Genomic landscapes reveal post-transcriptional modifier disruption in cholangiocarcinoma

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Abstract

Molecular variation of different geographic populations and cholangiocarcinoma (CCA) subtypes indicate CCA's potential genomic heterogeneity and novel genomic features. We analyzed exome-sequencing data of 87 perihilar cholangiocarcinoma (pCCA) and 261 intrahepatic cholangiocarcinoma (iCCA) from 3 Asian centers (including 43 pCCAs and 24 iCCAs from our center). Patients with iCCA presented higher tumor mutation burden and copy number alteration burden (CNAB) than pCCA patients, and CNAB indicated a poorer pCCA prognosis. In our newly identified 12 significantly mutated genes and 5 focal CNA regions, post-transcriptional modification-related novel driver genes METTL14 and RBM10 are prone to occur in pCCA. We demonstrated the tumor-suppressing functional role of METTL14, a major RNA N6-adenosine methyltransferase (m6A), and its loss-of-function mutation R298H may function through m6A modification on new driver gene MACF1. Our results may be valuable for better understanding of post-transcriptional modification effects CCA development, and similarities and differences between pCCA and iCCA.

Introduction

Cholangiocarcinoma (CCA) is a malignant primary hepatobiliary disease originating from every point of the biliary tree, from the canals of Hering to the main bile duct. In recent decades, the incidence and mortality of CCA have increased globally, especially in East Asia. In China, the incidence of CCA reached 6–7.55 per 100,000 individuals. The mortality of CCA is almost equal to its incidence due to resistance to common treatments and poor prognosis. The overall five-year survival rate is only 10%. Even for the patients with CCA who are amenable to surgical resection, and the corresponding five-year survival rate is only 25–35%, and high recurrence rates of 50 to 60% persist upon diagnosis, with no effective therapy.

CCA is regarded as a group of different diseases that are further divided into intrahepatic CCA (iCCA), perihilar CCA (pCCA), and distal CCA (dCCA) based on anatomical location. pCCA is the most frequent subtype, accounting for 50–60% of all CCA tumors. In general, the incidence and mortality rates of iCCA were reported rising worldwide in the past decade, whereas the incidences of pCCA seem to be stable or decreasing. Besides the burden of HCV infection has been linked with the incidence of iCCA, infection with liver flukes and HBV-related liver diseases have shown a stronger association for iCCA in East Asia. Primary sclerosing cholangitis (PSC) is considered as a predominant risk factor for CCA, especially for pCCA. The pronounced geographical and etiology heterogeneity of iCCA and pCCA indicated the potential diverse cancer-initiating cell in these two subtypes of CCA. In detail, iCCA is considered to originate two different cell of origin (mucin-secreting cholangiocyte or hepatic progenitor cell), due to the significant inter-tumor heterogeneity. Unlike iCCA, pCCA only originate from mucin-secreting cholangiocyte.
To date, previous studies of the genomic alterations in a variety of bile duct cancer, especially for iCCA, have demonstrated some known commonalities in mutation of CCA. These studies identified a number of driver genes, but most of them represented the genomic features of iCCA, such as TP53, KRAS, SMAD4, and ARID1A. Notably, the latest integrative genomic analysis of Caucasian extrahepatic CCA (eCCA) (including the pCCA and dCCA) revealed distinct molecular characterization of eCCA in western. However, the epidemiological profile of CCA and its subtypes shows enormous geographical variation, indicating underlying genomic heterogeneity across different regions. The prevalence of CCA is highest in East Asia and the subtype distribution is similar. Thus, integration analysis in sequencing data from Asian countries may find new genomic features ignored by previous studies. Here, we performed whole-exome sequencing on 43 pCCA and 24 iCCA, and analyzed an additional 44 pCCA and 237 CCA to investigate the genomic landscapes of pCCA and iCCA, respectively.

