LE STUDIUM
Loire Valley
Institute for Advanced Studies

LE STUDIUM Multidisciplinary Journal
ISSN 2669-7807
www.lestudium-ias.com

FELLOWSHIP FINAL REPORT

Investigating the Heterogeneity of the Crosstalk Between Cancer Cells and the Tumor-Microenvironment Using Calcium Profiling

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REPORT INFO

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Period of residence in region Centre-Val de Loire: March 10, 2022 – March 9, 2023

Keywords :
Cancers, Tumor heterogeneity, calcium profiling, machine learning

ABSTRACT

Cancers are highly heterogeneous. This intra-tumoral heterogeneity contributes to the tumor development by confering new features such as drug resistance or aggressiveness. Understanding how this heterogeneity arises and how to detect it is a promising challenge to improve diagnosis and therapies. Aberrant regulation of calcium homeostasis in cancer cells has been extensively described. However, the relationship between calcium homeostasis and tumor heterogeneity remains to be explored.
In this project, we hypothesized that the profile of calcium homeostasis could be indicate of the phenotype of the cancer cell. We aim to develop a workflow allowing to classify cancer cells according their profile of calcium responses. We generated a database of single cell calcium responses elicited by various molecules in a panel of colorectal and prostate cancer cell lines. Using unsupervised classification algorithms, we successfully develop a model defined several profiles of calcium responses. Using this model, we were able to distinguish the origin of cancer cells.
These results suggest that calcium profiling could be an effective tool to discriminate different sub-populations of cancer cells. Further experiments will be required to develop and improve this model.

1- Introduction

Cancers are heterogenous diseases. The intra-tumoral heterogeneity is essential in tumor development by confering it multiple features such as aggressiveness or chemotherapy resistance. With the dawn of modern era of genomic technologies, it became possible to genetically explore this tumoral heterogeneity and identify several markers allowing to distinguish different sub-population of cancer cells with distinct genotype (1).

However, the functional heterogeneity of cancer cells and in particular the heterogeneity of their interaction with the extracellular micro-environment remains largely unexplored due to the lack of assay allowing to discriminate subpopulations of cancer cells on the basis of their functions.

Calcium is an essential second messenger involved in a large variety of signaling pathways. Variations of its cytosolic concentrations are tightly regulated by the coordinated action of several ion channels and transporters (2). Interestingly, oscillations of

C. Caussette, E. Cheikh Mohamed Ewe, M. Guéguinou, K. Mahéo, C. Vandier, T. Brouard, D. Crottès. Investigating the Heterogeneity of the Crosstalk Between Cancer Cells and the Tumor-Microenvironment Using Calcium Profiling. *LE STUDIUM Multidisciplinary Journal*, **2023**, 7, 1-7
https://doi.org/10.34846/le-studium.234.02.fr.03-2023

cytosolic concentrations of calcium have been observed in response to the stimulation of various receptors located at the surface of cells by extracellular ligands (2, 3). Several investigation have suggested that the profile of calcium oscillations may encode the information leading to the appropriate genetic response to a particular event (2, 4–6). In cancer cells, aberrant regulation of calcium homeostasis has been extensively documented and have been associated to all hallmarks of cancer (7–9).

Thus, we hypothesized that the profile of the variations of cytosolic calcium homeostasis could be correlated to the phenotype of a cancer cell. In particular, this calcium profile may reflect the intra-tumoral heterogeneity and potentially discriminate cancer cells with different phenotype at single cell level.

Machine learning recently emerges as a powerful approach to classify data into natural groups according their similarities (10). In cancers, machine learning has been used to provide more comprehensive analysis of genomic data (11–14). Recent advances in computer sciences has made possible the use of machine learning for the processing of temporal series and the classification of dynamic features.

Here, we generated a collection of calcium profiles by recording variations of the cytosolic calcium concentration elicited by different molecules in a panel of cancer cell lines with distinct origins. Using unsupervised clustering algorithms, we classified these profile of calcium responses into distinct clusters that allow to discriminate individual cells according their origins.

2- Experimental details

Cell culture :

Human prostate cancer cell lines, 22RV1, LNCap, C4-2 are cultured in RPMI 1640 supplemented with 10 % FBS. Human metastatic prostate cancer cell line PC3 are cultured in RPMI 1640 supplemented with 5% FBS. Human normal prostate cell line RWPE-1 is cultured in keratinocyte SFM media supplemented with Bovine Pituitary Extract, Epidermal Growth Factor (EGF) (10 ng/ mL) and 10 % FBS. Human colorectal cancer cell lines (HCT-116, Lovo and SW48) are cultured in McCoy media supplemented with 10 % FBS. All culture media are supplemented with 1 % of Penicillin/Streptomycin.

