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Immunohistochemical Detection of Phosphoproteins and Cancer Pathways

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Abstract

The immunohistochemical detection and quantification of phosphoprotein levels can provide valuable information regarding the activation of cell signaling pathways within tumor samples, with potential implications for prognosis and treatment selection. However, phosphoprotein levels may be affected by clinical tissue handling, hypoxia and other cellular stressors prior to tissue fixation. The preservation of phosphoproteins may also heavily ...

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Abstract The immunohistochemical detection and quantification of phosphoprotein levels can provide valuable information regarding the activation of cell signalling pathways within tumor samples, with potential implications for prognosis and treatment selection. However, phosphoprotein levels may be affected by clinical tissue handling, hypoxia and other cellular stressors prior to tissue fixation. The preservation of phosphoproteins may also heavily depend on the method of fixation. Overall, there is a need to optimise and standardise these variables to help ensure that the phosphoprotein levels detected through immunohistochemistry are in fact representative of the status of the tumor itself.

Keywords Phosphoproteins • Ischemia time • Tissue fixation • Immunostaining • Western blotting

FREQUENTLY ASKED QUESTIONS

- 8.1. Why evaluate phosphoprotein levels in tumor samples?
- 8.2. To what extent does clinical tissue handling affect phosphoprotein levels?
- 8.3. To what extent does ischemia time affect phosphoprotein levels?
- 8.4. Does ischemia time affect some phosphoproteins more than others?
- 8.5. How does ischemia time affect total protein levels?
- 8.6. Is the preservation of phosphoproteins affected by the size of the tissue sample?
- 8.7. What is the optimum fixation time for the preservation of phosphoproteins?

- 8.8. Is the preservation of phosphoproteins affected by the choice of fixative?
- 8.9. Do studies show a difference in phosphoprotein detection between frozen and formalin-fixed paraffin-embedded tissue samples?
- 8.10. Is the addition of inhibitors recommended to help preserve phosphoproteins?
- 8.11. Is it possible to establish a clinically meaningful quantification of phosphoproteins in immunohistochemically stained tissue?

8.1 Why Evaluate Phosphoprotein Levels in Tumor Samples?

The phosphorylation of cell signalling proteins is a key mechanism in the activation of signalling pathways, including those implicated in cancer growth and progression. Accordingly, the development of different tumor types has been associated with the phosphorylation of a variety of cell signalling proteins, including Akt, extracellular signal-regulated kinases (Erks) and signal transducer and activator of transcription proteins (STATs) [1, 2]. Of particular note, Akt is involved in many growth factor signalling pathways, and evidence suggests that the presence of phosphorylated (p)-Akt in the cell nucleus is associated with the development of acute myeloid leukemia, as well as lung, breast, prostate and thyroid cancers [3].

Consequently, the assessment of levels of phosphorylated forms of cell signalling proteins may have important applications in oncology [1, 4]. Tumor phosphoprotein levels may function as prognostic markers. For example, a phosphorylated form of the repressor of mRNA translation, 4E-BP1, alone or in combination with cytoplasmic p-Akt, has been shown to have an adverse prognostic significance in lung adenocarcinoma [5]. In addition, information regarding the activation state of specific signalling pathways may predict tumor sensitivity to treatments that target those pathways. For example, phosphorylated forms of epidermal growth factor receptor (EGFR) or human EGFR-2 (HER-2)/neu in

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tumor samples have been found to be predictive of progression-free survival in patients with metastatic breast cancer who are subsequently treated with the anti-HER-2/neu monoclonal antibody trastuzumab [6]. Finally, research suggests that phosphoprotein levels in post-treatment tumor samples may be indicative of the efficacy of targeted treatments. For example, the EGFR tyrosine kinase inhibitor erlotinib has been found to reduce tumor p-EGFR and phosphorylated mitogen-activated protein kinase (p-MAPK) levels in patients with non-small cell lung cancer (NSCLC) who also derived clinical benefit from treatment [7].

The development of phosphorylation state-specific antibodies has enabled immunohistochemical detection of phosphoproteins through techniques that include Western blotting and immunohistochemical staining, but false-negative reactions can be an issue [1]. In order to counteract the problem of false-negative reactions, there are several important considerations in maintaining the quality of the tissue specimen and optimising the sensitivity of phosphoprotein detection, which are outlined in the following questions and answers.