**Methods And Materials**

Detailed information about the clinical sample collection and follow-up; DNA preparation, DNA capture, and sequencing; Next-generation sequencing; Sequencing alignment and detection of somatic variants; Mutational burden and signature analysis; Identification of CCA driver genes and comparison between the iCCA and pCCA; Somatic copy number estimation; Highly amplified/deleted regions identification; Pathway enrichment analysis; Annotation of genomic alterations upon clinical actionability; Cell culture and siRNA transfection; Establishment of stable METTL14R298H and METTL14wt cells and functional assays; RNA N6-methyladenosine (m6A) Quantification; RNA m6A immunoprecipitation assay and m6A sequencing; M6A sequencing analysis; Measurement of RNA lifetime; Western blotting; RNA isolation and quantitative real-time PCR (qPCR); Immunohistochemistry staining; Evaluation of immunostaining; Immunofluorescence analysis and statistical analysis are available in the supplementary material.

**Results**

**Mutational spectrum of pCCA and iCCA**

We conducted integration analysis of exome-sequencing data from 87 pCCA cases (including 43 xxx Medical University (xMU) cases and 44 International Cancer Genome Consortium (ICGC) cases) and 261 iCCA cases (including 24 xMU cases, 135 ICGC cases, and 102 cases from Zou et al.’s study). The general information and clinical features of patients from the xMU study are listed in Supplementary Table 1. The mean sequencing depth of the xMU study was 94.4× in tumor tissues and 91.8× in normal tissues dissected adjacent to the tumor. Detailed sequencing coverage and depth information are listed in Supplementary Table 2. We detected a total of 91,995 somatic mutations (Supplementary Table 2) in all the cases, and the median number per case was 120.5. The median tumor mutation burden (TMB) was 2.0 megabase per case (/Mb) and the burden was comparable between pCCA (2.0/Mb) and iCCA (2.0/Mb) (Wilcoxon rank-sum test \( P = 0.48 \), Supplementary Fig. 1A). Among these mutations, we defined 28,254 non-synonymous mutations, for which the median was 33 Single Nucleotide Variants (SNVs) and
3 small insertion and deletions (INDELs) per patient. Interestingly, the non-synonymous mutation burden (median 0.61/Mb) of iCCA was significantly higher than that (median 0.47/Mb) of pCCA (Wilcoxon rank-sum test $P = 9.6 \times 10^{-4}$, **Fig.1A**). Similar to the non-synonymous mutation pattern, iCCA had a higher copy number alteration burden (CNAB) than pCCA (iCCA median = 25.0%, pCCA median = 8.4%, Wilcoxon rank-sum test $P = 2.4 \times 10^{-4}$), no matter the amplification or deletion (**Fig. 1C**).

Next, we investigated the genomic mutational signatures, which provide clues for the carcinogenesis of CCA. Firstly, we classified all the mutations into six classic subtypes. Consistent with previous studies\(^ {17,18}\), C>T/G>A substitutions accounted for the predominant type of all SNVs in all of the patients (**Supplementary Fig. 1B**). The proportion of the mutation subtypes was similar between pCCA and iCCA (Wilcoxon rank-sum test $P = 0.46, 0.09, 0.67, 0.62, 0.51, \text{and} 0.29$ respectively for C>A, C>G, C>T, T>A, T>C, and T>G, **Supplementary Fig.1B**). We further analyzed mutational signatures using R package SignatureEstimation, which accurately reconstructs the mutation profiles of all pCCA and iCCA patients based on a predefined mutational spectrum of 30 COSMIC signatures\(^ {22}\). COSMIC signature 1 (Age, 44.71%), signatures associated with mismatch repair deficiency (MMR, Signatures 6, 15, and 26, 31.99%), signatures associated with activated APOBECs (Signatures 2 and 13, 6.48%), and signature 8 (6.41%) were dominant (proportion > 5%) in pCCA patients (**Fig. 1B**). For iCCA patients, COSMIC signature 1 (27.19%) and signature MMR (26.74%) were also identified as dominant, but the proportion was slightly lower than in pCCA patients. In addition, signature 4 related to smoking behavior (10.13%), signature 22 (9.96%) related to aristolochic acid, and signatures specific to liver cancer (Liver, Signatures 12, 16, and 24, 8.7%) were predominant in iCCA. Interestingly, signature Liver were more common in HBV-related iCCA (HBV: 26.0%, non-HBV: 3.8%, **Supplementary Table 3**). Aristolochic acid-related mutations were mainly found in iCCA patients from Zou et al.’s study, but this signature was also observed in pCCA patients (**Supplementary Table 4**). In addition, signature 3, related to homologous recombination deficiency (4.26%), signature 10, related to altered POLE activity (2.44%), and signature 7, related to ultraviolet (UV) light exposure (2.17%), were only identified in iCCA (**Fig. 1B**), which suggested that multiple types of DNA damage occurred during the carcinogenesis progress of CCA.

