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Original Article

Impact of warm ischemia on phosphorylated biomarkers in head and neck squamous cell carcinoma

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Abstract: Objectives: To quantitatively and visually characterize changes in phosphorylated biomarker expression in head and neck squamous cell carcinoma specimens from excision through 90 minutes of warm ischemia. Materials and Methods: Tissue biospecimens were procured prospectively. Head and neck squamous cell carcinoma specimens from 5 patients were subdivided into three parts upon excision, exposed to warm ischemia of 15, 30, or 90 minutes, and routinely biobanked. Relative change in biomarker expression of *p*-Akt, *p*-ERK, and *p*-Stat3 was measured by immunoblot densitometry. Immunofluorescent stains were performed to visually supplement the quantitative analysis. Results: From 15 to 30 minutes of ex vivo ischemia, there was a significant decrease in *p*-Akt ($p = 0.045$) as the mean intensity fell by 44.9%. This decrease in *p*-Akt remained significant at the 90 minute time point ($p = 0.015$). From 15 to 30 minutes of ischemia, there was a trend toward a decline in *p*-ERK, which became significant by 90 minutes of ex vivo warm ischemia ($p = 0.008$). These changes were supported by qualitative differences in *p*-ERK fluorescence at 0 and 90 minutes warm ischemia. Conclusion: Some phosphorylated biomarkers of HNSCC remain highly dynamic during the period of ex vivo warm ischemia after surgical excision but before biobanking. These findings have critical implications for studies that attempt to correlate protein phosphorylation with clinical outcome. We conclude that ex vivo warm ischemia time is a major determinant of tissue quality that may explain inconsistent results from biomarker research in head and neck squamous cell carcinoma.

Keywords: Biobanking, biomarkers, phosphorylated biomarkers, head and neck squamous cell carcinoma, warm ischemia

Introduction

Head and neck cancer is the sixth most common cancer in the world with an incidence rate over 600,000 [1]. With aggressive treatment, five-year survival in the United States is roughly 60%, as patients often present with late-stage tumors [2]. Poor survival is also related to high frequency of local recurrence, second primary tumors, and distant metastases. In head and neck squamous cell carcinoma (HNSCC), there has been limited progress in identifying relationships between particular biomarkers and clinical outcomes. HPV status remains the only clinically established biomarker, indicating better overall survival and cure rates compared to HPV-negative squamous cell carcinomas [3]. In recent decades, HNSCC has made only modest gains in survival compared to other cancer types which now have biomarkers that indicate targeted therapies, such as HER-2/neu and estrogen receptor positivity in breast cancer [2, 4]. The discovery and validation of new biomarkers could lead to the delivery of personalized medicine and better prognostication for HNSCC patients.

As tyrosine kinase inhibitors become the most promising targeted pharmacological treatments for cancers such as HNSCC, it is now increasingly important to recognize the value of proteomics and activity of relevant oncogenic pathways [5, 6]. Examples include the PI3K/AKT/mTOR and ERK intracellular signalling pathways which commonly harbor mutations in HNSCC and lead to overactivation and tumor