8.2 To What Extent Does Clinical Tissue Handling Affect Phosphoprotein Levels?

The clinical handling of a tissue specimen can affect phosphoprotein levels as early as the point of surgical excision. This has been clearly demonstrated by a study of cancerous liver tissue from patients undergoing surgery for colorectal cancer (CRC), in which levels of phosphorylated forms of several proteins involved in Akt and MAPK signalling were lower in samples taken 10–45 min after tissue resection than those obtained by endoscopy at the beginning of surgery. The reduction in phosphoprotein levels may have resulted from stress conditions during surgery, as indicated by a corresponding increase in levels of heat-shock protein 27, which is a known response to cellular stress. Therefore, it is important to be aware that cellular stresses evoked by clinical tissue handling during surgery may affect whether subsequent immunohistochemical analyses of phosphoproteins are truly representative of the actual tumor state.

8.3 To What Extent Does Ischemia Time Affect Phosphoprotein Levels?

Pre-analytical factors that can impact on phosphoprotein levels in tumor samples include the duration of ischemia time, which refers to the time that a tissue is without oxygen between excision and fixation. During this period, cell signal-

ling pathways persist in a dynamic state, with protein phosphorylation regulated by the opposing effects of kinases and phosphatases. Therefore, it is essential that tissue is processed and fixed promptly to minimise the potential for post-excision protein phosphorylation or dephosphorylation as a reaction to prolonged hypoxia or other cellular stresses [8, 9].

The effects of an extended ischemia time on phosphoprotein levels can vary depending on the tumor type and the phosphoproteins of interest, but evidence suggests that levels may increase, decrease, increase then decrease, decrease then increase, or remain relatively constant [9–11]. This can result in an inaccurate representation of phosphoprotein levels and signalling pathway activation in the tumor itself.

We have shown that immunostaining of phosphorylated mammalian target of rapamycin (p-mTOR) in CRC tissue becomes stronger with increasing ischemia time, with a substantial increase in staining levels after an ischemia time of 45 min (Fig. 8.1) [12]. In contrast, other studies have indicated that extended ischemia time may lead to an underestimation of levels of phosphoproteins in the tumor, and could contribute to false-negative results. For example, in a study of colon cancer xenografts, Western blotting indicated that p-Akt levels decreased with time when the samples were left at room temperature prior to fixation, with a half-life of 20 min (Fig. 8.2) [13]. In a study of breast cancer samples, delayed freezing was associated with weaker detection of p-HER-2/neu by Western blotting, with the signal disappearing at 24 h post resection [14]. In a study of adrenal tumor samples, storage at room temperature before and after freezing was associated with weaker detection of p-Erk by Western blotting [15]. Overall, such studies highlight the importance of minimising and standardising ischemia time to reduce the impact on phosphoprotein levels.

8.4 Does Ischemia Time Affect Some Phosphoproteins More Than Others?

Some proteins may be more susceptible to changes in their phosphorylation status due to ischemia time than others. This has been indicated by a study that used immunohistochemical staining to assess levels of p-Akt, p-mTOR and p-MAPK in breast cancer, CRC and NSCLC samples that were left for an ischemia time of 10, 30, 60 or 90 min prior to fixation. In this study, large proportions of the tumor samples demonstrated significant variations in phosphoprotein levels between the different ischaemic time points, but the degree of instability was different for each phosphoprotein. More specifically, percentage instability scores were 43 % for p-MAPK, 55 % for p-mTOR and 59 % for p-Akt, suggesting that prolonged ischemia time may have had the greatest effect on p-Akt levels in this context [11].

