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ORIGINAL ARTICLE – CANCER RESEARCH

# Malignant ascites-derived organoid (MADO) cultures for gastric cancer in vitro modelling and drug screening

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## Abstract

**Purpose** Malignant ascites (MA) is a common manifestation in advanced gastric cancer with peritoneal carcinomatosis and usually indicates a poor prognosis. However, lack of in vitro models that can faithfully recapitulate the characteristics of tumour cells in ascites hinders related researches. Tumour organoids have emerged as a robust in vitro model for tumour research and drug screening. Hence, we aimed to generate a 3-D in vitro organoid cultures from malignant ascites of gastric cancer for disease modelling and drug screening.

**Methods** Eleven MADOs were generated from the MA tumour cells of gastric cancer patients. We made comparisons between MADOs and original MA tumour cells in histopathology by immunohistochemistry and genomics by whole-exome sequencing. In order to evaluate MADOs as functional in vitro disease models, we tested whether MADOs could be used for drug sensitivity screens.

**Results** Eleven MADO cultures from human gastric cancer were established. MADOs demonstrated divergent growth characteristics and morphologies. MADO cultures preserve the histological architecture, genomic landscape of the corresponding MA tumour cells. MADOs exhibited heterogeneous responses to standard-of-care chemotherapeutics.

**Conclusions** We generated MADOs modelling characteristics and mutated genes of MA tumour cells. A broad range of intrinsic MADO response to conventional chemotherapeutics suggests MADOs are amenable to drug screening.

**Keywords** Gastric cancer · Malignant ascites · Organoid · Drug screening · Personalised medicine

## Abbreviations

| | |
|---|---|
| CNV | Copy number variation |
| GSEA | Gene set enrichment analysis |
| HIPEC | Hyperthermic intraperitoneal chemotherapy |
| H&E | Haematoxylin and eosin |
| ICGC | International cancer genome consortium |
| IHC | Immunohistochemistry |
| MA | Malignant ascites |
| MADO | Malignant-ascites derived organoid |
| NCCN | National comprehensive cancer Network |
| PBMC | Peripheral blood mononuclear cell |
| PCA | Principal component analysis |
| PDO | Patient-derived organoid SNV: single-nucleotide variants |
| WES | Whole-exome sequencing |

## Background

Approximately 10% of gastric cancer patients are affected by malignant ascites (MA), which means nearly 100,000 cases suffered from MA worldwide in 2013 (Fitzmaurice et al. 2015; Maeda et al. 2015). The appearance of MA, which is considered a manifestation of end-stage events due to the peritoneal dissemination of tumour cells, usually provides a sign of poor prognosis with an average survival of only 5.2 months in gastric cancer (Fang et al. 2014). Besides, several symptoms such as abdominal pain and bloating caused

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by peritoneal fluid collection seriously impair the patients' quality of life (Cavazzoni et al. 2013).

Management of MA needs multidisciplinary approaches based on the treatment of the primary tumour with antineoplastic therapy (Barni et al. 2011). Although aggressive therapeutic options in recent years like cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) (Randle et al. 2014), VEGF targeted therapy (Mateescu et al. 2014) and catumaxomab (Ott et al. 2012), the trifunctional bispecific antibodies binding EpCAM on tumour cells and CD3 receptors on T-cells have been applied in clinic and showed bright prospects, conventional chemotherapy is still considered a priority. Multidrug resistance, however, is one of the crucial reasons leading to being unresponsive to chemotherapy, which makes palliative measures such as paracentesis and diuretics the only approaches (Jehn et al. 2015; Jia et al. 2013).

Besides the reversal of drug resistance, another feasible solution is to predict the efficacy before treatment in order to enrich right patients to the right drugs. An emerging 3-D in vitro culture system termed "tumour organoids" has been devoted to capturing the characteristics of in vivo cancer cells and testing the sensitivities of candidate drugs. Patient derived organoid (PDO) cultures for cancer modelling have been established from colorectal cancer (van de Wetering et al. 2015), breast cancer (Sachs et al. 2018), gastric cancer (Yan et al. 2018), liver cancer (Broutier et al. 2017), pancreatic tumour (Huang et al. 2015), oesophageal adenocarcinoma (Li et al. 2018), prostate cancer (Gao et al. 2014), bladder cancer (Lee et al. 2018), ovarian cancer (Hill et al. 2018) and glioblastomas (Ogawa et al. 2018). Notably, a co-clinical study of PDOs from metastatic colorectal and gastroesophageal cancer patients has verified the feasibility of predicting clinical response with drug screening on PDOs in vitro (Vlachogiannis et al. 2018). Organoids derived from gastric cancer have already been initiated and gastric cancer organoids related drug sensitivity test has been conducted recently (Nanki et al. 2018; Seidlitz et al. 2019; Yan et al. 2018). Yet, the potential of using the malignant ascites-derived organoids (MADOs) for disease modelling and drug screening remains an open question.

On the basis of our previous work on normal gastric and gastric cancer organoid cultures, we established cultures of MA-derived organoids (MADO). Here we demonstrated the proof of concept that MADOs recapture the features of tumour cells in MA. Besides, we explored the potential of MADOs as a drug screening model to optimise chemotherapy in the treatment of gastric cancers with MA.

