Transform Alpha-II Spectrin (SPTAN1) to a Prototype Platform for Tissue Quality Assessment

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INTRODUCTION

Variable degrees of molecular degradation unavoidably occur in clinical tissue specimens. No accurate tool exists for degradation assessment and specimens are therefore analyzed without quality control.

Using proteomic comparison tools and an experimental Cold Ischemic Time (CIT) tissue degradation model, we have identified 27 Tissue Degradation Indicators (TDIs) for quantitative degradation measurement.

Alpha II spectrin (SPTAN1), one of the TDIs, undergoes continuous and dynamic conversion from its intact form to its breakdown forms during tissue degradation. The quantitative ratio between SPTAN1 intact and breakdown forms strongly correlates with the impact of CIT.

In the present study, we proceed with validation of SPTAN1 as a TDI in a large cohort of specimens and explore the transformative potential of SPTAN1 to be a tissue quality assessment tool.

MATERIALS AND METHODS

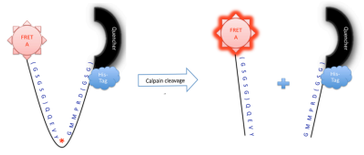
1. **Validation of SPTAN1 as a Tissue Degradation Indicator (TDI):**
   8 human cell lines and 62 surgical/autopsy specimens were exposed to 23°C at defined periods of time prior to specimen collection and procurement. This model is referred to herein as experimental Cold Ischemic Time (CIT)-dependent tissue degradation model. Proteins of each group were subjected to Western analysis for observation of the association between spectrin breakdown and the overall stage of tissue degradation.

2. **Mechanism study on SPTAN1 breakdown:**
   Protease inhibitory assay was performed to identify the protease responsible for the spectrin breakdown during degradation. Antibody specific to caspase-mediated SPTAN1 cleavage was used in Western analysis to further specify the protease that is responsible for degradation-induced spectrin breakdown.

3. **Development of an "In-tube" calpain-mediated spectrin cleavage reaction:**
   A 80 kDa spectrin fragment that contained calpain cleavage site and GST tag was expressed in E. coli and purified. To mimic the dynamic process of endogenous spectrin cleavage observed in the degradation process of native specimens, purified spectrins were incubated with calpain to develop an "in-tube" enzymatic cleavage assay.

4. **Development of an "*in-situ*" SPTAN1 cleavage model by injecting purified spectrin into tissues:**
   The 80 kDa spectrin peptides were injected into tissue specimens (cell pellets) followed by CIT exposure. The cleavage status of extrinsic spectrins was examined by Western blotting. Correlation analysis was performed to assess the association between extrinsic spectrin cleavage (mediated by intrinsic calpain) and respective CIT impact.

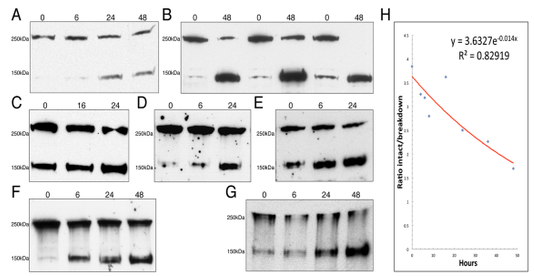
5. **Application of Fluorescence Resonance Energy Transfer (FRET)-based spectrin peptide to "in-tube" and "*in-situ*" models for transforming spectrin TDI discovery to tissue degradation assessment tool:**
Degradation-dependent and calpain-mediated cleavage occurs between Tryosine1176 and Glycine1177 on spectrin. Based on this cleavage site, a 11-amino acid spectrin peptide was synthesized with addition of fluorophore QXL-520 and 5-FAM as a "quencher": N'-QXL520-QQEVY*GMMPRD-5FAM-C'; control peptides include: N'-QXL520-QQEVY*GMMPRD-C' and N'-QXL680-QQEVY*GMMPRD-C'. These FRET-peptides were subjected to aforementioned "in-tube" and "*in-situ*" models and further analyzed with fluorescence detectors.



Acknowledgement: Support from Dr. Morrow's laboratory in spectrin studies.

RESULTS

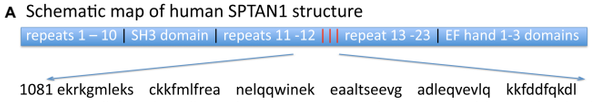
1. SPTAN1 breakdown correlates with tissue degradation status (CIT exposure).



