A PIK3CA-mutant breast cancer metastatic patient-derived organoid approach to evaluate alpelisib treatment for multiple secondary lesions

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Introduction
Breast cancer (BC) is a powerful example of the intra- and interpatient heterogeneity of tumours; thus, there remain several grey areas in BC treatment approaches. This is especially true for advanced/metastatic disease (mBC) [1–3]. Organoid cultures are suitable models for studying the histological complexity and genetic heterogeneity of parental tumours and can be used to assess treatment strategies for challenging diseases such as mBC. Here, we report a proof-of-concept test case using organoid cultures from metastatic BC specimens to rapidly test drug sensitivity and molecular-lesion-driven treatments. Based on the relevant impact of PIK3CA gene mutations on breast cancer progression and resistance to therapy [4], we assessed the efficacy of alpelisib (BYL719; Novartis Pharma AG, Basel, Switzerland), a specific PI3K-α inhibitor, on organoid cultures derived from multisite mBC samples carrying specific PIK3CA gene mutations.

Results
An 82-year-old woman was diagnosed in 2018 with invasive ductal carcinoma (IDC) of the right breast, grade 3, ER-/PR-/HER2 3+, that had metastasized to the lymph nodes and bone. In 2019, contrast-enhanced brain computed tomography (CT) revealed a 3.9 cm cerebellar lesion that was surgically removed (Fig. 1A-C). We derived organoid cultures from this brain metastatic material (mOGs) by using a slightly modified organoid growth medium (OGM) containing neuregulin-1 to match the aberrant expression of ERBB family members in the starting material (Fig. 1D) [5, 6]. Using NGS-customized panels containing breast cancer-specific hotspot regions for 63 genes, we identified the PIK3CA H1047L mutation in both the patient-derived organoid (PDO) and the metastatic sample tissue, with variant allele frequencies (VAFs) of 45.4% and 56%, respectively (Fig. 1E and Suppl. Table 1).

PIK3CA hotspot mutations drive oncogenic progression in several cancer settings [4]. These mutations occur in 30–40% of breast cancers and are associated with resistance to therapy and metastatic progression [7, 8]. We found that a very minor fraction of genes assessed in the mBC-PDOs exhibited different expression levels when compared with the brain metastatic sample of origin (Fig. 1F). This finding strongly...
validated the accuracy of the mBC-PDO model. Additionally, mutational analysis and Gene Set Enrichment Analysis (GSEA) of the paired metastatic lesion and the mBC-PDOs revealed that PI3K-related pathways were enriched (Fig. 1G).

We first evaluated 105 genes representative of the PI3K pathway by RNAseq (MSigDB, https://www.gsea-msigdb.org) to assess the relationship between the mutation status of PIK3CA and the activation of the PI3K pathway. We found the levels of PI3K genes to be significantly higher in PIK3CA-mutant brain metastases than in PIK3CA-wild-type brain metastases; the latter sample was obtained from a 53-year-old woman diagnosed in 2019 with IDC of the right breast ER+/PR+/HER2- (Suppl. Fig. 1A and Suppl. Table 2). The difference in the levels of the PI3K-related genes was even more evident in the mPDO cultures (Suppl. Fig. 1B). We stained brain metastasis-derived PDOs with an anti-AKT-phospho-S473 antibody. The flow cytometry data analysis revealed an enrichment of AKT-phospho S473-positive cells in the PIK3CA mutant PDOs compared with those in the PIK3CA wild-type PDOs. Altogether, these results suggest that the PI3K pathway was activated due to the experimental conditions (Suppl. Fig. 1C). Finally, a breast cancer TCGA cohort analysis further validated our results. In breast cancer samples bearing mutated PIK3CA, despite a nonsignificant modulation of the transcriptional levels of PI3K pathway genes, both AKT, AKT-phospho S473, AKT-phospho T308 and PRAS40-phospho T246 protein levels were significantly increased compared to those in wild-type PIK3CA samples (Suppl. Fig. 1D-E).

To test the functional relevance of PI3K pathway activation in PIK3CA-mutant PDOs, we treated the organoids with alpelisib, a clinical trial grade PI3K-α inhibitor [9–11], at pharmacologically relevant doses. This compound affected the mBC-PDO number (images and graph) and size (area graph) and reduced the number of live cells in the PIK3CA-mutant mBC-PDOs, as assessed by flow cytometry (Fig. 1H-I). To further relate the efficacy of alpelisib treatment to the presence of the PIK3CA mutation, we similarly treated non-PIK3CA-mutated brain metastasis-derived PDO cultures (ctl-mBC-PDO) with alpelisib. Treatment of the ctr-mBC-PDO cultures with 10 µM alpelisib had little to no effect on PDO number (images and graph) or size (area graph), and no changes in the number of live cells was observed by flow cytometry (Fig. 1J-K). Taken together, these findings further suggest the dependency of mBC-PDOs on aberrant PI3K-propagated signalling for their maintenance.