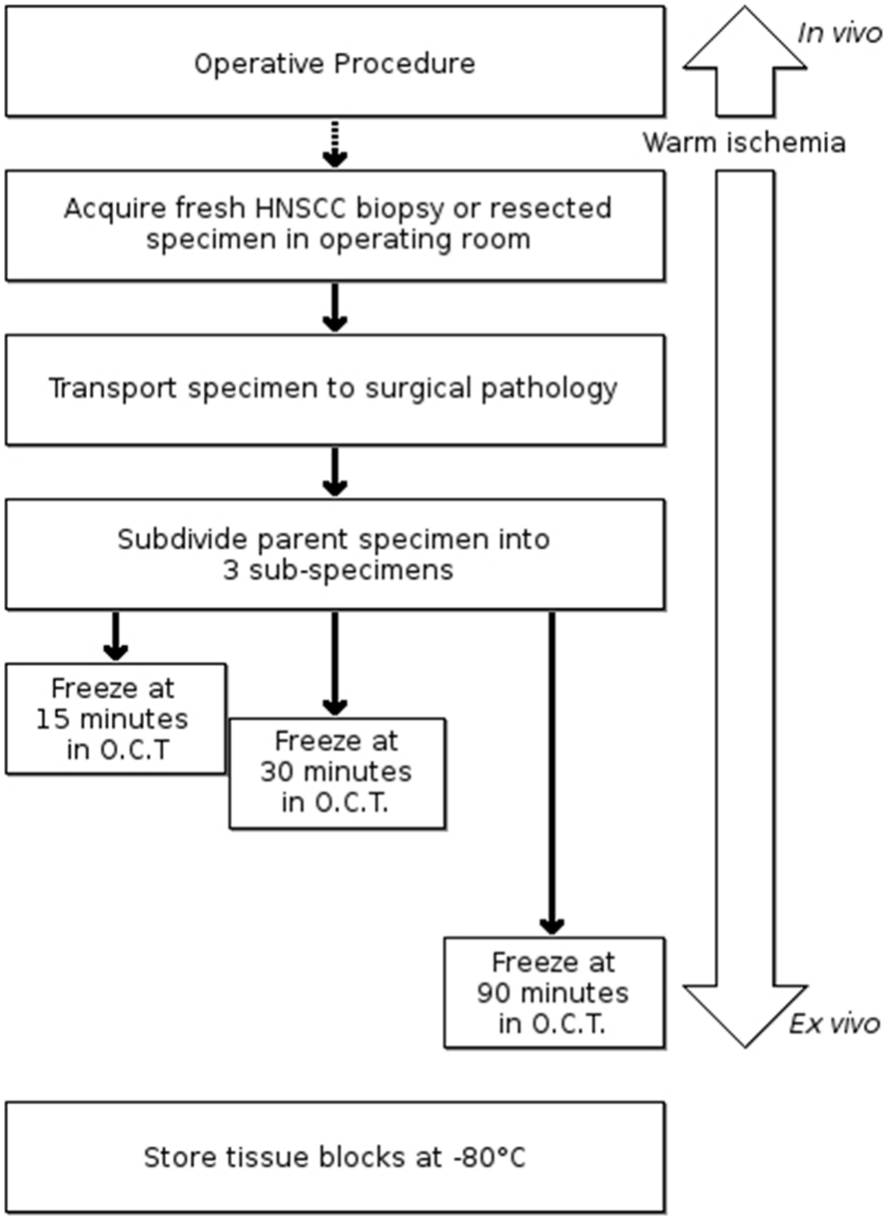

Figure 1. Tissue handling procedure. All tissues analyzed in this study were collected prospectively under the protocol displayed here. Four of the 5 specimens were collected as biopsy, which exposed the samples only to ex vivo warm ischemia, bypassing resection and in vivo ischemia. Warm ischemia, by our definition, ended with O.C.T. freezing after subdivision, and processing in surgical pathology.

growth [7]. Although genomic profiling and transcript profiling have been investigated as informative sources for clinical decision-making and patient classification [8], these modalities do not provide the rich information about the state of signaling pathways within the cell that proteomics can offer [9-11]. To develop targeted therapies, it will be necessary to assess the effectiveness of target inhibition *in vivo*, which will rely on accurate measurement of biomarker phosphorylation status-an indicator of activation [12]. This approach will depend on the use of preserved human tissue; however tissue protein biomarkers will not advance the diagnostic, prognostic and therapeutic aspects of cancer care until biomarker instability is addressed [9].

Tissue procurement and storage is being increasingly recognized as being of central importance to the future of such biomarker breakthroughs, as preanalytical changes can have a dramatic effect on the measurement of tissue biomarkers [13]. Phosphorylated biomarkers are particularly susceptible to *ex vivo* variability, such as prolonged ischemia, that may cause time-dependent hyperphosphorylation or dephosphorylation [14]. The effect of *ex vivo* ischemia has never been studied in squamous cell carcinomas of the head and neck, despite recent interest in phosphorylated proteins as potential biomarkers in HNSCC [15-21].

