VALIDATION OF A PREDICTIVE MOLECULAR SIGNATURE IN NEUROENDOCRINE TUMORS

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1. Summary of the project

Management of advanced pancreatic neuroendocrine tumors (PanNETs) poses challenges despite improved survival rates over the past decade. Our study aimed to analyze the potential identification of predictive biomarkers, and for this purpose we assessed the predictive capability of a PanNET molecular classification in patients receiving chemotherapy and everolimus treatment, previously included in the SEQTOR clinical trial. We analyzed paraffin-embedded tumor blocks from advanced grade 1 or 2 PanNET patients undergoing both everolimus and streptozocin/5FU (STZ/5FU) chemotherapy, administered upfront and upon progression respectively.

We conducted mutational analysis for a specific gene set using next-generation sequencing at the Center for Applied Research on Cancer at the University of Verona. Subsequently, gene expression was examined by TempOSeq at the Institute of Cancer Research, UK.

Finally, we analyzed clinical and molecular data using statistical and bioinformatic tools to establish correlations between molecular signatures and clinical outcomes.
The study aimed to correlate molecular data with information obtained from the clinical trial, identifying connections between expression patterns and outcomes like progression-free survival, response rates, and overall survival. We anticipate that this molecular classification of PanNETs could offer predictive insights into treatment responses with everolimus or STZ/5FU chemotherapy for patients with grade 1 or 2 PanNETs.

The main goals of our project were:

Primary Objective:

- To determine progression free survival according to our molecular subtype classification for first-line treatment with STZ-5FU or everolimus.

Secondary Objectives:

- To determine progression free survival according to our molecular subtype classification for second-line treatment with STZ-5FU or everolimus
- To determine response rate according to our molecular subtype classification for first/ second-line treatment with STZ-5FU or everolimus

2. Results obtained

Results analysis alongside the Reporter Library File analysis

The slides from each block, totaling 50 from the SEQTOR trial and 15 from another study, underwent analysis using TempOSeq, a probe-based capture and RNA sequencing technology, in two separate batches. TempOSeq profiles 22,533 genes, covering almost the entire human transcriptome. Quality control assessment was performed for library preparation, and samples with fewer than 30% of genes having fewer than 5 reads were considered poor quality and excluded from further analysis. In the pilot samples 2 out of 15 failed this criterion, while in our cohort 16 samples failed primarily due to insufficient material in the blocks. Additionally, 7 samples were flagged for further quality evaluation and potential resequencing. The remaining 27 samples underwent batch correction.

Of the 27 samples that passed quality control, we successfully classified them into four subtypes of PanNET: MLP-1 (n=4), MLP-2 (n=9), intermediate (n=9), and insulinoma (n=5).

To classify our data into four PanNET subtypes (MPL, insulinoma-like, and intermediate), MLP-1 and MLP-2 were grouped together in a complementary analysis. This grouping is justified by biological similarities shared by these two groups as described in the original PanNET subtype classification (Cancer Discovery, 5; 1296-1313, 2015).

Progression-free survival, overall survival, and overall response rate were analyzed for each PanNET subtype alongside oncologic treatment using Kaplan-Meier estimations, log-rank tests, and Cox regression models. A significance level of 0.05 was set for all hypothesis testing. Additionally, a piecewise constant Hazard model was adjusted as a Bayesian approach to study the same variables.

Assignment of molecular subtype and clinical data correlation

We have made progress in assigning molecular subtypes using 77 out of 228 genes that proved robust in classifying PanNET samples into four molecular subtypes (previously identified genes; Sadanandam et al., Cancer Discovery 2015). These

subtypes comprise insulinoma (21%), metastases-like primary (MLP-1; 21%), MLP-2 (21%), and intermediate (37%).

Continued efforts are underway to refine our comprehension of the remaining genes to unveil subtype-specific characteristics. Furthermore, we intend to incorporate additional samples into our study to bolster our analysis.

We examined the correlation of these four molecular subtypes of RNA expression with three clinical outcomes ("clinical outcomes"): progression-free survival, overall survival, and overall response rate, with adjustments made according to the type of treatment. Due to singularity problems, no other variables were included in the model. The insulinoma subtype demonstrated a lower risk of events compared to other PanNET subtypes for the progression-free survival endpoint. However, for overall survival and overall response rate endpoints, the analysis of PanNET subtypes did not reveal significant differences between groups.

Results obtained using a Bayesian approach were consistent with those obtained using frequentist methods.

Gene mutation and clinical correlation analyses:
Individual mutational screening of a selected set of genes relevant to this disease was analyzed and correlated with clinical endpoints.

Among the 56 sequenced samples, all but two with borderline results exhibited low tumor mutational burden (TMB) and were microsatellite-stable (MSS). Classic mutations were observed in ATRX, DAXX, MEN1, and PTEN. ATRX, DAXX, and PTEN mutations were always accompanied by MEN1, except for two cases. Several other mutations occurred in one or two samples each. MEN1 was mutated in 63% of the samples and 41% in arms A / B, respectively, ATRX in 26% and 22%, DAXX in 26% and 19%, and PTEN in 16% and 11% of samples analyzed. Copy number variations included homozygous deletions in CDKN2A and CDKN2B, as well as other variations like loss of heterozygosity (LOH) in several genes. Notably, a TERT amplification and a large deletion in WT1 were observed in individual cases.

