Diabetic Complications Consortium

Application Title: Predictive and Diagnostic Biomarkers for Diabetic Foot Ulcers

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1. Project Accomplishments:

In this project we evaluated two potential predictive and diagnostic tissue biomarkers for diabetic foot ulcers (DFU), c-myc and β-catenin. During this study we have also developed a statistically rigorous study plan and analytical methods for the clinical validation of these proteins as clinical biomarkers with predictive and diagnostic value. Through a streamlined process of evaluation, 'GO/NO GO' decisions and data analyses from 57 DFU samples received from 5 clinical sites, we *identified* ***c-myc*** *as highly significant potential biomarker* strongly meriting prospective validation. Specifically: 1) baseline nuclear presence of c-myc highly correlated with the percent change in wound size at week 4 ($p=0.022$) and the persistence of nuclear presence of c-myc over 4 weeks correlated with non-healing outcome ($p=0.011$) ; 2) conversely, low overall staining for c-myc at baseline predicts 4 week wound size improvement ($p<0.0001$) and likelihood of healing ($p=0.0001$; aROC of 0.79). This study also revealed that β-catenin, although may be a good predictor, does not qualify as a tissue biomarker due to the reproducibility and reliability of the antibody (new optimization needed every time staining was performed). Data generated from this study supported application for the NIDDK Diabetic Foot Consortium (DFC) Biomarker study application. Further analysis of c-myc as predicative and diagnostic biomarker through the consortium will allow for center based effects (from all DFC clinical research units).

2. Specific Aims:

Specific Aim 1. To develop a Core Management Plan that will design and prepare the implementation and oversight of all aspects of large clinical trial for biomarker(s) validation.

Results: As proposed we have streamlined the process of diabetic foot ulcer tissue collection, shipment and processing from multiple clinical sites (University of Miami-UM, NYU Winthrop and South Shore Hospital) involving multiple attending physicians from various specialties. Optimized operational protocol outlined below will be implemented in all aspects of the ongoing DFC biomarker validation. We have also optimized and standardized quality assurance and assessment of the received specimens as well as data acquisition and analyses. Tissue collection for the biomarker quantification was performed from the wound edge specimens collected during routine debridement at the initial visit (W0) and after 4 weeks (W4) of SOC at the UM, NYU Winthrop and South Shore Hospital by multiple attending physicians from various specialties. Weekly wound size measurements were performed as SOC and the extent of wound closure at W4 was used as a surrogate outcome of healing.

Importantly Specimen shipment was optimized for the wider implementation. Initially, specimens were sent as either formalin fixed or snap-frozen (**Figure 1**). Next we assessed and compared formalin fixed to paraffin embedded tissue at the harvest site. Due to associated cost of shipping frozen tissue as well as quality of tissue we decided to proceed with formalin fixed tissue.

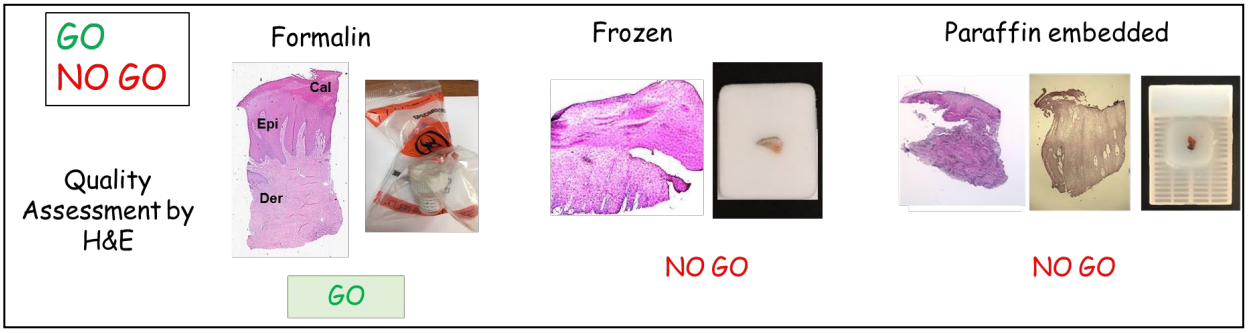

Figure 1. Optimization of the tissue collection and shipment. Shipping of formalin fixed samples without further processing at the collection site was determined as optimal.

It was determined that results were more reproducible when specimens were shipped in formalin to UM, where embedding and immunohistochemistry staining were both completed in the uniform fashion assuring proper tissue orientation, resulting in formalin fixed tissue as a "GO" decision. Histopathology was assessed for presence of epidermis and dermis required for biomarker quantification. When collected specimens failed quality assessment feedback was provided to the tissue harvest sites, which proved very helpful. In addition, a conference call discussing optimization of the sample collection was communicated with each individual physician by Drs Tomic-Canic and Kirsner and implemented modifications resulted in successful tissue collection for more than 80% of samples. In summary the goal of this aim was achieved as we established and successfully tested the operational protocol for maximum efficiency including confirming, optimizing and validating best tissue procurement and shipment methods, for the biomarker analyses and quantification.