Importantly, these studies of phosphorylated proteins in HNSCC have yielded inconsistent results. Sometimes expression of the same biomarker will appear to indicate a favorable prog-

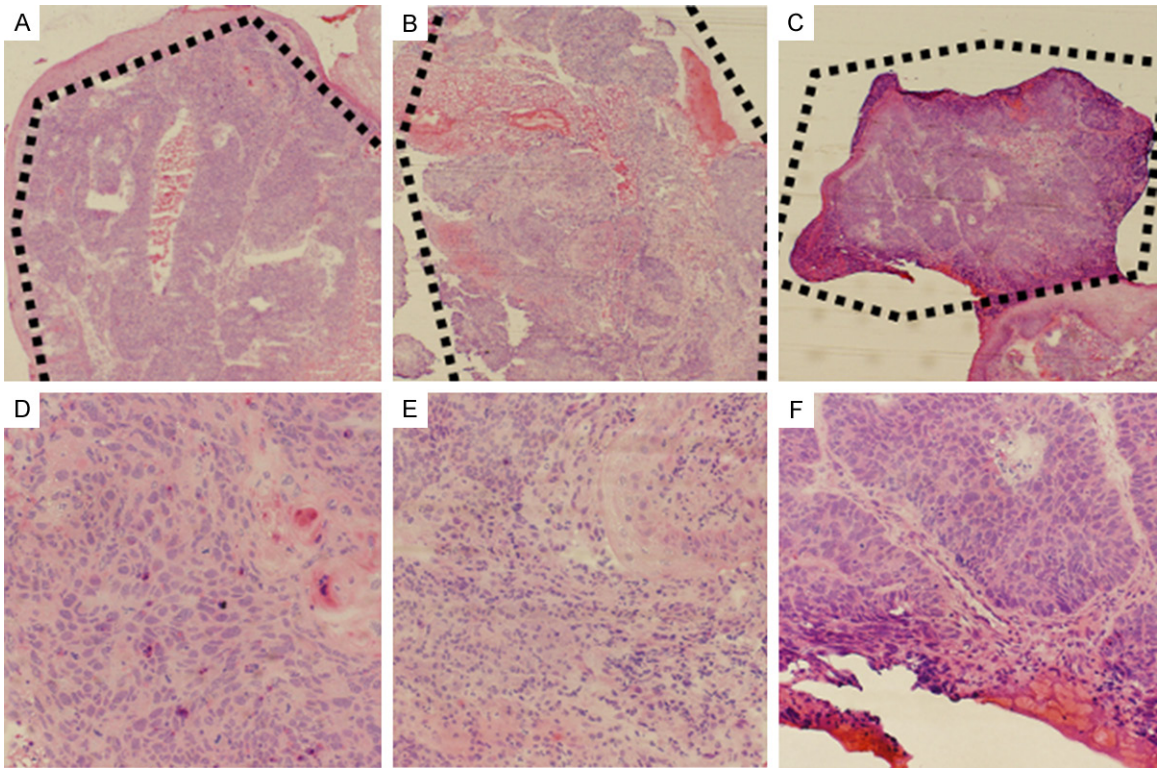

Figure 2. Hematoxylin and eosin staining of Tissue Sample 5. Areas of > 60% squamous cell carcinoma are outlined where appropriate. Tissue for analysis was taken only from these areas of frozen tissue blocks. A-C. 15, 30, and 90 minute ex vivo warm ischemia subdivisions of Tissue Sample 5 under 4x magnification. D-F. 15, 30 and 90 minute ex vivo warm ischemia subdivisions of Tissue Sample 5 under 20x magnification.

nosis [22], and other times unfavorable [23]; most frequently similar studies will fail to find significant relationships where others have done so [24-26] (see Discussion). These studies seldom report *ex vivo* warm ischemia, which our research suggests can dramatically effect biomarker expression and may explain some of these differences in findings. We report here that *p*-Akt and *p*-ERK biomarkers, identified as critical links in the PI3K and MAPK pathways, are sensitive to *ex vivo* warm ischemia in head and neck squamous cell carcinoma.

Materials and methods

Patients and tissue samples

For phospho-protein expression assays, 33 tissue samples from 11 squamous cell carcinomas of the head and neck were collected prospectively at The University of Chicago Medical Center for protein extraction in this study. The patients gave written informed consent, and the study was approved by the University of Chicago Institutional Review Board. Upon excision a stopwatch was started, and specimens were immediately transferred to surgical pathology at room temperature. The parent tissue sample was subdivided into thirds by scalpel, and then each subdivision was incubated at room temperature before being frozen at 15, 30, and 90 minutes, respectively. Each specimen was placed in a cryomold with Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA) immediately prior to freezing and stored at -80°C. Tissue flow is outlined in **Figure 1**. Ex vivo ischemia time is the only variable that was manipulated among specimen subdivisions.

