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POST-TRANSLATIONAL MODIFICATION

Identification of Phosphorylation-Activated States in Proteins

WHITEPAPER

Identifying Akt/PKB and STAT3 phosphorylation states as prognostic factors in clear cell renal cell Carcinoma using QF-Pro®

Clear cell renal cell carcinoma (ccRCC) presents significant challenges in clinical management due to its resistance to conventional therapies and genetic heterogeneity. The QF-Pro® platform, based on an enhanced and simplified two-site Förster Resonance Energy Transfer (FRET) assay combined with Fluorescence Lifetime Imaging Microscopy (FLIM), enables precise quantification of post-translational modifications (PTMs).

The phosphorylation activation states of protein kinase B (PKB/Akt) and STAT3 were quantified to investigate their potential as prognostic biomarkers and their relevance to the development of personalized treatment strategies in patients with ccRCC.

1. Introduction

1.1 Background on ccRCC

Clear cell renal cell carcinoma is the most common and aggressive subtype of renal cell carcinoma. Due to its radioresistance, chemoresistance, and genetic variability, ccRCC presents substantial therapeutic challenges. With 40% of patients experiencing mortality within five years post-diagnosis, there is an urgent need for reliable prognostic markers to guide personalised treatment. Our research indicates that monitoring oncoprotein activation states—specifically PKB/Akt and STAT3—via QF-Pro® may provide a promising diagnostic tool with prognostic relevance.

1.2 Current limitations in ccRCC biomarker assessment

Traditional diagnostic methods largely rely on immunohistochemistry (IHC) to detect protein expression levels. While IHC provides a basic measurement, it does not reveal the activation state or the functionality of a protein, therefore missing relevant information about the biology of the disease.

Conversely, QF-Pro® enables the quantitative evaluation of oncoprotein activation states by measuring the interactions between fluorophore-labelled antibodies targeting specific phosphorylation sites, allowing for a more detailed and reliable analysis of biomarker functionality in disease progression.

2. QF-Pro® technology and methodology

2.1 Principles of QF-Pro®

FRET is a non-radiative energy transfer technique that occurs between two fluorophores (donor and acceptor) when they are within 10 nanometres. FRET-FLIM provides additional precision by measuring changes in fluorescence lifetime, providing precise and quantitative values.

In this study, QF-Pro®, based on our proprietary two-site amplified FRET-FLIM method, was used to assess the activation states of PKB/Akt and STAT3 through phosphorylation-specific antibodies targeting key activation sites. The two-site assay achieves enhanced specificity due to the intrinsic spectroscopic properties of the FRET signal. The 1-10nm working distance of QF-Pro®, coupled with the two-site labelling, eliminates false positive results.

2.2 Sample preparation and analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue microarrays (TMAs) from primary and metastatic ccRCC samples were analysed. Each sample underwent QF-Pro® analysis using secondary QF-Pro® probes tagged with ATTO 488 (donor) and Alexa594 (acceptor) fluorophores. QF-Pro® activation state scores were then calculated for each patient.

3. Results

3.1 PKB/Akt activation upon EGF stimulation in cells

The activation dynamics of Akt/PKB (measured via phosphorylation at threonine-308) in cells were evaluated following stimulation with epidermal growth factor (EGF) using QF-Pro® technology (Fig. 1A). QF-Pro® activation maps revealed significant differences in Akt activation between basal and EGF-stimulated conditions. Under basal conditions (non-stimulated), the QF-Pro® maps showed cells predominantly in blue, indicative of minimal Akt phosphorylation. However, after 10 minutes of EGF stimulation, these maps shifted markedly, with cells exhibiting green to yellow signals. Orange pixels indicate areas of higher Akt/PKB activation state occurring at the plasma membrane of these cells (Fig. 1B). This transition reflects the phosphorylation and subsequent activation of Akt/PKB, detected via the QF-Pro® signal.

Quantitative analysis of phospho-Akt activation over time further confirmed these observations. Measurements taken at 1, 2, 5, and 10 minutes post-EGF stimulation demonstrated a significant progressive increase in Akt phosphorylation, with the highest activation observed at 10 minutes (Fig. 1C). The high dynamic range and sensitivity of QF-Pro® allowed for precise visualisation and quantification of these activation states.

