Deciphering Genetic Alterations of Taiwanese Pancreatic Adenocarcinoma With Targeted Sequencing

Chi-Cheng Huang  
Taipei Veterans General Hospital

Chih-Yi Liu  
Cathay General Hospital SiJhih

Chi-Jung Huang  
Cathay General Hospital

Yao-Chun Hsu  
E-da Hospital

Heng-Hui Lien  
Cathay General Hospital

Jia-Uei Wong  
Fu-Jen Catholic University Hospital

Feng-Chuan Tai  
Cathay General Hospital

Wen-Hui Ku  
Taipei Institute of Pathology

Chi-Feng Hung  
Fu-Jen Catholic University

Jaw-Town Lin  
China Medical University Hospital

Ching-Shui Huang  
(✉️ cshuang@cgh.org.tw )  
Cathay General Hospital

Han-Sun Chiang  
Fu-Jen Catholic University

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Abstract

Purpose

Pancreatic adenocarcinoma (PAC) is the 8th leading cause of cancer-related death in Taiwan, and its incidence is increasing. The development of PAC involves successive accumulation of multiple genetic alterations. Understanding the molecular pathogenesis and heterogeneity of PAC may facilitate personalized treatment for PAC and identify therapeutic agents.

Materials and Methods

We performed next-generation sequencing (NGS) with targeted panels to explore the molecular changes underlying PAC in Taiwan. The Ion Torrent Oncomine Comprehensive Panel (OCP) was used for PAC metastatic lesions, and more PAC samples were sequenced with the Ion AmpliSeq Cancer Hot Spot (CHP) v2 panel.

Results

Five fresh-frozen paraffin-embedded (FFPE) specimens were successfully assayed with the OCP, and KRAS was the most prevalent alteration, which might contraindicate the use of anti-EGFR therapy. One PAC patient harbored a FGFR2 p.C382R mutation, who might derive benefit from FGFR tyrosine kinase inhibitor. Additional 38 samples assayed with CHP v2 showed 113 hotspot variants, corresponding to 54 COSMID IDs. The most frequently mutated genes were TP53, KRAS, HRAS, and PDGFRA, (29, 23, 14, 10 hotspot variants), impacting 11, 23, 14, and 10 PAC patients. Highly pathogenic variants including COSM22413 (PDGFRA, FATHMM predicted score: 0.88), COSM520, COSM521, COSM518 (KRAS, FATHMM predicted score: 0.98) were reported.

Conclusions

By using NGS with targeted panel, somatic mutations with therapeutic potential were identified. The combination of clinical and genetic information is useful for decision making and precise selection of targeted medicine.

Introduction

Pancreatic adenocarcinoma (PAC) is the 8th leading cause of cancer-related death in Taiwan, and its incidence is increasing [1]. Most PAC patients are diagnosed when the tumor is relatively large and has extended beyond the pancreas. There are several reasons for such delay: first, because of its anatomic location, pancreas is not easily accessible with conventional diagnostic imaging tools. Second, initial symptoms of PAC are usually not remarkable, and clinical workup often procrastinates until the onset of more suspicious signs. Third, cystic precursor lesions of PAC are not easily distinguishable from benign cysts and may represent a diagnostic dilemma that eventually delays the correct diagnosis [2]. PAC is often caught at late stages because patients are often asymptomatic, so having more validated genetic biomarkers can augment early diagnosis and proper treatment. Besides, PAC is the most lethal human
malignancy, with a dismal five-year overall survival rate of less than 5%. Even when the tumor seems confined to the pancreas at the time of surgery, the 5-year survival rate never exceed 15%. It is estimated that 10% of PACs show a familial aggregation consistent with a genetic susceptibility. But in most instances, the genetic basis for the familial aggregation of PAC has not yet been identified [3–4]. The development of PAC involves successive accumulation of multiple genetic alternations with significant heterogeneity. Understanding the molecular pathogenesis and heterogeneity of PAC may facilitate personalized treatment for PAC and yield potential therapeutic targets [5–7].

