Clinically Significant Shared and Distinct Genomic Alterations in Chinese and Western Patients with Intrahepatic Cholangiocarcinoma

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Intrahepatic cholangiocarcinoma, genomic alteration, population, clinical significance, driver gene, actionability
Abstract

**Background** The goal of this study is to disclose the clinically significant genomic alterations in patients with intrahepatic cholangiocarcinoma of the Chinese and Western populations.

**Methods** A total of 86 Chinese patients were enrolled in this study. Samples from those patients were sequenced for a panel of pan-cancer genes. Results were compared to a public dataset from a cohort of Western patients. The comparison between the two populations was conducted in the driver genes, actionability, and TMB.

**Results** The Chinese and Western cohorts had 38 and 12 driver genes, respectively. Seven driver genes (\textit{IDH1}, \textit{KRAS}, \textit{TP53}, \textit{BAP1}, \textit{PBRM1}, \textit{ARID1A}, and \textit{NRAS}) were shared by the two cohorts. For both cohorts, half of the patients had actionable mutations. The two cohorts shared most of the actionable genes but differed much in the frequency. Though \textit{KRAS} mutations were at the first and second actionable rank respectively for Chinese and Western populations, they were still at a relatively low level of actionable evidence. Four driver genes (\textit{SPTA1}, \textit{ARID2}, \textit{TP53}, and \textit{GATA1}) were found significantly correlated with the tumor mutation burden.

**Conclusions** The revealed genomic alterations with clinical significance could help to improve the treatment of intrahepatic cholangiocarcinoma.

**Introduction**

In contrast to the Chinese population, intrahepatic cholangiocarcinoma (iCCA) is rare in the Western population (Singal et al., 2011). However, the incidence of iCCA was increasing in the Western population. Though at a low incidence, iCCA regularly had a poor prognosis. The only potentially curative treatment for iCCA is surgery. In a cohort study, half of the patients who underwent curative resection had a 5-year survival rate at 19% and a median survival of 27.6 months (Dhanasekaran et al., 2013). With the advent of precision medicine, targeted therapy and immunotherapy would change the treatment of iCCA, especially for those unresectable or resistant patients.

The first step of precision medicine is to identify the genotype of patients. Zou et al. (2014) report a landscape of intrahepatic cholangiocarcinoma in the Chinese population by whole-exome sequencing. They identified 8 driver genes in the 103 iCCA patients. Those genes were \textit{TP53}, \textit{KRAS}, \textit{IDH1}, \textit{PTEN},
Another study in the Western population conducted by Lowery et al. (2018) compared intrahepatic and extrahepatic cholangiocarcinomas with a panel of 410 genes. By sorting the frequency of mutations in patients, they got another eight commonly mutated genes in intrahepatic and extrahepatic cholangiocarcinoma including \textit{IDH1}, \textit{TP53}, \textit{ARID1A}, \textit{BAP1}, \textit{KRAS}, \textit{PBRM1}, \textit{SMAD4} and \textit{ATM}. Besides, there was also a 30-patients study comparing different types of biliary tract cancer with a 22-gene panel by Hogdall et al. (2020). They identified three significantly mutated genes including \textit{ARID1A}, \textit{TP53}, and \textit{KRAS} in CCA (cholangiocarcinoma). All the above studies concentrated on the mutations in one population, neglecting the difference between populations. In considering the significant incidence difference between the Chinese and Western populations, it would be interesting to know the clinically significant shared and distinct genomic alterations.

In this study, we sequenced 86 samples from Chinese iCCA patients. Since different researches used different methods to call the driver genes, directly comparing the results from the multiple pieces of research could be problematic. Through the same analysis pipeline, driver genes, actionability and tumor mutation burden were estimated and then compared.

**Methods**

**Patient and sample preparation**

This study was approved by the ethical committee of the Affiliated Hospital of Qingdao University and Shandong Provincial Hospital Affiliated to Shandong First Medical University. A total of 86 Chinese patients with intrahepatic cholangiocarcinoma were enrolled (ORI dataset). Each participant had provided written informed consent. Samples were collected from surgery after diagnosis. We also collected another public dataset to validate our analysis. This dataset, MSK cohort (Zehir et al., 2017), was downloaded from cBioportal (Gao et al., 2013). It contained 87 patients with intrahepatic cholangiocarcinoma from the Western population.