The results mentioned above revealed the nature of genomic instability in CCA patients. We further investigated the association between TMB/CNAB and the overall survival of patients. We found that the patients carrying higher loads of mutations showed worse outcomes after adjusting for age, sex, and stage at diagnosis (Hazard ratio (HR) = 1.71, $P = 3.9 \times 10^{-4}$, **Fig. 1D**). The association was consistent in pCCA and iCCA patients (**Supplementary Fig. 1C**). When examining CNAB, we noticed that the survival of pCCA patients with higher CNAB was significantly worse than for patients with lower CNAB (HR = 2.70, $P = 3.6 \times 10^{-2}$, **Fig. 1E**); However, there was no significant difference between iCCA patients with distinct CNAB (HR = 0.91, $P=6.7 \times 10^{-1}$, **Fig. 1F**).
New driver genes

We identified 36 significantly mutated genes (SMGs, Fig. 2), also described as mut-drivers, in all patients by MutSig2CV and IntOGen, including 24 reported SMGs and 12 new SMGs not highlighted in previous CCA publications (Supplementary Table 5). Among these new SMGs, MACF1 (5.17%, 18/348) and AXIN1 (0.86%, 3/348, all of the three patients were with iCCA), which is commonly considered as one of drivers in hepatocellular carcinoma (HCC), are two interactive components of the $\beta$-catenin/Wnt signaling pathway (Fig. 2). Although the mutation rate of AXIN1 was low, we noticed that there were multiple non-synonymous mutations in two iCCA patients (Fig. 2). The mutations of PIK3R1, a component of the PI3K pathway, was displayed in 2.59% (9/348) of patients. Post-transcriptional modification genes RBM10 (3.16%, 11/348) and METTL14 (0.86%, 3/348) act as well-known alternative splicing regulators and m$^6$A writers. Importantly, we identified recurrent mutations (Supplementary Methods) in METTL14, and all mutations in this gene affected the same amino acid (Supplementary Fig. 2A), which were also predicted as potentially deleterious variants by several bioinformatic pathogenicity prediction tools (Supplementary Table 5). We observed the same mutation rate (1.44%, 5/348) of chromatin modifiers SMARCA4 and WHSC1. Interestingly, mutations in these genes were prone to co-occur in the same patient (Fisher's exact test OR = 660, $P = 2.29 \times 10^{-6}$, Fig. 2). In addition, classic cancer driver genes identified in other cancers were also identified as SMGs in this study, including ATM (3.45%, 12/348), BRCA2 (2.01%, 7/348), and MLLT4 (2.01%, 7/348). Although BRCA2, EPHA2 and ATM were mentioned in previously published work due to their same position recurrent inactivating mutations, our integrated analysis with more sample size firstly identified them as SMGs.

We further defined the genes with a distinct mutation rate between pCCA and iCCA (see Supplementary Methods). TP53, ARID1A, PBRM1, MACF1, EPHA2, ARID2, IDH1, PTEN, RB1, BRAF, NRAS, SLC8A1, AXIN, and MLLT4 were iCCA-enriched genes, while RBM10, TGFBR2, PIK3R1, ELF3, NACC1, and METTL14, were pCCA-enriched genes. The mutation rates of TP53 and IDH1 were significantly higher in iCCA (Fisher's exact test OR$_{TP53} = 1.3$, $P_{TP53} = 8.8 \times 10^{-3}$; OR$_{IDH1} = +\infty$, $P_{IDH1} = 4.4 \times 10^{-2}$, Fig. 2). Importantly, IDH1 only mutated in iCCA, but not in pCCA. RBM10, however, carried significantly more mutations in pCCA than in iCCA (Fisher's exact test OR$_{RBM10} = 3.8$, $P_{RBM10} = 3.2 \times 10^{-2}$).

