a highly secure database. It is a comprehensive, online survey that gathers medical histories and test results. The Registry meets the highest standards for privacy and patient protection and has been approved as a scientific study by the Genetic Alliance Institutional Review Board.
- Natural History Study of Rare Solid Tumors: <https://www.cancer.gov/pediatric-adult-rare-tumor/participate/natural-history>. A natural history trial taking place at the NIH Clinical Center in Bethesda, MD, USA. The purpose of the trial is to collect information and samples from people with rare tumours and their relatives, and to track their health history over a long period of time.

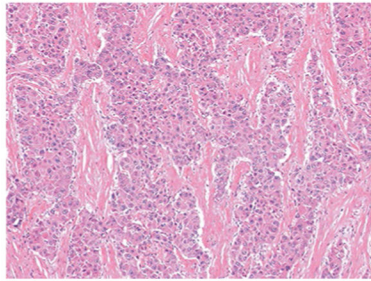


- [ClinicalTrials.gov](https://clinicaltrials.gov/), United States National library of Medicine: <https://clinicaltrials.gov/>. A database of privately and publicly funded clinical studies conducted around the world.
- European Union Drug Regulating Authorities Clinical Trials Database (EudraCT): <https://eudract.ema.europa.eu/>. The European database for all interventional clinical trials on medicinal products authorized in the European Union (EEA) and outside the EU/EEA if they are part of a Paediatric Investigation Plan (PIP) from 1 May 2004 onwards.

### Key points

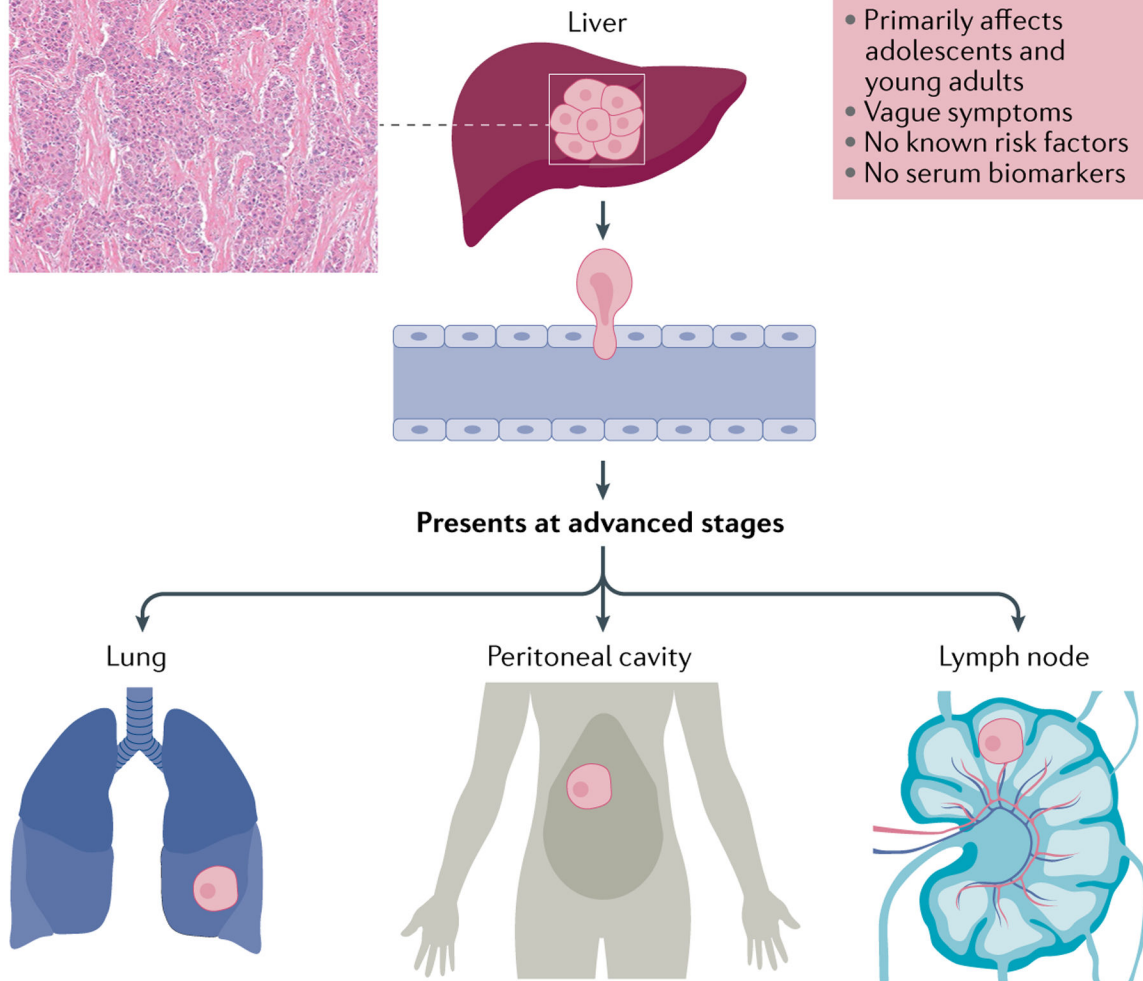
- Fibrolamellar carcinoma (FLC) is a rare but devastating disease that disproportionately affects adolescents and young adults and is characterized by the fusion oncogene *DNAJB1-PRKACA*.
- There is a critical need to establish new cellular and animal models of FLC to perform reliable functional and preclinical studies.
- Efforts to directly inhibit cyclic AMP-dependent protein kinase (PKA) catalytic activity for FLC therapy might be constrained by the important role of the normal enzyme in multiple tissues; a deeper understanding of mechanisms underlying cellular transformation by the oncogenic chimaera remains essential to defining tractable pharmacological targets.
- Advances in the genetic and molecular underpinnings of FLC, in part through the application of genome-scale techniques, reveal promising avenues for new targeted therapeutic strategies and motivate higher-resolution single-cell and spatial approaches.
- Immunotherapy for FLC must overcome the challenge of generating responses against a low-tumour mutational burden cancer, potentially by targeting the specific fusion oncoprotein as a neoantigen.
- Clinical trials must overcome obstacles to accruing sufficient patients and should utilize an evidence-based approach and collaborative networks tailored to rare cancers.