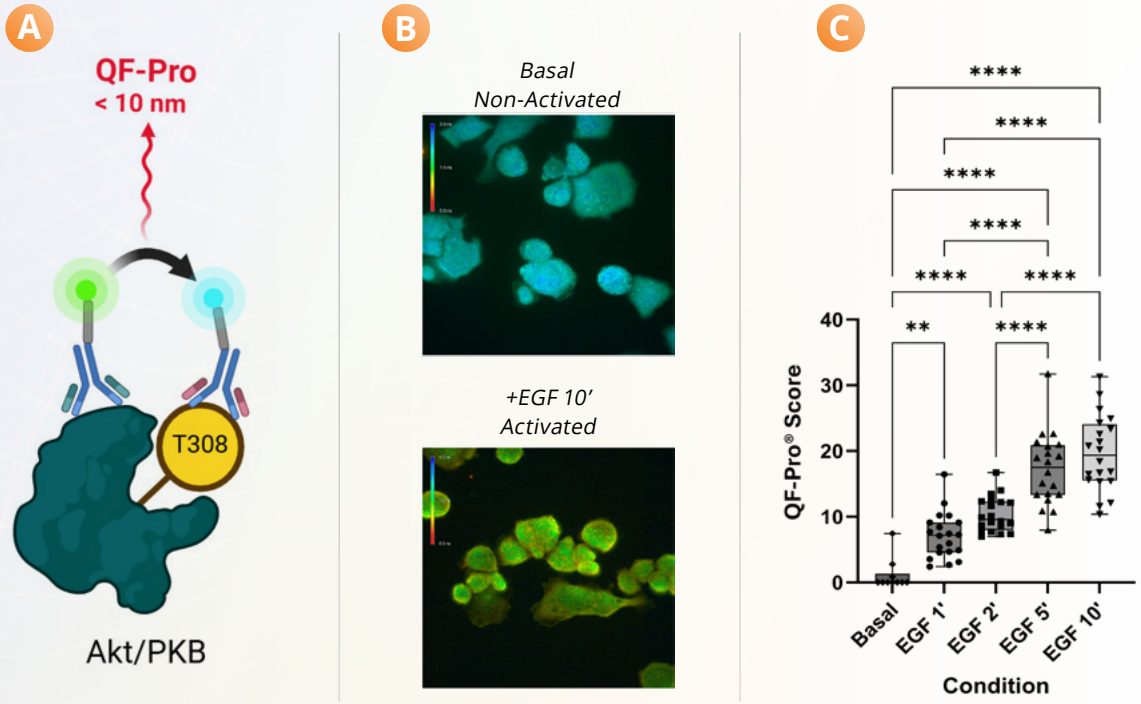


Figure 1A) *Illustration of QF-Pro® assay for detecting Akt/PKB activation.*

Figure 1B) *Representative QF-Pro® maps show minimal Akt/PKB activation under basal conditions and increased phosphorylation upon EGF stimulation for 10 minutes.*

Figure 1C) *Quantitative QF-Pro® scores across different EGF treatment time points demonstrate a significant increase in activation state with longer stimulation times ($p < 0.001$).*

3.2 PKB/Akt activation in ccRCC progression

The activation state of Akt/PKB in ccRCC TMAs was evaluated across renal control tissues, primary tumours, and metastatic samples using the QF-Pro® assay. QF-Pro® maps demonstrated distinct differences in Akt/PKB activation among these groups. Primary tumour samples exhibited low activation levels, with QF-Pro® maps predominantly green. In contrast, metastatic tissues showed an increase in Akt/PKB activation, indicated by the transition to yellow and red pixels, reflecting higher phosphorylation levels (Fig. 2A).

Quantitative QF-Pro® scores confirmed these findings. Renal control tissues exhibited minimal Akt/PKB activation, while primary ccRCC tumour samples showed a mild increase. However, only metastatic samples showed a significantly higher activation state compared to both renal control tissue and primary tumours (Fig. 2B). These results underscore the progressive activation of Akt/PKB during ccRCC progression and metastasis, suggesting its utility as a biomarker for disease aggressiveness and tumour evolution.