Specific Aim 2. To perform a "test run" to implement standardization and logistics of the experimental approach for biomarker quantification.

Results: This study provided collection of the thirty two additional DFU samples confirmed by H&E staining for the quality and pathology assessments, which together with previous studies allowed biomarker analyses on fifty seven tissue samples. Our previous studies analyzed both c-myc and β-catenin as biomarkers associated with non-healing DFU.
However, this study revealed inconsistencies in the antibody quality for detection of β-catenin challenging the reproducibility of staining and resulting in a "NO GO" decision for β-catenin for the purpose of the biomarker. Although β–catenin may be a good predictor, the antibody was found unreliable and new optimization was required every time new lot was purchased **(Figure 2**). Staining protocols for c-myc, image acquisition and automated biomarker quantification have been optimized and implemented during this study. Importantly antibodies

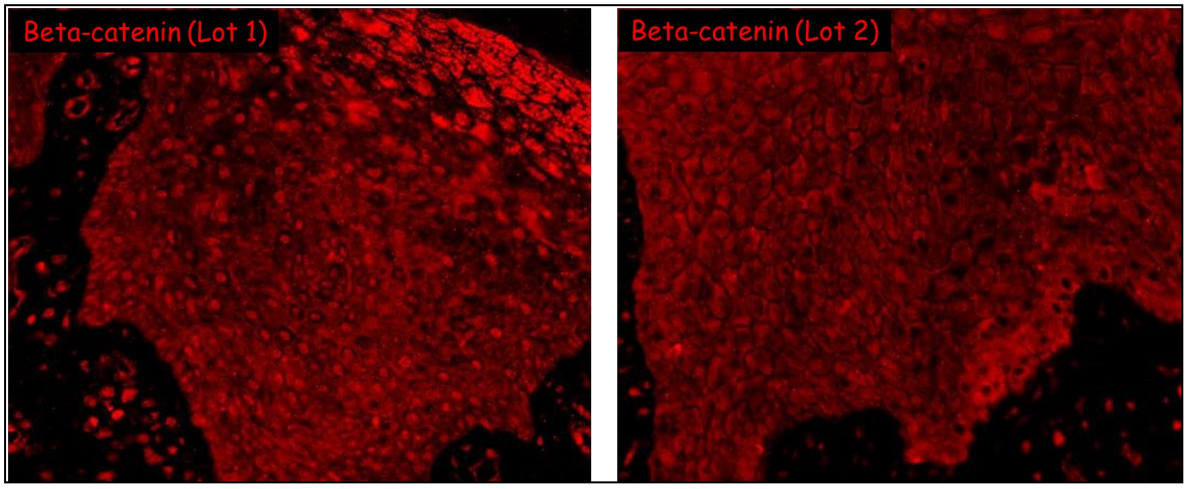

Figure 2.Varibality in the β –catenin antibodies and lack of reproducibility resulted in "NO GO" decisions for biomarker validation.

used for c-myc immunostaining have shown high consistency and reproducibility, despite the use of different lots, resulting in a GO decision. Prior to c-myc quantification each tissue was first assessed by histopathology for presence of epidermis and dermis. Once approved for biomarker testing, sectioned tissue was immunostained using specific anti-human c-myc antibody. For the high quality, reproducible, objective analysis of biomarker quantification and reliable clinical correlation we have optimized the use of newly designed bioimage analysis software QuPath (https://qupath.github.io; (Bankhead P, Sci Rep. 2017). We have developed a script in QuPath open source software that automates the counting and measuring of positively stained nuclei using peroxidase staining **(Figure 3**). First, a polygon object is drawn to represent the region of interest to be analyzed in order to remove cores which are unsuitable for analysis. This is followed by stain separation by color deconvolution and number of positive cells/mm^2 of tissue is automatically counted using a fast peak-finding algorithm which first estimates the full extent of each cell based upon a constrained expansion of the nucleus region, and calculates up to 33 measurements of intensity and morphology, including nucleus area, circularity, staining intensity for hematoxylin and DAB. Importantly, this method of quantification can be easily implemented at different sites participating in DFC.

