Integrated Genomic Characterization Reveals Novel, Therapeutically Relevant Drug Targets in FGFR and EGFR Pathways in Sporadic Intrahepatic Cholangiocarcinoma

Mitesh J. Borad1,2,3*, Mia D. Champion3,4,9, Jan B. Egan3,9, Winnie S. Liang5, Rafael Fonseca1,2,3, Alan H. Bryce1,2,3, Ann E. McCullough6, Michael T. Barrett2,5, Katherine Hunt1, Maitray D. Patel7, Scott W. Young7, Joseph M. Collins7, Alvin C. Silva7, Rachel M. Condjella3, Matthew Block3,8, Robert R. McWilliams3,8, Konstantinos N. Lazaridis3, Eric W. Klee3,4, Keith C. Bible8, Pamela Harris9, Gavin R. Oliver3,4, Jaysheel D. Bhavsar3,4, Asha A. Nair3,4, Sumit Middha3,4, Yan Asmann3,4, Jean-Pierre Kocher3,4, Kimberly Schahil9, Benjamin R. Kipp10, Emily G. Barr Fritcher10, Angela Baker5, Jessica Aldrich9, Ahmet Kurdoğlu5, Tyler Izatt5, Alexis Christoforides5, Irene Cherni5, Sara Nasser5, Rebecca Reiman5, Lori Phillips5, Jackie McDonald5, Jonathan Adkins5, Stephen D. Mastrian5, Pamela Placek5, Aprill T. Watanabe5, Janine LoBello5, Haiyong Han5, Daniel Von Hoff2,5, David W. Craig5, A. Keith Stewart1,2,3, John D. Carpten5

1 Division of Hematology/Oncology Mayo Clinic, Scottsdale, Arizona, United States of America, 2 Mayo Clinic Cancer Center, Scottsdale, Arizona, United States of America, 3 Center for Individualized Medicine, Mayo Clinic, Rochester, Minnesota, United States of America, 4 Department of Biomedical Statistics and Informatics, Mayo Clinic, Scottsdale, Arizona, United States of America, 5 Translational Genomics Research Institute, Phoenix, Arizona, United States of America, 6 Department of Pathology, Mayo Clinic, Scottsdale, Arizona, United States of America, 7 Department of Radiology, Mayo Clinic, Scottsdale, Arizona, United States of America, 8 Mayo Clinic Cancer Center, Rochester, Minnesota, United States of America, 9 Investigational Drug Branch, National Cancer Institute, Rockville, Maryland, United States of America, 10 Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, United States of America

Abstract

Advanced cholangiocarcinoma continues to harbor a difficult prognosis and therapeutic options have been limited. During the course of a clinical trial of whole genomic sequencing seeking druggable targets, we examined six patients with advanced cholangiocarcinoma. Integrated genome-wide and whole transcriptome sequence analyses were performed on tumors from six patients with advanced, sporadic intrahepatic cholangiocarcinoma (SIC) to identify potential therapeutically actionable events. Among the somatic events captured in our analysis, we uncovered two novel therapeutically relevant genomic contexts that when acted upon, resulted in preliminary evidence of anti-tumor activity. Genome-wide structural analysis of sequence data revealed recurrent translocation events involving the FGFR2 locus in three of six assessed patients. These observations and supporting evidence triggered the use of FGFR inhibitors in these patients. In one example, preliminary anti-tumor activity of pazopanib (in vitro FGFR2 IC50=350 nM) was noted in a patient with an FGFR2-TACC3 fusion. After progression on pazopanib, the same patient also had stable disease on ponatinib, a pan-FGFR inhibitor (in vitro, FGFR2 IC50≈8 nM). In an independent non-FGFR2 translocation patient, exome and transcriptome analysis revealed an allele specific somatic nonsense mutation (E384X) in ERRFI1, a direct negative regulator of EGFR activation. Rapid and robust disease regression was noted in this ERRFI1 inactivated tumor when treated with erlotinib, an EGFR kinase inhibitor. FGFR2 fusions and ERRFI1 mutations may represent novel targets in sporadic intrahepatic cholangiocarcinoma and trials should be characterized in larger cohorts of patients with these aberrations.

Citation: Borad MJ, Champion MD, Egan JB, Liang WS, Fonseca R, et al. (2014) Integrated Genomic Characterization Reveals Novel, Therapeutically Relevant Drug Targets in FGFR and EGFR Pathways in Sporadic Intrahepatic Cholangiocarcinoma. PLoS Genet 10(2): e1004135. doi:10.1371/journal.pgen.1004135

Editor: Marshall S. Horwitz, University of Washington, United States of America

Received July 26, 2013; Accepted December 6, 2013; Published February 13, 2014

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: This study was supported by the NIH Grant Number K12 CA90628 and the Center for Individualized Medicine. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: Dr. Kipp received grant funding from Abbott Molecular Inc, the suppliers of the FGFR2 FISH probe used in this study. The other authors have no conflicts to declare.

* E-mail: borad.mitesh@mayo.edu (MJB); jcarpten@gen.org (JDC)
9 These authors contributed equally to this work.
9 DWC and JDC also contributed equally.

Introduction

Biliary tract cancers (BTC) comprise malignant tumors of the intrahepatic and extrahepatic bile ducts. Known risk factors for BTC are the liver flukes O. viverrini and C. sinensis in high prevalence endemic regions in southeast Asia [1–3], as well as primary sclerosing cholangitis [4–7], Caroli’s disease [8], hepatitis

PLOS Genetics | www.plosgenetics.org 1 February 2014 | Volume 10 | Issue 2 | e1004135
Novel FGFR and EGFR Targets in Cholangiocarcinoma

Author Summary

Cholangiocarcinoma is a cancer that affects the bile ducts. Unfortunately, many patients diagnosed with cholangiocarcinoma have disease that cannot be treated with surgery or has spread to other parts of the body, thus severely limiting treatment options. New advances in drug treatment have enabled treatment of these cancers with “targeted therapy” that exploits an error in the normal functioning of a tumor cell, compared to other cells in the body, thus allowing only tumor cells to be killed by the drug. We sought to identify changes in the genetic material of cholangiocarcinoma patient tumors in order to identify potential errors in cellular functioning by utilizing cutting edge genetic sequencing technology. We identified three patient tumors possessing an FGFR2 gene that was aberrantly fused to another gene. Two of these patients were able to receive targeted therapy for FGFR2 with resulting tumor shrinkage. A fourth tumor contained an error in a gene that controls a very important cellular mechanism in cancer, termed epidermal growth factor pathway (EGFR). This patient received therapy targeting this mechanism and also demonstrated response to treatment. Thus, we have been able to utilize cutting edge technology with targeted drug treatment to personalize medical treatment for cancer in cholangiocarcinoma patients.

B and hepatitis C [9–14], obesity [13], hepatolithiasis [15,16] and thororast contrast exposure [17,18]. Surgical approaches such as resection and liver transplantation represent the only curative treatment approaches for BTC [19]. Unfortunately, most patients present with surgically unresectable and/or metastatic disease at diagnosis. Systemic therapy with gemcitabine and cisplatin has been established as the standard of care for patients with advanced disease, but is only palliative [20], emphasizing the imminent need for novel therapies.

Multiple studies have reported the presence of mutations/allelic loss of known cancer genes in BTC [21–39] and recently, a prevalence set of 46 patients was used to validate 15 of these genes including: TP53, KRAS, CDKN2A and SMAD4 as well as MLH1, ROBO2, RNF43, GNAS, PEG3, XIRP2, PTEN, RAD51, NCD80, LAMA2 and PCDHA13. Recent studies have also identified recurrent mutations in IDH1 (codon 132) and IDH2 (codons 140 and 172) with a prevalence of 22–23% associated with clear cell/ poorly differentiated histology and intrahepatic primary [40,41]. Fusions with oncogenic potential involving the kinase gene ROS1 have been identified in patients with BTC with a prevalence of 8.7% in a recent study [42]. Less frequently, mutations in sporadic BTC have been reported in EGFR [43,44], BRAF [45], NRAS [40,46], PIK3C4 [40,46,47], APC [40], CTNNB1 [40], AKT1 [40], PTEN [40], ABCB4 [48], ABCB11 [49,50], and CDH1 [51] as well as amplifications in ERRB2 [52].

Recently, two independent studies reported the presence of FGFR fusions in cholangiocarcinoma; a single case with FGFR2-AHCYL1 [53] as well as several cases identifying FGFR2-BICC1 fusions [33,54]. Arai et al. evaluated the presence of FGFR2 fusions in a cohort of 102 cholangiocarcinoma patients observing that the fusions occurred exclusively in the intrahepatic cases with a prevalence of 13.6% [53]. Due to the presence of known dimerization motifs in the fusion partners, Wu et al. conducted mechanistic studies that demonstrated the in vitro interaction of FGFR2-BICC1 and other fusions that was not observed in the presence of wildtype FGFR2 [54]. Furthermore, overexpression of the FGFR2-BICC1 and other selected fusions resulted in altered cell morphology and increased cell proliferation [54]. These data led to the conclusion that the fusion partners are facilitating oligomerization, resulting in FGFR kinase activation in tumors possessing FGFR fusions. In addition, in vitro and in vivo assessment of the sensitivity of cell lines containing an FGFR2 fusion to an FGFR inhibitor demonstrated sensitivity to treatment only in the fusion containing cells [53,54], suggesting the presence of FGFR fusions may be a useful predictor of tumor response to FGFR inhibitors.