Characterized by fibrous bands and eosinophilic foci



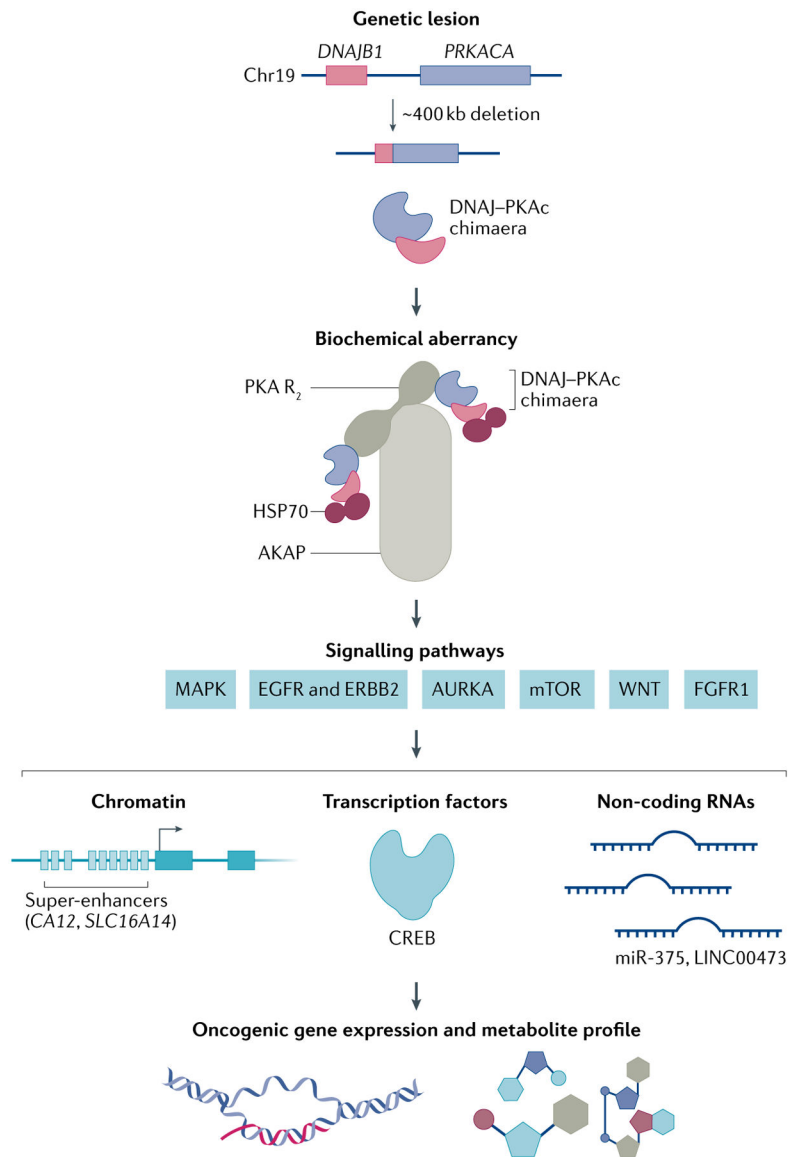
Typical clinical features

- Primarily affects adolescents and young adults
- Vague symptoms
- No known risk factors
- No serum biomarkers



**Fig. 1 | Fibrolamellar carcinoma.**

Tumour characteristics (left) and typical clinical features (right). Fibrolamellar carcinoma (FLC) presents, often with vague symptoms, in adolescents and young adults. It is associated neither with liver cirrhosis nor with other factors known to predispose to hepatocellular carcinoma. Primary FLC tumours often grow relatively slowly, but they are highly aggressive and invasive. The cure rate is very low. Common metastatic sites include local lymph nodes, lung and peritoneum. Less frequently observed locations include bone, ovary and intracranial<sup>166</sup>. Diagnosis often occurs only after extensive tumour growth, due to non-specific symptoms and a lack of non-invasive biomarker assays. Image (top left) courtesy of M. Torbenson, Mayo Clinic, USA.



**Fig. 2 | Molecular mechanisms of fibrolamellar carcinoma.**

The hallmark genetic lesion of fibrolamellar carcinoma (FLC) is a ~400 kb heterozygous deletion on chromosome 19 that leads to the in-frame fusion of exon 1 of *DNAJB1* and exons 2–10 of *PRKACA*<sup>35</sup>. The translated protein is referred to in this article as the DNAJ–PKAc chimaera. The chimaera recruits heat shock protein 70 (HSP70), conferring additional protein stability<sup>76</sup>. This molecular complex is recruited to A-kinase anchoring protein 13 (AKAP13; also called AKAP-Lbc). A key consequence is activation of multiple downstream signalling cascades including the MAPK pathway and a number of others, as shown. It is not yet known which of these are most critical to oncogenesis or most amenable to therapeutic intervention (see the section ‘Additional therapeutic targets?’). These biochemical alterations lead to notable changes in the wiring of chromatin (enhancers and super-enhancers), as well as in the activity of transcription factors (for example, cAMP response element binding protein (CREB)) and non-coding RNAs (for example, miR-375

and LINC00473), which together shape the aberrant molecular profiles that promote FLC tumour phenotypes. AURKA, aurora kinase A; EGFR, epidermal growth factor receptor; FGFR1, fibroblast growth factor receptor 1; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; PKA, cyclic AMP-dependent protein kinase; PKA R<sub>2</sub>, PKA regulatory subunit homodimer.

