

RELEVANCE OF ANNEXIN A1 AND CD70
IMMUNO-ONCOLOGICAL MARKERS IN SMALL
CELL LUNG CANCER

PhD thesis

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1. Introduction

Small cell lung cancer (SCLC) is one of the malignancies with the worst prognosis, limited therapeutic options, and no major therapeutic advances for decades. Its 5-year survival rate is approximately 7%, and the majority of patients are diagnosed at advanced stage, when chemotherapy remains the main therapeutic approach, with the recent addition of immune checkpoint inhibitors.

Unlike in non-small cell lung cancer (NSCLC), immunotherapy targeting the programmed cell death 1 (PD-1)/programmed death ligand 1 (PD-L1) pathway has not revolutionized the treatment of SCLC. Moreover, SCLC lacks actionable driver mutations but is characterized by a high mutational burden and plasticity, allowing recurrence after chemotherapy as therapy resistant disease. Therefore, identification of novel biomarkers and therapeutic vulnerabilities is critical.

Although previously considered a homogeneous entity, several distinct subtypes have been identified in SCLC, including ‘classical’ versus ‘variant’, neuroendocrine (NE)-high versus -low and non-NE tumors, as well as ‘immune oasis’ and ‘immune desert’ phenotypes, corresponding to dominant transcriptional drivers that define at least four molecular subtypes. Non-NE subtypes exhibit higher immune infiltration and are expected to respond better to immunotherapy, however, intratumoral heterogeneity complicates classification and reliable predictive biomarkers are still missing.

When characterizing the tumor microenvironment (TME) of SCLC, subtype-specific expression patterns of immunomodulatory molecules have been identified. Annexin A1 (ANXA1) was highly expressed in neuroendocrine-low tumors irrespective of immune infiltration, while cluster of differentiation 70 (CD70) was upregulated in NE-low, immune-oasis tumors. These findings highlighted ANXA1 and CD70 as potentially relevant molecules in SCLC and provided a rationale for their further investigation.

ANXA1 is a calcium-dependent phospholipid-binding protein with context-dependent physiological and pathological roles. Its best characterized function is a glucocorticoid-like anti-inflammatory effect in the adaptive immune system, while its roles in other biological processes are more variable. In cancer, aberrant ANXA1 expression has been associated with several hallmarks of malignancy, including uncontrolled proliferation, therapy resistance, metabolic alterations, and epithelial–mesenchymal transition (EMT). However, depending on the tumor type and cellular context, ANXA1 may be either upregulated or downregulated and can function as both a tumor promoter and a tumor suppressor. In SCLC, ANXA1 has been linked to brain and bone metastasis, but it had not been comprehensively characterized.

CD70 and CD27 form a co-stimulatory immune checkpoint pathway involved in the regulation of T cell activation and survival. In cancer, dysregulation of this

axis may result in aberrant T cell stimulation, immune exhaustion, and recruitment of regulatory T cells, thereby promoting an immunosuppressive tumor microenvironment and immune evasion. Since immune checkpoint pathways beyond the PD-1/PD-L1 pathway are of increasing interest in SCLC, characterization of the CD70–CD27 pathway may also be of relevance.

2. Objectives

Therapy resistance in SCLC remains poorly understood, highlighting the need for highlighting the need to explore the underlying mechanisms and identify biomarkers for patient stratification. Based on prior findings, ANXA1 and the CD70–CD27 axis may contribute to an immunosuppressive, tumor-promoting microenvironment. Therefore, we aimed to comprehensively characterize ANXA1 in SCLC using *in vitro* models, *in silico* analyses, and patient-derived data, and to evaluate the *in situ* expression and prognostic relevance of ANXA1 and the CD70–CD27 axis. To address Objectives 1 and 2 outlined below, we established an *in vitro* model where ANXA1 expression is stably silenced in three NE-low, ANXA1-high SCLC cell lines dominant transcription drivers Yes-associated protein 1 (YAP1) and POU class 2 homeobox 3 (POU2F3) as a platform for our experiments.

2.1. Functional effects of ANXA1 expression in SCLC.

To evaluate the impact of ANXA1 silencing on proliferation, chemosensitivity to standard cytotoxic

agents; malignant phenotypic traits, specifically expression of metabolic and mesenchymal markers; and immune evasion-related mechanisms as indicated by PD-L1 and CD47 immune checkpoint expression.

2.2. Potential roles of ANXA1 in SCLC non-NE phenotype-related changes and TME composition.

To investigate whether ANXA1 silencing affects dominant transcriptional driver expression in the three NE-low ANXA1-high cell lines, and if differences occur in the cellular composition of the TME of ANXA1-high and ANXA1-low tumors.

2.3. ANXA1 and the CD70/CD27 axis in the clinical context of SCLC.

To assess the *in situ* expression of ANXA1 and the CD70–CD27 axis in SCLC patient cohorts, including association of CD70 and CD27 expression with immune cell infiltration, and descriptive analysis of the spatial co-localization of ANXA1, CD70 and CD27 with TME-specific markers, and to evaluate the potential prognostic significance of ANXA1, CD70, and CD27.

3. Methods

3.1 Cell lines and *in vitro* experiments

In vitro experiments were performed using three NE-low, ANXA1-high SCLC cell lines (NCI-H841, SW1271, NCI-H1048). ANXA1 silencing was achieved using lentiviral shRNA transduction, with a non-targeting shRNA control and puromycin selection maintained throughout the experiments. Knockdown efficiency was confirmed at the protein level with Western blot.

