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FULL LENGTH ARTICLE

Stathmin as a surrogate marker of phosphatidylinositol-3-kinase pathway activity: Towards precision medicine in HPV-negative head & neck squamous cell carcinoma

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Abstract In order to assess Stathmin as an immunohistochemical (IHC) indicator of phosphatidylinositol 3-kinase (PI3K) pathway activity in HPV-negative head & neck squamous cell carcinoma (HNSCC), we compared Stathmin IHC to expression of other pathway components. We also evaluated the relationship between Stathmin IHC and the mutational status of four key pathway genes. Finally, we ascertained whether Stathmin IHC correlates with tumor grade or primary site. Correlation exists between high Stathmin expression and high pAKT1 expression, indicating a role for Stathmin IHC as a marker of pathway activity. Our analysis did not show correlation between Stathmin IHC and mutation of the four genes evaluated. We also observed an association between high Stathmin expression and oropharyngeal primary site. Our results suggest utility of Stathmin IHC as an indicator of PI3K pathway activity, and thereby demonstrate potential relevance of Stathmin IHC in the context of HNSCC.

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Introduction

Increased signal transduction by the phosphatidylinositol 3-kinase (PI3K) pathway occurs in many cancers, including head & neck squamous cell carcinoma (HNSCC). Dysregulation of this pathway in HNSCC occurs by various mechanisms.[1,2] Increased signaling may result from activating mutations of oncogenes such as *PIK3CA*, inactivating mutations of tumor suppressors such as *PTEN*, or copy number gains or losses affecting these and/or other loci.

Patients whose tumors harbor alterations that upregulate PI3K cascade activity may benefit from drugs that target this pathway. This prospect underscores the importance of detecting these variants. Despite increased understanding of molecular events that dysregulate the pathway, detection of these changes remains cumbersome, due to the diversity of different activating alterations. Immunohistochemistry (IHC) represents an inexpensive, well-established, and near-universal method of morphometric analysis. Accordingly, we sought to identify and characterize an immunohistochemical marker that indicates PI3K pathway activation. Several groups have evaluated IHC for PI3K pathway components, including PTEN and phosphorylated AKT1 (pAKT1), in HNSCC. In some instances, these studies have produced conflicting results.[3–9] The variability of these findings suggests IHC for these pathway members is suboptimal for assessment of overall signaling activity. This limitation possibly results from downstream effectors that modulate the output of factors such as PTEN and pAKT1. As opposed to these cascade components, an alternative surrogate IHC marker may provide more adequate indication of pathway status.

Stathmin, a phosphoprotein that functions in several aspects of cellular proliferation and carcinogenesis, appears to represent one such surrogate. This protein modulates microtubule dynamics (and, accordingly, turnover of the mitotic spindle); more broadly, Stathmin coordinates output from multiple signal transduction pathways, interacts with cell cycle control proteins such as TP53 and RB1, and participates in cell cycle control at the G1-S and G2-M checkpoints.[10] Stathmin's activity depends on/toggles with its phosphorylation status, and various kinases phosphorylate Stathmin. These kinases include PAK1 (p21-Activated Kinase 1), which is phosphorylated by pAKT1 (Fig. 1[11,12]). This indirect interaction with pAKT1, however, comprises only one aspect of Stathmin's complex regulation.

Saal et al. (2007)[13] reported "a gene expression signature of PTEN protein loss" using tissue from breast cancer specimens. Their "signature" incorporated multiple mechanisms of PI3K pathway activation. These authors identified a role for Stathmin as "a robust surrogate marker for the signature." In the current study, we assessed Stathmin as an indicator of PI3K pathway activity in HPV-negative HNSCC. We compared Stathmin IHC to pAKT1 and PTEN IHC, as these markers represent well-established and extensively studied pathway components, shown to respectively promote and suppress PI3K pathway activity. We also ascertained whether PTEN, pAKT1, or Stathmin IHC correlates with tumor grade and/or primary tumor site.

