Functional proteomics characterization of residual triple-negative breast cancer after standard neoadjuvant chemotherapy

J. Sohn[1], K. A. Do[2], S. Liu[1], H. Chen[1], G. B. Mills[3], G. N. Hortobagyi[1], F. Meric-Bernstam[4] & A. M. Gonzalez-Angulo[1,3]*

Departments of [1]Breast Medical Oncology; [2]Biostatistics; [3]Systems Biology; [4]Surgical Oncology (FMB); The University of Texas MD Anderson Cancer Center, Houston, USA

Background: In this study, we used functional proteomics to determine the molecular characteristics of residual triple receptor-negative breast cancer (TNBC) patients after neoadjuvant systemic chemotherapy (NCT) and their relationship with patient outcomes in order to identify potential targets for therapy.

Patients and methods: Protein was extracted from 54 residual TNBCs, and 76 proteins related to breast cancer signaling were measured by reverse phase protein arrays (RPPAs). Univariable and multivariable Cox proportional hazard models were fitted for each protein. Survival outcomes were estimated by the Kaplan–Meier product limit method. Training and cross validation were carried out. The coefficients estimated from the multivariable Cox model were used to calculate a risk score (RS) for each sample.

Results: Multivariable analysis using the top 25 proteins from univariable analysis at a false discovery rate (FDR) of 0.3 showed that AKT, IGFBP2, LKB1, S6 and Stathmin were predictors of recurrence-free survival (RFS). The cross-validation model was reproducible. The RS model calculated based on the multivariable analysis was $-1.1086 \times$ AKT $+ 0.2501 \times$ IGFBP2 $- 0.6745 \times$ LKB1 $+ 1.0692 \times$ S6 $+ 1.4086 \times$ stathmin with a corresponding area under the curve, AUC $= 0.856$. The RS was an independent predictor of RFS (HR $= 3.28$, 95%CI $= 2.07$–5.20, $P < 0.001$).

Conclusions: We found a five-protein model that independently predicted RFS risk in patients with residual TNBC disease. The PI3 K pathway may represent potential therapeutic targets in this resistant disease.

Key words: neoadjuvant chemotherapy, molecular characterization, residual disease, resistance, triple receptor-negative breast cancer

introduction

Triple receptor-negative breast cancers (TNBCs) are characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and HER2, comprising ∼12% to 17% of invasive breast cancers [1]. Patients with TNBCs have relatively poor outcomes and are not eligible to be treated with endocrine therapies or anti-HER2 targeted therapies [1]. Neoadjuvant (preoperative) or adjuvant systemic chemotherapy has been shown to improve survival in early disease. Pathologic complete response (pCR) after neoadjuvant chemotherapy (NCT) is known as a surrogate marker for long-term survival in TNBCs [2]. Patients with TNBCs can attain pCR rates of 30%–40%; however, patients with residual disease are at greater risk of relapse with dismal outcomes compared with other subtypes of

breast cancer [3]. Despite the significant impact of residual breast cancer after NCT on outcomes, no standard and more importantly effective therapy exists for this population. Therefore, there is a critical need for better understanding the molecular characteristics of such resistant tumors, and to identify novel targets that can be pursued for a more effective, personalized intervention to improve outcome.

To have a more precise assessment of the consequences of residual disease, investigators at our institution developed the residual cancer burden (RCB) as a continuous index combining pathologic measurements of the primary tumor and nodal metastases, and tested it as an independent predictor of distant relapse-free survival (DRFS) [4]. We and other investigators previously have shown the utility of reverse phase protein arrays (RPPAs) as a high-throughput platform to identify protein biomarkers [5].

In this study, we used RPPAs to determine the molecular characteristics of residual TNBC patients after NCT and their relationship with patient outcomes in order to identify potential targets for therapy.

*Correspondence to: Dr Ana M. Gonzalez-Angulo, Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Unit 1354, 1515 Holcombe Boulevard, Houston, TX 77030-4009, USA. Tel: +1-713-792-2817; Fax: +1-713-794-4385; E-mail: agonzalez@mdanderson.org

patients and methods

We obtained tumors from 54 patients diagnosed with primary TNBC and treated uniformly with taxane and anthracycline-based NCT at MD Anderson Cancer Center (see Supplementary File 1, available at *Annals of Oncology* online, for complete patient and methods section). RCB was calculated [5]. The Institutional Review Board approved the laboratory protocol and the waiver of informed consent for all included cases.