We further identified 22 regions frequently altered by focal copy number alteration (CNAs) in CCAs (Fig. 3A, Supplementary Table 6), including 16 regions overlapping with previously reported peaks, and six new significantly altered regions (amplification at 7q31.2, 22q11.21, and deletion at 1p36.13, 2p24.1, 7q35, and 12q24.33, Fig. 3A). The cancer related genes located at these peaks were considered to be CNA-driver genes. The gain of a 0.8-Mb region at 7q31.2 occurred in 45 samples (26.6%), which contains the classic
MET oncogene in the RTK/RAS pathway. However, the amplification of 7q31.2 was found rare previously in Caucasian population.23 The loss at 1p36.13 involved tumor suppressor gene SHDB and affected 76 samples (45.0%). DNA Polymerase Epsilon (POLE) at 12q24.33, where was also considered lost in HCC24, lost one copy in 40 patients (23.7%). In addition, important DNA methylation factor DNMT3A (2p24.1) and chromatin methylation factor EZH2 (7q35) also show loss in 20 (11.8%) and 19 (11.24%) patients (including iCCA and pCCA), respectively. Whereas these two regions were also only considered deleted in iCCA based on a previous single nucleotide polymorphism array(SNP-array) analysis25. We only observed heterogeneous loss in CCA patients (Fig. 3A). Among all of the regions, the frequencies of amplification/gain at 8q24.21 (MYC), 1q21.3 (S100A7), 5p15.33 (SDHHA), and 7q31.2 (MET) were significantly higher in iCCA (Supplementary Table 6). The frequency of high-level amplification was significantly higher in pCCA than in iCCA, although the frequency of amplification/gain was comparable across the subtypes (Fig.3A). In addition, we found that copy number loss and nonsynonymous mutations of classic tumor suppressors (TP53, CDKN2A, SMAD4, PTEN, and ATM) were prone to co-occur in the same patients (Fisher's exact test OR = 8.4, P = 8.2 × 10^-6, Fig. 3B).

Next, we performed pathway enrichment analysis on all driver genes identified in this study, which included SMGs (mut-drivers) and cancer genes in the frequently altered focal CNA regions (CNA-drivers) described above, and found that the driver genes of CCA were significantly enriched in the RTK-RAS, Wnt, PI3K, Cell Cycle, TP53, TGF-beta, and HIPPO pathways (Fig.3C). Including the newly identified MET amplification, 34.3% of CCA patients harbored mutations and CNAs in oncogenes from the RTK-RAS pathway, and these alterations occurred in a mutually exclusive manner, as reported in other cancers (Fig. 3D).