For a separate immunofluorescent staining experiment, an additional "butterfly" biopsy was collected from an exophytic squamous cell carcinoma tumor of the head and neck. This biopsy was hemisected: one half was immediately placed in formalin, the other half was placed in formalin after 90 minutes of ex vivo warm ischemia. After overnight fixation, the two specimens were embedded in paraffin with

their hemisected surfaces accessible for future sectioning. These specimens were processed in parallel, with the only difference being duration of ex vivo warm ischemia.

Protein and RNA extraction, western blot, and RIN analysis

Frozen tissue samples were cut in a cryostat for the preparation of hematoxylin and eosin slides which were then evaluated by an expert pathologist for areas containing > 60% squamous cell carcinoma cells. The areas of > 60% carcinoma cells identified on hematoxylin and eosin slides were used to provide a guide of areas eligible for tissue protein extraction (**Figure 2**) using AllPrep RNA/Protein kit (Qiagen, Valencia, CA). Eligible areas were cut to a shallow depth from O.C.T. blocks using a scalpel. After homogenization on ice by motorized pestle (Argos Technologies, Elgin, IL, USA), all steps of extraction were performed according to the manufacturer's instructions. Protein concentrations were measured as part of a BCA test protocol (Thermo Fisher Scientific, Rockford, IL) in a microplate reader (BioTek Instruments, Winooski, VT). Protein samples of 30 µg were submitted for SDS-PAGE in 10% acrylamide gel. Following electrotransfer to nitrocellulose membrane, the following antibodies were used in Western blot analysis for phospho-proteins (all Cell Signaling Technology, Beverly, MA, USA): a rabbit antiphosphorylated Akt antibody (Thr 308) (1:1000), a rabbit antiphosphorylated p44/42 MAPK (ERK1/2) (Thr 202/Tyr 204) antibody (1:2000), and a rabbit antiphosphorylated Stat3 (Tyr 705) antibody (1:2000). After applying antibodies for phospho-proteins, total protein levels were assayed with the following antibodies: a mouse anti Akt antibody (1:1000), a mouse anti ERK1/2 antibody (1:1000), and a mouse anti Stat3 antibody (1:1000). A mouse anti α-actinin was applied as a loading control. Secondary fluorescent antibodies for anti rabbit and anti mouse used 1:20,000 dilutions. Quantification was achieved by densitometry using a quantitative fluorescent imaging system (LI-COR Biosciences, Lincoln, NE).

A NanoDrop ND-1000 (Thermo Fisher Scientific, Rockford, IL) was used to measure RNA concentration. For samples with suitable concentration, the integrity of RNA isolated from tissue samples was assessed with an Agilent 2100 Bioanalyzer by RIN score (Agilent Technologies, Santa Clara, CA).

Statistical analysis

Statistical analysis was performed on the densitometry data collected via image analysis. Each band quantification was normalized to its respective α-actinin loading control. The change in phosphorylation at 30 minutes and 90 minutes was recorded as a percent change from baseline at 15 minutes. A paired student's T-test was performed, to compare protein phosphorylation in corresponding tissue samples at 15 and 30 minutes *ex vivo* warm ischemia, and 15 and 90 minutes *ex vivo* warm ischemia. To assess change in RNA quality, the same statistical method was used to compare RIN scores along the time points.

Immunofluorescence

Sections of the "butterfly" FFPE biopsy samples were cut 5 µm thick. These sections were then deparaffinized and rehydrated in successive solutions of citrisolv, 100% ethanol, 95% ethanol, 70% ethanol, and distilled water. After heat induced epitope retrieval, sections were blocked in 10% goat serum. A primary antibody solution of the following was applied overnight at 4°C: a mouse IgG1 anti-pan-cytokeratin antibody (clone AE1/AE3; dilution 1:200; Serotec), and a rabbit IgG anti-phosphorylated p44/42 MAPK (ERK1/2) (polyclonal; dilution 1:200; Cell Signaling, Beverley, MA, USA) in antibody diluent (Dako, Carpinteria, CA, USA). A secondary antibody solution of the following was applied for 45 minutes at room temperature: a goat anti-mouse IgG1 Alexa Fluor 555 antibody (dilution 1:200; Invitrogen) and a goat anti-rabbit IgG Alexa Fluor 488 antibody (dilution 1:200; Invitrogen). DAPI and antifade mounting medium were added for mounting. Stained sections were viewed with the Leica SP2 confocal microscope under the same conditions, at the same time. All steps of processing took place in parallel with no preferential treatment given to samples from either condition.