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Table 1 |

Currently available models for fibrolamellar carcinoma research

Model	Type	Species	Method	Strengths	Limitations	Refs
<i>Published work</i>						
M-DP-1	In vivo	Mouse	CRISPR-Cas9-based genome editing Transposon-mediated somatic gene transfer	In vivo tumour growth Mimics many features of FLC	Slow tumorigenesis Incomplete penetrance Lacks fibrous bands Lacks molecular features of FLC that are primate-specific	58
M-DP-2	In vivo	Mouse	CRISPR-Cas9-based genome editing	In vivo tumour growth Mimics many features of FLC	Slow tumorigenesis Incomplete penetrance Lacks fibrous bands Lacks molecular features of FLC that are primate-specific	57
ZF-DP-1	In vivo	Zebrafish	Zebrafish DNAI-PKAc over-expression	In vivo model	Evolutionarily more distant from humans than mice Lacks molecular features specific to mammals and primates	96
PDX-1	In vivo	Human	Xenograft of patient-derived FLC tumour cells in immune-deficient mice	In vivo tumour growth Mimics most/all major features of FLC Can be maintained stably in spheroid (3D) culture	Generally, tumour grown in host flank, not liver (non-orthotopic model) Not metastatic from flank	36
PDX-2 to PDX-7	In vivo	Human	Xenograft of patient-derived FLC tumour cells in immune-deficient mice	In vivo tumour growth Mimics most/all major features of FLC Derived directly from primary Liver tumours and metastases	Generally, tumour grown in host flank, not liver (non-orthotopic model) Not metastatic from flank	97
HepG2-DP	In vitro	Human	Lentiviral DP over-expression	All human cells Straightforward to culture and manipulate	No presence of cells from microenvironment Hepatoblastoma background Transformed cells	33,50
C3A-DP	In vitro	Human	Lentiviral DP over-expression	All human cells Straightforward to culture and manipulate	No presence of cells from microenvironment HCC background Transformed cells	33
AML12-DP	In vitro	Mouse	CRISPR-Cas9-based genome editing	Primary hepatocytes Straightforward to culture and manipulate	Mouse not human origin Transgenic for human TGF $\alpha$	50,76,89
<i>Unpublished or preliminary work</i>						
FLX-1 cell line	In vitro	Human	Cell culture from PDX-1 xenograft model	(Nearly) all human tumour cells Established line that retains molecular features of FLC for multiple passages Amenable to treatment and transfection	Limited (if any) microenvironmental signalling Relatively slow growth	N. Bardeesy (personal communication)
Huh7-DP	In vitro	Human	Lentiviral DP over-expression	All human cells Straightforward to culture and manipulate	No presence of cells from microenvironment HCC background Transformed cells	P.S., unpublished work

Model	Type	Species	Method	Strengths	Limitations	Refs
HEK293-DP	In vitro	Human	CRISPR– Cas9-based genome editing	All human cells Straightforward to culture and manipulate	No presence of cells from microenvironment Non-hepatic background Transformed cells	<sup>95</sup> (a preprint article)

All of these 16 in vitro and in vivo models currently in use for fibrolamellar carcinoma (FLC) research offer some strengths but also have key limitations, pointing to the critical need for improved models. DP, *DNAI1-PRKACA*; HCC, hepatocellular carcinoma; TGF $\alpha$ , transforming growth factor- $\alpha$ .

Table 2 |

## Fibrolamellar carcinoma research Roadmap

Unmet need	Actions	Current status or feasibility	Challenges
<b>Define the FLC patient base</b>			
Accurate diagnosis and determination of global incidence of FLC	Standardize diagnostic criteria and establish a unique ICD code	FLC recognized under ICD-10, but included under same numerical code as HCC Identification by pathologists might be inconsistent outside the most experienced groups Molecular testing for fusion gene is feasible to help verify diagnoses <sup>28</sup>	Achieving agreement among pathologists on diagnostic criteria Broader implementation of molecular testing for <i>DNAJB1-PRKACA</i> fusions Clarification of frequency and significance of FLC and mixed FLC/HCC tumours with <i>BAP1</i> mutations, lacking fusion gene