Functional recurrent mutations in METTL14

Among the potential driver genes mentioned above, we identified a new potential driver gene, METTL14, the main factor involved in aberrant m6A modification of various cancers, with recurrent and deleterious mutations26,27 (Supplementary Fig. 2A, Supplementary Table 7). All three mutations affected the same 298 amino acid residue (METTL14 p.R298H and p.R298C) and two of them were in the same genomic position (two patients from xMU and ICGC respectively) (Supplementary Fig. 2A). We further conducted sanger sequencing in an independent pCCA cohort with extra 40 subjects from xMU cohort and identified an additional p.R298H carrier (Supplementary Fig. 2B & C). In the COSMIC database, we also found that the same p.R298H also occurred in three pancreatic ductal carcinomas (Supplementary Fig. 2A) and all the three patients were East Asian.
Crystal structures of the METTL3-METTL14 complex have revealed that p.R298 lies close to the putative RNA-binding groove of the complex which may have a complex role to affect methylation activity\textsuperscript{28}. The recurrent and deletions mutant p.R298H suggested us its possibly positive selection and the need of METTL14's normal action in antitumor progress. However, the relevance of this hotspot mutation and m\textsuperscript{6}A mRNA methylation to the CCA has not yet been established. Dysfunction of METTL14, the key catalytic protein forming the core m\textsuperscript{6}A methyltransferase complex, has shown fundamental biological effects in cancer initiation and progression\textsuperscript{28, 29}. Thus, we hypothesized that CCA could be associated with METTL14 that regulated m\textsuperscript{6}A mRNA methylation. Hence, we first examined METTL14 expression in 69 CCA pairs and matched adjacent normal tissues. The results of qRT-PCR and western blot revealed that expression of METTL14 was significantly downregulated in tumors ($P < 0.01$; Fig. 4A & B). The immunohistochemistry (IHC) staining on tissue microarray also confirmed that METTL14 staining was decreased in CCA at protein level (Supplementary Fig.3A) and its downregulation displayed a marginal significant association with poor cancer-specific survival in CCA (Log-Rank $P = 0.08$; Supplementary Fig.3B). Consistently, we found that the m\textsuperscript{6}A level of total RNA was significantly decreased in CCA tissues (Fig. 4C). Interestingly, we also observed the m\textsuperscript{6}A modification level was significantly decreased in METTL14 low expression CCA group compared with METTL14 high expression CCA group (Supplementary Fig. 3C). These results suggested that METTL14 and it mediated m\textsuperscript{6}A modification were frequently down-regulated or disturbed in CCA.

To determine the “driver” role of METTL14 and R298H mutations during CCA development, we first confirmed the transfection efficiency of lentiviral constructs expressing METTL14\textsuperscript{wt} and METTL14\textsuperscript{R298H} in RBE and HCCC9810 cell lines (Fig. 4D and Supplementary Fig. 3D&E). In contrast to the cells stably overexpressing METTL14\textsuperscript{wt}, cells overexpressing METTL14\textsuperscript{R298H} showed significantly decreased overall m\textsuperscript{6}A modification ability (Fig. 4E and Supplementary Fig. 3F). Subsequently, while overexpression of wild-type METTL14 decreased cell proliferation, overexpression of the mutation had no noticeable effect on cell proliferation (Fig. 4F, G & H and Supplementary Fig. 3G, H&K). In addition, overexpression of METTL14\textsuperscript{wt} resulted in a decrease in cell migration and invasion, and METTL14\textsuperscript{R298H} remarkably reversed the gene's ability to inhibit migration and invasion in CCA cells (Fig. 4I & Supplementary Fig. 3I & J). Taken together, these results provided us evidence for loss of function caused by METTL14\textsuperscript{R298H} mutation, suggesting that METTL14\textsuperscript{R298H} mutation could reduce the tumor-suppressing effect of METTL14\textsuperscript{wt}.

**New driver gene MACF1 served as the target of METTL14**

We then conducted RNA-Seq and MeRIP-Seq assays in negative control, METTL14\textsuperscript{wt}, and METTL14\textsuperscript{R298H} cells. A total of 2,601 peaks involving classic transcripts of 1,586 genes were robustly identified by
exomePeak2 in all cells. The most common m\textsuperscript{6}A motif, GGAC, was significantly enriched in the m\textsuperscript{6}A peaks identified (Supplementary Fig. 4A), and the m\textsuperscript{6}A peaks were especially enriched in the vicinity of the stop codon. Next, we performed differential m\textsuperscript{6}A-methylation analysis between control and METTL14\textsuperscript{wt} cells, as well as between METTL14\textsuperscript{wt} and METTL14\textsuperscript{R298H} cells. Because of the writer role of METTL14 during the m\textsuperscript{6}A methylation modification, we included only m\textsuperscript{6}A peaks (712) with increased abundance in METTL14\textsuperscript{wt} cells as compared to control cells, and peaks (990) with decreased abundance in METTL14\textsuperscript{R298H} as compared to METTL14\textsuperscript{wt} cells. A total of 237 peaks were shared by both analyses, including four peaks on the two driver genes mentioned above (MACF1, MET) (Fig. 5A & B). MACF1 was also the new driver gene identified in this study (Supplementary Table 5). We then verified whether m\textsuperscript{6}A-modified MACF1 was susceptible to decay. The lifetime of MACF1 was prolonged in METTL14\textsuperscript{R298H} cells and shortened in METTL14\textsuperscript{wt} cells after actinomycin D treatment (Fig. 5C). We further immunoprecipitated m\textsuperscript{6}A from the RNAs of METTL14\textsuperscript{wt} and METTL14\textsuperscript{R298H} cells and found that METTL14\textsuperscript{R298H} significantly decreased the amount of MACF1 modified by m\textsuperscript{6}A compared to METTL14\textsuperscript{wt} (Fig. 5D). Because MACF1 was a very large gene (involving 22Kb of exons), we applied immunofluorescence assays to further confirm that METTL14\textsuperscript{wt} mediated MACF1 degradation, and METTL14\textsuperscript{R298H} showed increase expression of MACF1 compared to METTL14\textsuperscript{wt} (Fig. 5E). We also found that METTL14\textsuperscript{wt} cells decreased the expression of MACF1, and no noticeable effect on MACF1 expression was observed in METTL14\textsuperscript{R298H} cells using qRT-PCR (Supplementary Fig. 4B).