Upon performing automatic quantifications by two independent sites, NYU Wintrop and UM data were subjected to statistical analyses. To validate c-myc as a biomarker, percent of nuclei positive for c-myc was quantified at the initial visit (W0) and after 4 weeks of standard of care n tissue obtained from 57 patients and correlated with the surrogate outcome of healing. Clinical outcomes data and biomarker quantification data were sent to Dr

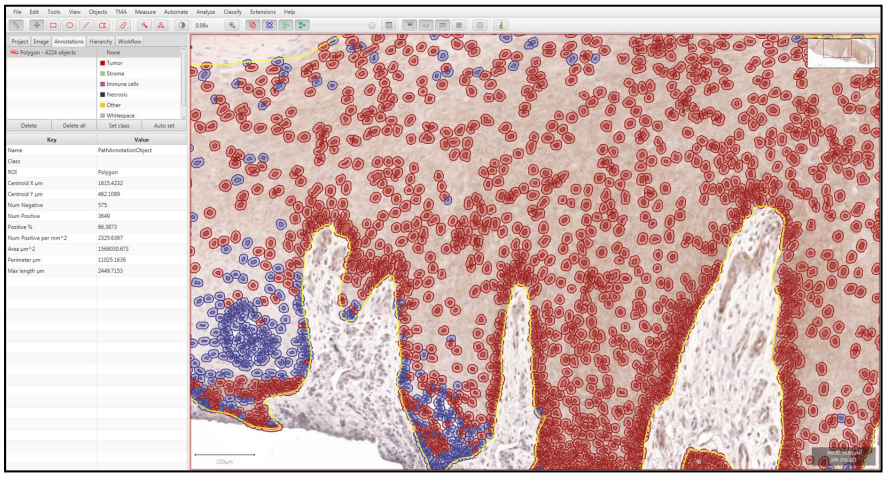

Figure 3. c-myc quantification using open source software QuPath. Red selects c-myc positive nuclei, whereas blue selects c-myc negative nuclei.

Margolis at UPenn to perform statistical analyses. We found that either no change or an increase in c-myc expression over 4 weeks correlates with non-healing outcome (test for trend p=0.011, p=0.03 comparing decreased to no-change or increase) confirming c-myc as a likely biomarker for non-healing DFU. The non-healing outcome was based on a change in wound size, used in previous studies as a surrogate for the likelihood that a wound will heal. Next, we analyzed if c-myc may serve as a predictive biomarker by quantifying its nuclear presence only in W0 specimens. It was highly correlated with the percentage change in wound size at week 4 (r2= 0.30, p=0.022). Furthermore, the percentage of cells staining for c-myc at week 0 was highly associated with wounds that were most likely to heal e.g. a 50% or greater decrease in wound size by week 4) (p=0.001) (e.g. wounds most likely to heal). Our hypothesis was that low % of nuclear c-myc (<39%) at W0 will associate with healing DFUs, whereas high Nuclear c-myc staining (≥ 39%) at W0 will associate with non-healing (**Figure 4**). Our results confirmed the hypothesis. c-myc percentage at week 0 can also correctly differentiate between an individual who is most likely to heal versus one unlikely to heal with high confidence (area under the receiver operating curve (aROC) of 0.79). Dihotomizing c-myc as low staining (<39%) and high staining (≥ 39%) revealed that low staining was also associated with change in wound size at week 0 (p<0.0001) and those most likely to healed (p=0.001). The aROC for c-myc low staining rom the nuclei from our wound edge specimens 0.82.

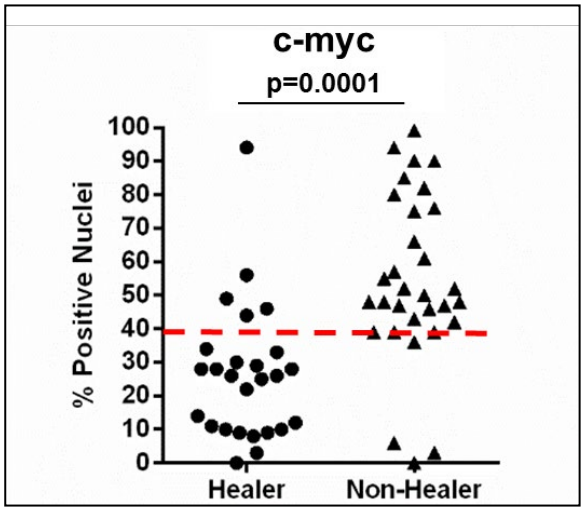

Figure 4. c-muyc as predictive biomarker for DFU healing outcomes.

From this pilot study we conclude that quantification of c-myc provides is an excellent predictor of healing outcomes at the initial visit and that no change or an increase in c-myc over 4 weeks significantly correlates with non-healing outcome, confirming that c-myc is a very strong candidate for predictive and diagnostic biomarker for DFUs. Based on the data generated during this Pilot Study we proposed to validate c-myc as a predicative and diagnostic biomarker in a multicenter clinical trial by the clinical research units of the recently formed NIDDK Diabetic Foot Consortium.

3. Publications:

The manuscript summarizing data generated in this study on c-myc as predictive and diagnostic tissue biomarker for DFU is in preparation. In addition we have published the review with guidelines on DFU tissue collection and validation:

Pastar I, Wong LL, Egger AN, Tomic-Canic M. Descriptive vs mechanistic scientific approach to study wound healing and its inhibition: Is there a value of translational research involving human subjects? Exp Dermatol. 2018 May;27(5):551-562.