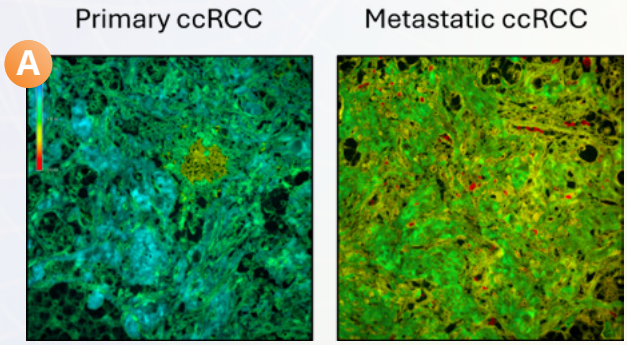


Figure 2A) *Representative fluorescence lifetime images, primary ccRCC tissue shows moderate Akt/PKB activation (blue/green) and metastatic ccRCC tissue exhibits increased activation (yellow/red).*

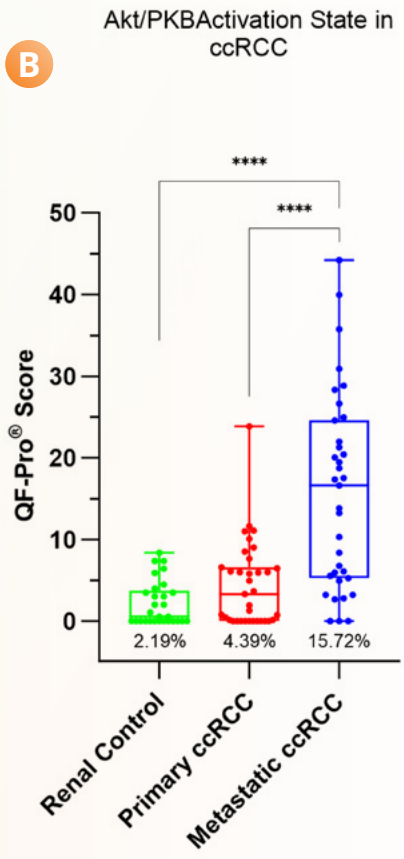


Figure 2B) *QF-Pro® Score demonstrated a significant difference between non-cancerous, primary, and metastatic samples ($p < 0.001$), confirming that Akt activation state, rather than mere expression levels, correlates with clinical prognosis in ccRCC patients.*

3.3 Prognostic precision of QF-Pro® versus IHC

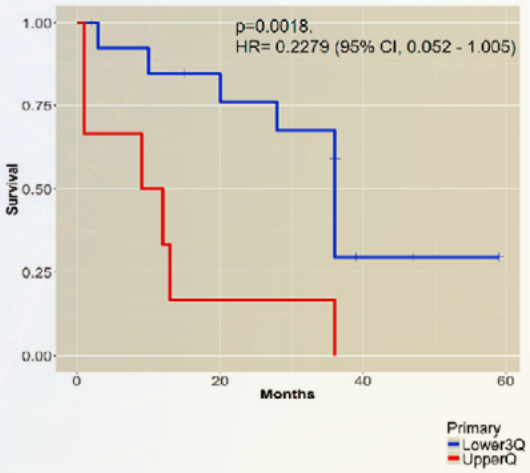
QF-Pro® technology demonstrated superior prognostic precision compared to traditional IHC in detecting variations in Akt/PKB activation states across patient samples. This was evident from the survival analysis, where Kaplan-Meier survival curves based on QF-Pro® scores provided a significantly stronger prognostic correlation compared to protein expression levels measured by IHC intensity.

In the QF-Pro® analysis, patients with higher Akt/PKB activation (upper quartile, red line) exhibited significantly poorer overall survival compared to those with lower activation levels (lower three quartiles, blue line) (Fig. 3A). This finding indicates that increased Akt/PKB activation is associated with worse survival outcomes, underscoring its role as a marker of tumour aggressiveness and outcome.

Conversely, survival analysis based on the IHC intensity of pT308 Akt/PKB failed to show a significant association with survival, highlighting its lower prognostic value (Fig. 3B). These differences can be attributed to the fundamental limitations of IHC, which measures only the expression level of a protein without providing insights into its activation state. Moreover, being a one-site assay, IHC is susceptible to the false positives that arise from the lack of specificity of primary antibodies.

These findings highlight the ability of QF-Pro® to assess activation states in FFPE tissues, accurately correlating high Akt/PKB activation with poorer survival, highlighting its value for understanding tumor behavior and guiding clinical decisions in ccRCC.

