Genomic Analysis of Pancreatic Juice DNA Assesses Malignant Risk of Intraductal Papillary Mucinous Neoplasm of Pancreas

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INTRODUCTION

Intraductal papillary mucinous neoplasms (IPMNs), described for the first time in 1982 (Ohashi K 1982, Lee, Kim et al. 2016) and posteriorly defined by the World Health Organization, are pancreatic tumors with unique characteristics including hyper-production of mucin in tall columnar epitheliums, and dilatation and papillary growth inside the pancreatic ducts(Santini, Campione et al. 1995, Sohn, Yeo et al. 2001, Hruban, Takaori et al. 2004, Sohn, Yeo et al. 2004). After the establishment of its diagnosis, the incidence of IPMN has been rapidly increasing, and it is now understood that IPMN can be classified along the spectrum of adenoma to carcinoma, and it is can be a precursor of pancreatic cancer(Patra, Bardeesy et al. 2017). Its prognosis, compared with pancreatic ductal adenocarcinoma (PDAC), is relatively better after surgical resection (Mino-Kenudson, Fernandez-del Castillo et al. 2011). However, IPMN is histologically very heterogeneous and some parts can progress from low to high-grade dysplasia and finally to invasive adenocarcinoma, which shows as poor prognosis as PDAC (Maire, Hammel et al. 2002). Furthermore, PDAC is sometimes coincident with IPMN(Yamaguchi, Ohuchida et al. 2002). Therefore, it is clinically important to assess the risk of pancreatic cancer progression and development in IPMNs in order to take the decision of tumor resection(Salvia, Fernandez-del Castillo et al. 2004, Moris, Damaskos et al. 2017) and it is essential to develop other non-invasive approach to assess the malignant potential of IPMNs based on biomarkers or genomic alterations. Cell-free DNA shed by tumor cells is a rich source of tumor-specific biomarkers and genomic analysis, as shown in studies on cell-free DNA derived from plasma(Diehl, Li et al. 2005, Crowley, Di Nicolantonio et al. 2013, Ono, Fujimoto et al. 2015), urine(Togneri, Ward et al. 2016), and cerebrospinal fluid (CSF)(Wang, Springer et al. 2015). Therefore pancreatic juice could also be an ideal material for assessing malignancy risk of IPMN and pancreatic tumors as well. Moreover, due to its liquid nature, pancreatic juice has potential to overcome the heterogeneity inherent to biopsy specimens from IPMN. Until now, several studies had analyzed pancreatic juice for driver mutations such as *KRAS* and *GNAS* (Eshleman, Norris et al. 2015, Yu, Sadakari et al. 2017). However, it is still unclear whether these mutations are useful as a marker of IPMN malignancy. More comprehensive genomic analysis of pancreatic juice cfDNA (PJD) may help discover better markers of malignancy and overcome the intratumoral heterogeneity of IPMN. Here, we performed deep exome sequencing for PJDs from 40 IPMN patients and found that mutational burden and copy number alterations detected in PJDs could evaluate the malignancy of IPMN.

RESULTS

Analysis flowchart of this study is shown in **Figure 1**. To identify somatic mutations of IPMNs in PJD, we collected pancreatic juice and blood of 40 IPMN cases and extracted DNA from these samples. The RIKEN SNP research center constructed next generation sequencing libraries from a small amount of PJD (5-10ng) and performed deep exome sequencing of PJD together with blood DNA for 40 IPMN cases. The median number of sequence reads were 163 million reads for PJD, and the median depth on target was 168x after duplication removal. For blood samples, the median number of sequence reads were 82 million reads, and the median depth on target was 112x after duplication removal. The median of duplication rate was 18.7% in exome of PJD and was higher than that in blood DNA exome (5.4%). This may be caused by low-input DNA for the library preparation. After removing one case that was revised and diagnosed as PDAC and not IPMN, seven cases with OxoG and/or high residual variance were removed from the remaining 39 cases, and therefore we called somatic mutations for the remaining 32. Totally, 627 somatic non-synonymous mutations affecting 561 genes were detected. Interestingly, the whole-exome mutation burden in PJD was associated with the histologic grades of IPMN (**Figure 2)**. The median number of mutations was 18 in grade 1, 21.5 in grade 2, and 71 in grade 3. Spearman correlation coefficient between the histologic grade of IPMN and the number of mutations was 0.417 ($p = 0.018$). This result indicated