To comprehensively explore the genetic basis of sporadic intrahepatic cholangiocarcinoma (SIC), with emphasis on elucidation of therapeutically relevant targets, we performed integrated whole genome and whole transcriptome analyses on tumors from 6 patients with advanced, sporadic intrahepatic cholangiocarcinoma (SIC). Notably, recurrent fusions involving the oncogene FGFR2 (n = 3) were identified. A patient whose tumor presented with an FGFR2-MGEA5 fusion has demonstrated preliminary evidence of anti-tumor activity manifest as stable disease accompanied by CA19-9 reduction and tumor necrosis to ponatinib, a pan-FGFR inhibitor (in vitro FGFR1 IC_{50}=24 nM, FGFR2 IC_{50}=8 nM, FGFR3 IC_{50}=8 nM and FGFR4 IC_{50}=34 nM). In another patient whose tumor possessed an FGFR2-TACC3 fusion, preliminary anti-tumor activity of pazopanib (in vitro FGFR2 IC_{50}=350 nM) was also noted. After progression on pazopanib, the same patient also responded to ponatinib and again demonstrated tumor shrinkage. Additionally, a non-FGFR fusion patient was found to have allele-specific preferential expression of a loss of function mutation in ERRF1, a direct negative regulator of EGFR activation. Similarly, rapid and robust disease regression was noted in the patient with an ERRF1 mutant tumor when treated with erlotinib, an EGFR kinase inhibitor. Results suggest that these novel targets in the EGFR and FGFR pathways may be therapeutically relevant in patients with sporadic cholangiocarcinoma.

Results

Genomic landscape

We identified 327 somatic coding mutations, with an average of 55 mutations/tumor (range 34–112) within our cohort (Table 1, Figure 1). Nonsynonymous single nucleotide variations were the predominant class in all of the patients. Patients 1 and 2 accumulated a high number of synonymous mutations in comparison to the other patients. Patient 5 carried the most stops gained likely contributing to a higher number of pseudogenes in comparison to the others and was also the only patient to carry several predicted high impact mutations affecting splice site acceptor regions (Figure 1, light green, percentage <5%). In addition, patient 6 also carried a codon change plus insertion variation. Sequencing statistics are provided in Table 2. Genes with mutations in more than one case included CSPAN4 (n = 2), GRK3A (n = 2) and PLXBN3 (n = 2) (Table S1); with half of these predicted to be potentially damaging by SIFT [55], Polyphen [56], Mutation Assessor [57] and Mutation Taster [58]. While there was overlap in the somatic landscape of SIC with liver-fluke associated cholangiocarcinoma, hepatocellular cancer and pancreatic cancer, most of the aberrations detected in our study were distinct (Table 3). More importantly, using previously published methods [59], we identified molecular fusions involving FGFR2 that were felt to be therapeutically relevant in 3 patients. Additionally, these fusions were validated with a break apart Fluorescent In situ Hybridization (FISH) assay (Figure 2). Notably, the patients who did not harbor the FGFR2 fusions were negative using the same assay. Two of the three patients with FGFR2 fusions (Patients 4 and 6) were treated with FGFR inhibitors while the third patient
(Patient 5), experienced clinical decline prior to the availability of results and as such did not receive any further therapy. Furthermore, overexpression of an SNV in \textit{ERRFI1} (E384X), a negative regulator of EGFR, was detected in a non-FGFR2 translocation patient’s tumor. Taken together, our results constitute important therapeutically actionable alterations in patients with advanced SIC ([Text S1]).

Pathway analysis

Comparative pathway analysis of genes carrying small scale nucleotide variations (SnSVs) has implicated several major pathways, possibly interacting as a network, that are predicted to underlie disease in all of our studied biliary carcinoma patients. These shared pathways include EGFR, EPHB, PDGFR-beta, Netrin-mediated and Beta1 integrin mediated signaling pathways (Figure 3 and Tables S2 and S3). Interestingly, most of these pathways have known roles in mediating epithelial-to-mesenchymal cell transitions, which occur frequently during development as well as tumorigenesis. Cell growth and motility is inherent to the successful progression of both biological processes. Studies of the nervous system and lung development have shown that Netrins act to inhibit FGF7 and FGF10 mediated growth or cell guidance [60]. In addition, Netrin-1 has a known role in mediating cell migration during pancreatic organogenesis [60]. Furthermore, Netrin-1 acts as a ligand for \textit{\(a_3b_1\)} and \textit{\(a_6b_4\)} integrins, both of which are involved in supporting adhesion of developing pancreatic epithelial cells with Netrin-1 although it is thought that \textit{\(a_6b_4\)} plays the principle role during this process [60]. Interestingly, \textit{\(a_3b_1\)} has been hypothesized to play a role during the process of angiogenesis, when chemotacticants and chemorepellents act to guide filopodia during migration [60]. The \textit{\(a_3b_1\)} integrin receptor may act together with additional pathways proposed to play a role during angiogenesis such as VEGF, PDGFR-beta [61], and EphrinB [62] as well as tumorigenesis [60]. Patients 3 and 4 also shared several genes acting in cadherin signaling pathways (Tables S3, S4), which are important for maintaining cell-cell adhesion and are known to be intimately integrated with EGFR and FGFR signaling pathways [63].

![Figure 1. Sequence variation effects.](https://doi.org/10.1371/journal.pgen.1004135.g001)

Table 1. Summary of mutation type by patient.

|                     | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
|---------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Nonsynonymous coding| 20        | 30        | 31        | 44        | 101       | 34        |
| Synonymous coding   | 13        | 12        | 0         | 0         | 0         | 0         |
| Insertions/deletions| 1         | 4         | 0         | 6         | 0         | 2         |
| Stop gained         | 0         | 3         | 3         | 2         | 6         | 2         |
| Start gained        | 0         | 1         | 0         | 0         | 0         | 0         |
| Codon insertion     | 0         | 1         | 0         | 0         | 0         | 1         |
| Codon deletion      | 0         | 0         | 0         | 0         | 0         | 1         |
| Splice site donor   | 0         | 0         | 0         | 1         | 0         | 1         |
| Splice site acceptor| 0         | 0         | 0         | 0         | 4         | 0         |
| Total               | 34        | 51        | 35        | 52        | 112       | 42        |

**doi:**10.1371/journal.pgen.1004135.t001
Table 2. Sequencing metrics of 6 advanced, sporadic biliary tract cancer patients.

| Patient | Exome | Whole Genome | RNA Seq |
|---------|-------|--------------|---------|
|         | Tissue | Aligned Reads (Millions) | Mean Target Coverage | % Target Bases 10× | # of Functional Coding Variants | Aligned Reads (Millions) | Aligned Bases (Billions) | Physical Coverage | Aligned Reads (Millions) | Aligned Bases (Billions) |
| 1       | N      | 161          | 100      | 94%          | -                        | 266                  | 22                   | 37                   | -                        | -                        |
|         | T      | 156          | 112      | 94%          | 21                       | 228                  | 18                   | 35                   | 100                      | 8.1                      |
| 2       | N      | 176          | 74       | 94%          | -                        | 179                  | 14                   | 5                    | -                        | -                        |
|         | T      | 202          | 81       | 94%          | 34                       | 370                  | 30                   | 10                   | 341                      | 26                       |
| 3       | N      | 226          | 110      | 58%          | -                        | 296                  | 24                   | 50                   | 163                      | 13                       |
|         | T      | 195          | 92       | 58%          | 52                       | 321                  | 26                   | 50                   | 101                      | 8.1                      |
| 4       | N      | 167          | 80       | 95%          | -                        | 317                  | 26                   | 42                   | -                        | -                        |
|         | T      | 202          | 93       | 96%          | 52                       | 163                  | 13                   | 12                   | 264                      | 20                       |
| 5       | N      | 257          | 146      | 96%          | -                        | 335                  | 27                   | 51                   | -                        | -                        |
|         | T      | 133          | 78       | 93%          | 250                      | 349                  | 28                   | 39                   | 401                      | 31                       |
| 6       | N      | 350          | 243      | 92%          | -                        | -                    | -                    | -                    | -                        | -                        |
|         | T      | 340          | 240      | 92%          | 43                       | -                    | -                    | -                    | 713                      | 31                       |
| Liver Control |       | -            | -        | -            | -                        | -                    | -                    | -                    | -                        | 118                      | 9.6                      |

N = Normal, T = Tumor.

doi:10.1371/journal.pgen.1004135.t002
In addition to the variations identified in genes acting in EGFR and/or FGFR signaling pathways, we also report multiple sSNVs and copy number variations (CNVs) (Figure 4) in genes such as HDAC1, TP53, MDM2 and AKT1, acting in interaction networks or regulatory pathways involving the fusion partner genes in patients 5 (BICC1), and 6 (TACC3) (Table 4). Known mutations in BICC1 have been shown to disrupt canonical Wnt signaling [64] and genes, such as BCL9, involved in this pathway are known to regulate a range of biological processes such as transcription and cell proliferation and carry variations in patient 5 (Table 4). CSPG4, a target that is being investigated for antibody-based immunotherapy in preclinical studies of triple negative breast cancer [65], is involved in the Wnt signaling pathway, and carries variations in both patients 1 and 2, however, it is not mutated in patient 5. TACC3 is known to mediate central spindle assembly and multiple genes including CDCA8, BUB1, and TACC1, belonging to the TACC3 interaction network exhibit aberrant copy number in patient 6 (Table 4). A recent study has also implicated TACC3 in EGF-mediated EMT when overexpressed [64], and we find that the PLCG1, MAP2K1, and MAPK3 genes, Table 3. Comparison of mutation frequency in cholangiocarcinoma, pancreatic and liver cancers.