Materials & methods

HNSCC cases

Tumor tissue from 30 HNSCC cases sampled or resected at New York Presbyterian Hospital from 1998 to 2009 were collected in accordance with institutional review board guidelines. Primary site was recorded. Tumors were graded as well-, moderately, or poorly differentiated (Table S1). As per standard operating procedure within the surgical pathology laboratory, carcinomas arising in the oropharynx were evaluated via p16 IHC (CINtec, Ventana) and/or HPV *in situ* hybridization (Ventana). Three cases that showed evidence of HPV association were excluded. We constructed a tissue microarray (TMA) containing 3 cores (19 cases), 2 cores (6 cases), or 1 core (2 cases) per specimen, from the remaining cases. Variability between numbers of cores per case resulted from differences in available amounts of tumor.

Immunohistochemistry

Staining for PTEN, pAKT1, and Stathmin was performed as previously described,[14,13] on an automatic staining workstation (Dako Autostainer Plus) using the Dako EnVision Flex + Visualization System, followed by counterstaining with hematoxylin.

For each tissue core, PTEN IHC was scored as "normal" (1) or "decreased" (0) (Fig. 2). Normal positive staining was defined as staining that distinguishes tumor cells/nests from surrounding stroma. For cores with focal borderline positivity, staining of tumor cells was compared to staining of inflammatory and endothelial cells. If the former was weak compared with the latter, staining was considered negative. If staining of tumor cells was comparable to staining of inflammatory/endothelial cells, staining was considered positive. Preliminary scores for pAKT1 were determined as follows: 0, light staining; 1, at least focal (but not diffuse) dark staining, overall less than staining of inflammatory/endothelial cells; 2, more than focal dark staining, approximately equal to staining of inflammatory cells (Fig. 2). To facilitate statistical analysis, pAKT1 scores were considered "high" (2) or "low" (<2). Preliminary Stathmin scores were calculated as per Saal et al. (2007)[13], as the product of staining intensity (0–2) and index of positively stained tumor cells (1: <25% of cells; 2: 25–50%;

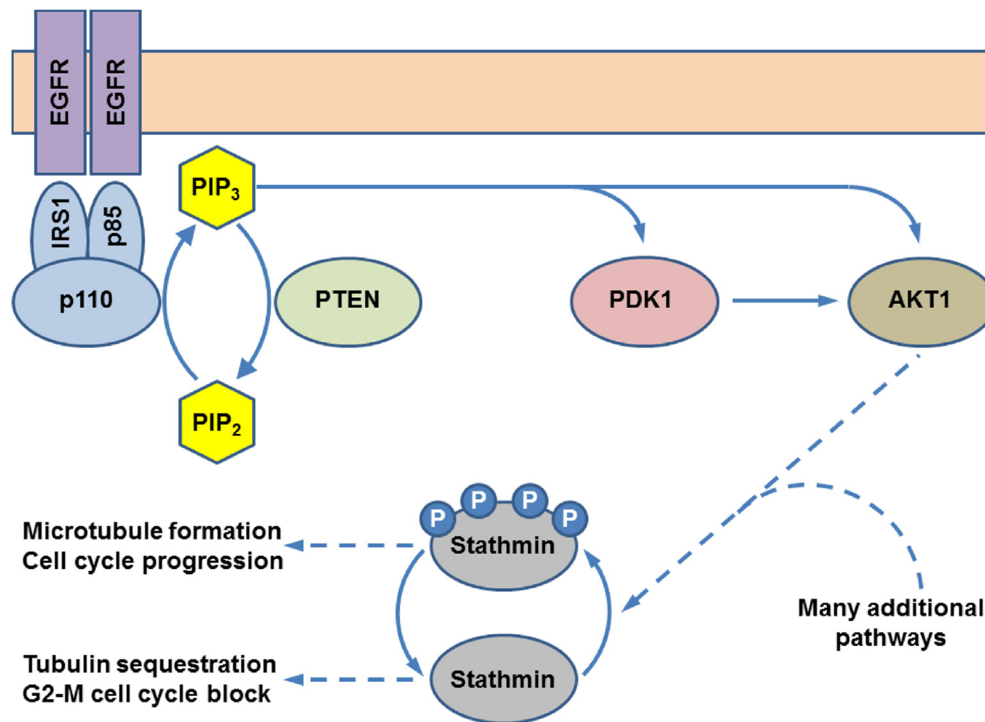

Figure 1 Interaction between the PI3K pathway and Stathmin. Solid lines represent direct interactions; dashed lines represent indirect or multifactorial processes. PI3K comprises three subunits: IRS1, p85, and p110. *PIK3CA* encodes the p110 subunit. "p" denotes a phosphate moiety.