	Improve data collection on paediatric and AYA cancers for US SEER database and globally	National Cancer Institute workgroup in place to improve data collection from registries International cooperative group support needed	AYA demographic is generally under-served, and tracking might be lost in transition between paediatric and adult practices Collection of similar data outside USA
	Source FLC data from patient-reported registries and natural history studies	Fibrolamellar Registry has large sets of patient-reported data Additional registries in development US National Cancer Institute MyPART Natural History Study of Rare Solid Tumors is at early stages of enrolment for FLC	Increasing participation across full diversity of patient population Collecting and sharing data internationally
	Capture extensive real-world data on FLC incidence and treatment <sup>58</sup>	Companies access large percentage of medical records in some areas Seek relevant data from ERNs in EU cross-border health-care initiative <sup>159</sup>	Global coverage beyond USA Search criteria must be defined and tested Who will bear cost?
<b>Develop targeted molecular therapies</b>			
Experimental systems widely accessible to the research community	Develop a research consortium to generate and distribute models and other resources that are routinely available for common cancers, but scarce for very rare ones, <sup>154-157</sup>	Biobanks of FLC tumour tissue exist at Rockefeller University and Massachusetts General Hospital; none identified outside USA Multiple PDX models (flank) now reported; orthotopic models could be tried with existing methods No 2D or 3D human FLC cell lines widely available No available animal model accurately replicates human FLC Genomic engineering of murine germline and/or liver cells, and organoids of normal human tissue to generate improved in vivo and culture models should be feasible	Slow growth of FLC tumours in PDX models and in cell culture Further genomic data on FLC required to better understand its oncogenesis and to determine mutations and/or focal lesions most likely to pair effectively with <i>DNAJB1-PRKACA</i> for FLC experimental models Biobank contribution from treating sites needs to gain momentum Achieving wide distribution of models to FLC research community
Drugs selectively targeting DNAJ-PKAc oncoprotein	Apply multiple modalities to target fusion protein but spare normal PKA	Potentially feasible approaches include design or screening for selective small-molecule inhibitors, RNAi <sup>143</sup> , PROTACs <sup>160</sup>	Reluctance of some biopharmaceutical companies to invest in therapeutics development for a very rare disease
Identification of additional, druggable therapeutic targets and/or drug sensitivities	Rigorously assess protein complexes through which DNAJ-PKAc acts and define biochemical pathways it activates	Underway in certain cell models	Confirmation needed in human FLC cells, which is limited by availability of PDX models and cell lines
	Determine comprehensive catalogue of molecular dependencies by genetic knockdown or knockout and	Dependency of FLC on candidate genes identified from genomic and epigenomic analyses can be assessed in low-throughput assays, even with the limited models currently available	For genome-wide studies culture models must grow robustly enough to carry out automated screens of libraries up to 20,000 genetic constructs (RNAi, CRISPR-Cas9)