## Methods

### Malignant ascites sample collection

MA was collected from gastric cancer patients undergoing palliative paracentesis. The primary cause of MA by gastric cancer was confirmed by concurrent endoscopic or previous surgical pathology. The protocol was approved by the Institute Ethics Board of Changhai Hospital.

### Establishment and culture of MADOs

MA obtained from gastric patients was centrifuged at 1200 rpm for 5 min. The supernatant was incubated at 56 °C for 30 min and filtered with 0.22 μm membrane. Meanwhile, isolated tumour cells from MA were collected, resuspended in 50 μl growth factor reduced Matrigel (Corning 356231). After the Matrigel was solidified on prewarmed 24-well cell culture plate at 37 ℃ for 15 min, 500 μl of complete organoid medium (Supplementary Table 1) was added to each well and plates transferred to 37℃/5% $CO_2$ incubators. Complete medium was subsequently refreshed every 3 days. In addition, different percentage of MA supernatant collected from the same patient was added to complete medium for MADOs culture. For passaging, MADOs were removed from the Matrigel, mechanically dissociated into small fragments and transferred to fresh Matrigel with fresh culture medium as described above.

### Histology and Immunohistochemistry

Centrifugal precipitation containing enriched Tumour cells of MA, which was confirmed by a pathologist, was fixed for 24 h at room temperature, washed with PBS, transferred to 70% ethanol and then processed for paraffin embedding. To make organoid sections, MADOs were fixed for 1 h at room temperature, washed and processed for paraffin embedding. Five-micrometre sections were prepared for Haematoxylin and eosin (H&E) staining. For immunohistochemistry staining, sections were stained with the following antibodies. Immunohistochemistry was performed using antibodies against Ki67. Primary antibodies were: P53(Rat mAb anti, No. 17070702, RX Bio); Ki67 (Rabbit mAb No. 18003, CD Bio); CDX2(Rat mAb anti, No. 18003, JH Bio). The DAB system was used for immunohistochemistry. Images were taken with MoticAE2000 microscope.

### Whole-exome gene sequencing and genomic analysis

Whole-exome gene sequencing was performed on MADOs and paired MA cells derived from six patients as previously

described (Zang et al. 2012). Exome sequencing was also performed on the corresponding peripheral blood mononuclear cells for comparison. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using Hiseq PE Cluster Kit (Illumina) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced on Illumina Hiseq platform and 150 bp paired-end reads were generated.

Quality control statistics including total reads number, raw data, raw depth, sequencing error rate, percentage of reads with Q30 (the perc ent of bases with phred-scaled quality scores greater than 30) and GC content distribution were calculated and summarised. Samtools mpileup and bcftools were used to do variant calling and identify SNP, InDels, as previously described (Li et al. 2009). ANNOVAR was performed to do annotation for Variant Call Format obtained in the previous effort (Wang et al. 2010). dbSNP, 1000 Genome and other related existing databases were applied to characterize the detected variants. The somatic SNV was detected by muTect (Cibulskis et al. 2013) and the somatic InDel by Strelka (Saunders et al. 2012). Control-FREEC was used to detect somatic CNV (Boeva et al. 2012).

### Drug screen

MADOs were harvested and dissociated into small fragments following the passaging procedure described above. 30 µl of growth factor reduced Matrigel containing 60–100 small fragments were seeded in standard 96-well cell culture plates (Corning), and plates were incubated for 10–15 min in a 37 ℃ and 5% $CO_2$ cell culture incubator; the Matrigel was solidified and overlaid with 100 µl of the complete human gastric organoid medium. Complete media was refreshed every 2–3 days. Media was removed and replaced by 100 µl of drug-containing complete MADOs medium at day 3 after seeding. Images of live MADOs cultures were obtained every 2–3 days with an MoticAE2000 microscope. 9 days after drug treatment, depending on the growth rate of the various organoid cultures, cell counting kit-8 (Dojindo, CK04) was used to measure the cell proliferation following the manufacturer's instruction. Six different concentrations per compound in these MADO lines were used to draw the dose–response curves, and 50% maximal inhibitory concentration (IC50) of each compound was used to measure the drug responses of different MADOs following the drug screen procedures. Images of live MADOs cultures were obtained every 2–3 days with Cytation 5 Cell Imaging Multi-Mode Reader. Organoid areas in each well were measured and calculated by Image J software. Relative viability of MADOs (areas of treatment groups/ control group) was used to measure the responses of MADOs to the compounds in drug screen. Each screen was conducted in triplicate.