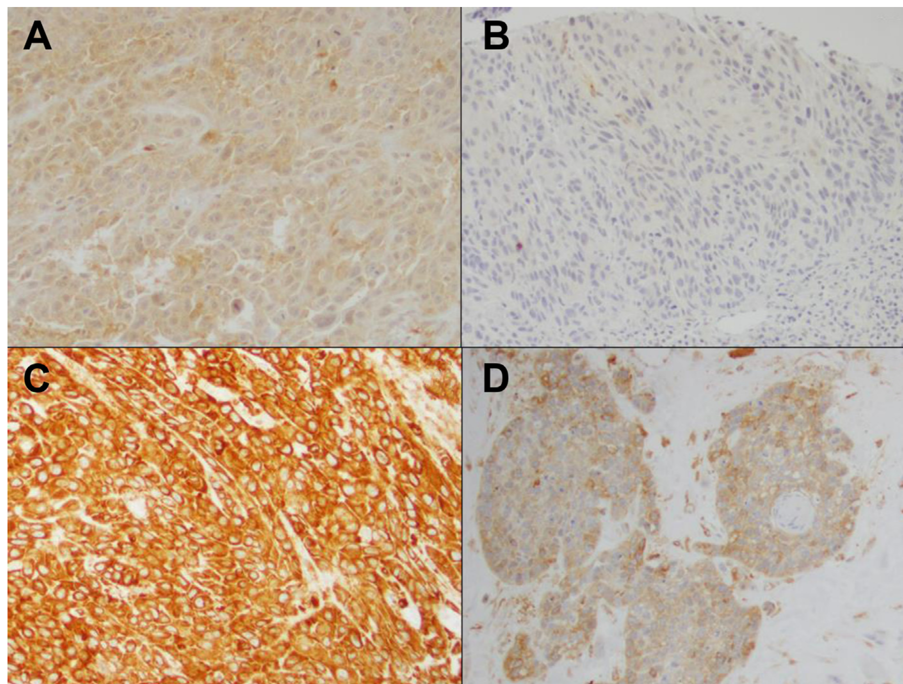

Figure 2 PTEN (**A,B**) and pAKT1 (**C,D**) immunohistochemical staining. For each tissue core, PTEN IHC was scored as "normal"/score = 1 (A) or "decreased"/score = 0 (B) (magnification: 200x). pAKT1 IHC was scored as 2 (C), defined as more than focal dark staining, approximately equal to staining of inflammatory cells, or 1 (D), defined as at least focal (but not diffuse) dark staining, overall less than staining of inflammatory/endothelial cells.

