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A 10-minute prototype assay for tissue degradation monitoring in clinical specimens

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ABSTRACT

We recently identified alpha II spectrin as a Tissue Degradation Indicator (TDI) and demonstrated that intrinsic spectrin-breakdown levels reliably reveal tissue degradation status in biospecimens. With the present study, we introduce an *in vitro* biological assay to mimic the endogenous spectrin-breakdown process and serve as degradation monitor (DM). By initiating the DM at the time of specimen collection and by attaching the DM to respective specimens, specimen degradation can be assessed by DM readout without specimen consumption.
Using a protease inhibitory assay and protease-targeted immunoassays, we identified calpain as the protease responsible for degradation-induced spectrin breakdown. To recapitulate spectrin degradation *in vitro*, we developed several enzymatic assays in test tubes by incubating recombinant spectrins and synthetic Fluorescence Resonance Energy Transfer (FRET)-based spectrin peptides with purified human and porcine calpains. The *in vitro* assays reliably performed in different environments for a limited time due to loss of calpain activity. To maintain longer calpain activity, we introduced cultured cells as calpain providers into the *in vitro* assays. Under a variety of degradative conditions, including 4 °C, 13 °C, 23 °C, 29 °C, 37 °C, freezing, and freeze-thaw steps, we compared the use of this prototype DM to the intrinsic spectrin cleavage assay (ISCA) in specimen degradation assessment using animal models. A strong correlation ($r = 0.9895$) was detected between the DM-revealed degradation and the ISCA-revealed degradation. Notably, the DM-based degradation assessment takes only 10 min and does not jeopardize the tissue itself, whereas the ISCA-based degradation assessment needs to sacrifice tissues and takes several hours to accomplish.
Our data suggests the application of an *in vitro* degradation monitor for fast, real time, and non-invasive assessment of specimen degradation. This observation could lead to a transformative product dedicated to biospecimen quality control. This study also addresses critical, yet unmet needs for developing a universal standard for specimen degradation measurement.

1. Introduction

Recent advances in molecular approaches have elevated clinical specimens to crucially important resources to characterize disease-associated molecular events (Hindorff et al., 2009). One area of great interest has been the use of clinical specimens in population genetic studies called GWAS (genome-wide association studies). Now over 1600 GWAS on human diseases have been published in the short "post genomic era" (Hindorff et al., 2013), including ours (Clark et al., 2013). However, numerous pre-analytical variables (PAVs) adversely affect specimens during sample handling and initiate variable molecular degradations in specimens. These molecular degradations may heavily impact laboratory results and hence mislead patient care (Albanell et al., 2009; Bartlett et al., 2012; Cappuzzo et al., 2004; Chivukula et al., 2008; Cross et al., 1990; De Cecco et al., 2009; Hammond et al., 2012; Moatamed et al., 2012; Pinhel et al., 2010; Sauter et al., 2009; Selvarajan et al., 2003; Striebel et al., 2008; Werner et al., 2000; Yamashita et al., 2005).

PAVs differ from specimen to specimen — there is no single PAV recorder universally suitable for all specimens. On the other hand, PAV-induced molecular degradation is a biological response and multiple PAVs may work together in a synergistic way to affect molecular integrity in tissue — therefore, mathematical aggregation of individual PAVs cannot reveal the true biological degradation stage in specimens. For instance, a specimen that underwent 4 °C exposure first and 23 °C exposure later, and a specimen exposed to the two temperatures in a reversed order, may exhibit different stages of molecular degradation. In reality, due to lack of measurement tools, clinical specimens are

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investigated without consideration of tissue degradation (Anagnostou et al., 2010; Bai et al., 2011; Tolles et al., 2011).

Using proteomic tools and a cold ischemic time (CIT)-dependent specimen degradation model, we have recently identified 27 intrinsic tissue degradation indicators (TDIs) (Li et al., 2013), which for the first time allow for a "biological" measurement of specimen degradation. Furthermore, we introduced the quantitative ratio between the intact forms and the respective breakdown form(s) of TDIs for particularly precise and sensitive degradation measurement. Alpha-II spectrin is especially attractive for degradation measurement because of the following features: (1) it is a housekeeping protein expressed in most mammalian cells, predicting broad application as a universal TDI; (2) the dynamic conversion between its intact form and its breakdown forms can be easily visualized by a single Western blot analysis; (3) the conversion lasts for at least 48 h in specimens at room temperature — this feature offers the assay a wide effective range to qualify the needs of analyzing multivariant clinical specimens; (4) our group in Yale Pathology is expert in spectrin biology (Brown et al., 1999; Fox et al., 1987; Glantz et al., 2007; Harris et al., 1988; Huh et al., 2001; Nedrelow et al., 2003; Simonovic et al., 2006; Stabach et al., 1997; Wang et al., 1998) and many in-house reagents and protocols have been developed in the laboratory over decades; and (5) the degradation-induced spectrin breakdown pattern resembles known proteolytic breakdown patterns conducted by calpains and caspases (Nakajima et al., 2011; Wang et al., 1998; Zhang et al., 2009) — this feature affords us the potential for future discovery using this TDI, including the present work.