### Statistical analysis

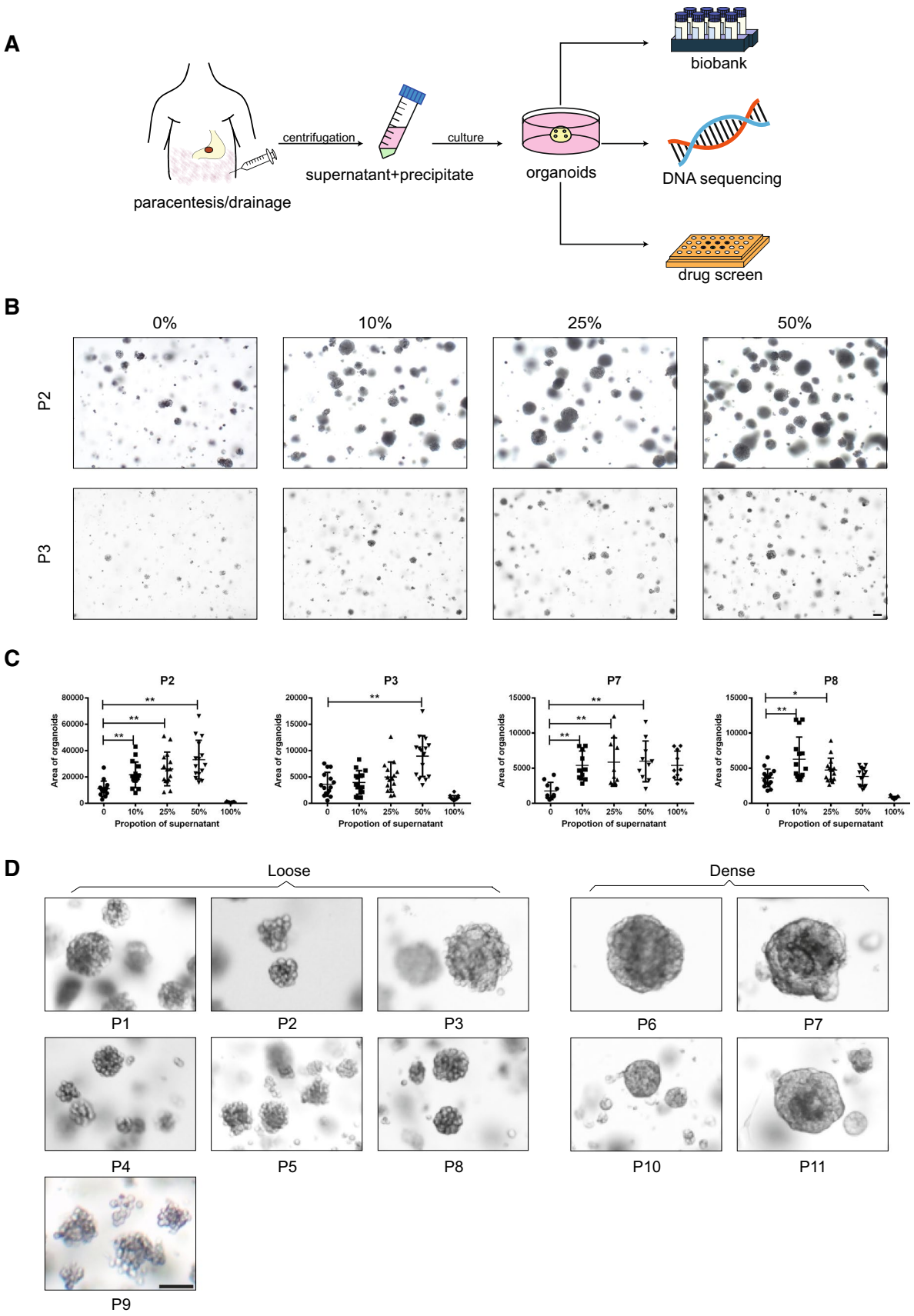
Statistical analyses were performed using Prism GraphPad. Student's *t* test was used to effect comparisons. All values are shown as mean ± standard error of the mean (s.e.m.). FDR ≤ 0.25 (for GSEA) or $p \leq 0.05$ was considered as statistically significant.

## Results

### Establishment of MADOs

Epithelial cells isolated from human normal gastric (Fig. S1a) or primary gastric cancer (Fig. S1b) were cultured in Matrigel with medium described in Methods part. In line with published data, those cells could grow into organoid (Bartfeld et al. 2015). To investigate whether the organoid culture system could be used for culture of ascites tumour cells from gastric cancer patients, we isolated and embedded the malignant ascites tumour cells into Matrigel with organoid medium. Unlike health gastric tissue-derived organoids, the number of MADOs varied between patient samples, with some MA rendering hundreds of organoids, whereas others yielded only 5–10 organoids. The difference in derivation likely reflects the various requirements for growth factors. During preliminary studies, we frequently experienced the slow-growing MADOs. In order to optimise the culture system for MADOs, we tried to add supernatant collected from MA to the corresponding MADOs culture. A total of 10%, 25% and 50% of supernatant were added to culture medium. Our data showed that the MA supernatant could significantly increase the organoid forming efficiency and organoid size (Fig. 1b, c). All these three concentrations of MA supernatant were beneficial to MADOs growth while the most efficient concentration is different as per each MADO line. We found that the excessively large proportion (100% here) of MA supernatant inhibited MADOs growth.

Using the optimised culture system, we established a collection of 11 MADOs with the successful rate of 92% (12 gastric cancer MA in total) and corresponding clinical data are shown in Supplementary Table 1. MADOs showed great diversity in growth rate and morphology. MADOs presented with a range of patient-specific morphologies, rang from loose spherical structures (P1, P2, P3, P4, P5, P8, P9) with protuberances resembling grape clusters to compact cystic structures (P6, P7, P10, P11) (Fig. 1d).