Protein lysates were arrayed and probed with 76 validated primary antibodies (supplementary Table S1, available at *Annals of Oncology* online) focused on markers currently used for breast cancer classification, treatment decision (ER, PR, HER2), targets implicated in breast cancer signaling and targets implicated in the signaling of other cancer lineages.

As the first exploratory analysis, we carried out unsupervised hierarchical clustering, using all 76 proteins. To identify proteins most related to survival, we carried out univariable Cox analysis as the first screening step, and selected the top 25 predictors from the univariable Cox analysis corresponding to a multiplicity FDR adjustment threshold of 0.3 (supplementary Table S2, available at *Annals of Oncology* online). Next, we used CoxBoost to construct a multivariable protein-marker of five proteins (AKT, IGFBP2, LKB1, S6 and Stathmin) (Figure 1A). We then developed a risk score (RS) for each patient, which is the sum of the estimated coefficients from the five-protein multivariable CoxBoost model multiplied

by their expression. To assess the robustness of the five selected proteins, a 'leave-one-out' cross-validation approach was employed.

results

Fifty-four patients with residual TNBC were included. Patient and tumor characteristics are listed in Table 1. The median age was 52 years (range 27–73). Most patients were Caucasians (44.4%) and African Americans (42.6%). Most patients had baseline clinical stage III disease (64.8%) and high nuclear grade (94.4%).

At a median follow-up of 24 months (range 7–145 months), there were 36 (66.7%) recurrences. The median RFS was 33 months (range 1–145 months). No clinical factor was significantly correlated with RFS in univariable analyses as shown in supplementary Table S4, available at *Annals of Oncology* online.

unsupervised global clustering

Unsupervised clustering of the 54 residual TNBC samples and 76 proteins split tumors into two groups. However, the
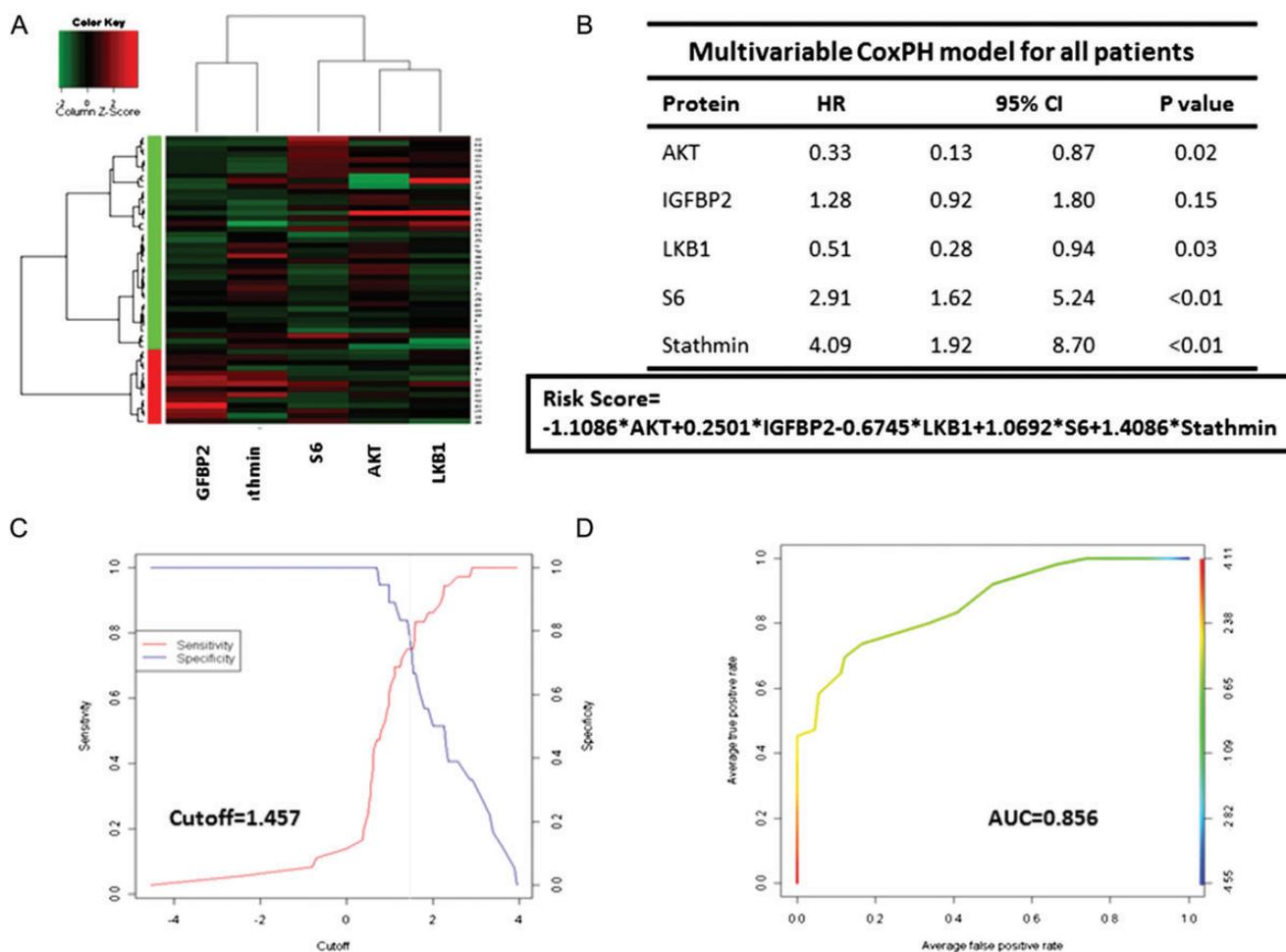