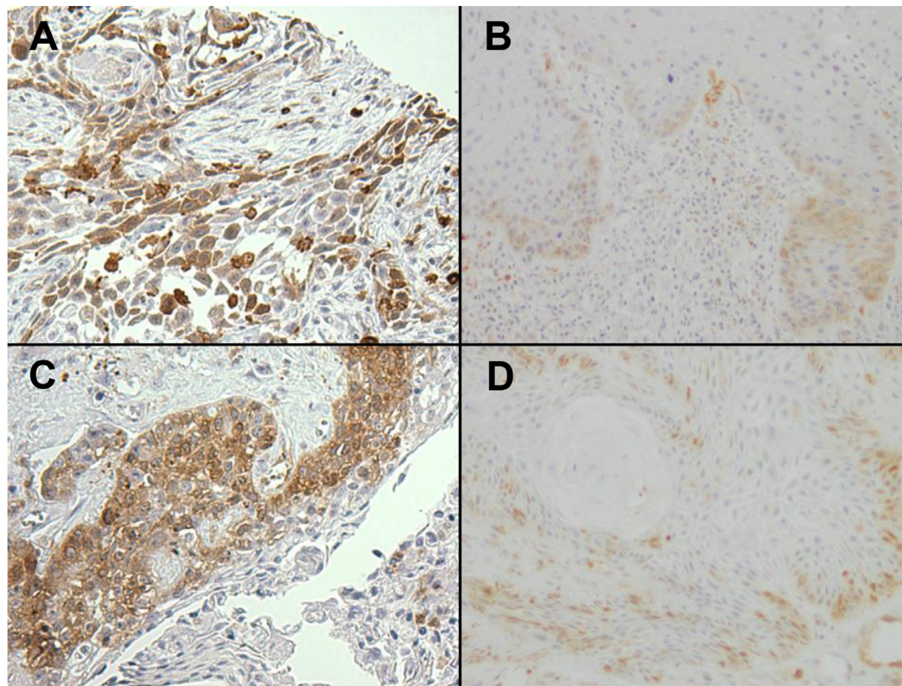

Figure 3 Stathmin immunohistochemical staining. For panels (**A, C**), Stathmin IHC score = 4/8 (intensity = 2; proportion = 2: positive staining in 25—50% of tumor cells). For panels (**B, D**), Stathmin IHC score = 2/8 (intensity = 1; proportion = 1: positive staining in <25% of tumor cells). Please note, percentage of stained cells was estimated by examining each entire core.

3: 50—75%; 4: >75%) (Fig. 3). Final Stathmin scores were classified as "high" (greater than overall mean score) or "low" (less than overall mean score). Two pathologists (ATT and EP) scored IHC results for all cores. Discrepancies between scores were resolved by joint review and discussion of relevant cores/cases.

Molecular analysis

Eighteen of twenty-seven cases yielded sufficient tissue for molecular analysis. We interrogated the mutational status of four PI3K pathway genes using the Illumina TruSeq Amplicon Cancer Panel assay. Targets of this panel include *AKT1* (exon 3), *EGFR* (exons 3, 7, 15, and 18—21), *PIK3CA* (exons 2, 5, 8, 10, 14, and 21), and *PTEN* (exons 1, 3, 4, 7, and 8). DNA was extracted from formalin-fixed, paraffin-embedded tissue on unstained glass slides. Sequencing entailed probe extension and ligation, followed by multiplexed PCR with adapters for Illumina sequencing, followed by sequencing on the Illumina MiSeq platform with reversible fluorescent terminators. Results for 15 of 18 specimens satisfied requisite quality metrics for sequence analysis, which was performed using the NextGENe viewer, version 2.3.4 (Softgenetics).

Statistical analysis

In order to appraise Stathmin as an indicator of PI3K pathway activity, we evaluated the relationship between Stathmin and pAKT1 IHC scores, and between Stathmin and PTEN IHC scores (as well as the relationship between pAKT1 and PTEN IHC scores). We also compared the mean Stathmin IHC scores between cases with and without mutations affecting hotspots of *AKT1*, *EGFR*, *PIK3CA*, and *PTEN*. Finally, we assessed possible correlation between scores for all IHC markers and tumor grade, as well as primary tumor site. All statistical analyses were performed using SPSS statistical software version 17.0 (SPSS Inc., Chicago IL, USA).

Results & discussion

Our analysis showed several associations between relative staining intensities with different IHC markers (Table 1). We detected correlation between high Stathmin scores and high pAKT1 scores: among 14 cases with high Stathmin expression, 11 also showed high pAKT1 staining ($p < 0.001$).

Table 1 Correlations between Stathmin IHC scores, pAKT1 IHC scores, PTEN IHC scores, and primary site.

	Stathmin (Low vs High)	pAKT1 (Low vs High)	PTEN (Normal vs Reduced)
Stathmin (Low vs High)	N/A	—	—
pAKT1 (Low vs High)	$P < 0.001$	N/A	—
PTEN (Normal vs Reduced)	$P = 0.001$	$P < 0.001$	N/A
Site (Oral vs Pharynx vs Larynx vs Other)	$P = 0.004$	$P = 0.819$	$P = 0.780$

High Stathmin scores were also associated with normal PTEN staining: 11 of 14 cases with high Stathmin scores exhibited normal PTEN IHC ($P = 0.001$). Finally, 11 of 16 cases with normal PTEN staining also demonstrated high pAKT1 expression, indicating a correlation between these two patterns ($P < 0.001$).

Next-generation sequencing demonstrated *PIK3CA* hotspot mutations in three cases, and deleterious (frameshift, nonsense, or splice site) mutations of *PTEN* in four cases. Interestingly, one case showed concomitant mutation of *PIK3CA* and *PTEN*. No cases showed *AKT1* or *EGFR* mutations within the exons interrogated by our panel. Mean Stathmin IHC scores were not significantly different between cases with (5.56) and without (5.23) mutations in these four genes ($P = 0.588$).

