Individualized drug screening based on next generation sequencing and patient derived xenograft model for pancreatic cancer with bone metastasis

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Abstract. The efficacy of traditional chemoradiotherapies for pancreatic cancer remains limited, and no effective targeted therapies or screening tests are currently available. Therefore more individualized drug screening is warranted for the clinical treatment of pancreatic cancer. A patient-derived xenograft (PDX) model of pancreatic cancer bone metastasis was established, and next-generation sequencing (NGS) was used to investigate the molecular characteristics of the cancer and screen for potential drugs. Immunohistochemical analysis was performed to validate that the PDX retained the molecular characteristics from the patient. Using NGS technology, 13 pancreatic-cancer-associated polymorphisms/mutations were identified out of 416 genes sequenced. Based on the sequencing results and associated literatures, AZD6244, a highly selective inhibitor against mitogen-activated protein kinase kinase 1 (*MEK1*), was chosen as a potential therapy. AZD6244, a highly selective MEK1 inhibitor, was evaluated as effective for the pancreatic cancer PDX model, and thus may provide potential efficacy in the clinical treatment of the patient with pancreatic cancer investigated in the present study. The feasibility of the novel NGS-PDX based drug-screening pattern was demonstrated, and has a potential to improve individualized treatment for cancer.

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Introduction

Pancreatic cancer is expected to be the second most lethal malignancy in the USA by 2020, and the 5-year survival rate for patients diagnosed with locally advanced or metastatic pancreatic cancer remains <3% (1,2). The efficacy of traditional chemoradiotherapies for pancreatic cancer remains limited (3-5). However, no effective targeted therapies or screening tests for pancreatic cancer are recently available, and no clinically comfirmed biomarkers are available for identifying subsets of patients who might benefit from chemoradiotherapies or targeted theprapies (6-9). Different from frequent liver and peritoneum metastases, the bone metastasis rate of pancreatic cancers is quite low but reaches higher of about 7.3% with the improvement of the diagnosis and treatment level (10,11). For pancreatic cancer patients, especially those in advanced or metastatic disease stages, individualized drug screening is urgently needed for the clinical treatment.

The lack of an appropriate *in vivo* model for preclinical studies has limited the mechanistic study of tumor resistance to anti-VEGF therapy. Patient-derived xenografts (PDXs), so-called Avatar models (12), have been increasingly widely used in various types of cancers for translational research in recent years, with the greatest advantage of its ability to better predict clinical tumor response (13). Accumulating evidence indicates that PDX is an reliable cancer research tool for drug screening and personalized medicine applications (14).

It is known that somatic genomic alterations alter the function of genes or pathways, thus resulting in tumorigenesis, metastasis, and resistance to therapies (15,16). Therefore, precise molecular profiles of tumors will help to predict drug

responses (17). Understanding the genomic landscape of CRC can contribute to drug screening (8,18-20). Large-scale sequencing projects has economically led to the rapid development and clinical popularization of next-generation sequencing (NGS) technologies (21). NGS can be a powerful tool to understand the genomic landscape of patients and mechanism of drug response, which thus might provide a more broad vision for clinically potential drug screening (22-24). Therefore, NGS technologies are being used by pharmaceutical companies throughout the drug discovery process (21).

In our previous studies, we established a series of PDX models of different tumor types and accumulated substantial experiences of drug evaluation, screening and mechanism exploration (25,26). While in the present study, we established a PDX model by pancreatic cancer bone metastasis tumor tissues for evaluation of potential drugs for pancreatic cancer patient. In our study, in order to select the optimal therapy for the patient, the NGS technology was used for investigating of tumor molecular characteristics and searching for potential drugs, which were finally evaluated in the corresponding PDX model. The aim of our study is to demonstrate the feasibility of the novel NGS-PDX based drug screening pattern which has a great potential to improve the cancer individualized treatment.

Materials and methods

Reagents and drugs. AZD6244 (cat. no. S1008) and Capecitabine (cat. no. S1156) were purchased from Selleck Chemicals (Shanghai, China). The antibodies against ki-67, CK19, CK7, PCNA, Caspase-3, ERK, p-ERK, and β-actin were purchased from Abcam (Cambridge, UK).