Results

Tissue collection

Tumor tissue from 11 patients was collected, and 33 subdivisions were created in total. Of

Table 1. Clinicopathological Characteristics of Specimens Undergoing Immunoblot Analysis

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Age	55	49	64	50	46
Sex	Male	Male	Male	Male	Female
Pathologic Diagnosis	SCC	SCC	SCC	SCC	SCC
Site	Larynx	Base of tongue	Larynx	Tonsil	Tonsil
Stage	T4N0Mx	T2N0Mx	T2N1Mx	T2N0Mx	T4N2bMx
Procedure	Staging panendoscopy and biopsy	Radical resection of recurrent cancer, with left parapharyngeal tumor excision	Staging panendoscopy and biopsy	Staging panendoscopy and biopsy	Staging panendoscopy and biopsy
Intra-operative warm ischemia?	No	Yes	No	No	No
Research Tissue Source	Biopsy	Resected specimen	Biopsy	Biopsy	Biopsy
Therapeutic History	Pre-chemo, pre-radiation	Post-chemo, post-radiation	Pre-chemo, pre-radiation	Pre-chemo, pre-radiation	Pre-chemo, pre-radiation
HPV Status	Positive	Positive	Positive	Positive	Positive
Specimen Temperature	RT	RT	RT	RT	RT

*SCC-squamous cell carcinoma, RT-room temperature.

the 11 parent tumors, only 7 provided tissue blocks with areas of adequate squamous cell carcinoma tumor content (> 60%). It is possible that this was due to positioning of specimens in the cryomolds that favored stroma visibility on hematoxylin and eosin staining. Of the 7 parent tumors, one had just a single subdivision eligible for analysis which excluded it from time-dependent comparative analysis. Another, upon tissue extraction had protein concentrations too low for SDS-PAGE. Therefore, 5 heterogeneous parent tumors of squamous cell carcinoma underwent full analysis, each with 3 time points (**Table 1**).

Time-dependent changes in p-Akt, p-ERK, and p-Stat3 expression

All tissue samples utilized in this study had positive expression of *p*-Akt, *p*-ERK, and *p*-Stat3 at the 15 minute baseline *ex vivo* warm ischemia time point. Overall, *p*-Akt expression fell rapidly in 4/5 tumors during warm ischemia, and by 90 minutes all tumors had a declined *p*-Akt expression relative to baseline. *P*-Akt was the most sensitive biomarker to ischemia as its mean expression among the HNSCC tumors fell by 44.9% between the 15 and 30 minute time points ($p = 0.045$, **Figure 3**). There was no further decline in mean expression of *p*-Akt by the 90 minute *ex vivo* warm ischemia time point, however it remained significantly less than the 15 minute baseline at 57.0% ($p = 0.015$). At the 90 minute time point, *p*-Akt expression levels in the tumors ranged from 23.4% to 80.0% of their original values on densitometry.

Phosphorylated ERK expression was also found to be sensitive to *ex vivo* warm ischemia. By 30 minutes of ischemia, the level of *p*-ERK remained at 63.6% compared to 15 minute baseline; however, this drop only trended toward significant ($p = 0.081$, **Figure 4**). By 90 minutes of warm ischemia, the level of *p*-ERK expression fell to 48.2% of its 15 minute baseline value ($p = 0.008$). Phosphorylated ERK followed the same trend as p-Akt during warm ischemia, with no tumor retaining its baseline level of biomarker expression as the duration of time spent waiting for freezing was extended. At the 90 minute time point, *p*-ERK expression levels in the tumors ranged from 21.9% to 75.4% of their original values on densitometry. Unlike *p*-ERK and *p*-Akt, there was no significant change in *p*-Stat3 expression over warm ischemia (data not shown).