| Gene   | Non-liver fluke CCA (n = 6) | Liver fluke associated CCA [111] (n = 54) | CCA [40] (n = 62) | PDAC [112] (n = 142) | HCC [113] (n = 149) |
|--------|-----------------------------|-------------------------------------------|-------------------|----------------------|---------------------|
| AKT1   | 0%                          | 0%                                        | 1.6%              | 0%                   | 0%                  |
| APC    | 0%                          | 0%                                        | 0%                | 0%                   | 1.3%                |
| ARID2  | 0%                          | 0%                                        | NA                | 2.1%                 | 6.0%                |
| BAP1   | 16.7%                       | 0%                                        | NA                | 0%                   | 0%                  |
| BRAF   | 0%                          | 0%                                        | 1.6%              | 0.7%                 | 0%                  |
| CDKN2A | 0%                          | 5.6%                                      | NA                | 2.4%                 | 7.4%                |
| CSF2L4 | 33.3%                       | 0%                                        | NA                | 0%                   | 0.7%                |
| CTNNB1 | 0%                          | 0%                                        | NA                | 0%                   | 34.9%               |
| DMXL1  | 0%                          | 0%                                        | NA                | 0%                   | 0%                  |
| EGFR   | 0%                          | 0%                                        | 0%                | 0%                   | 0%                  |
| ERRFI1 | 16.7%                       | 0%                                        | NA                | 0%                   | 0%                  |
| FLT3   | 0%                          | 0%                                        | 0%                | 0%                   | 0%                  |
| GNAT   | 0%                          | 9.3%                                      | NA                | 0.7%                 | 0%                  |
| GRIN3A | 33.3%                       | 0%                                        | NA                | 0%                   | 0%                  |
| IDH1   | 0%                          | 13%                                       | NA                | 0%                   | 0%                  |
| IDH2   | 16.7%                       | 0%                                        | 2%                | 0%                   | 0%                  |
| JAK2   | 0%                          | 0%                                        | 0%                | 0%                   | 0%                  |
| KIT    | 0%                          | 0%                                        | 0%                | 0%                   | 0%                  |
| KRAS   | 0%                          | 16.7%                                     | NA                | 66.2%                | 1.3%                |
| LAMA2  | 16.7%                       | 3.7%                                      | NA                | 0%                   | 0%                  |
| MLL3   | 16.7%                       | 14.8%                                     | NA                | 4.9%                 | 0%                  |
| NDC80  | 0%                          | 3.7%                                      | NA                | 0%                   | 0%                  |
| NLRP1  | 16.7%                       | 0%                                        | NA                | 0%                   | 0%                  |
| NOTCH1 | 16.7%                       | 0%                                        | 0%                | 0%                   | 0%                  |
| NRAS   | 16.7%                       | 0%                                        | 3.2%              | 0%                   | 0%                  |
| PDCDA13| 16.7%                       | 3.7%                                      | NA                | 0.7%                 | 0%                  |
| PAK1   | 16.7%                       | 0%                                        | NA                | 0%                   | 0%                  |
| PEG1   | 0%                          | 5.6%                                      | NA                | 1.4%                 | 0%                  |
| PKDCA  | 0%                          | 0%                                        | 0%                | 0%                   | 1.3%                |
| PLXNB3 | 33.3%                       | 0%                                        | 0%                | 0%                   | 0%                  |
| PTEN   | 0%                          | 3.7%                                      | 2%                | 0%                   | 0%                  |
| PTK2   | 16.7%                       | 0%                                        | NA                | 0%                   | 0%                  |
| RADIL  | 0%                          | 3.7%                                      | NA                | 0%                   | 0%                  |
| RNF43  | 0%                          | 9.3%                                      | NA                | 0%                   | 0%                  |
| ROND2  | 0%                          | 9.3%                                      | NA                | 1.4%                 | 0%                  |
| SMAD4  | 0%                          | 16.7%                                     | NA                | 11.3%                | 0%                  |
| TP53   | 33.3%                       | 14.4%                                     | 8%                | 23.2%                | 19.5%               |
| XRIP2  | 0%                          | 5.6%                                      | NA                | 3.5%                 | 0%                  |

CCA, cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma; HCC, hepatocellular carcinoma; NA, not assessed. doi:10.1371/journal.pgen.1004135.t003
which act in both FGFR and EGFR regulatory pathways, exhibit CNV in patient 6. We also note that the DNAH5 gene encoding a dynein protein which is part of the microtubule- associated motor protein complex carries two G→C missense mutations in patient 6 (Table S1). Several genes carrying more than one variation in either the same patient or different patients also included genes with known roles similar to genes in FGFR/EGFR pathways including axon guidance, invasive growth, or cell differentiation (NA13, LAMC3, PLXNB3, and PTPRK) (Table S1). In the case of patient 4, our studies suggest that the primary effect of the FGFR2-MGEA5 fusion is on FGFR2 related signaling, since changes in expression were observed in FGF8 (p<0.05) and the genome of this patient also carries a 4-bp insertion (GTGT) in the FGFR4 gene (Table S1).

FGFR2-MGEA5 as a putative therapeutic target

Patient 4 is a 62 year-old white female found to have a left-sided intrahepatic mass with satellite lesions, with metastasis to regional lymph nodes (Table 5). A biopsy of the liver mass revealed the presence of a poorly differentiated adenocarcinoma that was consistent with intrahepatic cholangiocarcinoma (CK7+, CEA+, CK20+, Hep-par 1+, TTF-1-) (Table 6). She received gemcitabine and cisplatin and obtained clinical benefit in the form of stable disease for 6 months, followed by disease progression. She was re-treated with gemcitabine and capcetabine systemic therapy and attained stable disease for 6 months, followed by disease progression. A clinical trial of pegylated hyalurondisace (PEGPH20) produced only stable disease for 4 months, followed again by disease progression. At this juncture, she underwent a liver biopsy to obtain tissue for whole genome characterization of her tumor. She was found to have an FGFR2-MGEA5 fusion (Figure 2, Table 7, and ponatinib monotherapy was pursued as salvage treatment. Evaluation of pre-treatment immunohistochemistry demonstrated increased expression of FGFR2 and FGFR3 (Figure 5) and Clinical Laboratory Improvement Amendments (CLIA) validation by quantitative PCR revealed increased expression of FGFR3 (Table S5). In order to further validate the activation of the receptor, we conducted immunohistochemistry (IHC) of pFRS2 Y436 and pERK (MAPK) that revealed strong expression of pFRS2 Y436 and pERK (Figure 6), thus confirming activation of the receptor.

Ponatinib was initiated at 45 mg given orally on a daily schedule. Approximately 6 weeks after initiation of therapy she was noted to have anti-tumor activity that was characterized by central necrosis of a caudate liver lobe mass, shrinkage of metastatic lymph nodes involving the right cardiophrenic angle, central necrosis and shrinkage of a metastatic supracleavicular axis lymph node (Figure 7) and reduction in CA 19-9 from 1408 U/ml to 142 U/ml. Per RECIST criteria, she exhibited stable disease with a 14% decrease in the sum of largest diameters but with tumor necrosis and reduction in the CA19-9 tumor marker (39.8%). While the evidence is preliminary in nature, it was felt that the combination of tumor shrinkage not meeting the RECIST criteria definition of partial response, tumor necrosis and reduction in CA19-9 constituted preliminary evidence of anti-tumor activity. She has experienced no treatment related toxicities thus far and remains on therapy of approximately 3.5 months duration thus far.

The FGFR2 fusion partner observed in this patient, MGEA5, is an enzyme responsible for the removal of O-GlcNAc from proteins [66]. Interestingly, soft tissue tumors myxoinflammatory fibroblastic sarcoma (MIFS) and hemosiderotic fibrolipomatous tumor (HFLT) both share a translocation event resulting in rearrangements in TGFBR3 and MGEA5 [67,68]. Associated with this translocation event is the upregulation of NPM3 and FGF8 [68], of which both genes are upregulated in this patient (fold change: NPM3 = 6.17865, FGF8 = 1.79769e+308). In breast cancer, grade III tumors had significantly lower MGEA5 expression than grade I tumors with a trend of decreasing expression observed with increasing tumor grade [66]. In summary, MGEA5 may play an important role in carcinogenesis as an FGFR fusion partner.

FGFR2-TACC3 as a putative therapeutic target

Patient 6 is a 43 year-old white female who underwent a right salpingo-oophorectomy and endometrial ablation in the context of a ruptured ovarian cyst (Table 5). Postoperatively she developed dyspnea and was found to have pulmonary nodules as well as a 5 cm left sided liver mass. Pathological evaluation of the liver mass was consistent with a moderately differentiated intrahepatic cholangiocarcinoma (CK7+, CK20+, TTF-1-) in the absence of any known risk factors (Table 6). She was treated systemically with gemcitabine and cisplatin and had stable disease for approximately 6 months, but was subsequently found to have disease progression. She was treated with FOLFOX for 7 months and again attained stable disease as best response to therapy but eventually experienced disease progression. Upon disease progression, she was enrolled on a clinical study with the multi-kinase inhibitor pazopanib that is FDA-approved for the
treatment of advanced renal cancer or sarcoma – and fortuitously has nanomolar activity against FGFR2 \((\text{in vitro IC}_{50} \approx 350 \text{ nM})\) [69]. Transcriptome analysis revealed the presence of an FGFR2-TACC3 fusion (Table 7, Figure 2). Evaluation of post-pazopanib tissue by immunohistochemistry revealed increased expression of FGFR2 and FGFR3 (Figure 5). Further evaluation of phosphorylation of downstream targets FRS2 Y436, and ERK/MAPK revealed strong expression of pFRS2 Y436, confirming activation of the receptor. She had been treated with pazopanib 800 mg orally daily for 4 months and demonstrated tumor shrinkage, which in retrospect, was postulated to be secondary to the FGFR2 inhibitory activity of pazopanib (Figure 8A). By RECIST criteria v1.1, the patient had a partial response to therapy as evidenced by a 71% decrease in the sum of diameters. Subsequently, the same patient was treated with a dedicated pan-FGFR inhibitor, ponatinib, (45 mg daily orally; \(\text{in vitro IC}_{50} : \text{FGFR}1\approx24 \text{ nM}, \text{FGFR}2\approx8 \text{ nM}, \text{FGFR}3\approx8 \text{ nM} \text{and FGFR}4\approx34 \text{ nM})\). She again attained minor tumor shrinkage (stable disease by RECIST criteria v1.1, decrease of 4% in sum of largest diameters) in multiple lesions after 2 months of therapy, despite undergoing a 50% dose reduction for abdominal pain felt to be related to drug (Figure 8B). She remains on therapy approximately 4 months since the initiation of ponatinib. As such, anti-tumor activity was obtained with two distinct FGFR inhibitors in the same patient.