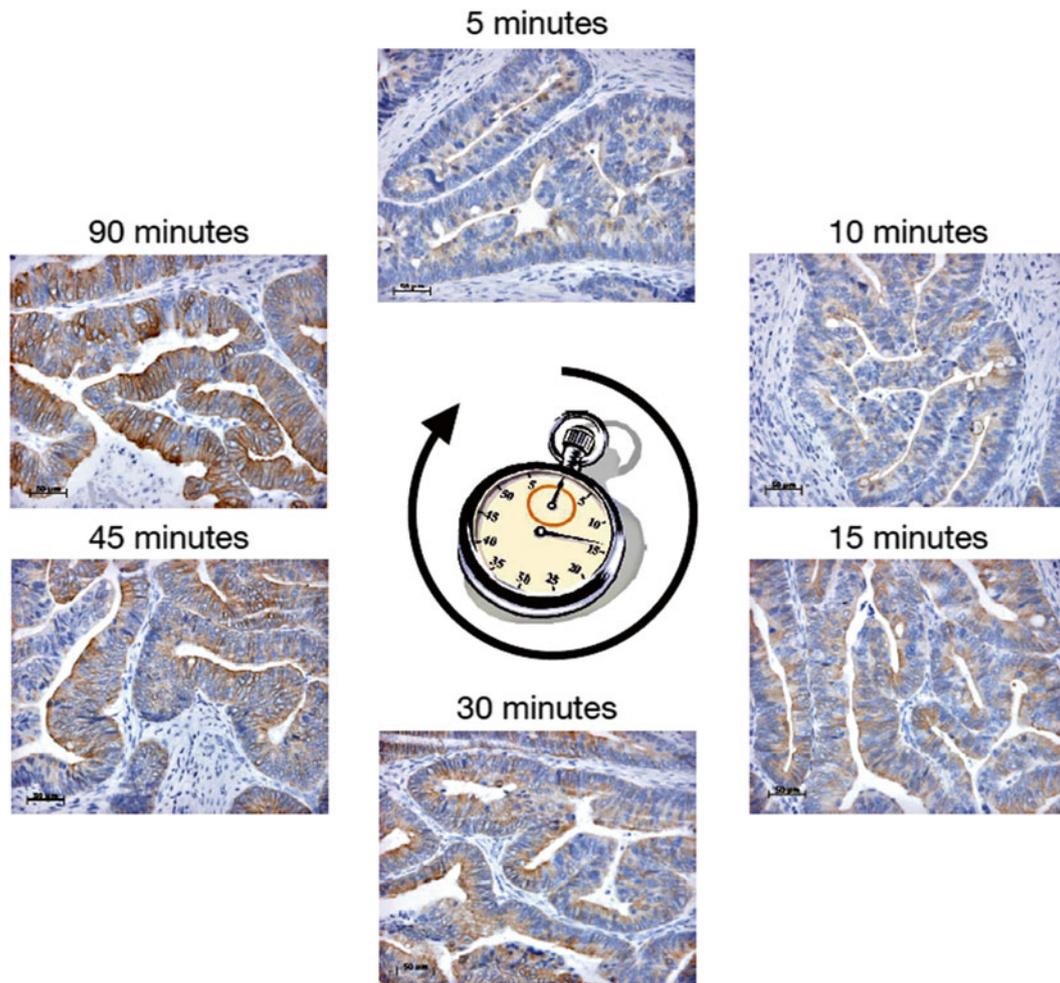


Fig. 8.1 Immunostaining of phosphorylated mammalian target of rapamycin (p-mTOR) in human colorectal cancer tissue following incubation in a wet chamber at room temperature for 5–90 min after surgical excision, and subsequent fixation in formalin for 24 h