Previous comprehensive exome sequencing of PAC had revealed that dozens of intragenic mutations accumulated in each cancer while most were infrequently mutated, and were passengers by themselves. But these studies also identified a number of recurrent aberrations, such as driver mutations which played critical roles during carcinogenesis, involving at least 12 cellular pathways implicated in PAC development [8–10]. However, all of these studies were conducted in Western countries, and no study ever assessed the molecular alternations of Taiwanese PAC. From past experiences, the patterns of driving mutations might be very diverse across different ethnic groups [11–12]. Therefore, we proposed to use state of the art next-generation sequencing (NGS) with targeted panels to explore the molecular alternations underlying PAC in Taiwan. The propose of NGS is to identify genetic alterations that might be targeted with existing drugs or used as biomarkers. PAC is among the most malignant neoplasms while research on PAC relies on clinical, pathologic, and molecular features for biomarker discovery and corresponding treatment. This study aimed to decipher genetic aberrations coupled with clinical characteristics. The combination of clinical and genetic information may help us for precisely selecting targeted therapeutics.

Materials And Methods

In summary, we used NGS to study genetic alternations of Taiwanese PAC. Tumor genomic DNA was extracted from fresh-frozen paraffin-embedded (FFPE) specimens with nucleic acid checked. The harvested DNA was sequenced by NGS with two targeted panels, and the information of genetic alterations was analyzed and correlated with clinical features.

In the first part, we took advantage of readily available FFPE specimens from deceased PAC cases to elucidate the feasibility of extracting adequate nucleic acid for NGS experiments. Pathological hematoxylin and eosin (H&E) stained slides were reviewed by one certified pathologist (CYL) to ascertain the presentation of adequate PAC cells. Nucleic acid was extracted from 5X5 µm FFPE sections, and the quality was checked by Qubit fluorimeter (Invitrogen, part of Thermo Fisher Scientific, Waltham, MA) and PCR of GADPH fragments to make sure that fragmentation was not too severe and was acceptable for amplification and sequencing. The Oncomine Comprehensive Panel (OCP, Thermo Fisher Scientific) was used as the initial screening tool detecting tumor genetic alterations which could be used as candidates for downstream analyses. There were 143 pre-selected genes on this panel, which were designed to interrogate somatic mutations including single/multi nucleotide variants (SNVs/MNVs), insertions/deletions (INDELs, 73 genes), copy number variation (CNV) including gain (49 genes) and loss (26 genes) recognized as being oncogenes or tumor suppressors recurrently altered in solid tumors with the potential for approved therapeutics or novel ones with near-term clinical relevance [13]. Besides DNA, we also performed RNA
extraction; once the RNA was adequate, the test of fusion genes was proceeded with the OCP, which interrogated 22 pre-selected onco-drive fusion genes.

In the 2nd part, more samples were collected to elucidate the oncogenesis of Taiwanese PAC, by using Ion AmpliSeq Cancer Hot Spot (CHP) v2 panel (Thermo Fisher Scientific), which was designed to amplify 207 amplicons covering approximately 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes [14–15]. Somatic mutations with clinical significance were identified from PAC with distinct precursors. After library generation, NGS experiments were conducted on Ion Torrent benchtop sequencers. The Ion PGM system operated by the Yourgene Health (New Taipei, Taiwan, for OCP) and Fu-Jen Catholic University (for CHP) was the platform where sequencing experiments were performed and Torrent Suite/Ion Reporter analysis (Thermo Fisher Scientific) was bound for calling variants. Partek Flow software (Partek Incorporated, St Louis, MO) was used as additional calling algorithm [16].

The whole study protocol had been reviewed and approved by IRB of Cathay General Hospital; informed consent of part I was waived from IRB of Cathay General Hospital while signed informed consent was obtained from all participants of part II. All methods were performed in accordance with the relevant guidelines and regulations.

Results

There were 6 FFPE PAC specimens retrieved and tested in part I of the study while 5 of which had adequate DNA/RNA for further NGS experiments. The nucleic acid quality was determined by Qubit fluorimeter to ensure that the specimen could be successfully amplified and sequenced by targeted panels (Table 1 for details).

| Sample ID | Tumor purity | Tumor source               |
|-----------|--------------|----------------------------|
| FJU01     | 15%          | Metastatic lymph node      |
| FJU02     | 10%          | Malignant effusion         |
| FJU03     | 25%          | Metastatic lymph node      |
| FJU04     | 15%          | Liver metastasis           |
| FJU05     | 20%          | Omentum metastasis         |
| FJU06     | 10%          | Malignant effusion         |