The FGFR2 fusion partner observed in this patient’s tumor, TACC3, is overexpressed in many tumor types with enhanced cell proliferation, migration, and transformation observed in cells overexpressing TACC3 [70]. Furthermore regulation of ERK and PI3K/AKT by TACC3 may contribute in part to epithelial-mesenchymal transition (EMT) in cancer [70], a significant contributor to carcinogenesis. Interestingly, TACC3 has been identified as a fusion partner to FGFR3 in bladder cancer, squamous cell lung cancer, oral cancer, head and neck cancer and glioblastoma multiforme [34].

ERRF1 as a putative therapeutic target

Patient 3 was a 50 year-old white male who presented with fevers and night sweats (Table 5). He was found to have a 4 cm tumor in his liver determined to be a poorly differentiated intrahepatic cholangiocarcinoma (CK7+, CK20−, TTF1−, CD56+, synatophysin−, Hep-par 1−) with sclerotic features (Table 6). No overt risk factors for cholangiocarcinoma were identified. A left hepatectomy was undertaken three months later. In addition to the primary tumor in segment 4, limited resections of segments 6 and 8 were undertaken to remove two tumor nodules. He was soon noted to have increased hypermetabolism in the left lower cervical, upper mediastinal and...
### Table 4. Stable fusion partner gene pathways.

| Patients | Gene in Interaction or Regulatory Network | Small-scale Variation (ssNV)/Copy Number Variation (CNV) | Associated Network | Associated Pathway |
|----------|------------------------------------------|----------------------------------------------------------|-------------------|-------------------|
| 4        | FGFR4                                     | ssNV                                                     | FGFR              | glucose homeostasis |
| 5        | RAF1                                      | CNV                                                      | EGFR/FGFR         | axon guidance      |
| 5        | RPS6KA5                                   | CNV                                                      | FGFR              | innate immune response |
| 5        | HGF                                       | CNV                                                      | FGFR              | mitosis            |
| 5        | FRS2                                      | CNV                                                      | FGFR              | ventricular septum development |
| 5        | FGFR2                                     | CNV                                                      | FGFR              | apoptotic process  |
| 5        | FGFR4                                     | CNV                                                      | FGFR              | glucose homeostasis |
| 5        | FGFR1OP2                                  | CNV                                                      | FGFR              | response to wounding |
| 5        | FGFR1                                     | CNV                                                      | FGFR              | transcription, DNA-dependent |
| 5        | ANTXR1                                    | CNV                                                      | BICC1             | actin cytoskeleton reorganization |
| 5        | ARL3                                      | CNV                                                      | BICC1             | cell cycle         |
| 5        | NXO3-1                                    | CNV                                                      | BICC1             | multicellular organismal development |
| 5        | WIF1                                      | CNV                                                      | BICC1             | multicellular organismal development |
| 5        | AXIN2                                     | CNV                                                      | BICC1             | negative regulation of cell proliferation |
| 5        | SFRP1                                     | CNV                                                      | BICC1             | negative regulation of cell proliferation |
| 5        | HDAC1                                     | CNV                                                      | BICC1             | negative regulation of transcription from RNA polymerase II promoter |
| 5        | HNF1A                                     | CNV                                                      | BICC1             | positive regulation of transcription, DNA-dependent |
| 5        | NRS2A                                     | CNV                                                      | BICC1             | positive regulation of transcription, DNA-dependent |
| 5        | IPO13                                     | CNV                                                      | BICC1             | protein import into nucleus |
| 5        | MAP3K7                                    | CNV                                                      | BICC1             | transcription, DNA-dependent |
| 5        | SLC6A20                                   | CNV                                                      | BICC1             | transmembrane transport |
| 5        | BTRC                                      | CNV                                                      | BICC1             | ubiquitin-dependent protein catabolic process |
| 5        | BCL9                                      | CNV                                                      | BICC1             | Wnt receptor signaling pathway |
| 5        | TP53                                      | ssNV                                                     | BICC1             | transcription, DNA-dependent |
| 5        | PLCG1                                     | CNV                                                      | EGFR/FGFR         | axon guidance      |
| 6        | MAP2K1                                    | CNV                                                      | EGFR/FGFR         | innate immune response |
| 6        | MAPK8                                     | CNV                                                      | EGFR/FGFR         | peptidyl-threonine phosphorylation |
| 6        | GAB1                                      | CNV                                                      | FGFR              | heart development  |
| 6        | ATF2                                      | CNV                                                      | FGFR              | innate immune response |
| 6        | MAPKAPK2                                  | CNV                                                      | FGFR              | innate immune response |
| 6        | RPS6KA5                                   | CNV                                                      | FGFR              | innate immune response |
| 6        | HGF                                       | CNV                                                      | FGFR              | mitosis            |
| 6        | FRS2                                      | CNV                                                      | FGFR              | ventricular septum development |
| 6        | FGFR2                                     | CNV                                                      | FGFR              | apoptotic process  |
| 6        | FGFR4                                     | CNV                                                      | FGFR              | glucose homeostasis |
| 6        | FGF17                                     | CNV                                                      | FGFR              | positive regulation of cell proliferation |
| 6        | FGF18                                     | CNV                                                      | FGFR              | positive regulation of cell proliferation |
| 6        | FGF20                                     | CNV                                                      | FGFR              | positive regulation of cell proliferation |
| 6        | FGFR1OP                                   | CNV                                                      | FGFR              | positive regulation of cell proliferation |
| 6        | FGFR1                                     | CNV                                                      | FGFR              | transcription, DNA-dependent |
| 6        | MDM2                                      | CNV                                                      | TACC3             | protein ubiquitination |
| 6        | E2F2                                      | CNV                                                      | TACC3             | apoptotic process  |
| 6        | GADD45A                                    | CNV                                                      | TACC3             | apoptotic process  |
| 6        | HMGB2                                     | CNV                                                      | TACC3             | apoptotic process  |
| 6        | RHOA                                      | CNV                                                      | TACC3             | axon guidance      |
| 6        | PEBP1                                     | CNV                                                      | TACC3             | brain development  |
abdomino-retroperitoneal lymph nodes related to metastatic disease from his cholangiocarcinoma. He was treated with gemcitabine and cisplatin for 9 months and obtained stable disease as his best response, followed by eventual progression. He received treatment with pegylated hyaluronidase (PEGPH20) in the setting of an investigational study for one month and had no response to therapy. A biopsy of a left supraclavicular lymph node was obtained two months prior to the initiation of PEGPH20 in the context of a clinical study employing whole genome analysis for putative therapeutic target selection.

Since our study goal was to identify potential therapeutically relevant events, the novel loss of function mutation in ERRFI1 (E384X) detected in Patient 3’s metastatic, recurrent/refractory SIC (Table S1) warranted additional examination. Specifically, the allelic fraction of the DNA mutation constituted only 11% of the sequencing reads, is consistent with tissue heterogeneity, and constituted 78% of the sequencing reads within the RNASeq data. Such allele specific expression of the mutated allele from the same tissue specimen suggests nearly complete loss of function of ERRFI1 in this patient’s tumor. Notably, the patient’s tumor did not harbor any mutations or amplifications in other EGFR signaling members such as EGFR and BRAF.

Upon availability of CLIA validated sequencing data (Table S5), the patient was treated with erlotinib 150 mg orally/daily. After 3 months, RECIST v1.1 partial response evidenced by a decrease of 58% in the sum of largest diameters was observed (Figure 9). Evaluation of pretreatment tumor tissue by immuno-histochemistry revealed increased expression of EGFR pathway members (Figure 10).

**Discussion**

Integrated analysis of sporadic intrahepatic cholangiocarcinoma (SIC) genomic and transcriptomic data led to the discovery of FGFR2 fusion products in three of six assessed patients (Table 7, Figures 4 and 11). Members of the FGFR family (FGFR1-4) have been associated with mutations, amplifications and translocation events with oncogenic potential [71]. FGFR fusions with oncogenic activity have been previously identified in bladder cancer (FGFR3) [72], lymphoma (FGFR1 and FGFR3) [73,74], acute myeloid leukemia (FGFR1) [75], multiple myeloma [76], myeloproliferative neoplasms [77], and most recently glioblastoma multiforme (FGFR1 and FGFR3) [78]. FGFR2, FGFR3 and FGFR4 have been found to be overexpressed in IDH1/IDH2 mutant biliary cancers [79], a context seen within Patient 1 in our study.