Patient and tumor tissues. Pancreatic cancer bone metastasis (diagnosed as adenocarcinoma) tissues were obtained at surgery from a 67-year-old female patient. A single bone metastasis was imageologically found at the right pedicle of L2 vertebral arch, which means a high risk of fracture and paraplegia. In addition, the patient urged for operation treatment. The study was done in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization and Good Clinical Practice guidelines. The Institutional Ethical Committee approved the current study.

Establishment of PDX model. BALB/c nude mice (3-to-4-week-old, female) were purchased from Shanghai Slaccas Laboratory Animal and housed in SPF laboratory animal rooms at laboratory animal center of Zhejiang University. Mice were acclimated to new environments for at least 3 days before use. Surgical tumor tissues were cut into pieces of 3 to 4 mm and transplanted within 30 min s.c. to mice. Additional tissues were snap-frozen and stored at -80˚C until use. Animals were monitored periodically for their weight with an electronic balance and tumor growth with a Vernier caliper twice every week. The tumor volume was calculated as formula $V=LD \times (SD)^2/2$, where V represents the tumor volume, LD and SD are the longest and the shortest tumor diameter, respectively. Tumors were then harvested, minced and re-implanted as described above for passaging. At each generation, tumors were harvested and stored in liquid nitrogen for further use. The usage of experimental animals was according to the Principles of Laboratory Animal Care (NIH #85-23, 1985 version). All animal studies were according to the Institutional Animal Care and Use Committee of Zhejiang University, and the approval ID was SYXK (ZHE) 2005-0072.

Table I. Next generation sequencing of the patient tumor.

Gene	AA Change	Type	Allele call	Abundance
BRCA2	N372H	SNP	Homozygous	
BRIP1	R439X	SNP	Homozygous	48%
CYP2D6	P34S	SNP	Homozygous	
CYP3A5	CYP3A5*3	SNP	Homozygous	
EGFR	R521K	SNP	Homozygous	
ERBB2	I655V	SNP	Homozygous	
ERBB2	P1170A	SNP	Heterozygous	
GSTM1		Deletion	Homozygous	
GSTT1		Deletion	Homozygous	
KRAS	G12D	SNP		5%
NQO1	P187S	SNP	Homozygous	
PTEN	R173C	SNP		37%
UGT1A1	6/7TA	SNP	Heterozygous	

Multiple gene mutation analysis by next generation sequencing. The sequencing including 416 gene exons was conducted by Geneseeq Technology Inc. (Nanjing, China). ctDNA was extracted from patient's tumor. The purified ctDNA is quantified by a Picogreen fluorescence assay using the provided lambda DNA standards (Invitrogen Life Technologies, Carlsbad, CA, USA). Then, library construction with the KAPA Hyper DNA Library Prep Kit, containing mixes for end repair, dA addition and ligation, were performed in 96-well plates (Eppendorf). Dual-indexed sequencing libraries are PCR amplified for 4-7 cycles. The 5'-biotinylated probe solution is provided as capture probes, the baits target 416 cancer-related genes. 1 μg of each ctDNA-fragment sequencing library is mixed with 5 μg of human Cot-1 DNA, 5 μg of salmon sperm DNA, and 1 unit adaptor-specific blocker DNA in hybridization buffer, heated for 10 min at 95˚C, and held for 5 min at 65˚C in the thermocycler. Within 5 min, the capture probes are added to the mixture, and the solution hybridization is performed for 16-18 h at 65˚C. After hybridization is complete, the captured targets are selected by pulling down the biotinylated probe/target hybrids using streptavidin-coated magnetic beads, and off-target library is removed by washing with wash buffer. The PCR master mix is added to directly amplify (6-8 cycles) the captured library from the washed beads. After amplification, the samples are purified by AMPure XP beads, quantified by qPCR (Kapa Biosystems, Inc., Wilmington, MA, USA) and sized on bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA). Libraries are normalized to 2.5 nM and pooled. Deep Sequencing is performed on Illumina HiSeq 4000 using PE75 V1 kit. Cluster generation and sequencing is performed according to manufacturer's protocol. Base calling was performed using bcl2fastq v2.16.0.10 (Illumina, Inc., San Diego, CA, USA) to generate sequence reads in FASTQ format (Illumina 1.8+ encoding).