Reagents :

All reagents were purchased from Fischer Scientific (Illkirch, France) except Cal520® purchased from AATBioquest. Thapsigargin and Cal520® are resuspended in DMSO at 1 mM and 5 mM respectively. Lysophosphatidic acid 18:1 (LPA), Acetyl-choline (Ach), Histamine (Hist.) are resuspended in PBS supplemented with 0,2 % of BSA. EGF and Prostaglandin E2 (PGE2) are resuspended in pure water at respectively 100 µg/mL and 10 µg/mL For experiments, LPA and Ach are added at 1 µM, Hist at 10 µM, EGF at 100 ng/mL, PGE2 at 10 ng/mL and Tg at 2 µM.

Calcium imaging :

The day prior experiments, 50 000 cells are plated on Fluorodish 35 mm² (World Precision Instrument, Germany). On the day of the experiments, each dish are loaded with the calcium dye Cal520® at 5 µM in PSS (NaCl 140 mM, KCl 4 mM, MgCl2 1 mM, CaCl2 2mM, D-Glucose 10 mM, HEPES 10 mM adjusted to pH 7,3 with NaOH). Acquisition are performed in PSS using a Nikon Ti Eclipse microscope equipped with DS-Qi2 camera, a combination of dichroic cube, excitation and emission filters at 488 and 515 nm (SemRock), and a solid state white light source (Sola). Images were acquired every 3 s for 15 min. To evoked cytosolic calcium responses, cells are stimulated with molecules after the first minute of acquisition. Fluorescence intensity of individual cells was obtained by defining a region-of-interest for each individual cell, substracting the background fluorescence and normalizing to the fluorescent intensity measured in the first image. All image processing was performed using ImageJ Software (NIH).

Machine learning analysis :

Each signal acquisition was considered as a temporal serie. Each temporal series was normalized and scaled using *scikit-learn* python library. Unsupervized clustering

algorithms such Hierarchical clustering (HCL), K-Means and Self-Organizing Maps (SOM) were applied using *MiniSOM*, *tslearn*, *scikit-learn* and *scipy* Python libraries.

3- Results and discussion

3.1 Acquisition of profiles of calcium responses.

To investigate the heterogeneity of calcium signaling, we successfully recorded the cytosolic calcium responses evoked by 7 different molecules in a panel of 8 colorectal (Lovo, SW48, HCT-116) and prostate (RWPE-1, 22RV1, C4-2, LNCap, PC3) cancer cell lines. Altogether, we acquired 9466 unique profiles of cytosolic calcium response. On each of these profiles, maximal amplitude and average intensity (AUC) of the calcium response were determined. We observed a strong heterogeneity in these profiles of calcium responses between cell lines but also at single cell-level with profiles displaying oscillations, long lasting responses, transient/adaptive responses or an absence of variations. Thus, our approach is able to produce heterogeneous data and suggest that it could be virtually possible to discriminate sub-populations of cells.

These heterogeneous profiles prevent the discrimination of cancer cell lines based on those restricted and limited read-outs (Amplitude, Intensity) **(Figure 1)**.

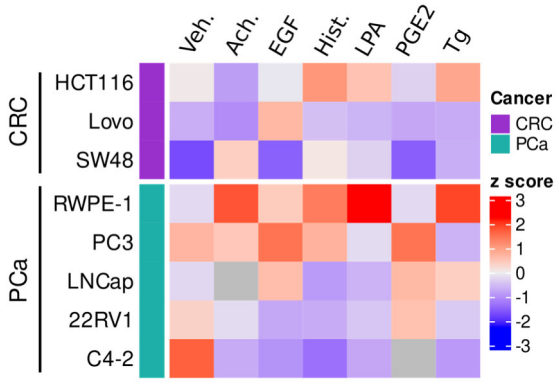

Figure 1. Heterogeneity of calcium responses elicited by different molecules in a panel of cancer cell lines. *Hierarchical clustering of cancer cell lines according the average intensity of the calcium response elicited by various molecules.*

3.2 Classification of single cell calcium responses

The heterogeneity of our data suggest that a more systemic approach should be used to identify possible patterns in the collection of profiles of calcium responses that may contribute to the classification of cancer cell lines according their characteristics. With the development of machine learning, the processing and classification of temporal and complex series become easier.

Here, we assayed the use of unsupervised classification algorithms on our dataset. The aim was to determine the number of unique profiles of calcium response contained in this large dataset independently of the cell line or the molecule tested. For that purpose, we assayed the classification of our temporal series using k-means, SOM, and Hierarchical clustering. Our preliminary results suggest that the SOM algorithm achieve the best performance and identified 36 different unique profiles.