Cell proliferation and chemosensitivity to etoposide and cisplatin were assessed over 120 hours using trypan blue exclusion and resazurin-based viability assays (Presto Blue™), respectively.

Protein expression (ANXA1, citrate synthase [CS], lactate dehydrogenase a [LDHa], β -catenin, vimentin, PD-L1, CD47, YAP1, POU2F3) was analyzed by Western blot.

3.2 Patient samples and *in situ* expression analyses

ANXA1 was analyzed in a small early-stage SCLC cohort (n = 9), while CD70 was evaluated in a large pathology-based cohort (n = 190). Formalin-fixed paraffin-embedded samples from SCLC patients were used to construct tissue microarrays (TMAs). Two cores per tumor were analyzed.

Immunohistochemistry and double immunofluorescence were used to descriptively assess ANXA1, CD70, and CD27 expression and their spatial relationships within the tumor microenvironment,

including immune (CD3, CD20, CD68) and stromal (vimentin) compartments.

Expression of CD70 and CD27 mRNA was evaluated using RNAscope® on TMA sections. Signal quantification was performed at single-cell level using digital image analysis, and expression groups were defined based on data-driven cutoffs.

3.3. Analysis of immune infiltration

To investigate the association of ANXA1 expression and tumor immune infiltration, bulk RNA-seq data from the UCologne2015 cohort (cBioPortal; n = 81) were analyzed. Tumors were stratified based on ANXA1 expression and TME composition was estimated using Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data (ESTIMATE) and Estimating the Proportions of Immune and Cancer cells (EPIC) algorithms.

RNAscope® results of the CD70 cohort were compared with previously published immunohistochemistry-based cell density data for CD45⁺ and CD8⁺ immune cells, separately assessed in tumor nests and stromal compartments.

3.8 Survival analysis

Overall survival was analyzed using Kaplan–Meier curves and Cox proportional hazards models for Annexin A1 expression in the UCologne2015 cohort and for CD70/CD27 in the pathology-based cohort.

3.4 Statistical analysis

Statistical analyses were performed using standard parametric or non-parametric tests as appropriate. A p-value < 0.05 was considered statistically significant.

4. Conclusions

Although SCLC was historically considered a relatively uniform disease, recent research has revealed substantial molecular heterogeneity and plasticity. Molecular subtyping may help identify therapeutic vulnerabilities, although subtype classification and stability remain under investigation. The immunosuppressive TME further contributes to disease complexity and treatment resistance.

In this work, we focused on two immune-related factors with potential relevance in SCLC biology: ANXA1 and the CD70–CD27 immune checkpoint axis. Using *in vitro* models, *in silico* analyses, and patient cohorts, we addressed three main aspects: **the functional effects of ANXA1 perturbation, its association with non-NE phenotype-related changes and TME composition, and ANXA1 and the CD70–CD27 axis in the clinical context.**

ANXA1 can act as either a tumor promoter or tumor suppressor depending on cancer type and context and its role in SCLC had not been comprehensively characterized prior to our study. The CD70–CD27 axis is

an emerging immunoregulatory pathway involved in tumor immune evasion and represents a potential therapeutic target in CD70-expressing malignancies.

Regarding the functional effects of ANXA1 perturbation, ANXA1 silencing produced cell line-dependent effects in three non-NE SCLC cell lines. Proliferation was unchanged in H841, decreased in SW1271, and increased in H1048 cells, while chemosensitivity to cisplatin and etoposide was generally reduced following ANXA1 silencing. Mesenchymal markers were also differentially affected, with decreased vimentin and β -catenin expression in H1048 and patterns consistent with a more mesenchymal phenotype in SW1271. CS and LDHa expression were altered in a cell line-dependent manner. In SW1271 cells, these changes suggested a possible Warburg-like metabolic shift, whereas in the other two cell lines, CS expression decreased without a concomitant increase in LDHa. ANXA1 silencing did not significantly alter PD-L1 or CD47 levels, suggesting that ANXA1 is unlikely to be a major upstream regulator of these checkpoint molecules in the SCLC models tested.

Regarding the association of ANXA1 with non-NE phenotype-related changes and TME composition, the non-NE phenotype-related markers YAP1 and POU2F3 were markedly upregulated following ANXA1 silencing in H1048 cells. No similar changes were observed in the other cell lines, suggesting that ANXA1–driver interactions may depend on the molecular context. Bioinformatic analyses further showed that ANXA1-high tumors were associated with increased immune infiltration, particularly with TAMs.

In the clinical context, ANXA1 was detected in both tumor nests and stroma and co-localized with CD45-positive immune cells and CD68-positive macrophages. CD70 was predominantly expressed in tumor nests, while CD27 expression was mainly stromal, and higher stromal CD27 expression associated with lower immune cell infiltration. High ANXA1 expression was associated with poorer survival in a small retrospective cohort, although the limited sample size precluded firm conclusions regarding its prognostic significance. High CD70 expression was significantly associated with worse overall survival, whereas CD27 showed no prognostic effect.

Overall, our study demonstrates the context-dependent roles of ANXA1 in SCLC, with possible associations with phenotypic heterogeneity and treatment response, although its prognostic significance requires further validation. High CD70 expression may serve as a prognostic marker, while its predictive and therapeutic relevance remains to be established. Together, these results underscore the contribution of both tumor-intrinsic and microenvironmental factors to SCLC heterogeneity.

5. Bibliography

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