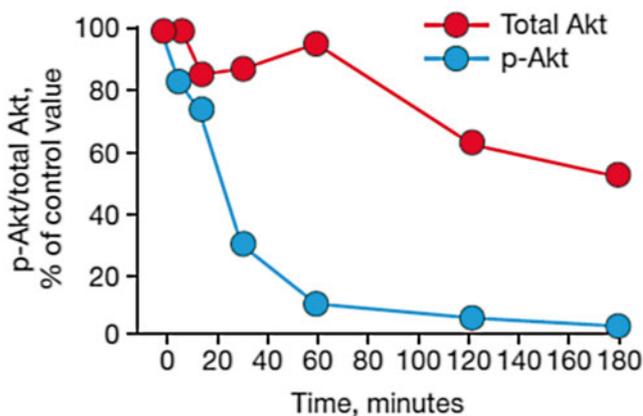


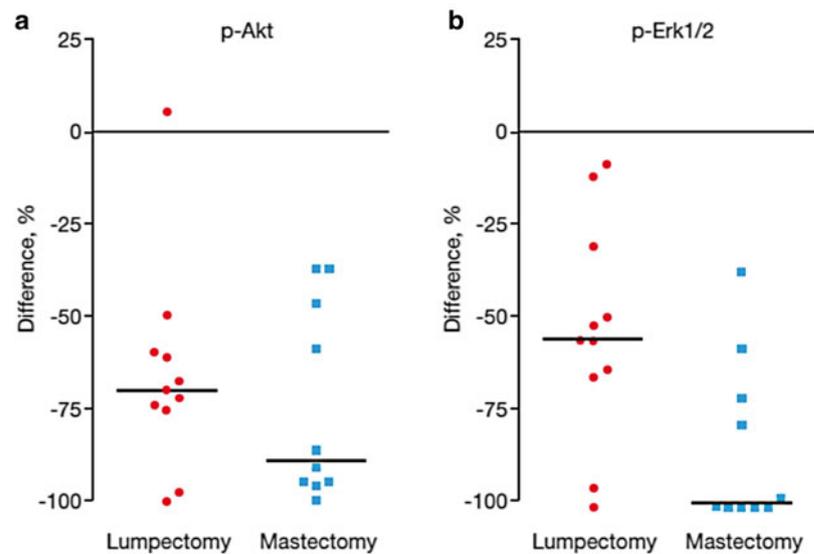
Fig. 8.2 Time course of the loss of phosphorylated (p)-Akt and total Akt in HT-29 human colon cancer xenografts excised from severe combined immunodeficiency mice and kept at room temperature for varying times shown prior to snap freezing in liquid nitrogen for Western blotting [25]. Figure originally published in Baker JW et al. *Allergy Asthma Proc.* 2013;34:162–169. Reproduced with permission. No further reproduction permitted. For additional reproductions visit <http://www.rockwaterinc.com>

8.5 How Does Ischemia Time Affect Total Protein Levels?

Several studies have indicated that ischemia time has little impact on total protein levels. For example, in a study of adrenal tumor samples, increasing ischemia time was associated with reductions in the detection of p-Erk, while total Erk levels appeared unaffected [15]. Even relatively long ischemia times may have limited impact: in a study of xenograft tumor samples, immunohistochemical staining of p-PRAS40 (which plays a role in Akt-mediated signalling) was already weaker when formalin fixation was delayed by just 1 h, whereas total PRAS40 levels were maintained even after a delay of 24 h [9].

Nonetheless, in a study of breast cancer and NSCLC biopsies, while total mTOR levels remained consistent over increasing ischemia time, total Akt and total MAPK levels appeared to change in some cases. However, it was considered that this may have been an artefact resulting from

Fig. 8.3 Percentage difference in (a) phosphorylated (p)-Akt and (b) p-extracellular signal-regulated kinase (Erk)1/2 levels between resection samples and core biopsies from primary breast cancer lumpectomy and mastectomy specimens [17]. Figure originally published in Pinhel IF et al. *Breast Cancer Res.* 2010;12:R76



heterogeneous protein expression across the tumors [11]. It is important to consider that heterogeneous expression can occur and may have a confounding effect when attempting to establish differences in protein or phosphoprotein levels.

8.6 Is the Preservation of Phosphoproteins Affected By the Size of the Tissue Sample?

The size of the tissue sample may have important implications for fixation time, which can in turn affect the preservation of phosphoproteins. In studies of breast cancer, the immunohistochemical detection of p-Akt and p-Erk1/2 was significantly reduced in routinely fixed resection samples compared with core biopsies, with a greater difference following mastectomy vs. lumpectomy (Fig. 8.3) [16, 17]. This is suggested to reflect the fact that fixatives penetrate small-volume core biopsies much more quickly than larger main resections, which may therefore be subject to a longer ischemia time before penetration is complete.

Similarly, in a study of samples from gastro-esophageal junction tumors, immunohistochemical staining of p-Akt was only achieved in biopsy tissue and not in any surgically resected samples. In this case it is suggested that postoperative surgical samples may be of limited value for the detection of phosphoproteins because of the implications of the prolonged time to fixative penetration [13]. However, it is also important to note that a core biopsy may not be representative of the entire tumor as there may be intrinsic heterogeneity in protein expression, as discussed above.

8.7 What Is the Optimum Fixation Time for the Preservation of Phosphoproteins?

The fixation process involves protein cross-linking by formaldehyde, which takes time [18]. Once the fixative has penetrated the tissue, the optimum fixation time can depend on the overall methodology and choice of fixative, with under-fixation or overfixation potentially leading to false-negative results. Perhaps the best established guidelines for fixation relate to the detection of estrogen and progesterone receptors in breast cancers, recommending that specimens are fixed in 10 % neutral-buffered formalin (NBF) for 6–72 h before processing, with complete tissue fixation usually requiring 24 h [19]. Similarly, in research studies reporting the immunohistochemical detection of phosphoproteins, fixation times have been reported of 24 h for breast cancer, CRC and NSCLC surgical specimens fixed in 10 % NBF [11]; 24–48 h for xenograft tumor samples fixed in 10 % NBF [9]; and 16–24 h for xenograft tumor samples fixed in Streck's tissue fixative (STF), 10 % NBF or 4 % paraformaldehyde [20].