A~G: Western blots detect SPTAN1 breakdowns (CIT hours are labeled on top of each lane). **A:** in a non-tumorous human kidney specimen**; B:** in 3 human meningioma specimens; **C:** in a human fibroid specimen; **D-G**: mouse uterus, intestine, lung, and bladder tissues; **H**: an exponential trend line indicates the overall association between the spectrin intact/breakdown ratio and the CIT impact.

2. Mechanism of degradation-induced spectrin breakdown

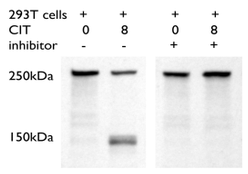
A Schematic map of human SPTAN1 structure



1081 ekrkgmleks ckkfmlfrea nelqqwinek eaaltseevg adleqvevlq kkfddfqkdl
1141 kanesrlkdi nkvaedlese glmaeevqav **q** QQEVY*GMMPRD etdsktas pwksarlmvh
1201 tvatfnsike lnerwrslqq laeersqllg sahevqrfhr dadetkewie eknqalntdn

Full length: 2474 AA ~ 285 KDa
Calpain cleavage sequence: QQEVY*GMMPRD between 1176-1177 AA
N' breakdown product: 1176 AA ~ 150 KDa; C' breakdown product: 1279 AA ~ 145 KDa

B Calpain-inhibitor assay

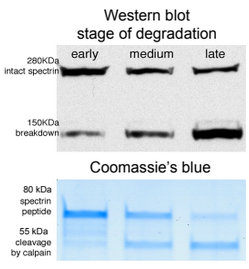


Western analysis detects SPTAN1: by applying a calpain inhibitor to 293T cell degradation model, we have successfully blocked degradation-induced SPTAN1 breakdown.

These data suggest that the degradation-induced SPTAN1 breakdown is calpain-mediated.

We could not detect caspase-dependent SPTAN1 breakdown on Western blot using a caspase cleavage-specific SPTAN1 antibody – this excludes the possibility that caspase is responsible for the degradation-induced SPTAN1 breakdown.

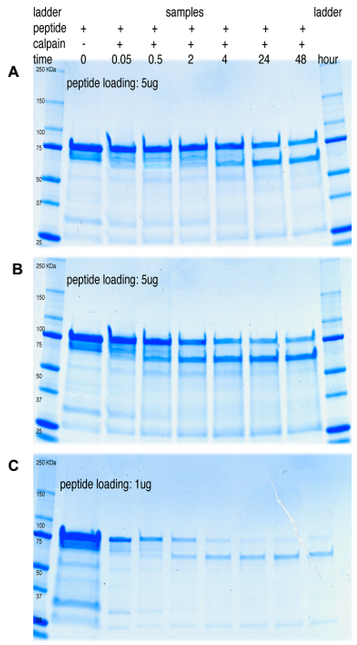
C Validation of degradation-induced calpain-mediated SPTAN1 cleavage in test-tube



(**upper panel**) Western blot detects dynamic SPTAN1 cleavage in a cancer specimen during degradation process. The intact SPTAN1 is 280 kDa; the breakdown product is 150 kDa.

(**lower panel**) Coomassie's blue staining demonstrates the success of using an "in-tube" enzymatic reaction to imitate the degradation-induced spectrin cleavage that occurs in native specimens. The purified spectrin fragment is 80 kDa; its breakdown product is 55 kDa.

3. "in-tube" SPTAN1 cleavage is CIT-dependent.



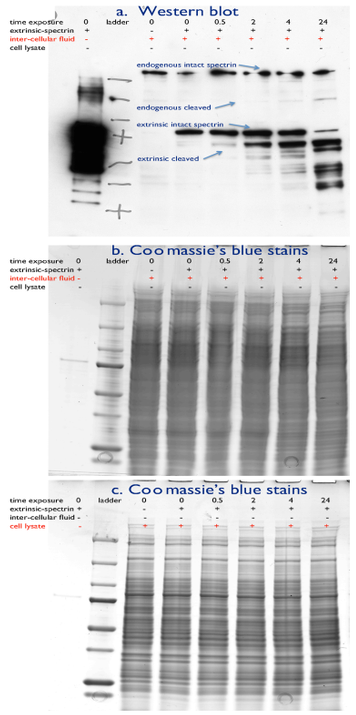
"In-tube" SPTAN1 cleavage reveals the impact of ambient temperature and the time length of exposure experience.

A: "In-tube" SPTAN1 cleavage at 4°C (Coomassie's blue staining). The time consumed for 50% SPTAN1 cleavage is about 24 hours.