| Patients | Gene in Interaction or Regulatory Network | Small-scale Variation (sSNV)/Copy Number Variation (CNV) | Associated Network | Associated Pathway |
|----------|------------------------------------------|----------------------------------------------------------|--------------------|-------------------|
| 6        | EVI5                                     | CNV                                                      | TACC3              | cell cycle        |
| 6        | CDC68                                    | CNV                                                      | TACC3              | cell division     |
| 6        | CKAP5                                    | CNV                                                      | TACC3              | cell division     |
| 6        | PPP1CC                                   | CNV                                                      | TACC3              | cell division     |
| 6        | BUB1                                     | CNV                                                      | TACC3              | cell proliferation|
| 6        | GTSE1                                    | CNV                                                      | TACC3              | DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest |
| 6        | TACC1                                    | CNV                                                      | TACC3              | microtubule cytoskeleton organization |
| 6        | KIF20A                                   | CNV                                                      | TACC3              | microtubule-based movement |
| 6        | KIF2C                                    | CNV                                                      | TACC3              | microtubule-based movement |
| 6        | NCPH                                     | CNV                                                      | TACC3              | mitosis           |
| 6        | NSUN2                                    | CNV                                                      | TACC3              | mitosis           |
| 6        | AKAP9                                    | CNV                                                      | TACC3              | mitotic cell cycle |
| 6        | KIF23                                    | CNV                                                      | TACC3              | mitotic cell cycle |
| 6        | MCM9                                     | CNV                                                      | TACC3              | mitotic cell cycle |
| 6        | NPM1                                     | CNV                                                      | TACC3              | negative regulation of cell proliferation |
| 6        | CEBP5                                    | CNV                                                      | TACC3              | negative regulation of transcription, DNA-dependent |
| 6        | MKI67                                    | CNV                                                      | TACC3              | organ regeneration |
| 6        | AURKAIP1                                 | CNV                                                      | TACC3              | positive regulation of proteolysis |
| 6        | AKT1                                     | CNV                                                      | TACC3              | protein ubiquitination |
| 6        | BRCAl                                    | CNV                                                      | TACC3              | protein ubiquitination |
| 6        | KLHL13                                   | CNV                                                      | TACC3              | protein ubiquitination |
| 6        | KLHL9                                    | CNV                                                      | TACC3              | protein ubiquitination |
| 6        | TTF2                                     | CNV                                                      | TACC3              | regulation of transcription, DNA-dependent |
| 6        | RACGAP1                                  | CNV                                                      | TACC3              | signal transduction |
| 6        | TDRD7                                    | CNV                                                      | TACC3              | spermatogenesis    |
| 6        | PRKACA                                   | CNV                                                      | TACC3              | transmembrane transport |
Table 5. Clinical characteristics of 6 advanced, sporadic biliary tract cancer patients.

|                        | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Age (years)            | 64        | 66        | 50        | 62        | 50        | 43        |
| Gender                 | F         | M         | M         | F         | F         | F         |
| Location of Primary Tumor | Intrahepatic | Intrahepatic/ Gallbladder | Intrahepatic | Intrahepatic | Intrahepatic | Intrahepatic |
| Stage                  | III       | IV        | IV        | IV        | IV        | IV        |
| CA19-9 (Units/ml)      | WNL       | 1008      | WNL*      | N/A       | 56        |
| Sites of Metastasis    | Abdominal Lymph Nodes | Cervical/Thoracic, Abdominal, Pelvic Lymph Nodes | Abdominal, Pelvic Lymph Nodes, Liver | Abdominal, Pelvic Lymph Nodes, Liver | Abdominal, Pelvic Lymph Nodes, Liver | Abdominal, Pelvic Lymph Nodes, Liver |
| Underlying Etiology    | Unknown   | Unknown   | Unknown   | Unknown   | Unknown   | Unknown   |
| Liver fluke            | No        | No        | No        | No        | No        | No        |
| Hepatitis B            | Unknown   | Unknown   | Negative  | Unknown   | Unknown   | Unknown   |
| Hepatitis C            | Unknown   | Unknown   | Negative  | Unknown   | Unknown   | Unknown   |
| Prior Surgical Resection | No       | Yes       | No        | Yes       | No        | No        |
| Prior Radiation Therapy | No        | No        | No        | No        | No        | No        |
| Systemic Chemotherapy  | Gem/Cis   | Gem/Cis, Capecitabine | Gem/Cis | Gem/Cis, Gem/Cape, PEGPH20 | Gem/Cis, 5-FU/Carbo | Gem/Cis, FOLFOX, Pazopanib |
| Survival Status        | Alive     | Dead      | Dead      | Alive     | Dead      | Alive     |
| Survival Duration from biopsy (months) | 14.5+ | 8.8 | 9.0 | 9.3+ | 4.1 | 5.5+ |

F = female; M = male; WNL = Within Normal Limits; Gem/Cis = Gemcitabine and Cisplatin; Gem/Cape = Gemcitabine and Capecitabine; PEGPH20 = pegylated hyaluronidase; 5-FU/Carbo = 5-Fluorouracil and Carboplatin; FOLFOX = 5-FU, Leucovorin and Oxaliplatin.

* = WNL at baseline but 1408 U/ml prior to therapy and N/A = Not Available.

doi:10.1371/journal.pgen.1004135.t005
Importantly, one critical study has shown that FGFR carboxy-terminal deletion mutants induce ligand-independent transformation and clonogenic growth [84]. This is important because all of the fusion events within our study would lead to loss of the carboxy-terminus of FGFR2. Furthermore, a very recent study that described FGFR fusions in solid tumors illustrated that FGFR fusion partners in SIC resulted in dimerization domains, and suggested that activation occurred through ligand independent dimerization and oligomerization [54]. It is likely that both loss of the carboxy terminus and the addition of dimerization domains leads to oncogenic FGFR2 activity in these tumors.

Comparative pathway analysis of genes carrying mutations/aberrant in copy number identified additional potential therapeutic targets belonging to, or intimately integrated with, the EGFR and FGFR signaling pathways (Figure 3, Tables S2, S3, S4). Interestingly, most of these pathways also have known roles in mediating epithelial-to-mesenchymal cell transitions, which occur frequently during development as well as during tumorigenesis [60]. Patients 3 and 4 harbored aberrations in several genes acting in cadherin signaling pathways (Tables S3, S4), which are important for maintaining cell-cell adhesion [63].

The preliminary anti-tumor activity noted in a patient with FGFR2-MGEA5 (Patient 4) and FGFR2-TACC3 fusion (Patient 6) represent the first reports of application of FGFR inhibitors to the treatment of patients with cholangiocarcinoma harboring these alterations. These results suggest that oncogenic activation of FGFR2 represent a potential therapeutically actionable event. The FGFR tyrosine kinase inhibitors (TKI) dovitinib [85] and NVP-BGJ398 [86] are currently in clinical development and the FGFR TKI ponatinib [75,87] was recently approved by the FDA for use in treating T315I mutant chronic myelogenous leukemia. FGF7 (keratinocyte growth factor) has been previously linked to poor

### Table 6. Pathological characteristics of 6 advanced, sporadic biliary tract cancer patients.

| Grade/differentiation | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Biopsy Procedure       | U/S Guided Liver Biopsy | U/S Guided Liver Biopsy | Excisional Biopsy Lymph Node | U/S Guided Liver Biopsy | U/S Guided Liver Biopsy | Excisional Lung Biopsy |
| %Necrosis ( aliquots)  | 5 (1)     | 0 (2)     | 0–35 (7)  | 0 (3)     | 0–5 (3)   | 0         |
| %Tumor                 | 50        | 10–20     | 25–75     | 0–20      | 40–50     | 30        |
| %Stroma and normal elements | 50        | 80–90     | 25–75     | 80–100    | 50–60     | 70        |
| Histological Type      | NST*      | NST       | NST       | NST       | NST       | NST       |
| Clear Cell Histology (Yes/No) | No        | No        | No        | No        | No        | No        |

**U/S = Ultrasound.**

**NST: No special type.**

**Rare gland formation with expression of cytokeratin, polyclonal CEA, and MOC-31.**

All were adenocarcinomas of no special types and high grades as defined by the World Health Organization Classification of Tumors of the Digestive System (Lyon 2000). Degree of differentiation is based on the percentage of glands (defined as having visible lumens by visual estimate) as follow: 95% or more glands-well differentiated, 40–94% glands-moderately differentiated, 5–39% glands-poorly differentiated, <5% glands-undifferentiated.

doi:10.1371/journal.pgen.1004135.t006

### Table 7. Fusion events.

| Gene1                   | Gene2 | Gene1 break location | Gene2 break location | Predicted Reciprocal Translocation | Patient |
|-------------------------|-------|----------------------|----------------------|-----------------------------------|---------|
| FGFR2                   | MGEA5 | chr10:123243211      | chr10:103552699      | No                                | 4       |
| FGFR2                   | BICC1 | chr10:123237843      | chr10:603800614      | Yes                               | 5       |
| BICC1                   | FGFR2 | chr10:60227900       | chr10:123237848      | Yes                               | 5       |
| FGFR2                   | TACC3 | chr10:123243211      | chr4:1741428         | No                                | 6       |

doi:10.1371/journal.pgen.1004135.t007
Figure 5. Immunohistochemistry demonstrating FGFR2 and FGFR3 expression. A) Tumor stained with FGFR2 antibody. Patient 1 demonstrates moderate cytoplasmic positivity (solid arrows); background fibro-inflammatory tissue is negative (empty arrows). Patient 2 demonstrates moderate cytoplasmic expression for FGFR2; tumor nuclei are negative. Patient 3 demonstrates tumor cells with negative nuclear and weak cytoplasmic expression of FGFR2 (solid arrows) with cells demonstrating moderate basolateral or complete membranous staining as well. Patient 4 demonstrates weak/moderate cytoplasmic positivity with multi-focal weak/moderate membranous expression (solid arrows); background fibro-inflammatory tissue demonstrates negative/weak staining (empty arrows). Patient 5 demonstrates weak/moderate cytoplasmic positivity with multi-focal moderate/strong membranous expression (solid arrows); background fibro-inflammatory tissue is negative (empty arrows). Patient 6 demonstrates moderate/strong cytoplasmic positivity (solid arrows); background lymphocytes are negative (empty arrows).