Time-dependent changes in Akt, ERK, and Stat3 expression

On average, the expression of ERK declined significantly by 26.7% ($p = 0.044$) from 15 minutes to 30 minutes of warm ischemia, but this change was not maintained at the 90 minute time point where the drop was calculated to be only 12.3% ($p = 0.23$, N.S.). The declines in total Akt and Stat3 expression over the period of *ex vivo* warm ischemia were not statistically significant.

Immunofluorescence of p-ERK1/2 in HNSCC sections

In order to visually assess phosphorylated biomarker stability over time, neighboring 5 μm thick sections from within the same tumor were stained for *p*-ERK. The sections differed in preparation only by *ex vivo* warm ischemia duration. The qualitative difference in *p*-ERK staining can be appreciated throughout the entire sections, and representative fields demonstrate a robust *p*-ERK stain from the 0 minute *ex vivo* warm ischemia specimen compared to minimal staining of the 90 minute specimen at both the edge and center of the tumor (**Figure 5**).

Time-dependent changes in RNA quality were not observed

RIN values were measured in all 12 subdivisions of 4/5 parent tumors. Statistical analysis found no significant difference in mean RIN values between any of the time points (data not shown). The RINs of the nucleic acid extracts ranged from 5.20-10, with a mean of 9.00. Ex vivo warm ischemia times did not appear to adversely affect RNA quality.

Discussion

Human biospecimens are subject to variable factors related to procurement, processing, and storage that can alter the molecular profile of a sample, and as a consequence the experimental outcomes of investigations that depend on them [27]. In this study we have shown quantitatively with five samples of human head and neck squamous carcinoma that for two

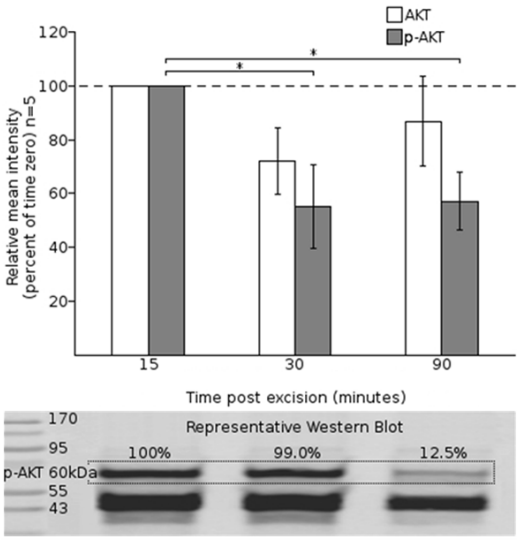

Figure 3. Time dependent changes in *p*-Akt. Mean changes in expression of Akt and *p*-Akt over the 90 minute time course. Mean intensities at 30 and 90 minutes are normalized relative to the 15 minute baseline. *indicates statistical significance when compared by Student's paired t-test ($p < 0.5$).

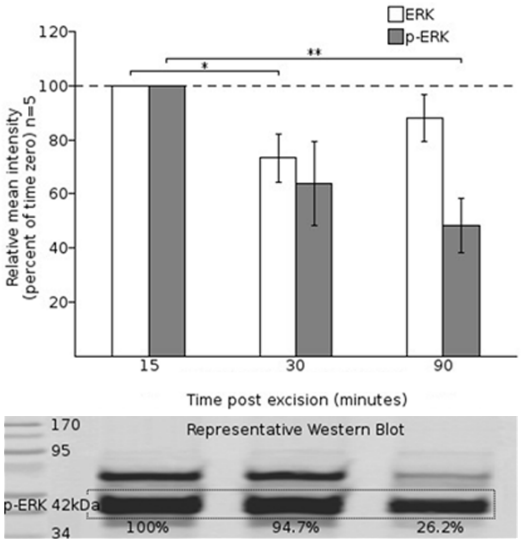

Figure 4. Time dependent changes in *p*-ERK. Mean changes in expression of ERK and *p*-ERK over the 90 minute time course. Mean intensities at 30 and 90 minutes are normalized relative to the 15 minute baseline. *indicates statistical significance when compared by Student's paired t-test ($p < 0.5$) (**indicates $p < 0.1$).

potential clinical biomarkers, Akt and ERK, phosphoprotein expression is unstable immediately after excision and before biobanking. Additionally, the immunofluorescence experiment demonstrates visually how a *p*-ERK positive tumor sample could be mistakenly classified as *p*-ERK negative after undergoing an extended period of *ex vivo* warm ischemia before fixation.