A
biobank
centrifugation
culture
supernatant+precipitate
organoids
DNA sequencing
paracentesis/drainage
drug screen
B
0%
10%
25%
50%
P2
P3
C
P2
P3
P7
P8
Area of organoids
Propotion of supernatant
D
Loose
Dense
P1
P2
P3
P6
P7
P4
P5
P8
P10
P11
P9

◂**Fig. 1** Study design and establishment of the biobank of MADOs. **a** Flow diagram of the establishment of MADOs and performed assays. **b** Representative bright-field images of two MADOs (P2, P3) indicating that growth of MADOs was increased by adding indicated proportion of parental supernatant (0, 10%, 25%, 50%). Scale bar, 100 µm. **c** Statistical scatter plots presenting growth area of four MADOs (P2, P3, P7, P8) with the incubation of indicated proportion of supernatant (0, 10%, 25%, 50%, 100%). Data indicated mean ± S.D. and analysed by Student's *t* test (*$p<0.05$; **$p<0.01$). **d** Bright-field images showing two phenotypes of MADOs. Scale bar, 100 µm

## Histological Characterization of MADOs

To analyse whether MADOs retain the histological characteristics of MA tumour cells, we performed histopathological analysis of H&E stained MA tumour cells and MADO sections. Abundant cells with cellular and nuclear atypia (such as enlarged and polymorphic nuclei, increased karyokinesis and signet ring cell) were judged as tumour cells. MADOs consist of clearly malignant cells with similar typical cancerous features and almost do not contain other common cell types in ascites, such as mesothelial cells and immune cells. Without the extracellular matrix, most MA tumour cells were dispersed in ascites, but they grew into 3D structures when cultured *in vitro* with extracellular Matrigel (Fig. 2). The H&E staining on MADOs sections showed the distinct interior structure of loose and dense phenotypes (Fig. S2a).

Immunohistochemistry staining showed that both MA tumour cells and MADOs displayed high expression level of tumour-associated markers Ki67 and P53. As reported, CDX2 is commonly used in ascites cytology of gastrointestinal and pancreatic malignancies (Kobayashi et al. 2006). Figure 2 shows that MADOs also could retain the CDX2 expression of the parental MA tumour cells.

## MADOs recapitulate parent tumour cell mutation spectrum

To analyse whether MADOs retain the mutational landscape of MA tumour cells, genomic DNA from six paired MADOs, MA tumour cells and peripheral blood mononuclear cells (PBMC) were subjected to a detailed whole-exome gene sequencing (WES) analysis. Principal component analysis (PCA) revealed the discrepant mutation spectrum of individual samples which showed heterogeneity of those tumours. MADOs displayed high similarity with paired MA tumour cells on mutation landscape (Fig. S3a, b). We compared exome-wide CNVs of MADOs and paired MA tumour cells and found that DNA copy number losses and gains were retained throughout the entire exome (Fig. 3a). Not surprisingly, total mutational load and point mutation type were similar between MADOs and corresponding MA samples (Fig. 3b, c). By means of comparing publically available gastric cancer cohorts in International Cancer Genome Consortium (ICGC), we found 87.21% on the average mutational overlap between MADOs and corresponding MA samples (Fig. 3d).

Importantly, MADOs showed mutations in many of the most related gastric cancer genes (Cancer Genome Atlas