Figure 1. (A) Clustering into green and red groups depending on the expression levels of AKT, IGFBP2, LKB1, S6 and stathmin 54 residual triple-negative breast cancers (TNBCs). (B) Multivariable Cox proportional hazard model and calculated risk score (RS). (C) Optimal cut-off point at 1.457 (sensitivity versus 1-specificity for the RS in all 54 cases. (D) Receiving operating curve of the RS model (AUC = 0.856).

Kaplan–Meier plot shows that these two groups had no significant difference in RFS, ($P = 0.471$) (supplementary Figure S1, available at *Annals of Oncology* online).

biomarker identification

Twenty-five proteins out of 76 had a false discovery rate (FDR) of 0.3. The univariable analysis results are presented in supplementary Table S2, available at *Annals of Oncology* online. Multivariable analysis implemented with CoxBoost showed that 5 out of the 25 proteins AKT, IGFBP2, LKB1, S6 and Stathmin were predictors for RFS. After hierarchical clustering of all 54 tumors with the five proteins, tumors split into two distinct

Table 1. Patient and tumor characteristics

Characteristic	Number	Percentage
Age (years)		
≤50	26	48.1
>50	28	51.9
Menopausal status		
Pre-menopause	19	35.2
Peri-/postmenopause	35	64.8
Ethnicity		
White	24	44.4
African American	23	42.6
Hispanic	6	11.1
Asian	1	1.9
Baseline stage		
I and II	19	35.2
III	35	64.8
Pathologic stage		
I and II	22	40.8
III	32	59.2
Nuclear grade		
2	3	5.6
3	51	94.4
Residual cancer burden (RCB)		
II	13	24.1
III	41	75.9
Lymphovascular space invasion		
Yes	33	61.1
No	21	38.9
Surgical therapy		
Breast-conserving therapy	10	18.5
Mastectomy	44	81.5

groups (Figure 1A). We then defined the RS for each patient as the estimated coefficients from the five-protein multivariable CoxBoost model multiplied by their expression ($RS = -1.1086 \times AKT + 0.2501 \times IGFBP2 - 0.6745 \times LKB1 + 1.0692 \times S6 + 1.4086 \times stathmin$). An optimal cut-off point (1.457) (Figure 1B) was obtained for the RS and corresponded to the value that simultaneously optimized sensitivity and specificity (Figure 1C), and the resulting area under the receiving operating curve was calculated to be 0.856 (Figure 1D).

risk score model for recurrence-free survival

The RS was applied to all patients, and they were classified as high and low risk of relapse with significantly different 3-year RFS estimates (7.14%, 95% CI = 1.27–40.1% versus 48.4%, 95% CI = 32.3–72.6%, $P = 0.001$) (Table 2, Figure 2A). The RS was then applied to the leave-one-out cross-validation group and patients at a high and a low risk of recurrence showed significant differences in 3-year RFS estimates ($P = 0.037$) (Table 2, Figure 2B).