**Sequencing**

Sequencing was conducted in a CLIA/CAP-compliant Molecular Diagnostics Service laboratory of Origimed Co., Ltd. Briefly, tissue samples were collected for each patient. KAPA Hyper Prep Kit (Illumina) was used to extract DNA. At least 50 ng of double-stranded DNA was required for further library construction. Barcodes were added to the segments for multiplex sequencing to reduce the
false discovering rate. From both segments ends, 151 bp nucleotides were read on an Illumina Novaseq 6000 (Illumina, San Diego, CA).

**Mutation Calling**

A total of 579 pan-cancer genes (Yuansuo® from Origimed incorporation, Shanghai, China) were captured by targeted amplification. Adaptors were trimmed from raw DNA reads by cutadapt (https://github.com/marcelm/cutadapt, version 1.18) and de-duplicated with an in-house pipeline. High-quality reads were mapped to the UCSC hg19 reference sequences using BWA MEM (version 0.7.9a) (Li and Durbin, 2009). GATK (McKenna et al., 2010) was used to recalibrate base quality. Variants were called by Varscan (Koboldt et al., 2009) with the default parameters and Mutect2 with a tumor only mode (Cibulskis et al., 2013). The variants with allele frequency (VAF) < 0.1% were filtered out according to the databases of 1000 Genomes (Genomes Project et al., 2010), ExAC (Karczewski et al., 2017), ESP6500 (https://evs.gs.washington.edu) and gnomAD (Lek et al., 2016). High-confident variants were further annotated by ANNOVAR (Wang et al., 2010) with RefSeq (version 2017/06/01).

**Driver Gene Identification**

In order to remove the false positive mutations or passenger mutations, the driver genes were identified by MutSigCV (Lawrence et al., 2013) using the GenePattern web service (Reich et al., 2006). The pre-build full exome coverage file, covariate file and genome file for hg19 from GenePattern web service were used. The p-values for all the panel genes were inferred by MutSigCV. Significantly mutated genes were determined by the p-values adjusted by the Benjamini & Hochberg method.

**Pathway Analysis**

The enriched KEGG pathway (Kanehisa and Goto, 2000) and gene ontology were analyzed using the DAVID web service (Huang da et al., 2009). The significant items were determined by the false discovery rate with a threshold at 0.05.

**Clinical Actionability Annotation Of The Somatic Mutations**

Clinical actional mutations were annotated with a python software MafAnnotator.py published by OncoKB (Chakravarty et al., 2017). The parameter, disease type, was set “CHOL”. Mutations were annotated with six levels, they were, “Level_1”, “Level_2A”, “Level_2B”, “Level_3A”, “Level_3B” and “Level_4” (Chakravarty et al., 2017).

**Tumor Mutational Burden Analysis**
Tumor mutational burden (TMB) was calculated as the number of mutations multiplied by an adjust factor. The adjust factors were 1.1152 and 0.9738 for the two assays from the MSK dataset, and 0.7875 for the ORI dataset. The TMB difference between mutated and wild type patients was compared by the Mann-Whitney U test. The false discovery rate (FDR) was used to adjust the p-values.

Results
The landscape of somatic mutations among the Chinese and Western populations
A total of 86 Chinese patients with primary intrahepatic cholangiocarcinoma were enrolled in this study (ORI dataset). The characteristics of these patients were listed in Table 1. The median diagnosed age of Chinese patients was 59, ranging from 18 to 83 years old. For comparison, we also included a cohort dataset (MSK) curated by Zehir et al. (2017), which consisted of 87 iCCA patients from the Western population. The median diagnosed age of Western patients was 65 years old, ranging from 37 to 79. The Chinese cohort had a younger age than the Western cohort at diagnosis (p-value = 0.024, Mann-Whitney U test).