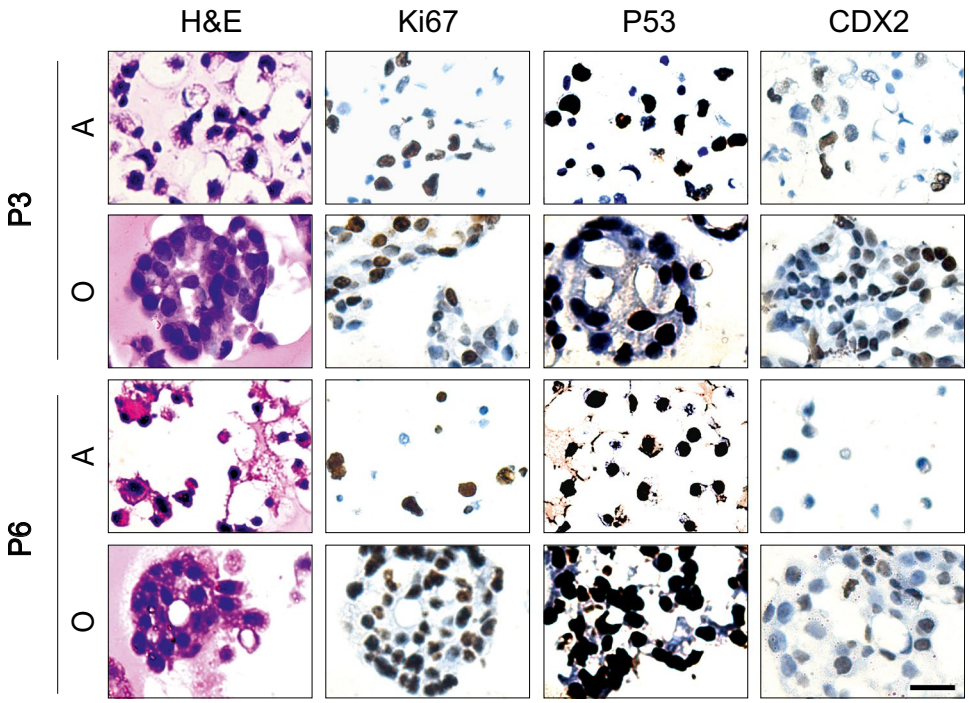
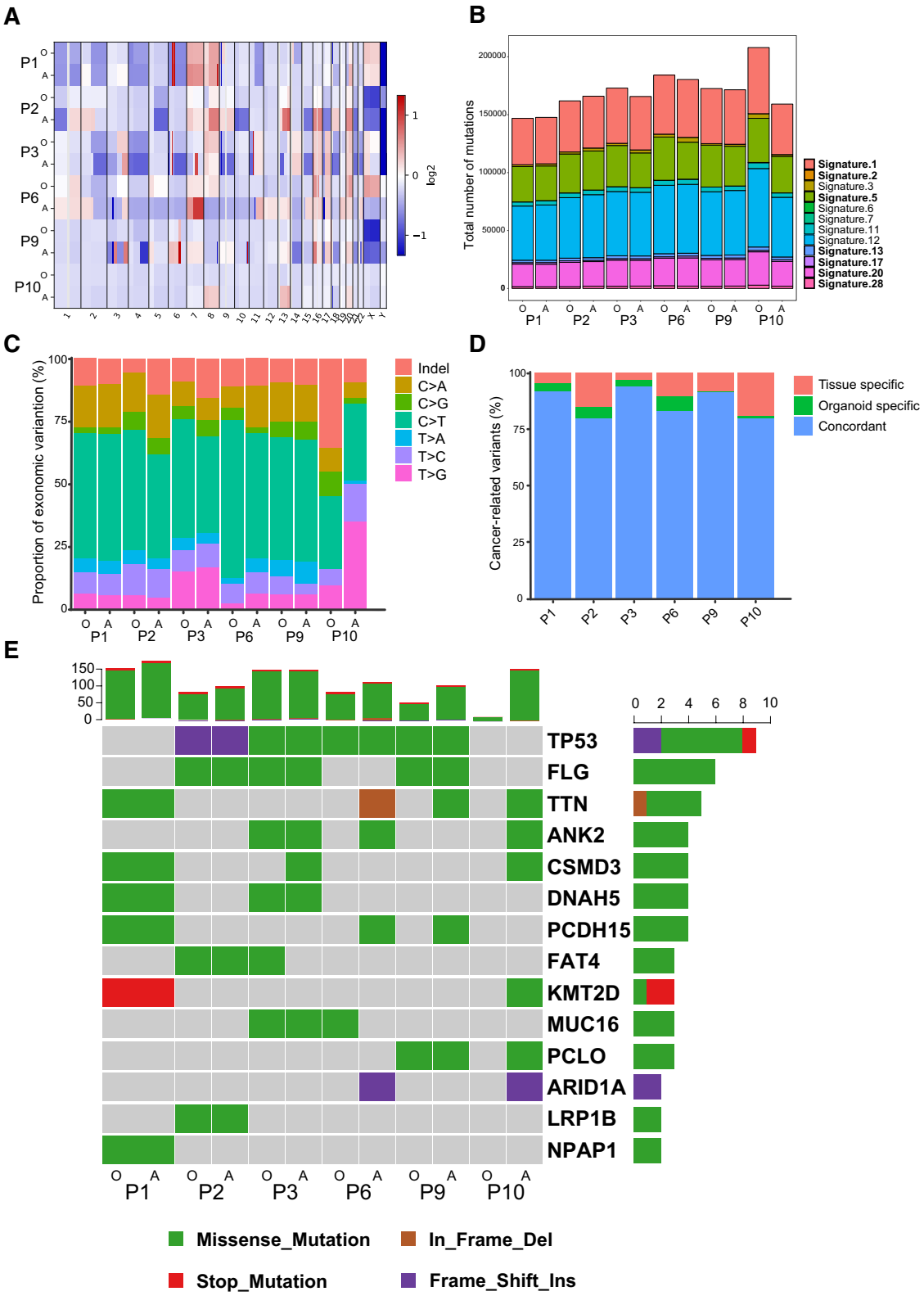



**Fig. 2** Histological characterization of MADOs. Hematoxylin and eosin staining (left column) and immunohistochemistry staining of P53, Ki67 and CDX2 (three columns on the right) comparing MADOs with corresponding tumour cells from MA are shown. *P3* Patient 3, *P6* Patient 6, *A* MA, *O* MADOs. Scale bar, 20 µm




Research 2014; Cristescu et al. 2015; Wang et al. 2014). For example, we found frequent loss-of-function mutation of cancer suppressor genes TP53 as well as CDH1 and activating mutation of oncogene PIK3CA (Fig. 3e, S3c). Especially, CDH family genes missense mutations were observed more commonly in loose MADOs than in the dense counterparts (Fig. S2b). In summary, we demonstrated that MADOs are composed of malignant cells with typical mutation landscape

◂**Fig. 3** Genetic characterization of MADOs. **a** Heatmap displaying Log2Ratio of copy number data for the whole-exome, across MADOs and corresponding MA samples. DNA copy number gains (red) and losses (blue) found in MA tumour cells are conserved in the MADOs. O, MADO; A, MA. **b** Stacked bar graphs showing the total mutation load per mutational signature per patient sample pair. Frequencies of 12 mutational signatures identified in this cohort normalised by the background frequency for each trinucleotide context. **c** Bar graphs showing proportions of exonic variants across MADOs and corresponding MA samples, the six types of SNVs and the indels are represented. **d** Bar plots indicating the concordance (%) between the cancer-related variants identified in the MADOs and derived tumour cells. **e** OncoPlot summary of recurrent mutated cancer-related genes. For each gene (row) indicated, MADOs and derived tumour cells (columns) with different mutational types are labelled in different colours. From the top, number of driver genes was indicated above each sample

and show that MADOs recapitulate the diverse genomic landscape of MA tumour cells including CNVs, mutation signatures and tumour mutation burden, as well as frequent cancer-related mutational genes.