When we successfully transfected CCA cells with siRNA pools targeting MACF1 (Supplementary Fig. 4D), significant inhibition of tumor metastasis and proliferation was observed (Fig. 5F, G, H, & I, and Supplementary Fig. 4E & F). Recent studies reported that MACF1 was involved in tumor metastasis and cytoskeleton, and that it played crucial roles in the nucleus translocation of β-catenin\textsuperscript{30,31}. In our findings, we first validated MACF1 upregulation in CCA using qRT-PCR (Supplementary Fig. 4C), and found that knockdown of MACF1 significantly reduced CCA cell proliferation and metastasis in vitro. Given the essential role of MACF1 in regulating the nucleus translocation of β-catenin, immunofluorescence assays showed that the increase of nuclear β-catenin was correlated with the expression of METTL14\textsuperscript{R298H} rather than METTL14\textsuperscript{wt} (Fig. 5J & K). To ascertain the role of MACF1 in METTL14-mediated nucleus translocation of β-catenin, we transfected MACF1 siRNA in METTL14\textsuperscript{R298H}-overexpressing cells, and observed that the nucleus translocation of β-catenin was decreased compared to only METTL14\textsuperscript{R298H}-overexpressing cells (Supplementary Fig. 4G).

Additionally, METTL14\textsuperscript{R298H} resulted in reversing the expression level of E-cadherin, N-cadherin, PCNA, and CyclinD1, which were the downstream targets of β-catenin, relevant to METTL14\textsuperscript{wt} (Fig. 5K). These
results implied that \textit{METTL14}-mediated m$^6$A modification repressed the MACF1/\textit{\beta}-catenin pathway in CCA, while \textit{METTL14}$^{R298H}$ mutation disrupted this mechanism.

\textbf{Discussion}

In this study, we included genomic data from 348 CCA samples (including 87 from pCCA and 261 from iCCA) to investigate the genomic landscapes of both pCCA and iCCA. We found the shared and distinct features between these two anatomical subtypes. Currently, there is no effective biomarker that can accurately predict the prognosis of CCA patients \textsuperscript{32,33}. Our study found that TMB has a good predictive effect on the prognosis of East-Asian CCA patients. It is worth noting that pCCA patients with higher CNAB showed worse survival outcomes compared to those with lower CNAB. These findings suggest that we can explore prognostic markers of patients with CCA from a more macroscopic genetic perspective.

In addition to several new driver genes in classic pathways, we identified driver genes participating in post-transcriptional modification (i.e., \textit{METTL14} and \textit{RBM10}), which carried more mutations in pCCA patients. With \textit{in vitro} experiments, we confirmed the role of \textit{METTL14}, as well as the role of this mutation in CCA development. We further determined that m$^6$A modification level and expression of new driver gene \textit{MACF1} could be regulated by \textit{METTL14}, which can influence the proliferation and metastasis ability of CCA cells.