The tumor genomic DNA sequenced with the OCP revealed genetic alternations: around 150 to 200 variants of different types were found in each sample (Table 2 and Supplementary Fig. 1). Although there were hundreds of variants found in these specimens, many of which were probable passengers. Most Taiwanese
PACs harbored *KRAS* mutations, as previous studies had shown [17]. There was one *FGFR2* mutant case. The distribution of CNVs found in this study is detailed in Fig. 1. To prove that the identified variants were not spurious, we also evaluated targeted sequencing results using the Partek Flow software and Sankey diagrams of the five assayed samples are detailed in Supplementary Fig. 2. It should be noted that SAM tools were used for variant calling, resulting in discrepancy in the number of variants harvested. Supplementary Fig. 3 shows variant impact heat map of 5 PAC samples assayed with the OCP.

| Sample ID | No. of SNVs/MNVs | No. of INDELs | No. of CNVs | No. of fusions | Total positive variants including SNVs / MNVs / CNVs / fusions | No. of SNVs / MNVs / INDELs with COSMIC IDs | No. of non-Synonymous Variants | Actionable mutations |
|-----------|------------------|---------------|-------------|---------------|-------------------------------------------------------------|---------------------------------------------|-------------------------------|---------------------|
| FJU01     | 154              | 19            | 11          | -             | 184                                                         | 1                                           | 59                            | *FGFR2* p.C382R       |
| FJU02     | 179              | 4             | 13          | -             | 196                                                         | 3                                           | 83                            | *KRAS* p.G12R         |
|           |                  |               |             |               |                                                              |                                              |                                | ATM deletion           |
| FJU03     | 137              | 10            | 1           | -             | 148                                                         | 2                                           | 44                            | *KRAS* p.G12V         |
|           |                  |               |             |               |                                                              |                                              |                                | *TP53* p.R273C         |
| FJU05     | 136              | 13            | 10          | -             | 159                                                         | 1                                           | 43                            | *KRAS* p.G12D         |
| FJU06     | 135              | 10            | 2           | -             | 147                                                         | 2                                           | 44                            | *KRAS* p.G12V         |
|           |                  |               |             |               |                                                              |                                              |                                | *TP53* p.248Q         |

In part II of the study, a total of 39 archived FFPE samples of PACs were recruited from the department of pathology, with their clinical data obtained through chart reviews. Initially both the quantity and quality of DNA extracted from outdated FFPE specimens were unsatisfactory for NGS experiments. A quality improvement program was pursued, and we followed the published protocols pertaining whole exome amplification methods; REPLI-g (Qiagen, Hilden, Germany)/GenomePlex (Sigma, part of Merck KGaA, Darmstadt, Germany) kits were adopted to enhance the yield of DNA from FFPE samples [16]. Re-extraction
DNA from FFPE samples was carried out with improved DNA concentrations by Qubit (data not shown). Finally, a total of 38 PAC FFPE samples were successfully sequenced by the ION AmpliSeq CHP v2 after excluding one heavily degraded sample.

Results of ION Reporter variant caller identified 1008 variants from 38 patients (0 variant from one PAC patient, P11). The range was between 15 and 56 variants per sample, with a median of 24. The number of impacted genes was 41 (1-152 variants/gene). The alleles sources from the 1008 variants were: 113 hotspots (54 COSMIC IDs) and 895 novel ones. For the 113 Hotspot regions, 34 out of 38 (89%) patients reported as least one hotspot variant, with a median of 3 hotspot regions per patient (range: 1–8). The most impacted 15 genes and the number of associated variants were tabulated in Table 3 (Supplementary Table 1 for the number of involved PAC subjects). The most frequently impacted genes were: KRAS (23 samples), HRAS (14 samples), TP53 (11 samples), and PDGFRA (10 samples, Table 3). The COSMIC database was consulted for annotation (Supplementary Table 2) and recurrent pathogenic variants are detailed in Supplementary Table 3. Precursor features of assayed PAC samples are detailed in Supplementary Table 4 with variant impact heat maps displayed in Supplementary Fig. 4.