### Drug screening

In order to evaluate MADOs as functional in vitro disease models, we tested whether MADOs could be used for high-throughput drug sensitivity screens. We chose seven clinical drugs for gastric cancer according to NCCN guidelines including oxaliplatin, 5-FU, cis-platinum, docetaxel, irinotecan, epirubicin and paclitaxel (Ajani et al. 2016). Using 6 concentrations per drug, we were able to generate reproducible dose–response curves and identify half-maximal inhibitory concentrations (IC50) (Fig. S4). Based on seven MADO lines, we tested several relative IC50 values for evaluated drugs. And average IC50 values for each drug were chosen to test the drug sensitivity of seven MADOs from the established bank, which were 100 μM oxaliplatin, 40 μM 5-FU, 25 μM cis-platinum, 6.25 nM docetaxel, 10 nM irinotecan, 1.25 μM epirubicin and 5 nM paclitaxel. Organoid size was measured after drug treatment and size changes compared to untreated control served as a measure of organoid survival. We found different responses to single drug using kinetic survival curves. As shown in Fig. 4a, b, all seven MADO lines displayed complete drug resistance to cis-platinum while they seemed to be most sensitive to epirubicin according to all these evaluated drugs. Interpatient variability responses were seen among the seven MADOs lines treated with the other five drugs. For individual MADO lines, like P6, the relative drug sensitivities counted from high to low is: epirubicin, 5-FU, docetaxel, irinotecan, cisplatin, paclitaxel and oxaliplatin. It is worth noting that individual MADOs were sensitive to single drug in the first 3–10 days after treatment and recovered in the next 10–18 days. So drug response should be constantly measured for about 18 days after drug treatment.

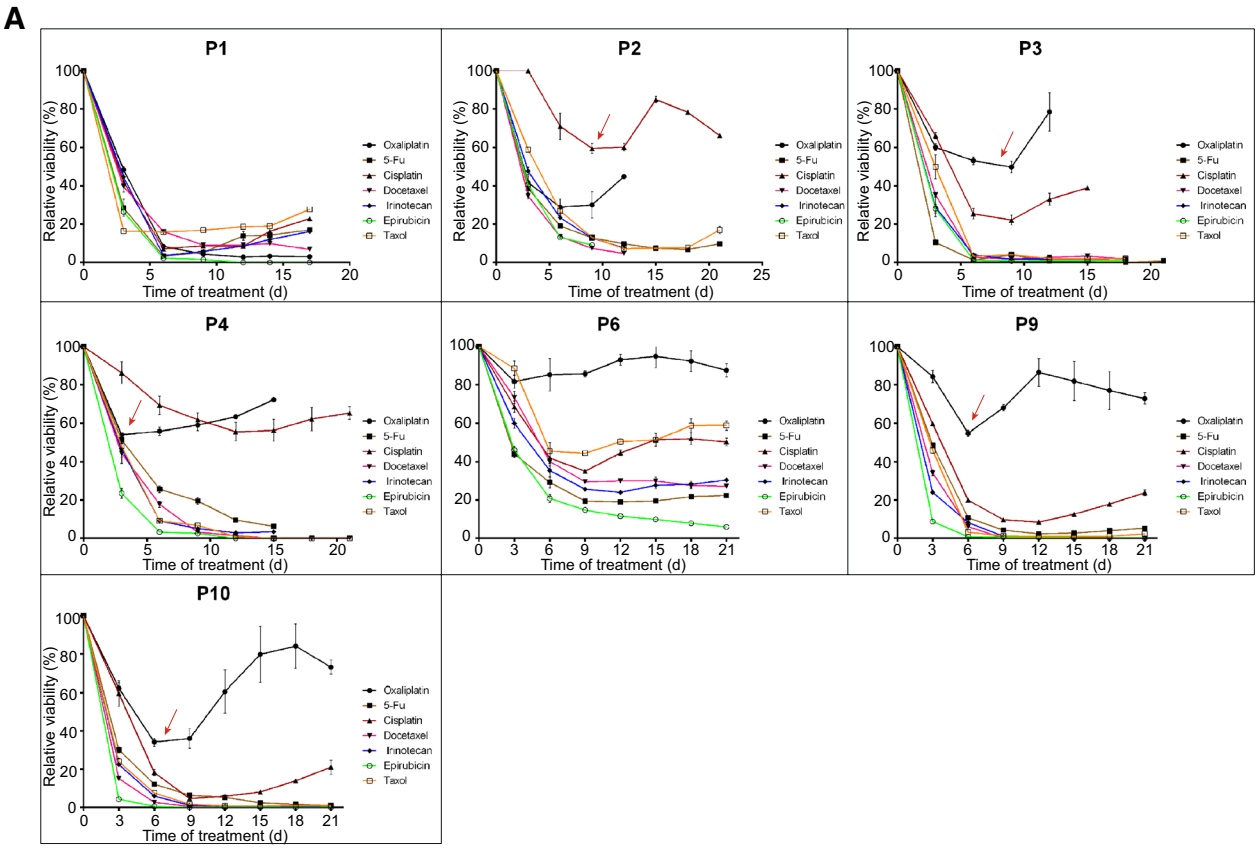
In summary, MADOs exhibited heterogeneous responses to standard-of-care chemotherapeutics in drug sensitivity screen assays and could be used as functional in vitro disease models.