that the mutation burden in PJD might serve as a marker for malignant potential of IPMN. In contrast with this association, the mutational signature had a similar distribution irrespective of grades. *GNAS* mutation was associated with male ($p = 0.019$) and with main duct type ($p = 0.018$), consistently with previous reports (Takano, Fukasawa et al. 2014, Lee, Kim et al. 2016). *KRAS* and *GNAS* were most frequently mutated (15 and 10 samples; 47% and 31%, respectively), which were also consistent with previous studies (Matthaei, Norris et al. 2012, Amato, Molin et al. 2014, Takano, Fukasawa et al. 2014, Yu, Sadakari et al. 2017). *TP53* and *RNF43* were mutated in five samples (15.63%). *RNF43* is a RING-type E3 ubiquitin ligase whose loss-of-function mutation can activate the Wnt/β-catenin signaling and is commonly mutated in IPMN and in other tumors (Yu, Sadakari et al. 2017, Fujita, Matsubara et al. 2018). Other recurrently mutated genes were *COL12A1*, which encodes the alpha chain of type XII collagen, and *MUC17* which encodes a protective membrane-bound mucin to gut epithelial cells and has been reported as highly expressed in PDAC as well as a marker for poor prognosis (Hirono, Yamaue et al. 2010). Both *COL12A1* and *MUC17* were mutated in three samples.

We called somatic copy number alterations (CNAs) from PJD exome data in our samples filtered from high residual variance and detected eleven significantly amplified regions (*1p31.2, 2p12, 2q31.2, 3p22.3, 3q13.11, 5q32, 6p12.1, 7q21.12, 8q24.22, 11q22.1* and *12q21.31*) and five significantly deleted regions (*1p36.31, 6p23, 16q12.2, 17p13.2* and *22q13.1*). The detected CNAs were evaluated for their potentials as malignancy markers (**Figure 3)**. We observed a significant association between the histologic grade and amplification of two regions (*7q21.12* and *8q24.22*). The *7q21* amplification was found in 1/7 (14.26%) in grade 1, 2/17 (11.76%) in grade 2, 7/11 (63.64%) in grade 3 ($p= 0.012$ by Fisher's exact test). *KIAA1324L* is located in this amplified region, but it has not been reported as associated with IPMNs and pancreatic cancer. The *8q24* amplification was found in 2/7 (28.57%) in grade 1, 1/17 (5.88%) in grade 2, 6/11 (54.55%) in grade 3 ($p = 0.011$). *MYC* is located in the *8q24* amplified regions in IPMNs, which is one of the most frequently altered genes by CNAs in pan-cancer analysis (Beroukhim, Mermel et al. 2010, Leiserson, Vandin et al. 2015) and whose amplification has been found in pancreatic acinar cell carcinomas (Bergmann, Aulmann et al. 2014, La Rosa, Bernasconi et al. 2018). The deletion region *17p13*, which contains *TP53* was also found to co-occur with *TP53* point mutations; three out of four malignant samples (grade 3) with *TP53* mutations, which might be consistent with the two-hit theory of tumor suppressor genes.