These encouraging findings prompted us to expand those observations to three additional cases of spine (case 2), lung (case 3) and skin (case 4) BC metastases, all with PIK3CA hotspot mutations. In detail, the case 2 sample was taken from a 61-year-old woman diagnosed in 2015 with pT2 pN3a IDC of the right breast, grade 3, ER+/PR+/HER2+, with a metastatic lesion to the spine (T4); the case 3 sample was taken from a 47-year-old woman diagnosed in 2014 with hormone-sensitive IDC breast cancer and multiple bilateral lung nodules, and the case 4 sample was taken from an 82-year-old woman with pT2 pN3 IDC of the left breast, grade 2, ER+/PR+/HER2+, with skin metastases on the left chest wall. All three patients had undergone previous Taxol and/or anastrozole treatment. The clinical and histopathological findings and representative magnetic resonance imaging (case 2) or computed tomography scans (cases 3 and 4) are shown in Fig. 2A. The receptor status, evaluated by immunohistochemistry, was ER+/PR-/HER2 2+ for the spine metastasis, ER+/PR+/HER2 2+ for the lung metastasis and ER+/PR+/HER2 2+ for the skin metastasis.
Fig. 1 (See legend on previous page.)
HER2- for the lung metastasis and ER+/PR-/HER2- for the skin-derived metastasis (Fig. 2B), indicating changes in HR expression in the metastases versus that in the primary tumours. NGS tumour sequencing of the three metastatic lesions revealed the following PIK3CA hotspot mutations: E545K in case 2, H1047L in case 3 and H1047R in case 4 (Fig. 2C).

We next analysed the bulk RNA-seq data of the metastatic lesions by comparing the normalized enrichment score (NES) of the PI3K pathway between the four cases harbouring PIK3CA mutations and the brain metastasis with no PIK3CA gene mutations to validate our previous findings. All the expressed genes were ranked after z score transformation, and enrichment analysis was conducted for each sample. As reported in Fig. 2D, the metastatic lesions harbouring PIK3CA mutations exhibited effective enrichment for the PIK3-driven pathway when compared to the PIK3CA wild-type lesions. We supplemented the basic OGM (neuregulin 7.5 nM, EGF 10 ng/mL, FGF2 50 ng/mL, B27, BSA 2%) with factors specific for the host tissue to generate mBC-PDO cultures from the PIK3CA-mutated metastases as follows: KGF (20 ng/mL) for skin metastases; FGF2 (50 ng/mL) for spine-derived metastases and an inverted ratio of FGF2 and EGF for lung-derived metastases (FGF2 20 ng/mL, EGF 40 ng/mL). The numbers of formed PDOs from the spine, skin and lung metastases were counted, revealing the effective formation of aggregates within 72 h after media addition. Additionally, the area of the PDOs showed the most limited response to alpelisib. This reduced effect could be partly due to the lower enrichment for PI3K pathway-related genes, despite the presence of PIK3CA mutation, as identified by NGS tumour sequencing (Fig. 2D). This finding echoes the current knowledge regarding the heterogeneity of metastatic lesions (Fig. 2F). Finally, we expanded this limited cohort of samples to include an additional sample derived from skin metastasis in an 86-year-old patient diagnosed with invasive ductal carcinoma (IDC) of the right breast, grade 3, ER+/PR-/HER2, harbouring the wild-type PIK3CA gene (Suppl. Fig. 2A). A viability assay performed on the PDO cultures obtained from this sample treated with the control (DMSO) or alpelisib revealed little to no PDO-derived cell death in response to the drug. This finding again strengthens the link between the response to alpelisib and the presence of a PIK3CA gene mutation (Suppl. Fig. 2B).

Discussion

Herein, we presented a test case evaluating six mBCs to demonstrate the possibility that an OGM based on tissue location may allow the effective growth and passage of mBC-PDOs. This test case is relevant because it may help address an unmet need in the field. The growth of organoids from breast cancer metastases has received less attention than the growth of organoids from primary tumours in recent years, as evidenced by the paucity of studies to date. The lack of studies may be due to the limited availability of metastatic material, as metastases are generally less amenable to surgical removal than primary lesions [12]; additionally, appropriate experimental conditions for PDO propagation are lacking. Our cohort of metastatic samples was also

(See figure on next page.)