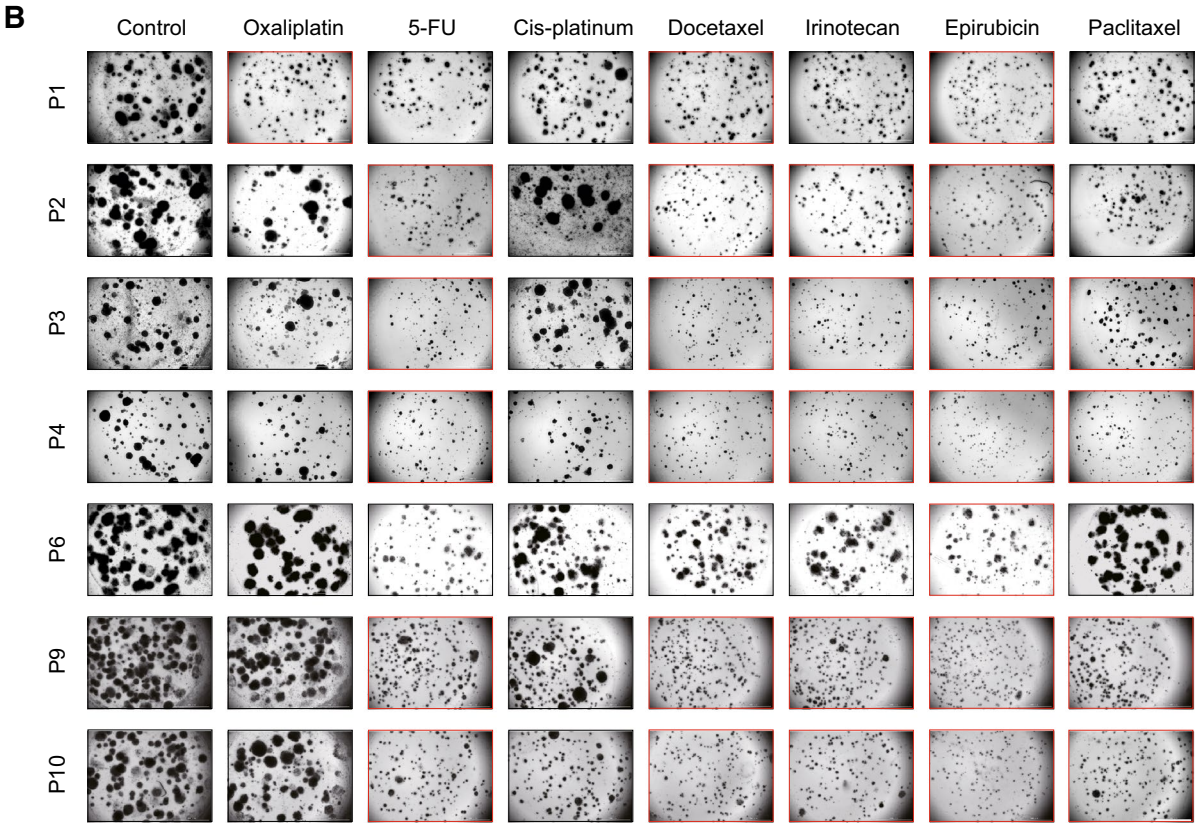
## Discussion

MA is often considered as a common complication tied to peritoneal implants and peritoneal carcinomatosis, while few research studies focused on MA tumour cells. Prior to this study, the viability of ascites-derived tumour cells has been verified. Trevor G Shepherd et al. established the in vitro culture system for primary epithelial ovarian cancer cells (Shepherd et al. 2007). Then, the protocol was used to ascites-derived pancreatic adenocarcinoma cell for drug screening. However, the consistency between primary and cultured cells needs to be explored and the cells could be only propagated for 5–6 passages long-term expansion (Golan et al. 2014).

Compared to tumour cell lines and patient-derived xenografts (PDX), the advantages of patient-derived organoids (PDOs) have been frequently mentioned in literature (Weeber et al. 2017). Research studies on PDOs have developed rapidly in recent years and organoids derived from different types of cancers have been established, including colorectal cancer (van de Wetering et al. 2015), prostate cancer (Gao et al. 2014), pancreatic cancer (Boj et al. 2015), liver cancer (Broutier et al. 2017), breast cancer (Sachs et al. 2018), bladder cancer (Lee et al. 2018), gastric cancer (Yan et al. 2018) and metastasis (Vlachogiannis et al. 2018). These organoids are used for research in carcinogenesis, translational medicine and so on. Most published tumour organoids are derived from surgical specimens or biopsy, which is non-reusable or harmful to patients. Here, we collected MA tumour cells from gastric cancer patients and established a robust in vitro model termed "MADO," which captures histopathological characteristics and molecular mutation patterns of original MA tumour cells. As far as we know, this is the first time to establish organoids from MA tumour cells of gastric cancer patients. Circulating tumour cells (CTCs) as part of liquid biopsy combined with tumour organoids has been reported before (Gao et al. 2014). Even more convenient and inexpensive than culture organoids derived from CTCs, MADOs allow conducting periodic organoid culture and drug screening to adjust regimens dynamically against acquired drug resistance could be realised.