| Characteristics | ORI | MSK |
|-----------------|-----|-----|
| Age             | Median(range) | 59(18–83) | 65(37–79) |
| Sex             | Male | 48  | 35  |
|                 | Female | 38  | 52  |
| Stage           | I    | 29  | 0   |
|                 | II   | 8   | 0   |
|                 | III  | 22  | 0   |
|                 | IV   | 3   | 58  |
|                 | Unknown | 24  | 29  |
| Histological grade | Low  | 2   | 0   |
|                 | Middle | 37  | 0   |
|                 | High  | 29  | 0   |
|                 | Unknown | 18  | 87  |

Supplemental Fig. 1. The enriched KEGG pathways of ORI driver genes
Samples from Chinese iCCA patients were sequenced by CLIA/CAP-compliant Molecular Diagnostics Service laboratory of Origimed Co., Ltd. Figure 1 showed the top frequent mutations in the Chinese population and Western population. The five top mutated genes in the Chinese population were TP53, KRAS, ARID1A, SMAD4, and PBRM1. The five top mutated genes in the Western population were IDH1, ARID1A, BAP1, TP53, and KRAS.

Driver Genes In Both Populations
To remove the possible false positive, we utilized MutSigCV (Lawrence et al., 2013) to identify the
driver gene in iCCA. The same full genome coverage file was used as a control for both populations. The calculation identified 38 and 12 significantly mutated genes for ORI and MSK datasets, respectively (Fig. 2A) (Supplemental Table 1). Seven mutated genes (\textit{KRAS}, \textit{TP53}, \textit{BAP1}, \textit{IDH1}, \textit{PBRM1}, \textit{ARID1A}, and \textit{NRAS}) were shared by them. There were also 31 ORI-specific driver genes and 5 MSK-specific driver genes. Most shared driver genes had a higher mutation allele frequency in both cohorts (Fig. 2B). Gene ontology analysis of the biological processes revealed enriched functions in macromolecule modification, regulation of cell proliferation and positive regulation of metabolic process for the ORI cohort (Fig. 2C). MSK cohort was enriched with the glyoxylate cycle, regulation of neuron death and regulation of cell proliferation (Fig. 2D). KEGG pathway analysis identified a significant pathway of melanoma, generic cancer and endometrial cancer in the ORI cohort (Supplemental Fig. 1) but none in the MSK cohort.

**Clinical Actionability Of Genomic Variations**

In order to reveal the treatment potential, mutations were annotated with the drug actionability into six levels of evidence proposed by OncoKB (Chakravarty et al., 2017). Each mutation was assigned the highest level according to the actionability proof strength. Then we summarized the best treatment for each patient. For the ORI cohort, 46.98\% of patients had actionable mutations (Fig. 3A). For the MSK cohort, 40.56\% of patients had actionable mutations (Fig. 3B). Among them, 23.26\% and 34.83\% of patients had standard care biomarkers predictive of response to an FDA-approved drug (Level_2A). Next, we studied whether the mutations in each gene had an equal actionability for both populations. The actionability for each mutation was summarized for each gene. The best highest actionable level was chosen for each gene in a patient. Then we counted the number of patients for each gene by the highest levels. ORI cohort had more actionable mutations in \textit{KRAS}, \textit{PIK3CA}, \textit{NF1}, and \textit{CDKN2A} (Fig. 3C). MSK cohort had more actionable mutations in \textit{IDH1}, \textit{KRAS}, \textit{PIK3CA}, \textit{NRAS}, \textit{IDH1} and \textit{ATM} (Fig. 3D). Both cohorts had a similar set of actionable genes, differing only in their frequency (Fig. 3CD).

**Driver Genes Correlated With High Tumor Mutation Burden**

Tumor mutation burden was found a significant biomarker for immunotherapy efficacy in many other
pieces of research. Next, we investigated the TMB distribution in both cohorts. First, the TMB was calculated based on the number of missense mutations across the covered genome length. The median TMB of the ORI cohort was 3.1, ranging from 0 to 50.2. The median TMB of the MSK cohort was 2.95, ranging from 0.98 to 27.88. No significant difference was found between the averages of the two cohorts (Fig. 5A). There were 11 and 5 patients with TMB > 10 mut/Mb for the ORI and MSK cohorts, respectively. Among them, two patients without actionable genes had TMB > 10 mut/Mb in each cohort. TMB was significantly correlated with *KMT2D, MUC16, SPTA1, ARID2, FAT4, FREX2, KMT2C, ACVR2A, LRP1B, and NF1* in ORI cohort (Fig. 5B-K) and with *TP53* and *GATA1* in MSK cohort (Fig. 5LM). Among those genes, *SPTA1, ARID2, TP53*, and *GATA1* were found as driver genes in their specific cohorts according to the analysis above.