We intended to translate our research findings into a user-friendly tool for degradation assessment in clinical environments. Since the Western-based intrinsic spectrin cleavage assay (ISCA) is often considered a tedious approach in clinical labs, other TDI assessment tools, such as ELISA- and IHC-based spectrin breakdown evaluations are currently being investigated. In addition to these assays that rely on intrinsic spectrin observation, the present work describes our exploration of an *in vitro* assay, in which synthetic spectrin peptides are cleaved into breakdown products following a degradative exposure-dependent manner and measured by quantitative fluorescence. We propose to attach such *in vitro* assays to fresh biospecimens to function as degradation monitors (DM) of these specimens. With this effort, we attempt to develop an objective standard, independent of endogenous TDIs, for tissue degradation measurement and ultimately deliver a prototype platform for degradation assessment in the clinic.

2. Materials and methods

2.1. Tissue specimens

Surgical specimens were collected by IRB-approved Yale Pathology Tissue Services (Yale Human Investigation Committee approved protocol 0304025173). All specimens underwent pathological review for diagnosis and histology confirmation. Animal organs were collected from adult mice with C57BL/6J genetic background at Yale Animal Resources Center (YARC). All animal care and experimental procedures were approved by the Yale Animal Care and Use Committee (YACUC) and were in accordance with all federal policies and guidelines governing the use of vertebrate animals.

2.2. Microarray analysis and exome-sequencing analysis

Five surgical brain meningioma specimens with variable ambient exposure were selected from Yale Brain Tumor Archive. Specimens had been embedded into O.C.T. compound (VWR International Inc., NJ), frozen, and stored in −80 °C freezers. On H&E-stained sections, areas of interest were identified (more than 80% tumor cells); unwanted regions such as inflammatory areas were excluded. DNA and RNA were prepared using the Allprep DNA/RNA/protein Mini Kit (Qiagen Science, MD). For microarray analysis, Illumina HumanHT12.v4 gene expression microarray chips were used. Merging of the data, background removal and normalization processes were performed using the limma R package. For exome-sequencing, Nimblegen/Roche human solution-capture exome arrays (Roche Nimblegen, Inc.) were used to capture the exomes of tumor samples according to the manufacturer's protocol. Sequencing of the library was performed on Illumina HiSeq instruments using 74 base pairs paired-end reads by multiplexing two tumor samples. Data quality parameters from microarray and exome-sequencing platforms were used to perform correlation studies with spectrin-indicated degradation stages of respective specimens.

2.3. Degradation assessment via evaluation of spectrin breakdown level

Western blot analysis and fluorescent immunostaining were carried out following standard protocols. In brief, protein extracts were prepared *via* cryostat-based tissue collection followed by conventional preparation using RIPA buffer with existence of protease inhibitor cocktail (Roche Applied Science, IN). 4–15% mini-Protean-TGX SDS PAGE gels (Bio-Rad, CA) were used for protein separation. For both Western blotting and fluorescent immunohistochemistry, overnight non-specific epitope blocking was performed at 4 °C, followed by 3-hour primary antibody incubation and 1-hour secondary antibody incubation at room temperature. Antibody information: for recognition of overall spectrins (intact and breakdown forms), mouse monoclonal anti-alpha spectrin (AA6, 1:2000) was used (obtained from EMD Millipore Corporation, MA). Signals in Western blots were developed at the exponential phase and the linear phase of the assay; Western signals were quantified by calculation of their densitometry density and exhibited area. The quantitative ratio between intact spectrin (285 kDa) and breakdown spectrin (150 kDa) was used to define the degradation stage of specimens. To exclude caspases' involvement in degradation-induced spectrin breakdown, a rabbit polyclonal anti-caspase-mediated spectrin breakdown fragment (Asp1185) (1:1000, Cell Signaling Technology, MA) was used to detect caspase-mediated spectrin breakdowns; in addition, an in-house developed, rabbit anti-spectrin breakdown product 1 antibody (BDP1, 1:2000) (Brown et al., 1999; Glantz et al., 2007; Huh et al., 2001; Nedrelow et al., 2003; Stankewich et al., 2010) was used to specifically detect calpain-medicated spectrin breakdowns.

2.4. Calpain inhibitory assay

Experimental specimens were homogenized in calpain reaction buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM NaCl, 30 μM $CaCl_2$, and 1 mM DTT. Tissue lysate was divided into two aliquots. 10-μM (final concentration) Calpain inhibitor III (EMD Millipore Corporation, MA) was added to one of the aliquots. Time zero lysates were then sampled from each aliquot and the remains were incubated under room temperature for an additional 8 h. The spectrin breakdown status was compared among experimental groups *via* Western blot analysis.

2.5. Cloning and recombinant spectrin production

Human alpha-II spectrin gene sequence, coding 882–1332 a.a., was cloned into pGEX 4T1 expressional vector (Pharmacia, MA) which yields a fusion product of N′-GST-spectrin-C′. This fusion protein contains the primary calpain cleavage site at 1176 tyrosine residue (*-marked location): QQQEVY*GMMP. Once cleaved by calpain, the intact 76-kDa fusion protein is split into a 58-kDa fragment and an 18-kDa fragment. Fusion protein was over-expressed in *Escherichia coli* and extracted with B-PER reagent (Thermo Scientific, IL). Protein was further purified with glutathione magnetic beads (Thermo Scientific, IL).