B) Tumor stained with FGFR3 antibody. Patient 1 demonstrates strong cytoplasmic positivity, variable nuclear expression and occasional moderate/strong membranous expression (solid arrows); background fibrous tissue is negative (empty arrows). Patient 2 demonstrates negatively staining background neutrophils (focally intraepithelial-far right) (empty arrows) and tumor cells with strong nuclear expression and moderate cytoplasmic positivity (solid arrows). Patient 3 demonstrates negatively staining background inflammation (empty arrows) and tumor cells with weak nuclear expression and moderate cytoplasmic positivity (solid arrows). Patient 4 demonstrates weak/moderate cytoplasmic positivity and variable nuclear expression; background fibro-inflammatory tissue demonstrates negative/weak positivity (empty arrows). Patient 5 demonstrates moderate cytoplasmic positivity, variable nuclear expression and strong multi-focal membranous expression (solid arrows); background fibrous tissue is negative. Patient 6 demonstrates diffuse/moderate/strong cytoplasmic and membranous positivity and variable nuclear expression (solid arrows); background lymphocytes are negative (empty arrows).

doi:10.1371/journal.pgen.1004135.g005
Figure 6. Immunohistochemistry demonstrating pFRS2 Y436, and pERK expression in Patients 1, 4, 5 and 6. A) Tumor stained with pFRS2 Y436 antibody. Patient 1 tumor cells demonstrating both strong cytoplasmic and nuclear expression of pFRS2 (solid arrows); background fibrous stroma is negative (empty arrows). Patient 4 tumor cells show strong nuclear expression and moderate to strong cytoplasmic positivity (solid arrows); occasional background fibrous stromal cells are negative for pFRS2 (empty arrows) and scattered tumor cells show basolateral/membranous staining as well (white arrows). Patient 5 tumor cells show intensely strong expression in both nuclei and cytoplasm (solid arrows); scattered background fibrous stromal cells are negative (empty arrows). Patient 6 tumor cells show negative nuclear expression of pFRS2, moderate cytoplasmic expression and basolateral or membranous expression of varying intensity (solid arrows); background fibrous stromal cells are negative (empty arrows). B) Tumor stained with pERK(MAPK) antibody. Patient 1 demonstrates negative/weak fibrous stroma (empty arrows) and tumor cells with strong nuclear and cytoplasmic expression (solid arrows). Patient 4 demonstrates negative inflammatory background (empty arrows) tumor cells with variable negative to strong nuclear expression and moderate to strong cytoplasmic positivity (solid arrows). Patient 5 demonstrates negative/weak fibrous stroma (empty arrows) and tumor cells with strong nuclear and cytoplasmic expression (solid arrows). Patient 6 demonstrates negative background lymphocytes/mononuclear inflammatory cells (empty arrows) and tumor cells with strong nuclear and cytoplasmic expression (solid arrows).
FDA-approved EGFR and FGFR tyrosine kinase inhibitors. Antibodies specific to FGFR2-IIIb have also shown preclinical efficacy and may serve as an additional platform for therapeutic development in this context [104]. Additional studies to characterize the prevalence of these aberrations in both sporadic and liver fluke associated BTC will need to be conducted. Nevertheless, our results suggest that prospective clinical studies designed to treat patient’s tumors harboring these novel genomic aberrations utilizing targeted agents on an individualized basis should be pursued more fully through larger clinical studies in order to explore the precise extent of clinical benefit that this tailored approach may have in patients with primary or advanced BTC.

Additionally, post-treatment biopsies to assess pathway downregulation in patients 4 and 6 (treated with FGFR inhibitors) and patient 3 (treated with EGFR inhibitor) are not available, as the treatment was not conducted in the setting of a protocol that would allow for the collection of additional research biopsies. Incorporation of post-treatment biopsies in carefully designed prospective studies will be critical towards defining the association between the use of FGFR and EGFR inhibitors in appropriately selected patients with relevant genomic aberrations.

Figure 7. Anti-tumor activity in Patient 4 harboring an FGFR2-MGEA5 fusion, to FGFR inhibitors. A) CT images of patient 4, whose tumor possessed an FGFR2-MGEA5 fusion, at baseline and 6 weeks demonstrate central necrosis of a caudate liver lobe mass (left arrow), 2.6 cm at baseline and 6 weeks, and shrinkage of a metastatic suprarenal axis lymph node (right arrow), 3.1 cm and 2.9 cm at baseline and 6 weeks respectively. B) CT images of patient 4 showing shrinkage of metastatic lymph nodes involving the right cardiophrenic angle (red circles), 1.3 cm and 0.5 cm at baseline and 6 weeks respectively.

Figure 8. Anti-tumor activity in Patient 6, harboring an FGFR2-TACC3 fusion, to FGFR inhibitors. A) CT images of patient 6, whose tumor possessed an FGFR2-TACC3 fusion, at baseline and after four months of pazopanib demonstrate significant tumor shrinkage (red arrows), 10.8 mm and 3.1 mm respectively. B) CT images of patient 6 at baseline and two months demonstrate significant tumor shrinkage (red arrows), 41.1 mm and 39.4 mm respectively after subsequent ponatinib treatment, 45 mg/daily, was begun.

Materials and Methods

Ethics statement and sample collection
Clinical information was assimilated from patient records from the Mayo Clinic. Informed consent was obtained for each patient on two ongoing research protocols approved by the Mayo Clinic Institutional Review Board (10-006180 and 10-002879). Clinico-pathological features collected included age, gender, stage, histological grade, sites of metastasis, tumor sample assessment for overall cellularity/necrosis and percent tumor cellularity, prior therapies and risk factors (hepatitis B and C, Caroli’s disease, obesity, hepatolithiasis and cholelithiasis, primary sclerosing cholangitis, thorotrast exposure and H. pylori, H. bilis, S. typhi and S. paratyphi infections). All patients were known to not have had prior exposure to liver flukes that have been implicated in biliary carcinogenesis (O. viverrini and C. sinensis). Tissue specimens were collected fresh frozen and maintained below −80°C until nucleic acid extraction. A board certified pathologist who is experienced in biospecimen studies, evaluated a portion of each specimen to confirm the presence of tumor, the degree of necrosis and the percent cellularity.

Whole genome sequencing

Patients 1, 3, 4, and 5. 1.1 µg genomic DNA was used to generate separate long insert whole genome libraries for each
sample using Illumina’s (San Diego, CA) TruSeq DNA Sample Prep Kit (catalog# FC-121-2001). In summary, genomic DNAs are fragmented to a target size of 900–1000 bp on the Covaris E210. 100 ng of the sample was run on a 1% TAE gel to verify fragmentation. Samples were end repaired and purified with Ampure XP beads using a 1:1 bead volume to sample volume ratio, and ligated with indexed adapters. Samples are size selected at approximately 1000 bp by running samples on a 1.5% TAE gel and purified using Bio-Rad Freeze ‘n Squeeze columns and Ampure XP beads. Size selected products are then amplified using PCR and products were cleaned using Ampure XP beads.

**Patient 2.** 300 ng genomic tumor and normal DNA was used to create whole genome libraries. Samples were fragmented on the Covaris E210 to a target size of 200–300 bp and 50 ng of the fragmented product was run on a 2% TAE gel to verify fragmentation. Whole genome libraries were prepared using Illumina’s TruSeq DNA Sample Prep Kit.

**Exome sequencing**

**Patients 1 and 3.** 1.1 μg genomic DNA for each sample was fragmented to a target size of 150–200 bp on the Covaris E210. 100 ng of the fragmented product was run on TAE gel to verify fragmentation. The remaining 1 μg of fragmented DNA was prepared using Agilent’s SureSelectXT and SureSelectXT Human All Exon 50 Mb kit (catalog# G7544C).

**Patient 2.** 50 ng genomic tumor and normal DNA was used to create exome libraries using Illumina’s Nextera Exome Enrichment kit (catalog# FC-121-1204) following the manufacturer’s protocol.

**Patients 4 and 5.** 1 μg of each tumor and germline DNA sample was used to generate separate exome libraries. Libraries were prepared using Illumina’s TruSeq DNA Sample Prep Kit and Exome Enrichment Kit (catalog# FC-121-1008) following the manufacturer’s protocols.

**Patient 6.** 3 μg of genomic tumor and normal DNA was fragmented on the Covaris E210 to a target size of 150–200 bp. Exome libraries were prepared with Agilent’s (Santa Clara, CA) SureSelectXT Human All Exon V4 library preparation kit (catalog# 5190-4632) and SureSelectXT Human All Exon V4+ UTRs (catalog# 5190-4637) following the manufacturer’s protocols.

**RNA sequencing**

**Patients 1, 2 and 3.** 50 ng total RNA was used to generate whole transcriptome libraries for RNA sequencing. Using the Nugen Ovation RNA-Seq System v2 (catalog# 7102), total RNA was used to generate double stranded cDNA, which was subsequently amplified using Nugen’s SPIA linear amplification process. Amplified products were cleaned using Qiagen’s QIAquick PCR Purification Kit and quantitated using Invitrogen’s Quant-iT Picogreen. 1 μg of amplified cDNA was fragmented on the Covaris E210 to a target size of 300 bp. Illumina’s TruSeq DNA Sample Preparation Kit was used to prepare libraries from 1 μg amplified cDNA.

**Patients 4, 5 and 6.** 1 μg of total RNA for each sample was used to generate RNA sequencing libraries using Illumina’s TruSeq RNA Sample Prep Kit V2 (catalog# RS-122-2001) following the manufacturer’s protocol.
Paired end sequencing

Libraries with a 1% phiX spike-in were used to generate clusters on HiSeq Paired End v3 flowcells on the Illumina cBot using Illumina’s TruSeq PE Cluster Kit v3 (catalog #PE-401-3001). Clustered flowcells were sequenced by synthesis on the Illumina HiSeq 2000 using paired-end technology and Illumina’s TruSeq SBS Kit.