Evidence suggests that there may be scope to reduce fixation time under certain temperature conditions. Heating can speed up the process of formaldehyde cross-linking, but this approach appears to result in uneven fixation and poor morphology in the interior of the tissue. However, a study has indicated that a fixation protocol involving a 2-h incubation with 10 % NBF at 4 °C followed by a second 2-h incubation at 45 °C can preserve phosphoproteins in xenograft and human tumor samples for immunohistochemical staining more effectively than when a 24-h incubation at room

temperature is used [21]. It is hypothesised that the initial cold incubation reduces phosphatase activity and inhibits the process of formaldehyde cross-linking to facilitate penetration of the tissue, and that the subsequent heated incubation speeds up the cross-linking process only after penetration is complete.

8.8 Is the Preservation of Phosphoproteins Affected by the Choice of Fixative?

While formalin is routinely used as a fixative for paraffin-embedded tissue samples in many laboratories, research has suggested that other fixatives may be better for the preservation of phosphoproteins. Specifically, a study using paraffin-embedded xenograft tumor samples and human surgical tumor samples has indicated that STF may be superior in preserving phosphoproteins compared with 10 % NBF or 4 % paraformaldehyde [20]. For example, staining of p-MET was scored as +++ for all five tested STF-fixed xenograft tumor samples, but was only scored as + for the majority of formalin- or paraformaldehyde-fixed samples ($p=0.0008$).

Newer alternatives to formalin are also becoming available, including a one-step room temperature process using biomarker and histology preservative (BHP), which has been suggested to be more effective than 10 % NBF in preserving phosphoproteins in paraffin-embedded tissue samples, although large-scale validation is required [22]. PAXgene Tissue Fix (PreAnalytix GmbH, Switzerland) has also been found to be effective in preserving phosphoproteins in paraffin-embedded tissue samples; although rather than being a formaldehyde replacement, it is suggested as an approach to facilitate the analysis of diseased tissues for which collection of frozen material for molecular analyses is difficult or not feasible [23].

8.9 Do Studies Show a Difference in Phosphoprotein Detection Between Frozen and Formalin-fixed Paraffin-embedded Tissue Samples?

Snap freezing in liquid nitrogen is an alternative method for preserving phosphoproteins, although it is expensive and unavailable in many clinics. There is evidence to suggest that snap freezing of large surgical specimens increases phosphoprotein detection compared with formalin fixation and paraffin embedding. This is probably because the duration of ischemia is not dependent on the time required

for the formalin to penetrate the tissue. However, it seems that similar results may be achieved with both methods if the tissue sample is small enough to ensure rapid penetration by formalin [11].

8.10 Is the Addition of Inhibitors Recommended to Help Preserve Phosphoproteins?

Kinase and phosphatase inhibitors can be useful to stabilise the activation status of proteins, preventing phosphorylation and dephosphorylation, respectively [8]. Sample preparation techniques may involve kinase and phosphatase inhibitors at different stages depending on the process and purpose. For example, they may be included with the fixative [22], incubated with the sample prior to freezing [8], added to protein samples following extraction from frozen sections [23], or added to lysed cells during protein extraction for Western blotting [14]. However, a study of breast cancer samples has indicated that the phosphatase inhibitor orthovanadate may not be effective in preserving phosphoproteins at room temperature [14].

8.11 Is It Possible to Establish a Clinically Meaningful Quantification of Phosphoproteins in Immunohistochemically Stained Tissue?

Manual scoring of immunohistochemically stained tissue is subject to interobserver variability, which can limit its usefulness in the clinical setting. However, several computer-based programs are available to automatically quantify immunohistochemical staining with a view to improving the reproducibility of results (e.g., Ariol, Aperio Image Analysis Toolbox, Bacus TMA Score), and some now have diagnostic applications in breast cancer [24].

However, as discussed throughout this chapter, results from the immunohistochemical detection of phosphoproteins may be subject to multiple pre-analytical variables, so regardless of quantitative analysis, they may not accurately reflect the status of the tumor. In the future, continued progress to standardise such variables will be helpful to optimise their clinical usefulness.

Acknowledgements Writing assistance was provided by Hannah FitzGibbon of Complete Medical Communications, with funding from Indivumed.

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