A ***QF-Pro® Analysis***



B ***IHC Analysis***

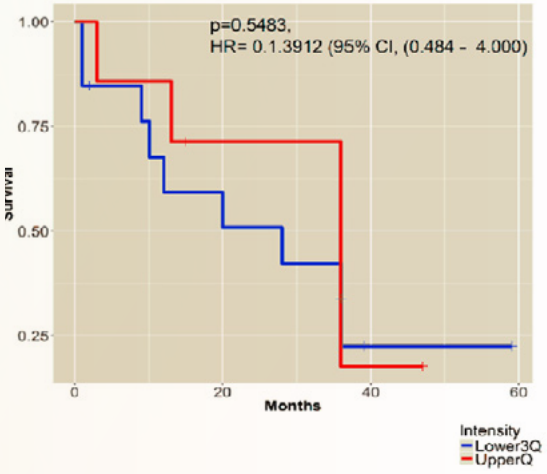


Figure 3 - *Akt/PKB activation state correlates with poor overall survival in ccRCC. Kaplan-Meier survival outcomes related to PKB/Akt activation as determined by QF-Pro® (A) or by conventional IHC (B).*

A) *QF-Pro® analysis revealed significantly poorer survival for patients in the upper quartile compared to the lower three quartiles.*

B) *No significant survival difference was observed using conventional IHC.*

3.4 STAT3 activation dynamics

Finally, the activation state of Tyr705-STAT3, a key phosphorylation site required for STAT3 activation and nuclear translocation, was analysed across renal control tissues, primary ccRCC tumours, and metastatic ccRCC samples using the QF-Pro® assay. The quantitative analysis demonstrated a progressive increase in STAT3 activation from non-cancerous renal tissue to metastatic tumours.

Renal control tissues showed minimal activation, while primary ccRCC samples exhibited a modest but statistically significant increase. Furthermore, metastatic ccRCC samples showed a marked elevation in STAT3 activation, highlighting the role of Tyr705-STAT3 phosphorylation in tumour progression and aggressiveness (Fig. 4).

These results underscore the potential of STAT3 activation as a biomarker for ccRCC prognosis, with higher activation correlating with more advanced disease stages and poorer patient outcomes.

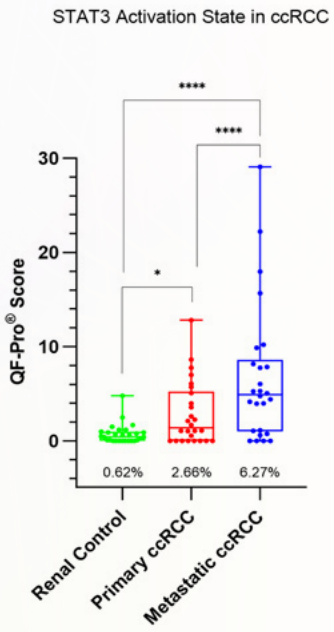


Figure 4) *Tyr705 activation is higher in metastatic ccRCC tumors in FFPE TMAs. Box and Whisker plots show the QF-Pro® scores of the three groups. Activation of Tyr705 is higher in metastatic cores than in primary cores. Both groups are significantly higher than the non-cancerous renal control tissue ($p < 0.001$).*

4. Conclusion

QF-Pro® technology represents a significant advancement in prognostic biomarker analysis by enabling the precise measurement of protein activation states rather than simple expression levels. This is a paradigm shift from traditional methods such as IHC, which are limited to detecting protein expression without assessing functionality. By focusing on post-translational modifications, such as phosphorylation, QF-Pro® offers unparalleled specificity and sensitivity, revealing critical insights into the signalling dynamics that drive tumour progression.

In the context of ccRCC, QF-Pro® has shown clinical utility by accurately assessing the activation states of two key biomarkers, PKB/Akt and STAT3. This technology offers valuable prognostic insights, linking biomarker activation to tumour progression and patient outcomes. By integrating multiple biomarkers, QF-Pro® enhances risk stratification and supports personalized treatment strategies.
With its ability to measure functional protein states and monitor therapeutic responses, QF-Pro® addresses the limitations of conventional approaches, paving the way for more precise and individualized cancer care in ccRCC.

Contact Us

Find out more information about our QF-Pro® services at:

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References:

1. *Miles J, Applebee CJ, Leboucher P, et al. Time resolved amplified FRET identifies protein kinase B activation state as a marker for poor prognosis in clear cell renal cell carcinoma. BBA Clin. 2017;8:97-102. doi:10.1016/j.bbacli.2017.10.002*
2. *Veeriah S, Leboucher P, de Naurois J, et al. High-throughput time-resolved FRET reveals Akt/PKB activation as a poor prognostic marker in breast cancer. Cancer Res. 2014;74(18):4983-4995. doi:10.1158/0008-5472.CAN-13-3382*