3.3 Correlation of the single cell classification with phenotype.

We then assayed how these 36 clusters defined by SOM distribute according the cancer cell line or the cancer type investigated.

For each cancer cell lines and cancer types, we calculated the percentage of cells associated to each of clusters defined by SOM. We then applied a HCL to classify clusters according cancer cell lines or cancer types.

For HCT-116, SW48, Lovo, PC3 and 22RV1, we observe that most of cells are associated to a restricted number of clusters defined by SOM allowing to clearly distinguish those cancer cell lines from each other. In parallel, we can observe that RWPE-1, C4-2 and LNCap uniformly distribute into all of the clusters defined by SOM **(Figure 2A)**.

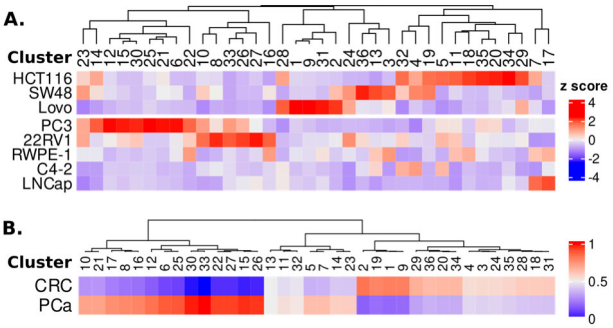

Figure 2. Distribution of cancer cell lines and cancer types in identified clusters. A. *Classification of cancer cell lines according different clusters identified in the catalogue of cytosolic calcium responses.* ***B.*** *Classification of cancer types according the percentage of each clusters identified in the catalogue of cytosolic calcium responses.*

Interestingly, we observed that the distribution of the percentage of cells associated to each cancer types in clusters defined by SOM reveals that some clusters are preferentially associated to colorectal cancer cells and others to prostate cancer cells. Thus, it appears that clusters defined by SOM allow to discriminate between profiles of calcium responses elicited by colorectal or prostate cancer cells **(Figure 2B)**.

3.4 Discussion:

In this study, we successfully generated a large dataset gathering the profile of intracellular calcium response of 9466 unique cells originated from different cancer cell lines and different cancer types stimulated by 7 different molecules.

We observed a strong heterogeneity in profiles of cytosolic calcium responses obtained. This heterogeneity is expected as a genetic heterogeneity of cancer cell lines has been previously described (15). Here we demonstrate that a functional heterogeneity can be observe at a single cell level using calcium profiling. Further studies are required now to demonstrate that this heterogeneity of calcium responses can be associated to the heterogeneity of the genetic and transcriptomic profiles of cancer cells.

In this study, we assayed if the heterogeneity of calcium responses can serve to discriminate cancer cells based on their lineage or origin. For that purpose, we assayed several unsupervised classification algorithms and were able to generate a first classification of all profiles of calcium responses into 36 different clusters. The distribution of cells according clusters defined reveal that our classification of calcium responses allow to discriminate cancer cells based on their origins. Thus, calcium profiling can be informative of some characteristic of cancer cells such as their origin. These preliminary results led us to perform a second set of experiments, pitting 9 algorithms against each other, on a base of 7807 calcium profiles obtained for 2 cancer types, 8 cell lines and 9 molecules tested: Affinity Propagation, Agglomerative Clustering, BIRCH, DBSCAN, K-Means, Mean Shift, OPTICS, Spectral Clustering and Mixture of Gaussians. All these algorithms come from the standard *sklearn* libraries available for the Python language. The processing of the results is in progress, but we can already see some interesting behaviors. We have also tested a supervised approach to predict cancer type based on calcium response. Here again, the preliminary results are encouraging, with more than 80% of correct predictions.

Heterogeneity of cancer cells have been described as one possible source of development of drug resistance (16, 17). It could be interesting to further investigate if the profile of calcium responses can be associated to a particular phenotype. This could potentially allow to discriminate cancer cells with distinct functional feature at single cell level.

Machine learning and classification algorithms are becoming more and more powerful and allow to identify complex patterns between variables and features of a dataset (10). However, it is important to note as in any machine learning project, our results are deeply related to the data used as input to the machine learning algorithms. There are therefore uncertainties in conclusions generated by our model (18, 19). To limit these bias and uncertainties, further studies will be required to comprehensively characterise, adjust and optimize our machine learning model. To develop and generalize this classification, it will require additional data to

extend the range of possible conditions used as input of our model.

4- Conclusion

In conclusion, the main goal of this study has been achieved. In this project, we successfully generate an experimental design allowing the calcium profiling of cancer cells, and generate a classification of those profiles that is representative of the origin of cancer cells. This project will pave the ground for future development of calcium profiling as a tool to detect and characterize subpopulations of cancer cells.