Although we were unable to control for exact percent-tumor content of individual samples, there was a consistent pattern of significantly reduced expression with prolonged *ex vivo* ischemia time across all tumors in *p*-Akt and *p*-ERK, but not total Akt or ERK. Although this study depends on a low number of parent tumor specimens, they are all unmistakably prone to the effects of warm ischemia on biomarker stability. The magnitudes of the changes are large enough to raise the concern that previous and future studies of phosphorylated biomarkers, which depend on pathologic tissue specimens, may be compromised by pre-analytical variability. Studies of *p*-Akt and *p*-ERK warrant immediate scrutiny, and further reserch is needed to quantify time-dependent changes in phosphorylation and inform best practices for tissue handling.

Phosphorylated Akt has been investigated as a prognostic biomarker in many HNSCC cohorts, but with equivocal results overall. Akt is a central player in the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and once activated, *p*-Akt mediates survival, proliferation, and growth. Studies of different patient cohorts from different institutions have produced conflicting results when trying to answer the same question: What is the prognostic significance of phosphorylated *p*-Akt in pathologic tissue specimens? One group, working with "immediately snap frozen" specimens, found that high *p*-Akt expression is correlated with *lack of node metastasis* and *better outcome* [22] whereas another, working with specimens of unreported warm ischemic time, found that higher *p*-Akt expression is an indicator for *node metastasis* and *worse prognosis* [23]. Other differences in methodology make it impossible to conclude that *ex vivo* warm ischemia accounts for the difference in results, but in light of the findings of the present study and consensus of biospecimen reporting standards [27], it must be considered a confounding methodological concern.

Studies that use immunohistochemistry (IHC) as a primary technique to investigate phos-