MA, which is considered as a hypoxia, inflammatory and immunosuppressive tumour environment, is composed of complexed cytokines, chemokines, growth factors, exosomes and various suspended cells like tumour cells, mesothelial cells as well as immune cells. Several studies of ovarian


A
P1
P2
P3
P4
P6
P9
P10
Relative viability (%)
Time of treatment (d)
Oxaliplatin
5-Fu
Cisplatin
Docetaxel
Irinotecan
Epirubicin
Taxol


B
Control
Oxaliplatin
5-FU
Cis-platinum
Docetaxel
Irinotecan
Epirubicin
Paclitaxel
P1
P2
P3
P4
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P9
P10

◂**Fig. 4** Response of MADOs to conventional chemotherapeutics. **a** Organoid size change curve of seven MADOs lines treated with seven chemical drugs, respectively. Red arrows point to the time that MADOs recover after drug treatment. **b** Representative bright-field images of MADOs after drug treatment in seven selected cases. Images were taken on day 18–21 after drug treatment. Relatively sensitive drugs (relative viability<10%) were marked with red frames. Scale bar, 100 μm

cancer demonstrated that some specific components of MA could promote the proliferation and the function of anti-apoptosis (Lane et al. 2010; Mills et al. 1990; Puiffe et al. 2007). To mimic tumour microenvironment further, we tried to add centrifugated supernatant into the medium. As expected, the growth promotion effects were observed in several MADOs, while the underlying mechanism needs to be explored. Interestingly, we noticed the medium composed of full supernatant impeded organoids growing, which is possibly related to exhaustion of nutriment.

We next started an analysis of the mutation landscape, which was considered as the most essential feature of cancer by whole-exome gene sequencing (WES). On account of MADOs derived from gastric cancer patients and few genetic analysis data of MA available, we utilized published genetic databases for gastric cancer to analyse mutation spectrum compared with derived tumour cells in MA. As expected, these analyses demonstrate MADOs recapitulate genetic features of original tumour cells.

Current pathological diagnosis provides limited information, and molecular classification of gastric cancer is insufficient to direct clinical treatment. High-throughput drug screening could be a feasible way to optimise existing therapeutic regimens including chemotherapy. Researches on drug screening by PDOs suggest a bright prospect in drug development and personalised drug testing. Ling Huang and his colleges observed responses of gemcitabine and several drugs targeting epigenetic regulators to ductal pancreatic cancer organoids (Huang et al. 2015). Two researches focusing on the intratumour heterogeneity and drug sensitivity by using colorectal cancer organoids revealed a possible mechanism underlying drug resistance (Roerink et al. 2018; Schutte et al. 2017). Although these studies had made surpassingly tentative exploration in PDOs which were served as a robust in vitro drug screening model, their efficacy of matching or predicting clinical responses was never evaluated efficiently. However, a co-clinical study of PDOs showed 100% sensitivity, 93% specificity, 88% positive predictive value and 100% negative predictive value in forecasting response to targeted agents or chemotherapy in metastatic gastrointestinal patients (Vlachogiannis et al. 2018). In this study, PDOs established from biopsies at baseline (BL), which means the time of best response [partial response (PR) or stable disease (SD)], were cleverly set as control. Another research of pancreatic cancer PDOs recently used area under the curves (AUC) of dose–response curves to evaluate the correlation between PDO therapeutic profiles and patient outcomes: the least responsive (resistant, top 34% AUC), the most responsive (sensitive, lowest 33% AUC) and those exhibiting intermediate response (middle 33% AUC) (Tiriac et al. 2018).

Here we conducted drug screening on MADOs and observed divergent responses to different compounds. In addition, rapid drug resistance was frequently observed in drug screening of MADOs. Hereby, we basically showed the feasibility of MADOs as a platform for drug sensitivity tests. To test whether MADOs could guide personalised medicine, parallel analysis of drug sensitivities between MADOs and derived patients' clinical response is needed.

This protocol could be extended to MA tumour cells from other types of cancer patients or pleural effusion tumour cells, such as ovarian and pancreatic cancer. This system might be helpful in personalised medicine.

## Conclusions

In summary, we established an in vitro organoid model of MA tumour cells and showed good concordance between MADOs and MA tumour cells in histopathology and genomics. We observed the divergent response of MADOs to chemotherapeutics. MADOs might be applicable for drug screening and optimise clinical treatment of MA in the near future.

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**Author contributions** JL: design, collection of samples and data. HX: data analysis and interpretation, manuscript writing. LZ: collection of data, revision of the manuscript. LS and LZ: revision of the manuscript and data analysis. XP, DF, MW and BW: collection of samples and data analysis. YZ: genetic analysis. XZ and GH: conception and design, study supervision, data analysis and revision of the manuscript.

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**Availability of data and materials** The data and materials supporting the conclusions of the current study are available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no competing interests.

**Ethics approval and consent to participate** This study was approved by the Ethics Committee of Changhai Hospital of Second Military Medical University (CHEC2016-157), and written informed consent was obtained from all patients or their guardians.

**Consent for publication** Informed consent was obtained from all participants for publication.

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