5- Perspectives of future collaborations with the host laboratory

Preliminary data generated by this work strengthen our hypothesis that the profile of calcium responses can be an indicator to discriminate sub-populations of cancer cells.

In collaboration with the host laboratory, we will continue this project by extending the dataset and adding new cancer cell lines with different characteristics (aggressiveness, chemoresistance, ...) or different origins (breast, ovarian or pancreatic cancers). We will develop a correlation analysis in order to establish relationship between the profile of calcium responses and the cancer phenotype. Finally, we will upscale this preliminary analysis by performing similar analysis *ex-vivo* on organotypic culture of human tumor slices.

Both the host laboratory and the fellow are committed to pursue this research project through grant applications, publication of research articles and valorization of the developed technology in the near future.

6- Articles published in the framework of the fellowship

Published articles:

Guéguinou M., Ibrahim S., Bourgeais J., Robert A., Pathak T., Zhang X., Crottès D., Dupuy J., Ternant D., Monbet V., Guibon R., Flores-Romero H., Lefèvre A., Lerondel S., Le Pape A., Dumas JF., Frank PG., Girault A., Chautard R., Guéraud F., García-Sáez AJ., Ouaissi M., Emond P., Sire O., Hérault O., Fromont-Hankard G., Vandier C., Tougeron D., Trebak M., Raoul W., Lecomte T. Curcumin and NCLX inhibitors share anti-tumoral mechanisms in microsatellite-instability-driven colorectal cancer. Cell Mol Life Sci. 2022 May 8;79(6):284. doi: 10.1007/s00018-022-04311-4. PMID: 35526196

Manuscripts in revision:

Cancel M., Crottès D., Bellanger D., Bruyère F., Mousset C., Pinault M., Mahéo K., Fromont G., Variable Effects of Periprostatic Adipose Tissue on Prostate Cancer Cells: Role of Adipose Tissue Lipid Composition and Cancer Cells Related Factors. 2023. *In revision in Cancers*

Manuscripts in preparation:

Robert Alison*, Crottès David*, Bourgeais Jérôme, Chevrolier Arnaud, Gueguen Naig, Dumas Jean-François, Servais Stephane, Chadet Stephanie, Herault Olivier, Lecomte Thierry, Raoul William, Guéguinou Maxime. The regulatory role of MICU2 in metabolic reprogramming and colorectal cancer progression.

Guéguinou M., Mahéo K., Fromont-Hankard G., Vandier C., Jan YN., Jan LY., and Crottès D. Comprehensive analysis of ion channels in pan-cancers.

7- Other disseminative actions in the framework of the fellowship

Organization of an international meeting:

Sept 21st-23rd 2022 – Le Studium Conference "Ion channels in pathological context, new methods and diagnosis tools" – Tours, FR.

Co-convenor with Pr. Vandier (Inserm UMR1069) and Pr. Petoud (CNRS CBM).

Oral presentation :

Dec 6th 2022 – Ion Channels in Cancer Meeting – Lille, FR.

Sept 22nd 2022 – Le Studium Conference "Ion channels in pathological context, new methods and diagnosis tools" – Tours, FR.

Students training :

2022-2023 – Amélie Bura, Pharmacy student 5cells accrodignth year (5 months, co-supervision with Pr. Karine Mahéo at Inserm U1069) : Role of calcium-activated chloride channels in prostate cancer cells.

2022 – Abigail Bentley, Medical School 4th year from Birmingham University (4 weeks, co-supervision with Dr. Maxime Guéguinou at Inserm U1069) : Contribution of mitochondrial exchangers in the store-operated calcium entry in prostate cancer cells.

2022 – Camille Caussette, Master 2 student (6 months, co-supervision with Pr. Karine Mahéo at Inserm U1069) : Regulation of calcium signaling of prostate cancer cells by hypoxia and fatty acids.

2022 – Jean-Baptiste Gourvennec, Master 2 student (Medical Intern 5th year, 6 months, co-supervision with Pr. Gaëlle Fromont at Inserm U1069) : Role of chlordecone on the phenotype of prostate cancer cells.

8- Acknowledgements

This work was supported by the Le Studium, Loire Valley Institute for Advanced Studies, Orléans & Tours, France (DC), Région Centre-Val de Loire APR-IR grant (CC and KM), Inserm PCSI (CV) and ARFMAD association (MG).

We acknowledge all lab members of Inserm U1069 and LIFAT for their help during the framework of this fellowship. We finally thank Le Studium Loire Valley Institute for Advanced Studies for this fellowship, for their welcoming, their wonderful organization and for providing the opportunity to organize an international consortium on this topic.

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