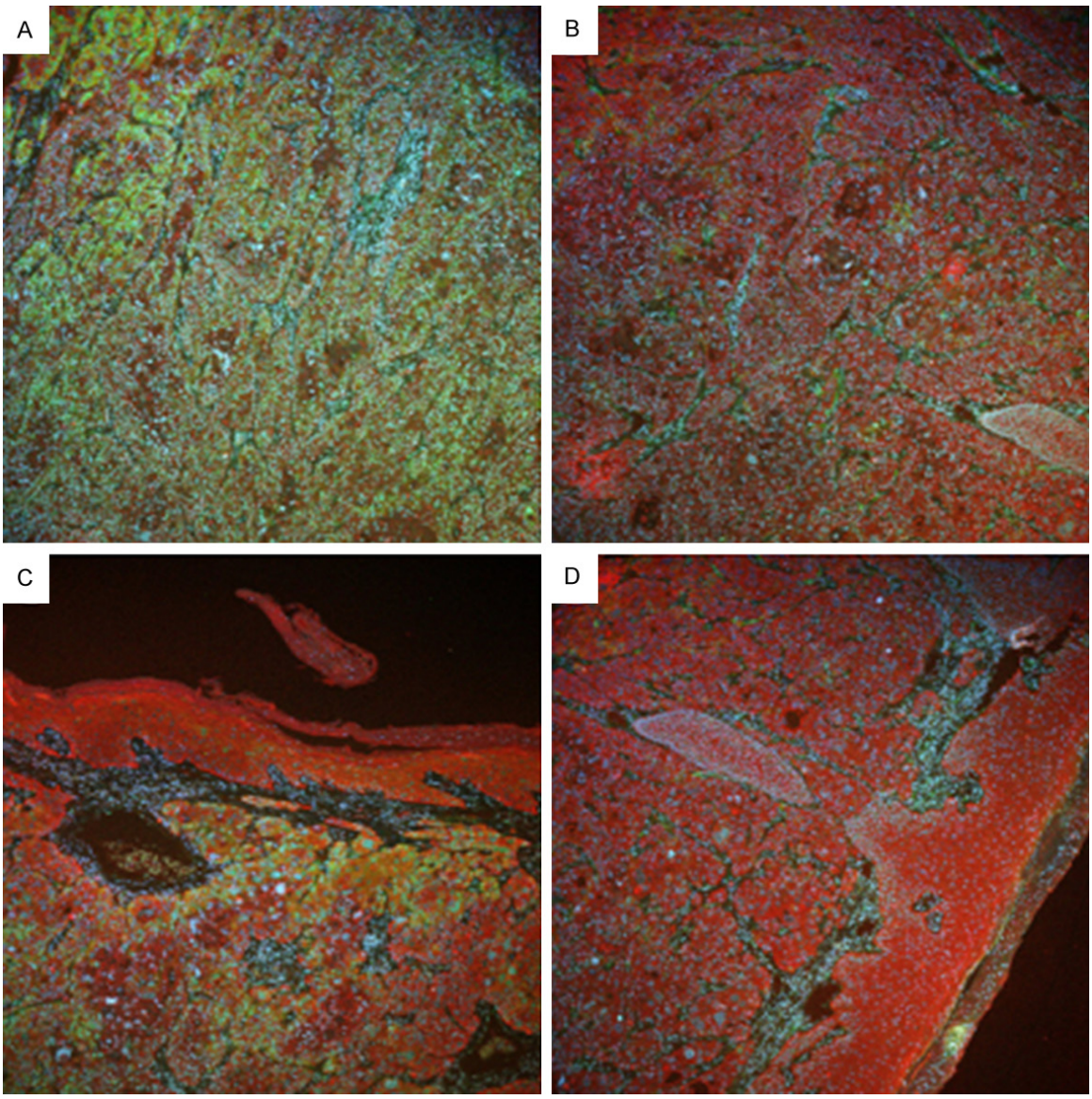

Figure 5. Immunofluorescent assay of *p*-ERK at 0 and 90 minutes ex vivo warm ischemia. Green-*p*-ERK; red-cytokeratin; blue-DAPI. The samples displayed in these panels are from the same parent tumor, and photographed at 10x magnification. The section displayed in panels (A and C) was from tumor fixed in formalin immediately upon resection, whereas the section displayed in panels (B and D) was from tumor fixed after 90 minutes warm ischemia. (A) Central tumor fixed at 0 minutes warm ischemia. (B) Central tumor fixed at 90 minutes warm ischemia. (C) Tumor edge with overlying skin fixed at 0 minutes warm ischemia. (D) Tumor edge with overlying skin fixed at 90 minutes warm ischemia.

phorylated biomarkers may be especially burdened by pre-analytical variability in tissue specimens because of its qualitative nature [12]. Although some additional studies have correlated elevated *p*-Akt with unfavorable prognosis [28-30], others using similar sample sizes have found no correlation between patient outcome and biomarker status [24-26]. Variability in fixation, ischemic times, treatment status, and procurement method of the tissues used within and among these studies likely contributed to irreproducibility. None of the studies cited in this paragraph comment on *ex vivo* warm ischemia. Standardization of biobanking practices may make it possible for optimization of tissue materials for future study purposes [13, 27], and easier reporting for comparability among similarly designed investigations.

By procuring pathological specimens for research purposes, head and neck surgeons

represent the first position in the chain of custody of biospecimens that will eventually be used for downstream investigative applications. Pre-analytical changes in specimens that effect biomarker reliability begin in the operating room and may progress while remaining on the sterile field or specimen cup before being sent for processing. In addition to *ex vivo* ischemia, *in vivo* ischemia may also affect phosphorylation status, particularly if resected specimens rather than biopsies are used for tissue analysis. This is the topic of ongoing investigation. In our observations, coordination is required between pathologists and the operative team to ensure timely fixation of any specimen designated for research. Although inconvenient, for future progress in development of phosphorylated proteins as clinical biomarkers it will likely be necessary to routinely process or stabilize excised research specimens within minutes of extirpation [24, 25].

In conclusion, potential clinical biomarkers of the head and neck cancer phosphoproteome including *p*-Akt and *p*-ERK are sensitive to *ex vivo* warm ischemia. In light of this finding, reporting on recorded or estimated *ex vivo* warm ischemia as well as the nature of specimen acquisition (biopsy versus resected specimen) should be done whenever possible in future research publications. Additionally, surgeons who resect specimens that will be preserved for future research should consider ensuring the fixation or freezing of specimens as quickly as possible to minimize detrimental effects of ischemia on biomarker stability. We conclude that *ex vivo* ischemia time is a major determinant of tissue quality that appears to be underappreciated and may explain inconsistent results from biomarker research in head and neck squamous cell carcinoma.

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Disclosure of conflict of interest

None.

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