Regarding copy number variations, 15 samples had homozygous deletions, 9 in *CDKN2A* and 8 in *CDKN2B.* Two samples exhibited LOH, 1 in each of *CDKN2A* and *CDKN2B.* One case had a *MEN1* homozygous deletion. Additionally, LOH was observed in MAP2K2, MEN1, MET, PMS2, RB1, and SETD2 in four cases each. Notably, one case showed TERT amplification, and another had a large deletion in WT1.

Finally, a structural variation involving a large deletion spanning exon 11-15 was detected in one case.

Regarding correlations with clinical outcomes variables, we were able to statistically analyze mutations in only four genes: PTEN, MEN1, DAXX and ATRX. Other genetic alterations occurred too infrequently to be studied effectively.

In the analysis of progression-free survival, patients with altered DAXX exhibited a higher risk of events (hazard ratio: 2.5 with a 95% confidence interval of 1.1 – 6). However, for overall survival, no significant differences were detected among these four mutations.

Regarding the response rate endpoint, patients with ATRX alterations showed a higher odds ratio (OR: 7.9 with a 95% confidence interval of 1.7 - 57) compared to those without alterations. Both Bayesian and frequentist approaches yielded consistent results.

We are still analyzing these results and their correlations to be able to delve into their potential clinical relevance.

3. Relevance and possible future implications

The significance of our current efforts lies in developing and validating a molecular classification capable of distinguishing prognosis and predicting treatment response beyond known clinical and pathological biomarkers. We have confirmed that the insulinoma-like subtype consistently yields a notably better prognosis regardless of treatment selection. This finding is crucial when faced with clinical dilemmas such as deciding between watchful waiting strategies versus more aggressive interventions.

Clinicians and patients, particularly in cases of low disease volume and mitigating prognostic factors, can feel more confident in opting for less aggressive approaches like somatostatin analogs rather than chemotherapy or more toxic therapies if molecular analysis confirms the low biological aggressiveness associated with the insulinoma-like subtype. While the limitation of our final statistical sample size has hindered further validation of the potential to discriminate treatment benefits of individual therapies, the fact that the technology and platform used have been clinically validated for prognostic potential opens the door for further refinements and confirmation of its predictive potential in future independent cohorts.

Confirmation that a molecular classification based on gene expression profiling holds prognostic information in an independent cohort within a prospective randomized clinical trial validates its use as a prognostic tool. It complements prior knowledge on other prognostic markers such as disease stage, grading, and proliferative Ki67 index. Additionally, it validates the technological approach and platform used, providing a basis to develop and refine signatures for predicting differential treatment benefits with individual therapies such as chemotherapy or targeted agents like everolimus.

4. Scientific bibliography generated

Manuscript preparation

For the clinical part of the study (SEQTOR study), we have a paper ready to be submitted:

Type of article: Original article

Title: A Randomized Study of Streptozotocin plus 5-Fluorouracil Followed by El Everolimus or the Reverse Sequence in Patients with Advanced Pancreatic Neuroendocrine Tumors (SEQTOR)

Authors:

Jaume Capdevila[1], Salvatore Tafuto[2], Merete Krogh[3], Alex Teulé[4], Rocio Garcia-Carbonero[5], Heinz Josef Klümpen[6], Birgit Cremer[7], Isabel Sevilla[8], Barbro Eriksson[9], Elizaveta Tabaksblat[10], Jean-Philippe Metges[11], Nicholas Simon Reed[12], Joerg Schrader[13], Valentí Navarro[14], Vicente Valentí [4], Jorge Hernando[1], Ana Maria Colao[2], Lene Vestermark[3], Carlo Carnaghi[15], Ulrich Peter Knigge[16], Paula Jimenez-Fonseca[17], Marta Benavent[18], Judith de Voos-Geelen[19], Marino Venerito[20], Alexander Von Werder[21],

Henning Jann[22], Anja Rinke[23], Denis Smith[24], Dieter Hörsch[25], Naureen Starling[26], Philippe Ruszniewski[27], Eric Baudin[28], Francois-Xavier Caroli-Bosc[29], José Luis Manzano[30], Marta Martín[31], Ramón Salazar[1]

We are still finishing analyzing results from the project for the preparation of a second manuscript publication.

Two abstracts have recently been submitted to ESMO 2024 Congress, one based on the results of this project and another with more clinical aspects.

Conference presentations:

- Subgroup analysis of ORR and PFS of the SEQTOR study (GETNE 1206): Everolimus followed by Streptozotocin (STZ)-5FU upon progression or the reverse sequence, in advanced progressive panNETs. J. Capdevila et al. Annual ENETs conference 2023. Vienna, Austria.
- Randomized open label phase III study comparing the efficacy and safety of everolimus followed by chemotherapy (CT) with Streptozotocin (STZ)-5FU upon progression or the reverse sequence, in advanced progressive panNETs: the SEQTOR study (GETNE 1206). Annals of Oncology, Vol 33(S7): S1412. DOI: https://doi.org/10.1016/j.annonc.2022.08.044. R. Salazar et al. ESMO Congress 2022. Switzerland.
- Randomized open label phase III study comparing the efficacy and safety of Everolimus followed by chemotherapy with Streptozotocin (STZ)-5FU upon progression or the reverse sequence, in advanced progressive panNETs: The SEQTOR study (GETNE 1206). R. Salazar et al. Annual ENETs conference 2022. Paris, France.