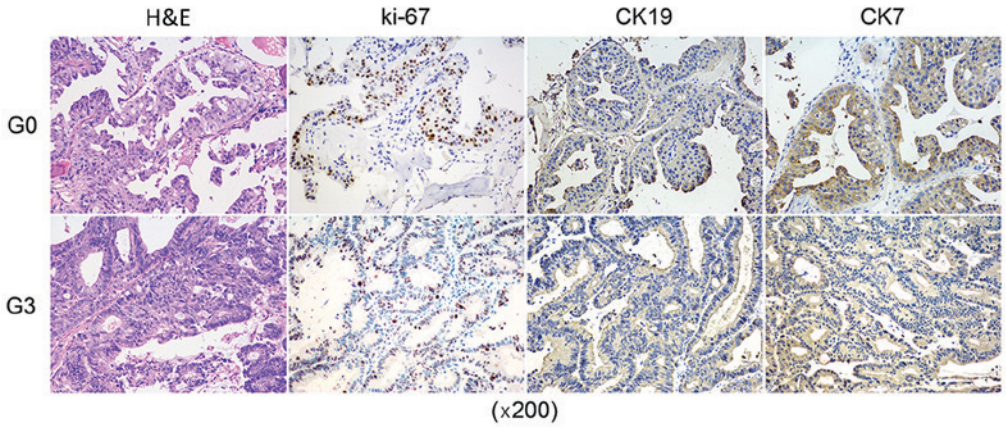

Figure 1. Immunohistochemical expressions compaired with PDX and patient tumor. The pathological characteristics of the third passage PDX xenograft was in accordance with the original patient sample. PDX, patient-derived xenograft; H&E, hematoxylin and eosin.

Quality control (QC) was applied with Trimmomatic (27). High quality reads were mapped to the human genome (hg19, GRCh37 Genome Reference Consortium Human Reference 37) using modified BWA aligner 0.7.12 (28) with BWA-MEM algorithm and default parameters to create SAM files. Picard 1.119 (http://picard.sourceforge.net/) was used to convert SAM files to compressed BAM files which were then sorted according to chromosome coordinates. The Genome Analysis Toolkit (29) (GATK, version 3.4-0) was modified and used to locally realign the BAMs files at intervals with indel mismatches and recalibrate base quality scores of reads in BAM files (30). Single nucleotide variants (SNVs) and short insertions/deletions (indels) were identified using VarScan2 2.3.9 (31) with minimum variant allele frequency threshold set at 0.01 and P-value threshold for calling variants set at 0.05 to generate Variant Call Format (VCF) files. All SNVs/indels were annotated with ANNOVAR, and each SNV/indel was manually checked with the Integrative Genomics Viewer (32) (IGV). Copy number variations (CNVs) were identified using ADTEx 1.0.4 (33). The 416 gene exons sequencing report from Geneseeq Technology Inc also provided the drug treatment suggestions.

Treatment protocol. From the 3rd generation, PDX tumors were permitted to grow to a volume of 150-200 mm^3, then mice were randomized (6 mice with tumors per group and housed in per rearing cage) and dosing was administrated (AZD6244, 50 mg/kg p.o. qd; Capecitabine, 1.0 mM/kg p.o. qd) for 4 weeks. Mice were weighed for signs of toxicity and tumor size was evaluated once per week. TGI (Relative tumor growth inhibition) was calculated using the following formula: (1-T/C)%, where T means the relative tumor volume of the treated mice, and C means the relative tumor volume of the control mice.

Immunohistochemistry. Specimen were fixed by 10 neutral formalin, then embedded in paraffin, sectioned (5 μm thick) and placed on slides for marker analysis. Sections were incubated with the primary antibodies overnight at 4˚C, after blocking nonspecific antibody bindings. The streptavidin-biotin peroxidase complex method (Lab Vision, Nairobi, Kenya) was used for immunohistochemistry. The slides were photographed using an Olympus BX60 (Olympus, Hamburg, Germany).

Statistical analysis. Results were presented as mean ± SD. Calculation and statistics were performed with Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA were used to analyze the significance of differences among groups. $P<0.05$ was considered statistically significant.