The previous study showed that there was lack of evidence that anatomical site determines molecular subtypes according to integration analysis of transcriptomic and methylation data \textsuperscript{34}. Our study identified four new driver genes that were related to chromatin and methylation modification (i.e., mut-drivers: \textit{SMARCA4} and \textit{WHSC1}; CNA-drivers: \textit{DNMT3A} and \textit{EZH2}), further supporting the finding that driver genes cause disruption at the transcriptional level. Consistent with the previous study’s findings, we found comparable frequency between these alterations of driver genes. However, our analysis first identified two genes as drivers (\textit{METTL14} and \textit{RBM10}) and found that they are related to post-transcription modification. Mutations in these two genes were more common in pCCA patients. This evidence suggested that the investigation of the difference between pCCA and iCCA should be extended to the post-transcriptional area. \textit{METTL14} engages in m$^6$A modification \textsuperscript{35,36}, which modulates alternative splicing, export, stability, and translation of mRNA \textsuperscript{37}. Interestingly, we found that two new driver genes (\textit{MACF1} and \textit{MET}) could be modulated by the m$^6$A alteration caused by \textit{METTL14} mutations. It was worthy of note that all of these genes had a higher mutation rate or copy number altered rate in iCCA than in pCCA. Thus, they were crucial driver genes in both iCCA and pCCA. However, they could be activated through different mechanisms. Although \textit{MACF1} and \textit{MET} could serve as shared therapeutic targets for pCCA and iCCA, we should not ignore the fact that \textit{METTL14} could modulate m$^6$A modification of broader genes other than the two drivers, which may lead to unexpected drug resistance or side effects,
and therefore, further investigation in pCCA patients is warranted. Another gene, RMB10, is an RNA binding protein and alternative splicing regulator frequently mutated in lung adenocarcinomas. Similar to lung cancer, the majority of RBM10 mutations (63.6%) in CCA truncate the protein and may alter downstream splicing of specific genes. Thus, the post-transcriptional events in CCA could be highly disturbed and further study may help determine new molecular subtypes and optimize therapeutic strategy in the clinical setting.

Our study also identified a number of new driver genes, involving multiple important pathways. MACF1 and AXIN1 were first identified as SMGs of CCA. They are involved in a complex that also contained CTNNB1, GSK3B, and APC, and can contribute to the activation of β-catenin/Wnt signaling pathways. Consistently, the mutations in the two genes were mutually exclusive. CNA-driver MET belongs to the classic RTK-RAS signaling pathway, and its inhibitor has been widely used in other cancers. However, MET amplification has never been reported in a genomic study of pCCA and reported with rare amplification frequency in Caucasian iCCA, suggesting that it may occur more frequently in Chinese patients. According to OncoKB, nearly 106 (30.5%) of all CCA patients carried actionable alterations in the RTK-RAS signaling pathway. PIK3R1, phosphatidylinositol 3-kinase regulatory subunit alpha, is the predominant regulatory isoform of PI3K and is frequently mutated in multiple cancers. ATM served as the activator of the TP53 tumor suppressor protein and somatic ATM mutations or deletions that are commonly found in lymphoid malignancies, pancreatic cancer, and lung adenocarcinoma. Notably, our results suggested that ATM could be affected by both truncating mutations and deletion, resulting in the bi-allelic inactivation. Several known tumor suppressors, such as TP53, SMAD4, PTEN, and CDKN2A, showed a similar bi-allelic loss pattern. Germline mutations in BRCA2, which encodes a BRCA-associated protein, were reported in familial CCA cases. Our study also suggested that BRCA2 could be affected by somatic mutations. MLLT4, also known as AF6, commonly fused with KMT2A in acute myeloid leukemia. It has also been identified as a SMGs in breast cancers. Few genomic alterations has ever been reported in NACC1, but it is activated in ovarian serous carcinomas and influences cell apoptosis, senescence, and cytokinesis in cancer cells. Integrated whole-exome analysis of Asian CCAs identified new CCA driver genes and emphasized the similarities and distinctions genomic characteristics between pCCA and iCCA. Importantly, our study also highlighted the effect of underlying post-transcriptional modification in CCA occurrence and development. These results provide a better understanding of the CCA mutational landscape that may drive improvements in clinical practice.

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