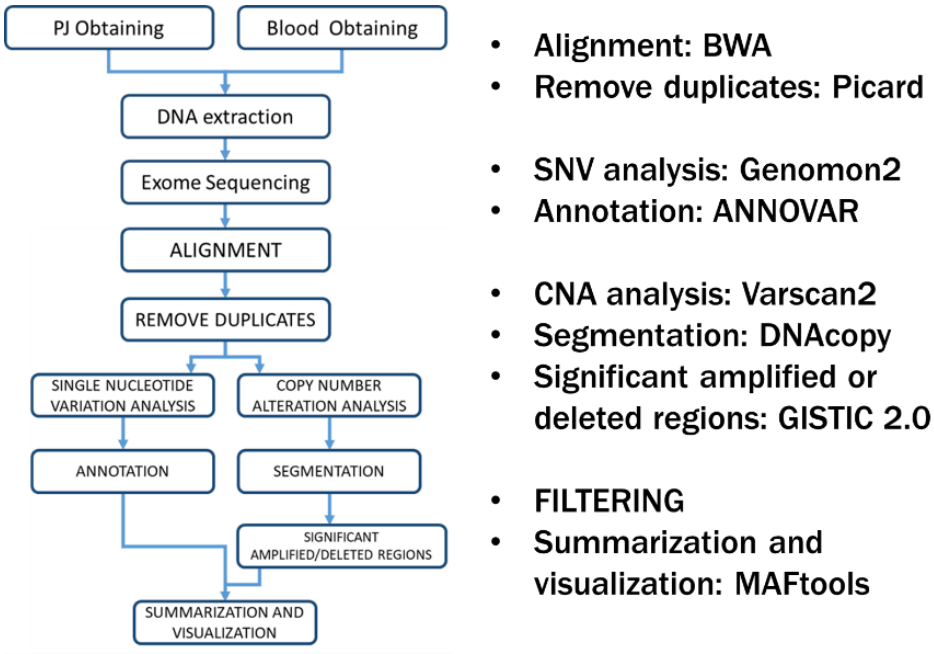

Figure 1: Workflow of deep exome sequencing and CNA analysis for PJD.