*Fig. 2* A Clinical features of cases 2, 3 and 4 metastatic lesions and relative magnetic resonance imaging (case 2) or computed tomography scans (cases 3 and 4). B Representative staining results from haematoxylin–eosin (HE) and immunohistochemistry analyses of ER, PR, HER2 and Ki-67 expression in the metastatic lesions from patients 2, 3 and 4. Scale bar = 200–500 μM. ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor. C Summary of the mutational profile of metastatic lesions of cases 2, 3 and 4. VAF refers to variant allele frequency. D Normalized enrichment score (NES) of the PI3K pathway among the four patient cases harbouring PIK3CA mutations (#1, #2, #3, #4) and the brain metastasis with an intact PIK3CA gene. All expressed genes were ranked after z score transformation, and enrichment analysis was conducted for each sample. The size of the circles reflects the percentage of genes in the core enrichment of pathway. E Representative bright-field microscopy images of organoids generated from metastatic lesions of patients 2 and 4 treated with 10 μM alpelisib for 7 days. Magnification: 10X. The number of organoids for each condition is plotted in the bar graph. F Flow cytometry of Helix NP blue-stained organoids generated from metastatic lesions of patients 2 and 4 after 7 days of treatment with 10 μM alpelisib. G Number of live cells of organoids derived from metastatic lesions of patients 2 and 4 after 7 days of treatment with 10 μM alpelisib.
Fig. 2 (See legend on previous page.)

| Case | Location | Time to metastasis (months) | Age | Diameter (cm) | ER  | PgR | Her2 | Ki67 |
|------|----------|-----------------------------|-----|---------------|-----|-----|------|------|
| 2    | Spine    | 51                          | 61  | 60%           | 60% | neg | NA   | 90%  |
| 3    | Lung     | 86                          | 47  | 90%           | 90% | 11% | neg  | 30%  |
| 4    | Skin     | 27                          | 82  | multiple, 3.4 max | 100% | 5%  | neg  | 30%  |

D. HALLMARK P13K AKT MTOR SIGNALING

E. #2 #4

F. 0 uM 10 uM

G. live cells

alpelisib uM
limited for these reasons. Growing metastatic material in OGM formulated for the primary tumour may not be ideal. Herein, we tested the hypothesis that if the metastatic material mimics the destination material, the formulation of OGM should be conducted based on the destination tissue instead of the “originating” tissue [13]. Additionally, we provided a test case in which mBC-PDOs from four patients retained sensitivity to alpelisib due to the PIK3CA mutation status of the originating lesion. Thus, we provide encouraging evidence that treating metastatic lesions based on the molecular status of the originating lesion may be logical and effective. Importantly, our reported findings require appropriate clinical studies for validation. TME components (cancer-associated fibroblasts, lymphocytes, macrophages) are rapidly underrepresented in epithelial organoids within a few passages [14, 15]. Therefore, we cannot evaluate the full contribution of the TME to the drug response. Future validation studies would greatly benefit from a multi-OMICs approach, including metabolomic, proteomic, transcriptomic and secretomic analyses. We believe that the data provided here establish a correlation between the genomic status of PIK3CA and PI3K pathway activation and provide initial evidence justifying such an approach. Nonetheless, the observations reported here still prove that metastatic material can be propagated in a way that mimics the bioarchitecture of the originating tissue by employing destination tissue-inspired media, thus allowing the evaluation of treatments targeted toward the molecular status of the lesion.

Conclusion
In summary, our data reveal an unprecedented method of cultivating mBC-derived PDOs, with the subsequent possibility of interrogating such structures with limited pharmacogenomic screening in real time. Additionally, our findings indicate that lesion-based treatment, such as for PIK3CA gene mutations, may override multisite breast cancer metastatic specificity.

Abbreviations
BC: Breast cancer; mBC: Metastatic breast cancer; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PIK3-α: Phospho-BC: Breast cancer; mBC: Metastatic breast cancer; PIK3CA: Phosphatidylinoside 3-kinase catalytic subunit alpha; IDC: Invasive ductal carcinoma; ER: Oestrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2; mOG: Organoid from metastases; OGM: Organoid growth medium; NGS: Next-generation sequencing; VAF: Variant allele frequency; PDO: Patient-derived organoid; mBC-PDO: Metastatic breast cancer patient-derived organoid; GSEA: Gene set enrichment analysis; TPM: Transcripts per million; EMT: Epithelial-mesenchymal transition; PDAC: Pancreatic ductal adenocarcinoma; MT’14: Metastatic breast cancer patient-derived organoid; GSEA: Gene set enrichment analysis; TF: Transcription factor; NES: Normalized enrichment score; RNA-seq: RNA sequencing; EGF: Epidermal growth factor; FGF2: Fibroblast growth factor 2; BSA: Bovine serum albumin; KGF: Keratinocyte growth factor.
Declarations

Ethics approval and consent to participate
The study was approved by the ethics committee and institutional review board of Regina Elena National Cancer Institute and appropriate regulatory authorities (approval no. IFO 1270/19); all patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and guidelines for Good Clinical Practice, as defined by the International Conference on Harmonization.

Consent for publication
All patients gave written informed consent.

Competing interests
The authors declare that they have no competing interests.

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