**Table 3**
The most impacted 15 genes and the number of associated variants among 113 hotspot regions (part II, n = 38).

| Gene symbol | No of variants | No. of PAC samples (patients) |
|-------------|----------------|------------------------------|
| TP53        | 29             | 11                           |
| KRAS        | 23             | 23                           |
| HRAS        | 14             | 14                           |
| PDGFRA      | 10             | 10                           |
| KIT         | 6              | 6                            |
| PTEN        | 6              | 4                            |
| SMARCB1     | 6              | 6                            |
| GNAS        | 4              | 2                            |
| MET         | 4              | 4                            |
| CDKN2A      | 2              | 2                            |
| CTNNB1      | 2              | 2                            |
| EGFR        | 2              | 2                            |
| IDH1        | 2              | 2                            |
| STK11       | 2              | 2                            |
| SMO         | 1              | 1                            |
Discussion

Long-term survival of PAC remains stubbornly low and there is an unmet need for early detection and efficient systemic treatment. Although treatment outcomes for many types of cancer has improved, PAC survival has lagged significantly behind in the last decades. One major limitation comes from very few treatment options for PAC. A better understanding of PAC may lead to new treatment options and improved clinical outcomes for this lethal disease [18]. In current study, we hypothesized that by using NGS, genetic alterations guiding the selection of targeted therapies could be identified. By predefining a set of relevant somatic alternations, targeted panels identified variants which could be linked to potentially therapeutic strategies [19–20]. Two commercialized panels, namely the OCP and CHP, were adopted to fulfill the purpose of NGS experiments and novel therapeutics identification. The OCP was designed for compatibility with routine FFPE tissues, with co-isolation of DNA and RNA. The OCP consisted of multiplexed PCR compatible with 20 ng of DNA and 15 ng of RNA input, further augmenting its clinical applicability. The CHP provided a more cost-effectiveness and scalable solution for routine practice as 10 ng of input tumor genomic DNA from FFPE tissues is adequate for targeted library construction.

Initially, 6 Taiwanese PAC FFPE samples were evaluated with metastatic lesion DNA extracted, and 5 with adequate nucleic acid were sequence by the OCP. Up to 150 and 200 alternations were found in each sample while most were probable bystanders with no relevant therapy. In part I of the study, there was at least one actionable mutation found in each PAC patient, making personalized therapy achievable. These actionable mutations corresponded to potentially matched treatments, which could be the targets of novel therapeutics.

The presence of KRAS mutation may be a predictive biomarker against the use of anti-EGFR antibody. In lung cancer treatment, KRAS is downstream in the EGFR pathway; therefore, tyrosine kinase-based treatment with gefitinib and erlotinib is ineffective when KRAS is constitutively activated [21–22]. However, many clinical trials which combine other tyrosine kinase inhibitors (TKIs) and chemotherapy are ongoing for these KRAS mutant PACs [23]. In addition, knockdown of mutant KRAS with RNA interference may be a potentially therapeutic strategy in near future [24]. Recently, a subset of KRAS wild-type younger PACs has been identified for whom the recognition of alternative oncogenic drivers which are also targetable is in eagerly need [25]. There was one FGFR2 mutant case (FJU01) and the NGS result showed the possibility of FGFR TKI treatment in the future as there are several FGFR TKI trials [26–27]. Although RNA sequencing showed no fusion gene in current study, there are more and more evidences suggesting that fusion oncogenes present not only in sarcoma, but also in carcinoma [28]. This kind of alternation could also be a potential biomarker for targeted therapy, such as crizotinib for treating lung cancer with EML4-ALK translocation [29]. Many CNVs were also found in this study while their meaning needs further investigation. Further studies to clarify whether the gain of oncogenes or loss of suppressors represent prognostic or predictive biomarkers for PAC are warranted.

The CHP was adopted for part II of the study with more PAC samples assayed. The choice of the CHP rather than OCP was based on economic consideration. The CHP v2 surveyed hotspot regions of 50 oncogenes and tumor suppressor genes, with wide coverage of KRAS, BRAF and EGFR genes, which were evidenced as
being PAC-relevant from part I of the study. It deserves notice that it’s also the CHP platform which was adopted in NCI’s MATCH trial [30]. An example of COSMIC 518 KRAS mutation involved in MAPK, EGFR1, IL2, IL3, IL5, and ErbB pathways with a high pathogenic score. Depending on the impacted subject/variant count, the most common actionable mutations came from KRAS (23 samples/variants), HRAS (14 samples/variants), TP53 (11 samples/29 variants), and PDGFRA (10 samples/variants). Supplementary Table 1 showed that up to 5 variants could be detected within the same subject. Although COSMIC database identified KRAS, PFGFRA and KIT as being pathogenic (Supplementary Table 2), only KRAS was predictive in terms of matched alternation-drug combination. A mutated KRAS gene is a biomarker for many cancer types, as this gene has control over cell cycle division and cancer cell growth.