B: "In-tube" SPTAN1 cleavage at 23°C (Coomassie's blue staining). The time consumed for 50% SPTAN1 cleavage is about 2 hours.

C: For obtaining the best sensitivity of "in-tube" reaction, systemic optimization was conducted (Coomassie's blue staining). Much clearer conversion between intact SPTAN1 and its breakdown is seen by adjusting the components in the reaction.

4. "*in-situ*" SPTAN1 cleavage is CIT-dependent.



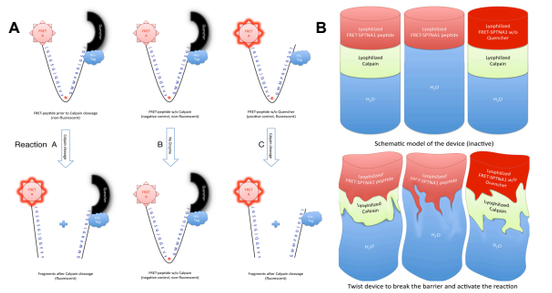
Purified 80 kDa spectrins were added to PBS to suspend cells during harvest. Cell pellets were collected via centrifuging and subjected to CIT exposure. Before sample collections, PBS was added to suspend the pellets and intercellular fluid (supernatant) was obtained after centrifuging.

a: Western analysis using intercellular fluid detects the dynamic conversion between the 80 kDa purified SPTAN1 and its 55 kDa cleavage product. To some extent, the endogenous (intracellular) 250 kDa SPTAN1 and its 150 kDa cleavage can also be revealed due to degradation-induced cell leakage. Data shows that the cleavage of extrinsic SPTAN1 is associated with the CIT impact.

b: Coomassie's blue staining demonstrates the overall protein quality of intercellular fluid;

c. Coomassie's blue staining demonstrates the overall protein quality of the cell pellets.

5. FRET-based "in-tube" SPTAN1 cleavage model

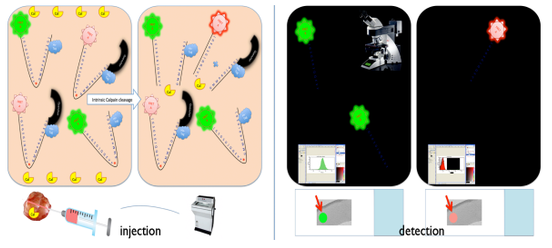


FRET is a mechanism describing energy transfer between two chromophores. A donor fluorophore (QXL-520 in our case) in its electronic excited state transfers energy to an acceptor/quencher fluorophore (5-FAM) through nonradiative dipole–dipole coupling if the latter located within a very short distance (10nm). Combined fluorescence emission will be distinctive from either the donor or the acceptor/quencher.

A: FRET-based "in-tube" SPTAN1 cleavage reaction with two controls: we have proved "in-tube" SPTAN1 cleavage can reveal the degradation stage of tissue. FRET technology is introduced to facilitate the observation of cleavage – when cleavage occurs, FAM-fragment will depart from QXL-fragment and FRET-effiect will thereby disappear and original QXL-fluorescence will be emitted (left). Negative control will persistently emit FAM-fluorescence (middle) and positive control will always emit QXL-fluorescence, independent to cleavage (right). *Percentage of cleavage = (experimental – negative)/positive.*

B: (Schematic illustration) Transform "in-tube" FRET cleavage to a "bend (for initiation of the reaction)-N-glow (for detection of the fluorescence)" ambient reporter: we propose to physically adhere this enzymatic system onto surgical specimens immediately after resection – by examine the fluorescence emitted, ambient impact on specimen degradation can be continuously monitored.

5. FRET-based "*in-situ*" SPTAN1 cleavage model



In parallel with in-tube cleavage that monitors ambient circumstance of specimen exposure, we propose an *in-situ* degradation reporter: FRET-spectrin and its positive control (N'-QXL680-QQEVY*GMMPRD-C') will be mixed and inject into surgical specimens *in situ* at resection. Two types of fluorescence can be revealed during degradation assessment: QXL-520 signal (representing FRET-spectrin being cleaved) and QXL-680 (representing total spectrin injected, independent to cleavage.
Percentage of cleavage = QXL-520/QXL680

CONCLUSIONS

This project builds upon our discovery of SPTNA1 as a Tissue Degradation Indicator (TDI). We intended to accelerate the transformation of this discovery toward a convenient final product by introducing FRET technology into SPTNA1 breakdown examination. The completion of this work will launch the first tool in the field for standardized degradation measurement.