Akt as a potential prognostic marker in neuroendocrine tumours: a possibility?

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Neuroendocrine tumours (NET) include intestinal and pancreatic NET, bronchiopulmonary NET, phaeochromocytomas, paragangliomas, neuroblastoma, medullary thyroid carcinoma, pituitary tumours, small cell lung cancer and Merkel cell tumours. The diversity and relative rarity of these tumours create important challenges to our understanding of their molecular biology, and also to identifying markers of prognosis or response to therapy. Although NETs are rare, the incidence has increased in recent years, at least in part due to improved diagnosis. Prognosis depends on the tissue of origin, tumour grade, and tumour stage. The proliferative index is considered in the grading since it contributes to poor prognosis. Often these tumours are slow growing and well differentiated with an indolent course, but some are aggressive and poorly differentiated.

Chemotherapeutic approaches have not shown consistent benefit for patients, and attention has turned to molecular targeted therapy. The PI3K/Akt/mTOR pathway holds great promise, at least for a proportion of NET. In brief, various G-protein coupled receptors, tyrosine kinase receptors and mutant *RAS* stimulate PI3K/Akt signalling. PI3K (phosphatidylinositol-3-kinase) phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP2), forming phosphatidylinositol (3,4,5) triphosphate (PIP3), which recruits Akt to the cell membrane. Akt is then activated by phosphorylation enabling it to phosphorylate a plethora of downstream targets including mTOR, and hence Akt drives key cell processes such as cell survival, cell cycle progression, angiogenesis, and cell migration.

Defects in the PI3K/Akt/mTOR pathway have been highlighted through exome sequencing of small intestinal NET (33%) and pancreatic NET (P-NET) (14%) [1]. Multiple tyrosine kinase receptors and G-protein coupled receptors that stimulate this pathway are overexpressed in NET [2]. Critically, the mTOR inhibitor everolimus (RAD001) increased progression-free survival over placebo in clinical trial, and has been approved for treatment of progressive advanced P-NET, non-functional (non-secreting) gastrointestinal and lung NET. Further clinical trials of everolimus and other mTOR inhibitors (either as single agents or in combination therapy) are underway or recruiting (https://clinicaltrials.gov). Of note, mTOR is only one of a large number of Akt target proteins, and drugs acting upstream through inhibiting tyrosine kinase receptors, PI3K and Akt are being evaluated as potential therapeutic options.

It is relevant to question whether activation of Akt, detected as phospho-Akt (p-Akt), can be used as a prognostic marker for NETs, or as a method or predicting response to treatment. Akt1 is considered to be “fully active” when phosphorylated at both threonine 308 (by PDK1) and serine 473 (primarily by mTOR complex 2: mTORC2). Expression of p-Akt in NETs has been demonstrated by immunohistochemical staining using antibodies to p-Akt(Ser473), for example in combined sets of gastroenteropancreatic NET (GEP-NET) [3-6]; bronchiopulmonary neuroendocrine tumours (BP-NET) [7, 8], pheochromocytomas [9, 10] and pituitary adenomas [11]. Interestingly, p-Akt may also be detected in tumour stroma and blood vessels supplying the tumours. Comparisons with corresponding normal cells are not made directly in all studies, but the general picture is indicative of p-Akt(Ser473) overexpression and/or overactivation in a large proportion of NET. These studies and others showing elevated p-mTOR or its downstream targets suggest frequent overactivation of this pathway in these tumours.

Few studies of p-Akt expression examined progression-free or overall survival of NET patients, possibly in part due to the extended follow-up needed due to the indolent course of many of these tumours. Fernandez et al [12] found that higher staining scores for p-Akt(Ser473) or phospho-S6 ribosomal protein (p-S6) (a downstream target of mTOR), predicted shorter median progression-free survival in a series of 25 metastatic neuroendocrine carcinomas of mixed anatomical sites. The same study also reported an association between staining for p-Akt(Ser473) and p-S6. In medullary thryoid carcinomas, studies showed no relationship between p-Akt(Ser473) and prognosis [13, 14], although there was an association between p-Akt(Ser473) staining and p-mTOR and p-S6K staining, suggesting that Akt is actively stimulating mTOR activity in these tumours [13]. In incompletely resected non-functioning pituitary adenomas, p-Akt(Ser473) expression was associated with recurrance (n=35) [15]. In a series of 210 BP-NET, tumorlets and low to intermediate grade carcinoids tended to have a higher percentage of p-Akt(Ser473) positive cells than high grade tumours [7], and although p-Akt positive staining correlated with lymph node status in a study of 110 BP-NET [8], there was no association with disease-free survival or overall survival. Qian et al [6] used a series of 195 GEP-NET to examine p-Akt(Ser473) and, although they observed association with p-mTOR, there was no association with prognosis.