It’s convenient to conduct summarized cohort analysis and subgroup analysis by the method of variant impact heat maps, as moderate sample size of part II made whole cohort and subgroup clustering analyses possible. Variant impact heat map of PACs with mucinous and pancreatic intraepithelial neoplasm (PIN) precursor showed roughly comparable distributions of genetic alternations in TP53, APC, SMAD4, PTEN, PIK3CA and CDKN2A (Supplementary Fig. 4). Although Ryan et al. highlighted that more than 90% of PAC were associated with activating KRAS mutation, the frequency was much higher for intraepithelial neoplasm (> 90%) then mucinous neoplasm (40–65%). Other suppressors such as CDKN2A, TP53 and SMAD4, the aberrant rate increased with higher nuclear grade, albeit alterations in tumor suppressor genes rarely led to targeted therapy. The purposed more GNAS oncogenic mutation was not observed in PAC with mucinous precursor in current study, either [23].

Singhi et al. conducted by far the largest study on around 3,600 PACs and found that the most frequently mutated genes, i.e. KRAS, TP53, CDKN21, and SMAD4, can’t be targeted with readily available existing drugs [31]. In 2020, FDA approved olaparib for maintenance treatment of germline BRCA-mutated metastatic PAC whose disease has not progressed on at least 16 weeks of a first-line platinum-based chemotherapy, based on phase III POLO trial [32]. The most common pathogenic germline mutations are in BRCA1, BRCA2, and ATM, and more rarely, in PALB2, MLH1, MSH2, MSH6, PMS2, CDKN2A, and TP53, among others, for an aggregate frequency of 3.8–9.7% [33–34]. Although somatic alternations were interrogated in current study, several of aberrant DNA damage repair genes were reported from Taiwanese PAC patients and reflux germline testing should be indicated once alternations in tumor-only sequencing were evidenced.

There were some limitations of the study. First, the modest sample size might hamper externalization of sequencing results. The 50 targeted genes of the CHP had been covered by the OCP, so there was no concern of comparability. In addition to the limited sample size, it should be noted that only 15% of PACs were resectable at time of diagnosis, which means the majority of PAC genetic analyses were conducted on early stage disease [35–36]. In current study, part I samples were derived from metastatic lesions while for part II, all specimens came from primary pancreatic neoplastic tissue in an effort to broaden clinical scenarios of PAC [37]. Second, although PAC is characterized by diverse, largescale chromosomal changes with forms of amplifications, deletions, and rearrangement, only the five OCP assays interrogated CNVs and structural aberrations of the 38 CHP assays were left undetermined. Third, as PAC is hard-to-diagnose due to the difficulty in obtaining tumor samples from patients, the feasibility and prognostic value of circulating tumor DNA (ctDNA) in PAC is being tested rigorously [38]. The knowledge learned from current study and
other genetic studies may pave the way for future circulating biomarkers to screen, guide treatment, and monitor disease progress of PAC.

**Conclusions**

By using NGS with targeted panel, somatic mutations with therapeutic potential were identified. The combination of clinical and genetic information is useful for decision making and precise selection of targeted medicine.

**Declarations**

- **Ethical Approval and Consent to participate**

The whole study protocol had been reviewed and approved by IRB of Cathay General Hospital; informed consent of part I was waived while signed informed consent was obtained from all participants of part II.

- **Consent for publication**

All authors gave their consent for publication.

- **Availability of data and materials**

Genomic data of the study were secured by the primary investigators as requested by IRB and might be available in an anonymous manner upon reasonable request.

- **Competing interests**

All authors declared there was no conflict of interest.

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- **Authors’ contributions**

CCH initiated and drafted the manuscript. CYL and WHK ascertained pathological diagnosis and quality of assayed samples. CJH conducted NGS experiments. YCH revised and manuscript critically. HHL, JUW, and FCT contributed in enrollment of PAC subjects during part II of the study. CFH and JTL assembled series of panel discussion. CSH and HSC finalized and approved the manuscript.

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Figures
Overview of CNV Gain/Loss within Genes

Figure 1

CNVs per gene across Taiwanese PAC samples (part I, n=5).

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