Results

Patient characteristics and PDX model establishment. Pancreatic cancer bone metastasis (diagnosed as adenocarcinoma) tissues obtained at surgery from a 67-year-old female patient were subcutaneously implanted into BALB/c nude mice for the PDX model establishment. Tumors were re-implanted in new mice after reaching a volume of 1,000 mm^3 as model passaging. The PDX model was serially passaged in animals 3 times. In order to further evaluate the PDX xenograft, immunohistochemical test was performed to identify if the patient's characteristics were retained in the PDX. Immunohistochemical expressions of CK19, CK7, and ki67 as well as the H&E staining showed that the pathological characteristics of the third passage xenograft was in accordance with the original patient sample (Fig. 1).

Next generation sequencing for drug efficacy prediction. The sequencing of pancreatic cancer bone metastasis tissues of the patient tumor was conducted by Geneseeq Technology Inc. Totally, 13 pancreatic cancer-associated gene polymorphisms/mutations were found out of the 416 genes sequenced (Tables I and II). Based on the sequencing results and associated literatures, there were no under-clinical-trial targeted therapies of pancreatic cancer directly suitable for the genes detected. Therefore AZD6244 (AZD for short, also named as Selumetinib), a highly selective inhibitor against *MEK1*, was chosen as a potential therapy whose antitumor efficacy would then be evaluated in our PDX model.

Efficacy evaluation of AZD6244 based on PDX model. To test whether the PDX model of pancreatic cancer bone metastasis was sensitive to the suggested therapy, antitumor-growth ability of AZD6244 were evaluated (Capecitabine for positive control). Since tumors volume reached 150-200 mm^3,

Table II. 416 genes for analysis.

ABCC2	DMNT3A	KDR	RAF1
ACTB	DNM2	KIF1B	RARA
ADH1B	DOCK1	KIT	RASGEF1A
AIP	DOT1L	KMT2B	RB1
AKT1	DPYD	KMT2C	RECQL4
AKT2	DUSP2	KRAS	RELN
AKT3	EBF1	LEF1	RET
ALDH2	ECT2L	LMO1	RHBDF2
ALK	EED	LSP1	RHOA
AMER1	EGFR	LYN	RICTOR
AP3B1	EGR1	LYST	RNF146
APC	EP300	LZTR1	RNF43
AR	EPCAM	MAP2K1	ROS1
ARAF	EPHA3	MAP2K2	ROS1
ARID1A	ERBB2	MAP2K4	RPTOR
ARID2	ERBB3	MAP3K1	RRM1
ARID5B	ERBB4	MCL1	RUNX1
ASXL1	ERCC1	MDM2	SBDS
ATM	ERCC2	MDM4	SDHA
ATR	ERCC3	MECOM	SDHAF2
ATRX	ERCC4	MED12	SDHB
AURKA	ERCC5	MEF2B	SDHC
AURKB	ESR1	MEN1	SDHD
AXIN1	ETV1	MET	SERP2
AXL	ETV4	MGMT	SETBP1
B2M	EWSR1	MITF	SETD2
BAP1	EXT1	MLH1	SF3B1
BARD1	EXT2	MLL	SGK1
BAT3	EZH2	MLLT10	SH2D1A
BCL2	FANCA	MLPH	SLX4
BCL2L1	FANCB	MPL	SMAD2
BCL2L2	FANCC	MRE11A	SMAD3
BCORL1	FANCD2	MSH2	SMAD4
BIM(BCL2L11)	FANCE	MSH3	SMAD7
BLM	FANCF	MSH6	SMARCA4
BMPR1A	FANCG	MTHFR	SMARCB1
BRAF	FANCI	MTOR	SMC1A
BRCA1	FANCL	MUTYH	SMC3
BRCA2	FANCM	MYC	SMO
BRD4	FAT1	MYCL1	SOX2
BRIP1	FBXO11	MYCN	SPOP
BTG2	FCGR2B	MYD88	SRC
BTK	FGF19	MYNN	SRSF2
BTLA	FGFR1	NBN	STAG2
BUB1B	FGFR2	NCSTN	STAT3
c11orf30	FGFR3	NF1	STAT5A
CALR	FGFR4	NF2	STAT5B
CBL	FH	NFKBIA	STIL
CCND1	FIP1L1	NKX2-1	STK11
CCNE1	FLCN	NOTCH1	STMN1
CCT6B	FLT1	NOTCH2	STX11
CD22	FLT3	NPM1	STXBP2
CD274	FLT4	NQO1	SUFU

Table II. Continued.