Alignment and variant calling

Whole genome and whole exome. For whole genome and exome sequencing fastq files were aligned with BWA 0.6.2 to GRCh37.62 and the SAM output were converted to a sorted BAM file using SAMtools 0.1.18. BAM files were then processed through indel realignment, mark duplicates, and recalibration steps in this order with GATK 1.5 where dupsnp135 was used for known SNPs and 1000 Genomes’ ALL.wgs.low_coverage_vqsr.20101123 was used for known indels. Lane level sample BAMs were then merged with Picard 1.65 if they were sequenced across multiple lanes. Comparative variant calling for exome data was conducted with Seurat [105].

Previously described copy number and translocation detection were applied to the whole genome long-insert sequencing data [59] and these are made available through https://github.com/davcraig75/tgen_somaticSV. Copy number detection was based on a log2 comparison of normalized physical coverage (or clonal coverage) across tumor and normal whole genome long-insert sequencing data, where physical coverage was calculated by considering the entire region a paired-end fragment spans on the genome, then the coverage at 100 bp intervals was kept. Normal and tumor physical coverage was then normalized, smoothed and filtered for highly repetitive regions prior to calculating the log2 comparison. Translocation detection was based on discordant read evidence in the tumor whole genome sequencing data compared to its corresponding normal data. In order for the structural variant to be called there needs to be greater than 7 read pairs mapping to both sides of the breakpoint. The unique feature of the long-insert whole-genome sequencing was the long overall fragment size (~1 kb), where by two 100 bp reads flank a region of ~800 bp. The separation of forward and reverse reads increases the overall probability that the read pairs do not cross the breakpoint and confound mapping.

RNA. For RNA sequencing, lane level fastq files were appended together if they were across multiple lanes. These fastq files were then aligned with TopHat 2.0.6 to GRCh37.62 using ensembl63.genes.gtf as GTF file. Changes in transcript expression were calculated with Cuffdiff 2.0.2. For novel fusion discovery reads were aligned with TopHat-Fusion 2.0.6 [106] (patients 2, 3, 4 and 6). In addition, Chimerascan 0.4.5 [107] was used to detect

Figure 10. Immunohistochemistry of Patient 3’s tumor demonstrating activation of the EGFR pathway. A) Tumor stained with panAKT demonstrating diffuse cytoplasmic positivity with negative background lymphocytes (empty arrows). B) Tumor stained with pAKT demonstrating diffuse membranous staining and negative cytoplasmic expression; scattered background inflammatory cells showing strong cytoplasmic staining (empty arrows). C) Tumor stained with EGFR. Tumor cells are EGFR negative with background lymphocytes also negative (empty arrows). D) Tumor stained with pEGFR showing membranous positivity (solid arrows) with negative background lymphocytes (empty arrows). E) Tumor stained with MAPK/ERK1/2 demonstrating moderate to strong cytoplasmic staining of total MAPK with negative background lymphocytes (empty arrows). F) Tumor stained with pMAPK/pERK demonstrating increased expression compared to the negative background lymphocytes (empty arrows).

doi:10.1371/journal.pgen.1004135.g010
Figure 11. FGFR2-IIIb fusion events. Transcripts and hypothetical protein products are modeled to illustrate the potential functional impact of fusion events involving FGFR2 (A–C). The identified fusion events involving MGEA5 (patient 4) (A) and BICC1 (patient 5, reciprocal event) (C) are chromosome 10 intrachromosomal (D). In addition, patient 6 carried an interchromosomal fusion event (D) involving FGFR2 and TACC3 (B). The FGFR2 gene encodes for several isoforms with eleven representative transcripts and patients 4, 5, and 6 carry fusions involving the epithelial cell specific transcript isoform (FGFR2-IIIb). All identified fusion breakpoints are close in proximity and are predicted to occur within the last intron of the transcript and terminal to a known protein tyrosine kinase domain (A–C, gold domain). Predicted “Other” sites for all of the fusion protein models are the same and include the following: Casein kinase II phosphorylation sites, N-glycosylation sites, Protein kinase C phosphorylation sites, N-myristoylation sites, Tyrosine kinase phosphorylation sites, and cAMP-/cGMP-dependent protein kinase phosphorylation sites (A–C, grey triangle annotations). In all cases, fusions result in a predicted expansion of Casein kinase II phosphorylation and Protein kinase C phosphorylation sites. A protein product model is shown only for one of the reciprocal events involving the FGFR2 and BICC1 genes (FGFR2–BICC1, C). The fusion breakpoints of the reciprocal events effect Exons 1 and 2 of the BICC1 gene, which translates to a difference of a predicted phosphoserine site within the Casein kinase II phosphorylation region (C, purple triangle within red circle). The FGFR2 gene is located within a fragile site region (FRA10F) and is flanked by two ribosomal protein pseudogenes, RPS15AP5 and RPL19P16 (see D inset (*)), whose repetitive sequence content may also contribute to genomic instability at the FGFR2 initiation site.

doi:10.1371/journal.pgen.1004135.g011

Table 8. DNA and RNA validation of FGFR2 fusions in 3 patients with advanced sporadic biliary tract cancer.

| Fusion         | Annealing site | PCR input | Direction | Primer sequence             |
|----------------|----------------|-----------|-----------|-----------------------------|
| FGFR2-MGEA5    | FGFR2          | gDNA      | F         | 5’-CTGACTATAACCACGTCACC-3’  |
|                | MGEA5          | gDNA      | R         | 5’-AGGGAGAAATTAAAGAACTTGG-3’|
| FGFR2          | cDNA           | F         |           | 5’-TGATGATAGAGGAGCTTGG-3’   |
| MGEA5          | cDNA           | R         |           | 5’-GAGTTCTGTGTCACATTG-3’    |
| FGFR2-BICC1    | FGFR2          | gDNA      | F         | 5’-GGCAAGAAAGAAGTGGG-3’     |
| BICC1          | cDNA           | R         |           | 5’-ACTACTGAGTTGCCCAT-3’     |
| FGFR2          | cDNA           | F         |           | 5’-TGATGATAGAGGAGCTTGG-3’   |
| BICC1          | cDNA           | R         |           | 5’-GTGGATCTCAGGAATAAG-3’    |
| BICC1-FGFR2    | BICC1          | cDNA      | F         | 5’-CTGGAAGAGGAAGAAT-3’      |
| FGFR2          | cDNA           | R         |           | 5’-GTGGATCTAGGGAAG-3’       |
| FGFR2-TACC3    | FGFR2          | cDNA      | F         | 5’-TGACACCCCTAATCTAGTGC-3’  |
| TACC3          | gDNA           | R         |           | 5’-AACCTGCCATTGACCTCC-3’    |

F - forward, R - reverse.
doi:10.1371/journal.pgen.1004135.t008
Supporting Information

Table S1 Somatic point mutations, insertions and deletions identified in all samples. (DOCX)

Table S2 Gene Ontology (GO) functional classification of genes carrying Smallscale Nucleotide Variations (SnNVs) or in regions exhibiting Copy Number Variation (CNV). (XLS)

Table S3 Many genes carrying SnNVs act in key Cancer-associated pathways, and are differentially expressed in tumors from 6 patients with advanced sporadic biliary tract cancer. (XLS)

Table S4 Many genes identified in genomic regions exhibiting Copy Number Variation (CNV) functionally rank by significance to known Cancer-associated pathways. (XLS)

Table S5 CLIA validation of somatic mutations with therapeutic relevance in 6 patients with advanced, sporadic biliary tract cancer. (DOCX)

Table S6 Differential gene expression of fibroblast growth factor receptor pathway family members in 6 patients with advanced sporadic biliary tract cancer. (DOCX)

Text S1 Supplementary discussion. (DOCX)

Author Contributions
Conceived and designed the experiments: MJB AHB AKS RF DWC JDC DVH. Performed the experiments: WSL KH JAd RMC IC RR LP JM JAd. Conceived and designed the experiments: MJB AHB AKS RF DWC JDC DVH. Performed the experiments: WSL KH JAd RMC IC RR LP JM JAd. Analyzed the data: MDC JBE GRO DVH. Performed the experiments: WSL KH JAd RMC IC RR LP JM JAd. Wrote the paper: MJB MDC JBE JDC DWC.

References

1. Shin HR, Lee CU, Park JH, Seol SY, Chung JM, et al. (1996) Hepatitis B and C virus, Cronorhia sinensis for the risk of liver cancer: a case-control study in Pusan, Korea. International journal of epidemiology 25: 933–940.

2. Watanapa P (1996) Cholangiocarcinoma in patients with opisthorchiasis. The British journal of surgery 83: 1062–1064.

3. Watanapa P, Watanapa W (2002) Liver fluke-associated cholangiocarcinoma. The British journal of surgery 89: 962–970.

4. Bergquist A, Elkhom A, Olsson R, Kornfelt D, Loof L, et al. (2002) Hepatic and extrahepatic malignancies in primary sclerosing cholangitis. Journal of hepatology 36: 321–327.

5. Bergquist A, Glaumann H, Persson B, Broome U (1998) Risk factors and clinical presentation of hepatobiliary carcinoma in patients with primary sclerosing cholangitis: a case-control study. Hepatology 27: 311–316.

6. Burak K, Angulo P, Pasha TM, Egan K, Pena J, et al. (2004) Incidence and risk factors for cholangiocarcinoma in primary sclerosing cholangitis. The American journal of gastroenterology 99: 525–526.

7. Claessen MM, Vlieggar F, Tysgat KM, Siersema PD, van Buuren HR (2009) High lifetime risk of cancer in primary sclerosing cholangitis. Journal of hepatology 50: 130–164.

8. Visser BC, Suh I, Way LW, Kang SM (2004) Congenital choledochal cysts in adults. Archives of surgery 139: 855–860; discussion 860–852.

9. Huang AW, Zhang M, Rashad A, McGlynn KA, Wang BS, et al. (2008) Hepatitis B and C virus infection and the risk of biliary tract cancer: a population-based study in China. International journal of cancer journal international du cancer 122: 1849–1853.