The final multivariable Cox proportional hazard model confirmed that the RS was an independent predictor of RFS (HR = 3.28, 95% CI = 2.07–5.20, $P < 0.001$) after adjustment for other known significant patient and disease characteristics, including clinical stage at diagnosis, nuclear grade and RCB (a measurement of the volume of the residual disease at the time of surgery) (Table 3).

discussion

This biomarker identification study, using 76 antibodies to proteins related to breast cancer signaling for RPPA, showed that AKT, IGFBP2, LKB1, S6 and stathmin were predictors of RFS in 54 patients with residual TNBC after NCT. The obtained RS model based on the five identified proteins was found to be reproducible on leave-one-out cross validation. Multivariable analysis incorporating known important clinical and pathological factors of prognosis suggested that the RS was an independent predictor of RFS.

In breast malignancy, residual disease after NCT with anthracyclines and taxanes is considered to be resistant to standard chemotherapy; however, no further systemic therapy is indicated as no effective drugs have yet been identified. To our knowledge, our study is the first approach focusing on discovering molecular targets in these chemo-resistant residual
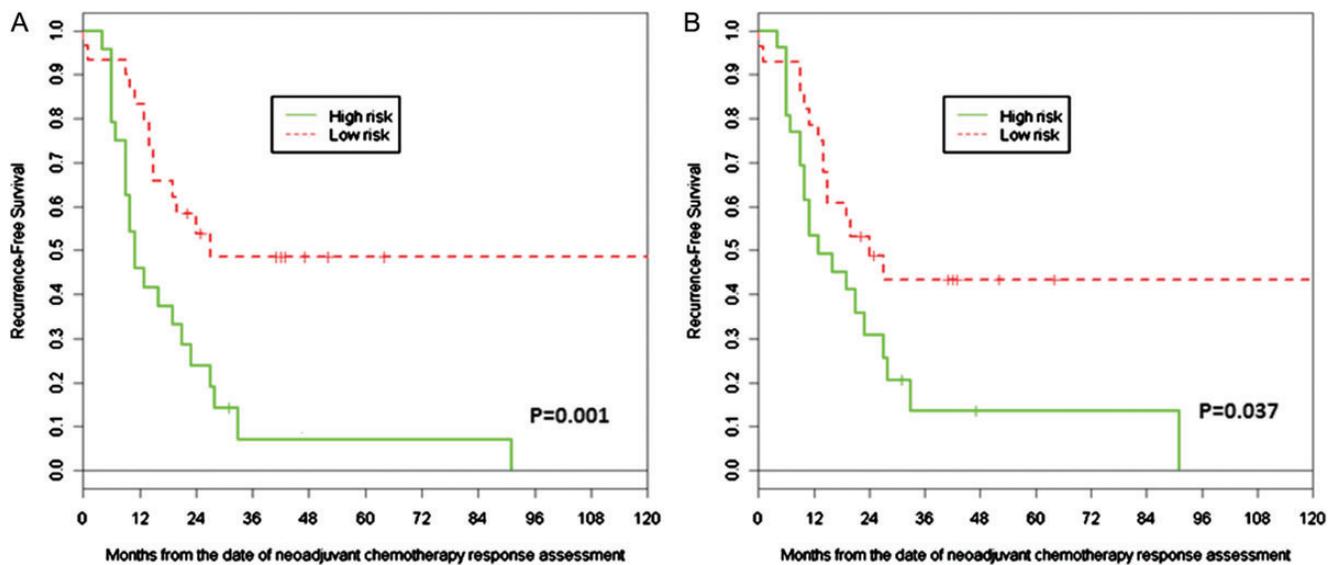
Table 2. Recurrence-free survival (RFS) estimates by risk group in training and cross-validation

	N	N Events	Median RFS (range) in months	3-year estimate	95% confidence interval	P value
Training						
All						
High-risk clustering	24	22	11 (4–91)	7.14%	(1.27%, 40.1%)	
Low-risk clustering	30	14	20 (0–145)	48.4%	(32.3%, 72.6%)	0.0014
Cross-validation						
All						
High-risk clustering	26	21	12 (4–91)	13.7%	(4.3%, 43.5%)	
Low-risk clustering	28	15	20 (0–145)	43.3%	(27.5%, 68.1%)	0.0367

Figure 2. (A) Kaplan–Meier survival curves by risk score (RS). (B) Kaplan–Meier survival curve by RC in leave-one-out cross validation.