ABCC2	DMNT3A	KDR	RAF1
CD58	GADD45B	NRAS	SUZ12
CD70	GATA1	NRG1	SYN3
CDA	GATA2	NSD1	TCN2
CDC73	GATA3	NT5C2	TEK
CDH1	GATA4	NTRK1	TEKT4
CDK10	GATA6	PAG1	TERC
CDK12	GNA11	PAK3	TERT
CDK4	GNA13	PALB2	TET2
CDK6	GNAQ	PARK2	TGFBR2
CDK8	GNAS	PAX5	TLE1
CDKN1B	GPC3	PBRM1	TLE4
CDKN1C	GRIN2A	PC	TMEM127
CDKN2A	GRM3	PDCD1	TMPRSS2
CDKN2B	GSTM1	PDCD1LG2	TNFAIP3
CDKN2C	GSTP1	PDGFRA	TNFRSF14
CEBPA	GSTT1	PDGFRB	TNFRSF17
CEP57	HBA1	PDK1	TNFRSF19
CHD4	HBA2	PHF6	TOP1
CHEK1	HBB	PHOX2B	TOP2A
CHEK2	HDAC1	PICK3R1	TP53
CKS1B	HDAC2	PIK3C3	TP63
CREBBP	HDAC4	PIK3CA	TPMT
CRKL	HDAC7	PIK3CD	TRAF2
CROT	HGF	PIK3R1	TRAF3
CSF1R	HNF1A	PIK3R2	TRAF5
CSF3R	HNF1B	PLCE1	TSC1
CTCF	HRAS	PLK1	TSC2
CTLA4	ID3	PMS1	TSHR
CTNNB1	IDH1	PMS2	TTF1
CUX1	IDH2	POLD1	TUBB3
CXCR4	IGF1R	POLD3	TYMS
CYLD	IGF2	POLE	TYR
CYP2B6*6	IKBKE	POT1	U2AF1
CYP2B6*6	IKZF1	PPP2R1A	UGT1A1
CYP2C19*2	IKZF2	PRDM1	UNC13D
CYP2C9*3	IKZF3	PRF1	VEGFA
CYP2D6	IL13	PRKAR1A	VHL
CYP2D6*3	IL7R	PRKCI	WISP3
CYP2D6*4	INPP4B	PTCH1	WRN
CYP2D6*6	INPP5D	PTEN	WT1
CYP3A4*4	IRF1	PTPN11	XIAP
CYP3A5*3	IRF2	PTPN2	XPA
DAB2	IRF4	PTPN6	XPC
DAXX	IRF8	PTPRO	XPO1
DDB2	JAK1	QKI	XRCC1
DDR2	JAK2	RAC1	YAP1
DDX1	JAK3	RAD21	ZAP70
DHFR	JARID2	RAD50	ZBTB20
DICER1	JUN	RAD51	ZNF217
DIS3L2	KDM2B	RAD51C	ZNF703
DLG2	KDM5A	RAD51D	ZRSR2