In terms of tumor differentiation, we did not observe significant differences in Stathmin, pAKT1, or PTEN staining between lesions of different grades. For all markers, we evaluated staining differences between well- versus moderately versus poorly differentiated tumors (Stathmin: $P = 0.234$; pAKT1: $P = 0.187$; PTEN: $P = 0.682$), well-differentiated *versus* all higher-grade (moderately and poorly differentiated) lesions (Stathmin: $P = 0.227$; pAKT1: $P = 0.410$; PTEN: $P = 0.756$), and poorly differentiated *versus* all lower-grade (well- and moderately differentiated) lesions (Stathmin: $P = 0.259$; pAKT1: $P = 0.410$; PTEN: $P = 0.555$).

We also evaluated the relationship(s) between Stathmin staining and primary tumor site (Table 1). We detected an association between high Stathmin expression and oropharyngeal origin, with 6 of 7 oropharyngeal-primary lesions showing high Stathmin expression ($P = 0.004$).

Upregulation of signal transduction via the PI3K pathway characterizes many malignancies, including HNSCC. pAKT1 and PTEN respectively promote and suppress activity of the PI3K signaling pathway. In the current study, we performed IHC for these two proteins as well as Stathmin, in order to evaluate the latter as an indicator of pathway function. In terms of relationships between IHC scores for different markers, we observed a correlation between high Stathmin expression and high pAKT1 expression. This finding suggests Stathmin IHC may provide a surrogate marker of PI3K pathway activation in HNSCC. We also detected a relationship between high pAKT1 staining and normal PTEN staining. This result is consistent with current concepts of PI3K pathway physiology, since pAKT1 functions downstream of PTEN, and increased pAKT1 activity could therefore drive oncogenesis regardless of PTEN status.

We also observed a correlation between high Stathmin expression and normal PTEN expression (as opposed to PTEN loss). Several explanations may account for this finding. First, PTEN IHC may not reflect PTEN hypofunction resulting from mechanisms other than genetic deletion and/or protein loss. In other words, IHC may not detect loss of PTEN function resulting from missense mutation, or other mechanisms. Second, increased signaling may result from dysregulation of pathway components downstream of PTEN, without affecting PTEN IHC.

Additionally, our observed association between high Stathmin and normal PTEN staining perhaps relates to the findings of Pattje et al.[15] and Snietura et al.[16] Both studies demonstrated worse loco-regional disease control in patients with PTEN-positive HNSCC. The latter study incorporated multivariate analysis, according to which positive PTEN IHC had stronger negative prognostic significance than any other variable (including nodal metastasis and EGFR overexpression). Interpreted together, these findings suggest that Stathmin expression may provide more adequate/reliable indication of PI3K pathway activation, compared to PTEN IHC, since normal PTEN expression (instead of PTEN loss) paradoxically correlates with disease progression in some studies.

Our inability to detect correlation between Stathmin IHC and pathway mutation by NGS may relate to scope of and capacity for molecular analysis. Our lab uses the TruSeq Amplicon Cancer Panel for routine clinical testing, for which it performs robustly, and for which reason it is readily available for our group's translational studies. However, the genes targeted by this panel represent an incomplete subset of genes comprising the PI3K pathway. Secondly, even within these four genes, the panel interrogates a limited number of exons. Furthermore, oncogenic molecular alterations such as copy number variation, structural chromosomal changes, et cetera, may not be detectable by DNA sequencing. The large number of diverse molecular events that may potentially increase PI3K signaling prevents exclusion of pathway activation based on DNA sequencing alone, and underscores our original rationale for this study.

Finally, these results also suggest a role for Stathmin in assessing carcinoma of unknown primary, since we observed a correlation between high Stathmin expression and oropharyngeal primary site. Prior studies have not demonstrated similar utility of PTEN or pAKT1 IHC, which do not appear to differ between HNSCC cases from different primary sites.[15,7,17]

Our findings demonstrate potential relevance of Stathmin IHC in the context of HNSCC, as previous studies of breast cancer have shown. We also provide further data regarding the significance of pAKT1 and PTEN IHC. Additional investigation of these two markers is valuable, since prior studies have yielded discordant results. These observations provide insight into the molecular mechanisms of this disease, and reflect analogous findings in other tumor types. Overall, these results provide further evidence of PI3K pathway activation in HNSCC tumorigenesis, and support the potential of this signaling cascade as a treatment target.

Conflict of interests

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2020.12.002.

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