Discussion

The significantly distinct incidence of iCCA between the Chinese and Western populations had driven us to disclose their genetic and actionable difference. Previously, driver genes were often identified by frequency among the populations previously. As suggested by Dees et al. (2012) and Lawrence et al. (2013), high-throughput sequencing studying could bring about many false-positive driver genes because of the mutational heterogeneity on the genome. To circumvent such errors, MutSigCV was employed. MutSigCV analysis had identified 32 significant mutated genes in the ORI cohort. The 10 most significant genes in the ORI dataset were *KRAS, TP53, SMAD4, BAP1, IDH1, PBRM1, ARID1A, SPTA1, NTRK3* and *STK11*, among which *IDH1, SPTA1, NTRK3*, and *STK11* were not found in the top 20 highly mutated gene list. Actually, *IDH1* mutation was very common in iCCA patients, accounting for 11% according to an integrated analysis of multiple studies (Xie et al., 2016).

Seven genes (*KRAS, TP53, IDH1, ARID1A, PBRM1, NRAS*, and *BAP1*) were shared between ORI and MSK cohort. Their mutation prevalence was higher than the median in both cohorts. We compared them to the results from other multiple publications (Zou et al., 2014, Lawrence et al., 2013). The sharing genes included *KRAS, TP53, IDH1*, and *ARID1A*, which were driver genes in both ORI and MSK cohorts. The other three genes (*PBRM1, NRAS*, and *BAP1*) were not reported in the research of Zou et al. (2014) and Lowery et al. (2018). Actually, *NRAS* is a very important gene involving many cancer-
related signaling pathways, such as MAPK, mTOR, and PI3K-Akt signaling pathways. Currently, NRAS mutation is actionable at level 3B. Patients with NRAS mutation could be treated with Binimetinib or Binimetinib + Ribociclib.

Most iCCA patients can benefit from targeted therapy. About 50 ~ 60% of patients had actionable mutations in the ORI cohort and MSK cohort. Among the actionable mutations, the MSK cohort had a higher actionable level than the ORI cohort, which implied the big potential in the treatment of iCCA for the Chinese population. The major gap between the actionability between the two cohorts lied in the two major actionable genes, KRAS and IDH1. ORI cohort had a higher percentage of KRAS mutation at actionable level 4 and less percentage of IDH1 mutation at the actionable level 2B. Considering the low evidence proof in KRAS mutations in both populations, drugs targeting KRAS mutations should be improved urgently.

Different mutations in the same gene could lead to different optimal treatments. ORI cohort had BRAF mutation V600E at a higher actionable level, 2B. V600E mutation in BRAF was recommended to be treated with Dabrafenib, Dabrafenib + Panitumumab + Trametinib, Dabrafenib + Trametinib, Encorafenib + Binimetinib, Encorafenib + Cetuximab + Binimetinib, Trametinib, Vemurafenib or Vemurafenib + Cobimetinib. MSK cohort had K601E mutation with a lower actionability (level 3B). K601E mutation in BRAF was recommended to be treated with PLX8394.