Unmet need	Actions	Current status or feasibility	Challenges
	testing of compounds <sup>161</sup>	First systematic drug repurposing screen of >5,000 compounds reported <sup>97</sup> Cancer Dependency Maps covering entire human genome are now feasible for all common cancers and some rare ones; large comparator datasets are available <sup>144,146,162,163</sup> Available chemical resources include libraries of a large percentage of compounds that have been utilized in human trials Specialized chemical libraries such as protein kinase inhibitors (Kinase Chemogenomic Set (KCGS)) are publicly available <sup>164,165</sup>	
<b>Develop targeted molecular therapies (cont.)</b>			
Characterization of the tumour ecosystem, including the stromal and immune microenvironment	Leverage state-of-the-art single-cell and/or spatial genomic and proteomic technologies	Rapidly developing technology best implemented at sites with appropriate facilities and expertise	Logistics of rapid collection of rare tumour biospecimens at widely dispersed institutions and transport to centralized study sites without degradation Longitudinal specimen collection to study tumour progression and responses to therapy High cost
<b>Develop immune therapies</b>			
Identification of cell surface antigens targetable by antibodies or CAR T cells	Find proteins expressed selectively in FLC tumours at sufficient levels and for which monoclonal antibodies can be obtained	Some candidates already reported from RNA data <sup>50</sup> Protein studies with mass spectroscopy and labelling methods, and antibody development are feasible	Beyond proof of concept, development of therapeutic products will probably require commercial partners
Assessment of DNAM1-PKAc as target for immune therapy	Characterize T cells from mouse models, patient biospecimens, clinical trials	Preclinical studies and one clinical trial of a therapeutic vaccine for the fusion protein are in progress	Focuses on a single immune epitope Tumour cells might become independent of DNAM1-PKAc, and escape by losing the antigen
<b>Devise effective clinical regimens</b>			
Creation of an international clinical forum of physicians who treat FLC	Share ideas and data towards goal of determining standard(s) of care for systemic therapy Devise and implement efficient clinical trial designs with strong correlative studies	Precedent exists for creation of multi-institutional consortiums for study of rare disease indications Lessons from other rare cancers <sup>159</sup>	Consensus recommendations might not yet be achievable for FLC in absence of data to support a standard of care for systemic therapy

AYA, adolescents and young adults; CAR T cells, chimeric antigen receptor T cells; ERN, European Reference Network; FLC, fibrolamellar carcinoma; HCC, hepatocellular carcinoma; ICD, International Code of Diseases; PDX, patient-derived xenograft; PKA, protein kinase A; PROTAC, proteolysis-targeting chimaera; SEER, Surveillance, Epidemiology, and End Results.