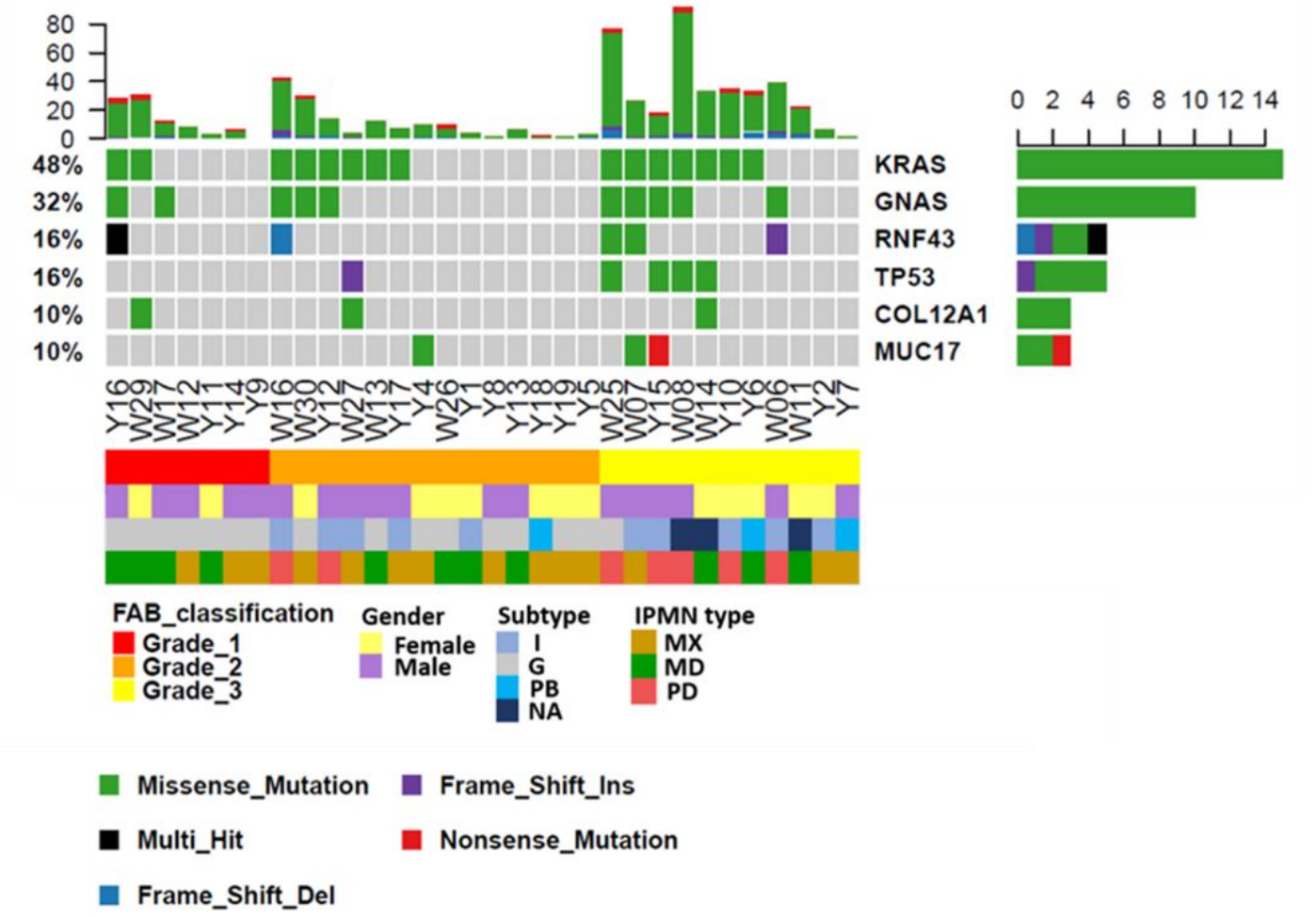

Figure 2. Somatic mutations detected in PDJ and the histologic grade of IPMN.

DISCUSSION

We here succeeded in genomic profiling of heterogeneous IPMNs by comprehensive genomic analysis of PJD and showed clinical usefulness of PJD sequencing. Pancreatic juice is a useful source for genetic profiling of IPMN and pancreatic tumors; it is less invasive than biopsy and deals better with the heterogeneity of the tumor. With this method, we not only detected critical mutations such as *KRAS* and *GNAS*, known markers in IPMN that prove to a certain extent the reliability of this sample source, but also found out that the mutation burden is significantly correlated with IPMN histological grade. This correlation of mutation burden with histological grade can be associated not only with the development of malignancy in IPMN, but also with the genomic heterogeneity inherent to advanced tumor samples and our distinct sampling method; different cell populations might possess singular mutations that could go undetected by traditional sampling, but they may be able to be observed on its entirety by the use of liquid samples, which has the potential to gather information from all the populations. We as well found multiple regions significantly deleted and amplified in our dataset. Finding *TP53* deleted in the malignant state of the IPMN also reinforced the reliability of our method. More interestingly, two of the regions (*7q21.12* and *8q24.22*) were significantly amplified in malignant samples, which lead us to discover the association of *MYC*, a known marker of malignancy, with the development of IPMN. The amplification and deregulation of *MYC* has been reported in numerous cancer studies, and its key role in metabolism, cell cycle, the biogenesis of ribosomes, and consequently, in cell growth and proliferation makes it the perfect target and marker for malignancy in IPMN