Mann-Whitney U test had revealed that TMB from both cohorts was not significantly different, which was consistent with the results of an abstract (Abdel-Wahab et al., 2019). The higher incidence of iCCA in the Chinese population did not come from a higher mutation rate. Immunotherapy could have similar efficacy in both populations. There were several successful cases of immunotherapy for iCCA. Mou et al. (2018) had reported a late-stage iCCA Chinese patient with a high TMB. After a combination of anti-PD-1 immunotherapy and chemotherapy, the patient showed a remarkable response. Another Western patient with a high TMB, as reported by Gbolahan et al. (2019), gained a good response by taking anti-PD-1 immunotherapy after chemotherapy resistance. In both cohorts, two patients who had no actionable mutation but had TMB > 10mut/Mb could benefit from immunotherapy. Besides, the identified genes that were highly correlated with TMB could be biomarkers for immunotherapy.
prognosis. For example, MUC16 (CA-125) is a very long protein (14,500 amino acids), which is easier to mutate in cancer cells. MUC16 was reported to associate with a higher TMB and a better immunotherapy outcome in Gastric Cancer (Li et al., 2018).

This study also had limitations. For example, TMB was calculated from different gene panels for the ORI and MSK cohort, which could weaken the results of the comparison. But according to a more restrict comparison, the conclusion still held between the Chinese and Western populations (Abdel-Wahab et al., 2019).

In summary, this study compared the Chinese and Western populations in the driver genes, actionability and TMB for iCCA patients. Shared and distinct driver genes were identified. KRAS and IDH1 mutations appeared in about 30% of patients with a significant frequency bias in the two populations. The drugs targeting KRAS need urgent improvement. Many driver genes are still not actionable. Both populations did not show a significant TMB difference.

Abbreviations
iCCA intrahepatic cholangiocarcinoma; CCA:cholangiocarcinoma; FDR:false discovery rate; TMB:tumor mutational burden

Declarations
Ethics approval and consent to participate
This study was approved by the ethical committee of the Affiliated Hospital of Qingdao University and Shandong Provincial Hospital Affiliated to Shandong First Medical University.

Consent for publication
Not applicable.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests

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Authors' contributions
YJZ and WR designed the study. SX, YG, and YWZ analyzed and interpreted the patient data. SX, YG, XZ, NF, ZZ, GR, YJZ, and WR collected the patient information and samples. SX, YG, YWZ, ZS, XZ, NF, ZZ, GR, YJZ, and WR drafted the manuscript. All authors read and approved the final manuscript.

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Figures

**Figure 1**

Mutational landscapes of iCCA patients from the Chinese and Western populations (A) The mutational landscape of iCCA patients from the Chinese population (ORI cohort). (B) The mutational landscape of iCCA patients from the Western population (MSK cohort).
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Mutational landscapes of iCCA patients from the Chinese and Western populations (A) The mutational landscape of iCCA patients from the Chinese population (ORI cohort). (B) The mutational landscape of iCCA patients from the Western population (MSK cohort).
Figure 2

Significantly mutated genes in both populations (A) The significantly mutated gene as revealed by MutSigCV analysis. (B) The frequency of mutations in ORI and MSK cohorts was plotted. (C) The biological process of gene ontology was enriched for ORI cohort. (D) The biological process of gene ontology was enriched for MSK cohort.
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Clinical actionability of somatic alterations (A) The percentage of patients was actionable in the ORI cohort. (B) The percentage of patients was actionable in the MSK cohort. (C) The number of patients was plotted against the best actionable gene of each patient in the ORI cohort. (D) The number of patients was plotted against the best actionable gene of each patient in the MSK cohort.
Clinical actionability of somatic alterations (A) The percentage of patients was actionable in the ORI cohort. (B) The percentage of patients was actionable in the MSK cohort. (C) The number of patients was plotted against the best actionable gene of each patient in the ORI cohort. (D) The number of patients was plotted against the best actionable gene of each patient in the MSK cohort.
Figure 4

The mutation distribution of the actionable genes in ORI and MSK cohort (A) The mutation distribution in BRAF. (B) The mutation distribution in KRAS. (C) The mutation distribution in IDH1.
Figure 4

The mutation distribution of the actionable genes in ORI and MSK cohort (A) The mutation distribution in BRAF. (B) The mutation distribution in KRAS. (C) The mutation distribution in IDH1.
Genes associated with tumor mutation burden (A) The tumor mutation burden (TMB) distribution in both cohorts was plotted. (B-K) Ten genes were significantly correlated with TMB in the ORI cohort (FDR<0.01). (LM) Ten genes were significantly correlated with TMB in the ORI cohort (FDR<0.1).
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