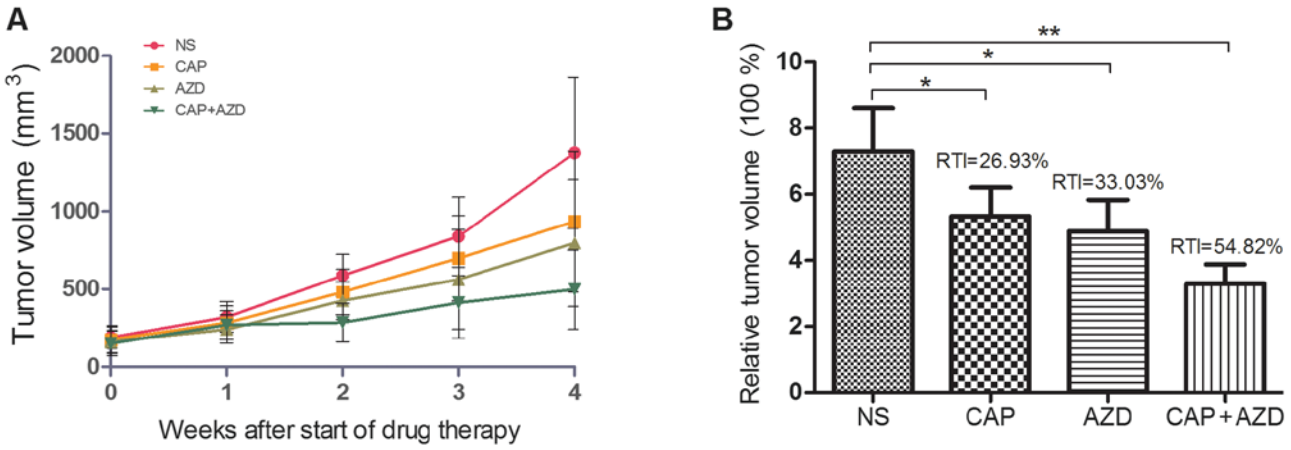

Figure 2. (A) Antitumor-growth ability of AZD6244. (B) The single AZD6244 exhibited better efficacy than Capecitabine, while the combination of both shown a significant synergistic effect.

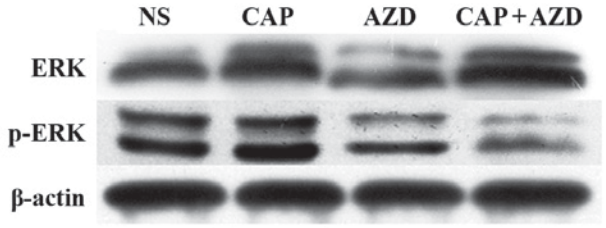

Figure 3. Western blot analysis for changes of ERK and p-ERK expressions in all groups. The p-ERK expressions were significantly suppressed in both single and combined AZD6244 groups. **P<0.01, *P<0.05.

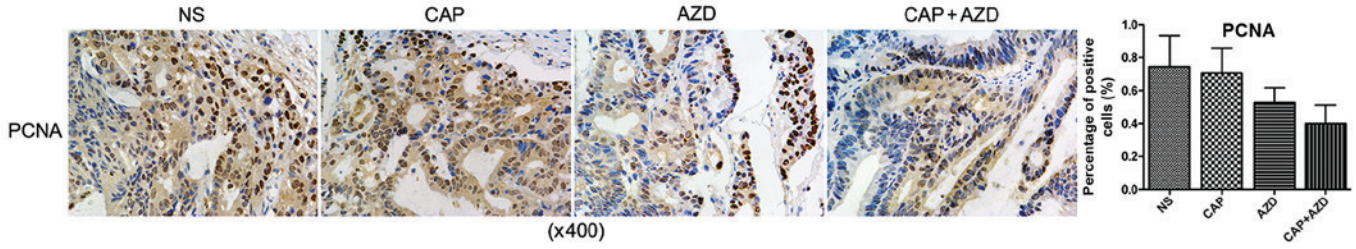

Figure 4. Immunohistochemical staining shown that PCNA expressions in the AZD6244-treated groups were significantly suppressed.

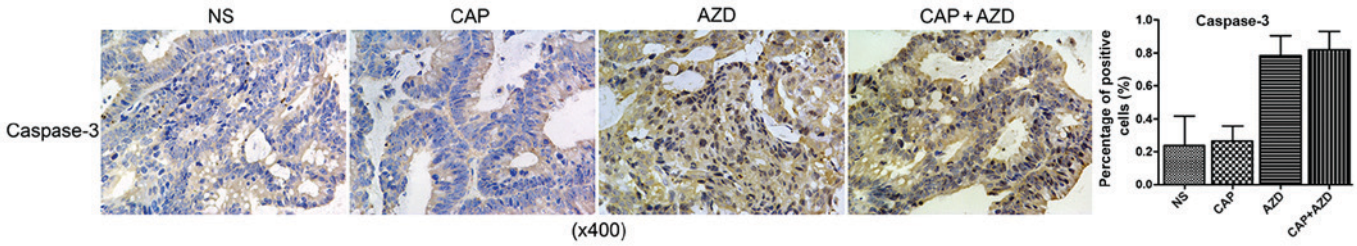

Figure 5. Immunohistochemical staining shown that caspase-3 expressions in the AZD6244-treated groups were significantly upregulated.