10. Kobayashi I, Ikeda K, Saitoh S, Suzuki F, Tsukuba A, et al. (2002) Incidence of primary cholangiocellular carcinoma of the liver in Japanese patients with hepatitis C virus-related cirrhosis. Cancer 88: 2471–2477.

11. Liu XF, Zhou SQ, Qin FZ (2003) Pathogenesis of cholangiocarcinoma in the porta hepatis and infection of hepatitis virus. Hepatobiliary & pancreatic diseases international : HBPD INT 2: 205–209.

12. Shaib YH, El-Serbag HB, Davila JA, Morgan R, McGlynn KA (2005) Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. Gastroenterology 128: 620–626.

13. Welzel TM, Graubard BI, El-Serbag HB, Shaib YH, Hsing AW, et al. (2007) Risk factors for intrahepatic and extrahepatic cholangiocarcinoma in the United States: a population-based case-control study. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 13: 1221–1228.

14. Yamamoto S, Kubo S, Sai H, Ueno T, Yamamoto T, et al. (2004) Hepatitis C virus infection as a likely etiology of intrahepatic cholangiocarcinoma. Cancer science 95: 592–595.

15. Donato F, Gelatti U, Tagger A, Farret M, Riberi ML, et al. (2001) Intrahepatic cholangiocarcinoma and hepatitis B and C virus infection, alcohol intake, and hepatolithiasis: a case-control study in Italy. Cancer causes & control : CCC 12: 959–964.

16. Lee CC, Wu CY, Chen GH (2002) What is the impact of coexistence of hepatolithiasis on cholangiocarcinoma? Journal of gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 17: 1015–1020.

17. Becker N, Liebermann D, Wesch H, Van Kaick G (2008) Mortality among Thorotrast-exposed patients and an unexposed comparison group in the German Thorotrast study. European journal of cancer 44: 1259–1268.

18. Travis LB, Hauptmann M, Gaul LK, Storm HH, Goldman MB, et al. (2003) Site-specific cancer incidence and mortality after cerebral angiography with radioactive thorotrast. Radiation research 160: 691–706.

19. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD (2005) Cholangiocarcinoma. Lancet 366: 1303–1314.

20. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, et al. (2010) Intrahepatic cholangiocarcinoma: a population-based case-control study. Gastroenterology 138: 1615–1622.
cholangiocellular carcinomas in Japan and Thailand. Moleculcarnogenesis 8: 312–318.
22. Ohashi K, Tsutsumi M, Nakajima Y, Noguchi O, Okita S, et al. (1994) High rates of K-ras point mutation in both intra- and extra-hepatic cholangiocarcinoma. Japanese journal of clinical oncology 24: 305–310.
23. Petruir S, Pinhal S, Thousunnugan A, Karakal A, Mijagan A (1998) K-ras oncogene and p53 gene mutations in cholangiocarcinoma from Thai patients. The Southeast Asian journal of tropical medicine and public health 29: 71–75.
24. Roa JC, Roa I, de Aretxabala X, Melo A, Faria G, et al. (2004) [K-ras gene mutation in gallbladder carcinoma]. Revista medica de Chile 132: 953–960.
25. Sturma PD, Baas IO, Clemen MJ, Nakeeb A, Johan G, et al. (1998) Alterations of the p53 tumor-suppressor gene and K-ras oncogene in perihilar cholangiocarcinoma from a high-incidence area. International journal of surgery 27: 619–624.
26. Tada M, Omata M, Ohto M (1990) Analysis of ras gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. Cancer research 50: 1121–1128.
27. Tada M, Omata M, Ohto M (1990) [Detection of ras gene mutations of primary hepatic malignant tumors by polymerase chain reaction and direct sequencing method]. Nihon Shokakibyo Gakkai zasshi = The Japanese journal of gastroenterology 87: 811–815.
28. Wattanasirichaigoon S, Tassanakajorph U, Jedsaparuttakul S (1998) The incidence of K-ras codon 12 mutations in cholangiocarcinoma detected by polymerase chain reaction technique. Journal of the Medical Association of Thailand = Chom Thong thangsaith 81: 316–323.
29. Boberg KM, Schrumpf E, Bergquist A, Broome U, Pares A, et al. (2000) Cholangiocarcinoma in primary sclerosing cholangitis: K-ras mutations and Tp53 dysfunction are implicated in the neoplastic development. Journal of pathology 192: 373–380.
30. Tanapatpinyo A, Wainans M, Greisler F, Schutz A, Katalinic A, et al. (2000) Mutations of p53 tumor suppressor gene, apoptosis, and proliferation in intrapancreatic cholangiocellular carcinoma of the liver. Digestive diseases and sciences 45: 317–321.
31. Tuffa A, D’Etiache AM, Honda K, Kelly MD, Habib NA, et al. (2000) New p53 mutations in hilar cholangiocarcinoma. European journal of clinical investigation 30: 798–803.
32. Hahn SA, Barsch D, Schroers A, Gallehrh D, Becker M, et al. (1998) Mutations of the DPC4/Smad4 gene in biliary tract carcinoma. Cancer research 58: 1124–1126.
33. Iacobuzzi-Donahue CA, Song J, Parmigiani G, Yeo CJ, Hruban RH, et al. (2004) Missense mutations of LDLR: characterization of the mutational hotspot amino acid and functional consequences in human tumors. Clinical cancer research : an official journal of the American Association for Cancer Research 10: 1597–1604.
34. Ahrendt SA, Eisenberger CF, Yip L, Rashid A, Chow JT, et al. (1999) Mutations of p53 and RB1 genes in human bile duct carcinoma. Journal of pathology 187: 462–467.
35. Tadokoro H, Shigihara T, Ikeda T, Takase M, Suyama M (2007) Two distinct pathways of p16 gene inactivation in gallbladder cancer. World journal of surgery 31: 723–728.
36. Taniai M, Higuchi H, Burgart LJ, Gores GJ (2002) p16INK4a promoter methylation reveals the role of p16 inactivation in human cholangiocarcinoma. Gastroenterology 122: 1552–1558.
37. Kraus MR, Clauin S, Pfister Y, Di Maio M, Ulinski T, et al. (2012) Two colorectal cancer-associated mutations in the APC gene. World journal of gastroenterology 18: 6929–6935.
38. Ahrendt SA, Eisenberger CF, Yip L, Rashid A, Chow JT, et al. (1999) Mutations of p53 and RB1 genes in human bile duct carcinoma. Journal of pathology 187: 462–467.
94. Anastasi S, Fiorentino L, Fiorini M, Fraioli R, Sala G, et al. (2003) Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene. 22: 4234–4244.
95. Zhang X, Pickin KA, Bore R, Jura N, Cole PA, et al. (2007) Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. Nature 450: 741–744.
96. Frosi Y, Anastasi S, Ballaro C, Varsano G, Castellani L, et al. (2010) A two-tiered mechanism of EGF inhibition by RALT/MIG6 via kinase suppression and receptor degradation. The Journal of cell biology 189: 557–571.
97. Wang P, Dong Q, Zhang C, Kuan PF, Liu Y, et al. (2012) Mutations in isocitrate dehydrogenase 1 and 2 occur frequently in intrabehiatic cholangiocarcinomas and share hypermethylation targets with glioblastomas. Oncogene. 32: 3091–100.
98. Maisonneuve C, Guilletard I, Vaud P, Weber T, Andre P, et al. (2009) Ricol D, Cappellen D, El Marjou A, Gil-Diez-de-Medina S, Girault JM, et al. (2006) Mig6 is a multifunctional adaptor protein with tumor suppressor-like activity in papillary thyroid cancer. The Journal of clinical endocrinology and metabolism 96: E534–563.
99. Reschke M, Ferby I, Stepiak E, Seitzer N, Horst D, et al. (2010) Mitogen-inducible gene-6 is a negative regulator of epidermal growth factor receptor signaling in hepatocytes and human hepatocellular carcinoma. Hepatology 51: 1393–1390.
100. Anastasi S, Fiorentino L, Fiorini M, Fraioli R, Sala G, et al. (2003) Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene. 22: 4234–4244.
101. Li Z, Dong Q, Wang Y, Qiu L, Qiu X, et al. (2012) Downregulation of Mig-6 in nonsmall-cell lung cancer is associated with EGF receptor signaling. Molecular carcinogenesis 51: 522–534.
102. Zhang YW, Staal B, Dykema KJ, Furge KA, Vande Woude GF (2012) Cancer-type regulation of Mig-6 expression by inhibitors of methylation and histone deacetylation. PLoS one 7: e38955.
103. Zhang YW, Staal B, Su Y, Swiatk P, Zhao P, et al. (2007) Evidence that Mig-6 is a tumor-suppressor gene. Oncogene 26: 269–276.
104. Bai A, Meetz K, Vo NY, Kollipara S, Maza EK, et al. (2010) GP369, an FGFR2-IIIb-specific antibody, exhibits potent antitumor activity against human cancers driven by activated FGFR2 signaling. Cancer Res 70: 7630–7639.
105. Chauvin-Artois D, Carpentier JD, Weiss GJ, Demeure MJ, Von Hoff DD, et al. (2013) Identification of somatic mutations in cancer through Bayesian-based analysis of sequenced genome pairs. BMC Genomics 14: 362.
106. Kim D, Salberg SL (2011) TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. Genome Biol 12: R72.
107. Iyer MK, Chinnaiyan AM, Maher CA (2011) ChimeraScan: a tool for identifying chimeric transcription in sequencing data. Bioinformatics 27: 2903–2904.
108. McPherson A, Hormozdiari F, Zayed A, Giuliany R, Ha G, et al. (2011) deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. PLoS Comput Biol 7: e1001135.