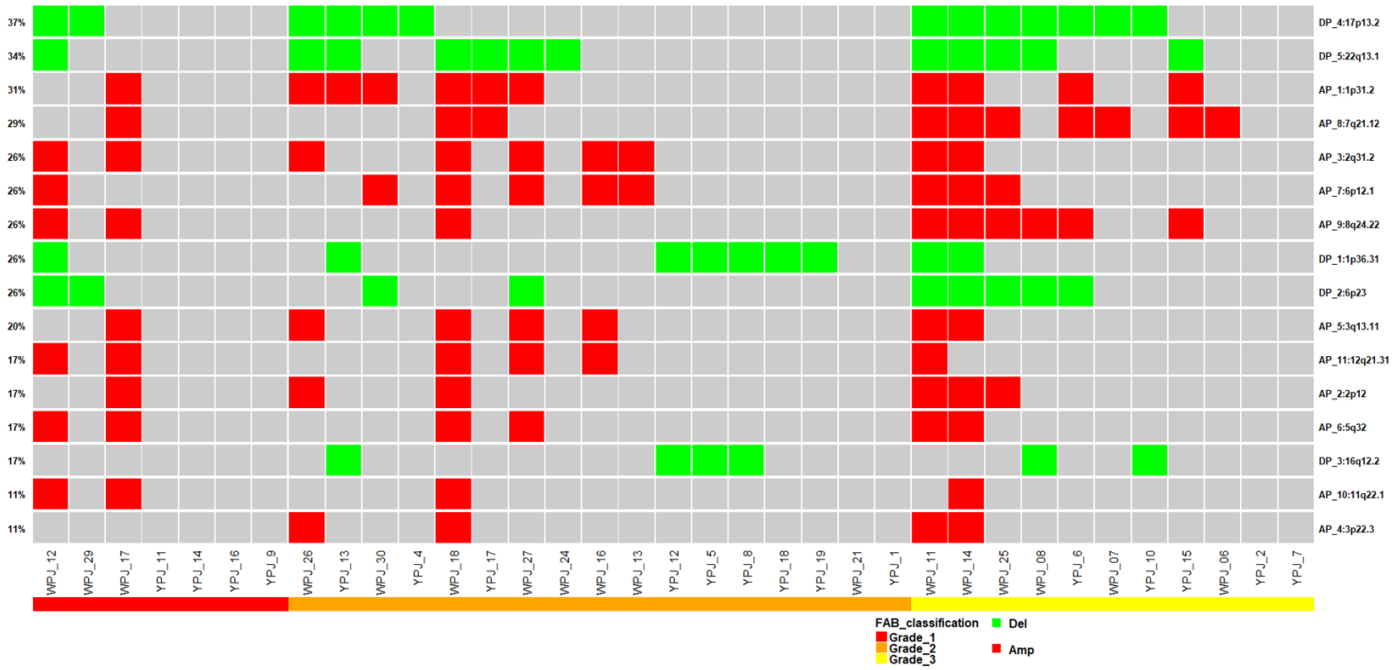

Figure 3. Significantly amplified regions (red) and significantly deleted regions (green) by patient and by grade The *7q21* amplification and *8q24* amplification (*MYC*) showed significant association with the histologic grade 3 (p= 0.012 and p = 0.011, respectively, by Fisher's exact test).

detection by PJD (Alitalo, Schwab et al. 1983, Dang 2012, Hsieh, Walton et al. 2015, Kalkat, De Melo et al. 2017). This finding, combined with the correlation of mutation burden with grade, have strong potential to predict the progression of IPMNs to malignancy, and exome sequencing or whole genome sequencing analysis of PJD is useful to assess the malignant risk of IPMNs.

Although we demonstrated the proof-of-concept of PJD exome testing, its clinical deployment has to overcome several issues. Firstly, the clinical procedure to obtain pancreatic juice remained controversial due to being prone to producing pancreatitis. It has not been recommended in international consensus guidelines of 2012 for the management of IPMN (Tanaka, Fernandez-del Castillo et al. 2012). Further study will be required to assess the risk and benefit of the PJD exome testing. Secondly, we had to exclude some of our PJD samples because their exome data contained high levels of noise. Three samples were affected by artifacts in SNVs that resembles OxoG, whereas four samples had extreme fluctuations in their copy number segments. In this study, we simply excluded these noisy samples from analysis. However, the yield should be improved through protocol optimization. Potential strategies are addition of antioxidants to prevent OxoG, and whole genome sequencing to reduce copy number noise. Furthermore, the presence of different tumoral subpopulations with different alterations, combined with the existence of non-tumoral cfDNA in PJ, can modify the final ratio of altered/normal reads, potentially making the signal of some of these alterations unable to reach the threshold defined for their detection, leading to the reduction or even the disappearance of them in the final output. Learning how to evaluate this kind of results, and rescue true outputs without reducing the specificity of the pipeline is the future direction of this research.