The regulation of the PI3K/Akt/mTOR pathway is complex, and is controlled at a number of levels. Akt is regulated by ubiquitination, sumoylation and glycosylation as well as phosphorylation, and therefore measurement of downstream substrate activation in conjunction with p-Akt measurement will give a clearer picture of its activation, and may provide further prognostic information. Assessment of inhibitors of this pathway is also potentially informative. PTEN and TSC2 are key suppressors of the PI3K/Akt/mTOR pathway, and reduced levels of TSC2, PTEN (or both) were found in 85% of P-NET tumours in tissue microarray analysis. Furthermore, low levels of each of these proteins were associated with shorter progression-free survival [16]. Whilst PTEN is classically downregulated by allelic loss, there is strong evidence that regulation via microRNAs play a role, as microRNAs that target PTEN are upregulated in NET [2].

Treatment with everolimus results in increased p-Akt, believed to be because elimination of negative feedback mechanisms downstream of mTOR complex 1 (mTORC1) results in stabilisation of IRS-1, a protein which potentiates PI3K signaling. Meric-Bernstam et al [17] used paired pre-treatment and on-treatment fine needle aspirates from 17 neuroendocrine carcinoma patients on a phase II trial of everolimus and octreotide (a somatostatin analogue) to determine whether baseline p-Akt would be predictive of response and whether induction of p-Akt by everolimus would engender resistance. In this study, p-Akt(Thr308) was detected using a reverse phase protein array (RPPA). Pre-treatment p-Akt(Thr308) was reported to be associated with progression free survival, but this was of marginal significance ($P = 0.0533$); however, patients who had a partial response to everolimus treatment were significantly more likely to show an increase in p-Akt(Thr308) than patients with stable or progressive disease. Moreover, on-treatment p-Akt(Thr308) showed an association with progression-free survival, suggesting that this is a marker of everolimus response . What is interesting is that no such association was detected using archival tumor blocks from 23 patients on the same trial, probably because use of fine needle aspirates and RPPA is a more sensitive technique. Monitoring p-Akt(Thr308) or both p-Akt(Thr308) and p-Akt(Ser473) together improved the prognostic significance of Akt activation in a series of 116 primary neuroblastoma samples by tissue microarray analysis compared with p-Akt(Ser473) alone [18]. In this study p-Akt, but not S6 ribosomal protein, was indicative of poor prognosis. Relatively few studies of NET have included measurement of p-Akt(Thr308), yet assessment of the dual phosphorylation status of Akt may prove to be a more robust marker of prognosis.

As stated above, mTOR inhibition results in elevated p-Akt through disruption of negative feedback. Critically, this is potentially a mechanism of drug resistance and reduced efficacy of mTOR inhibitors. Dual PI3K/mTOR inhibitors should prevent this escape mechanism, and therefore could perhaps be a more effective therapeutic approach. One such inhibitor, BEZ235, was put into Phase I trial after promising preclinical results, however toxicity proved problematic. The RIP1-Tag2 mouse develops P-NETs that show elevated p-Akt similar to human P-NETs and has proved useful in evaluating the effectiveness of everolimus [1]. Using this model, p110α PI3K was highlighted as the isoform contributing to carcinogenesis. Genetic and pharmacological inhibition of the p110α PI3K led to decreased p-Akt, increased tumour cell death, and reduced angiogenesis and metastatic dissemination [19].

Current research is hampered by the rarity and diversity of these tumours, and further research would perhaps benefit from larger more homogenous cohorts of patients. Further genomic, transcriptomic (including microRNAs) and proteomic analyses are needed to provide clearer molecular profiles to help define prognostic biomarkers. Using a single phospho-protein as a marker of pathway activation is perhaps over-simplistic: there is a move towards identifying a panel of phospho-proteins to provide a more rigorous assessment activation of the PI3K/Akt/mTOR pathway, and to evaluate compensatory pathways such as the MEK/ERK pathway. In addition, the bulk of current research utilises p-Akt(Ser473) as a marker of pathway activation, but it will be worthwile to include p-Akt(Thr308) as a potential marker.

In summary, current evidence suggests that p-Akt may be of prognostic value as part of a panel of phospho-proteins indicating activity of the PI3K/Akt/mTOR pathway in NET. There is promising evidence to suggest that such a biomarker panel will be useful in predicting and monitoring tumour response to therapies that target the signalling nodes of this pathway.

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