orally administration of AZD6244 (50 mg/kg), Capecitabine (1.0 mM/kg) or saline were then given once a day for 28 days. The mice were killed and excised tumors were measured. Then, relative tumor growth inhibition (TGI) was calculated as per the following formula: (1-T/C) %, where T is relative tumor volume of treated group mice, and C is relative tumor volume of control group mice. We found that single AZD6244 exhibited better efficacy (TGI, 33.03%) than Capecitabine (TGI, 26.93%), although without statistical significance. While the combination of both shown a significant synergistic effect, with TGI of 54.82% (Fig. 2). By western blotting, we evaluated the changes of ERK and p-ERK expressions in all groups, to

find that p-ERK expressions were significantly suppressed in both single and combined AZD6244 groups (Fig. 3). By immunohistochemical staining, we found that PCNA (proliferating cell nuclear antigen) expressions in the AZD6244-treated groups were significantly suppressed, while caspase-3 (one of apoptosis associated antigens) expressions were significantly upregulated (Figs. 4 and 5). Therefore, AZD6244 was evaluated effective for the pancreatic cancer PDX model, thus might provide potential efficay in the clinical treatment of the very pancreatic cancer patient.

Disscussion

Novel technologies contribute to the progress of the drug screening of pancreatic cancer during recent years. PDX models are being used for pancreatic cancer research in a series of studies (2,7,34,35), while NGS technologies contribute to the translational research of pancreatic cancer (36-38). Multiple clinical studies have showed NGS and PDX will ameliorate personalized medicine and will be necessary for discovering novel therapeutic targets and biomarkers (39). With the progress of these technologies, both are getting economically availble for patients. In our study, we combined PDX and NGS as an promising pattern of individualized drug screening to improve the clinical treatment of pancreatic cancer patients.

The PDX model of pancreatic cancer bone metastasis we established was comfirmed as highly molecularly stable with clinical patients in our study. Immunohistochemical expressions of CK19, CK7, and ki67 as well as the H&E staining showed that the pathological characteristics of the third passage xenograft was in accordance with the original patient tumor. Therefore, our PDX model could be considered as an 'Avatar' or a 'stand-in' of our pancreatic cancer patient, which would be a quite promising platform for drug screeing and evaluation.

In order to select the potential therapies customized for the pancreatic cancer patient, the bone metastasis tumor tissues were used for NGS detection (Geneseeq Technology Inc). However, based on the sequencing results and associated literatures, we found no under-clinical-trial targeted therapies of pancreatic cancer directly suitable for the genes detected. The sequencing report from Geneseeq Technology Inc also provided the alternative drug treatment suggestions, and MEK1 inhibitor was one of the most promising targeted therapies suggested. Then we concentrated on *MEK1*, a downstream gene *of KRAS,* which might be a potential target for treatment. Therefore we chose AZD6244, a MEK1 inhibitor, as a potential therapy which would then be evaluated in our PDX model.

In our study, we found that single AZD6244 exhibited better efficacy than Capecitabine, although without statistical significance. While the combination of both shown a significant synergistic effect, with TGI of 54.82%. AZD6244 significantly suppressed p-ERK expressions of the pancreatic cancer PDX model. AZD6244 significantly suppressed tumor cell proliferation and upregulated tumor cell apoptosis. Several studies have evaluated the effect of AZD6244 in pancreatic cancer in preclinical and clinical phase, and AZD6244 was shown to be effective in combination with EGFR/PIK3CA/STAT3 inhibitors in patients with pancreatic cancer (40-42). While we have shown that AZD6244 also has a synergistic effect in combination with Capecitabine. In addition, it was suggested that AZD6244 alone was mainly cytostatic, and apoptosis was mainly induced by combination therapies targeting multiple pathways (43). While here in the present study, we shown that AZD6244 also suppressed tumor cell proliferation as a sinlge agent. Therefore, AZD6244 was evaluated as effective for the pancreatic cancer PDX model, thus might provide potential efficay in the clinical treatment of this pancreatic cancer patient.

In our study, AZD6244, a highly selective MEK1 inhibitor, was evaluated as effective for the pancreatic cancer PDX model, and thus might provide potential efficay in the clinical treatment of this pancreatic cancer patient. Although only one targeted agent was evaluated, we have successfully shown PDX-NGS based drug screening as a novel promising pattern of individualized drug screening to improve the clinical treatment of pancreatic cancer patients.

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