Table 3. Multivariable model including the risk score (RS) and residual cancer burden (RCB)

Variables	Hazard ratio	95% confidence intervals	P value
Age at diagnosis (continuous)	0.99	0.96–1.01	0.320
Clinical stage (I and II versus III)	0.60	0.27–1.35	0.219
Nuclear grade (2 versus 3)	4.53	0.89– 22.93	0.068
RCB (continuous)	1.27	0.85–1.90	0.239
RS (continuous)	3.28	2.07–5.20	<0.001
RS only model			
RS (continuous)	2.718	1.855–3.988	<0.001

TNBCs based on a high-throughput protein array technique in the targeted therapy era.

Stathmin is a ubiquitous cytosolic phosphoprotein and a key regulator of cell division due to its depolymerization of microtubules in a phosphorylation-dependent manner. Its ability to remodel microtubule networks through tubulin polymerization indicates a role for stathmin in tumor cell migration and invasion [6]. Constitutive activation and overexpression of stathmin expression have been associated with a variety of human cancers, including breast, lung, gastric, ovarian, cervical, prostate, urothelial, hepatocellular and colorectal [7, 8]. In breast cancer cell lines, stathmin overexpression has been associated with reduced taxane sensitivity and increased resistance to taxane-based chemotherapy [9]. In patients with breast cancer, overexpression of stathmin messenger RNA has been correlated with high mitotic index, loss of hormone receptors and poor prognosis [10]. A study using traditional immunohistochemistry evaluated stathmin protein expression as a surrogate marker for a PTEN gene expression signature [11]. Investigators found that stathmin IHC staining scores were significantly higher in PTEN-negative tumors than in PTEN-positive tumors (P = 0.005), indicating

that loss of the tumor suppressor PTEN, and subsequent activation of PI3 K signaling were associated with increased stathmin expression. In addition, high-stathmin-expressing patients experienced significantly worse DRFS than low-stathmin-expressing patients [11].

Insulin-like growth factor (IGF) binding proteins (IGFBP) modulate interactions of the IGF ligand with the IGF-I receptor. Studies on IGFBP2 expression in breast cancer tissue are still limited in number. High IGFBP2 concentrations in blood or malignant cells and tissues have shown to be predictive of poor prognosis in many malignancies including colon, lung, ovary, prostate and other tumors [12–14]. The largest study on the prognostic role of IGFBP2 included tumor specimens from 4186 patients with breast cancer [12]. In this study, IGFBP2 was not prognostic in patients with ER-positive disease, but it was associated with a trend to worse breast cancer disease-specific survival in patients with ER-negative disease, (P = 0.068), which is consistent with our study that showed IGFBP2 as a poor prognostic factor. These results imply the possibility of IGFBP2 as a potential novel target for patients with residual TNBCs.

The ribosomal protein S6 (S6 kinase) represents an extensively studied effector of the TORC1 [TOR (target of rapamycin) complex 1], which possesses important roles in cellular and organismal physiology. TORC1 functions as an environmental sensor by integrating signals derived from diverse environmental cues to promote anabolic and inhibit catabolic cellular functions. mTORC1 (mammalian TORC1) phosphorylates and activates S6K1 and S6K2. The mTORC1-S6K1 axis regulates cell physiology by controlling fundamental cellular processes, including transcription, translation, protein and lipid synthesis, cell growth and cell metabolism [15]. Persistent inhibition of S6K1 has been shown to activate Akt via feedback inhibition of the PI3 K pathway, wherein S6K1 phosphorylates several sites on IRS-1 (insulin receptor substrate-1) and inhibits it [16–20]. The limited therapeutic efficacy of rapamycin and its analogs in some tumor types has been attributed in part to the activation of AKT via this negative feedback loop [16–19]. The S6 protein is a

downstream target of S6K1. The mechanism by which changes in total protein levels in S6 protein could alter patient outcomes remains to be determined.

LKB1 is a kinase-activating kinase and a number of LKB1 dependent phosphorylation cascades regulate fundamental cellular and organismal processes in at least metabolism, polarity, cytoskeleton organization and proliferation. The characterized substrate of LKB1 is adenosine monophosphate-activated protein kinase, which is the master regulator of cellular and organismal metabolism, providing a putative downstream pathway to LKB1-mediated tumor suppression [20]. Indeed, a high level of PI3 K activity can improve bioenergetics by increasing nutrient uptake as well as through other mechanisms.

All together, these results suggest that residual TNBC is characterized by activation of components of the PI3 K pathway. Further, other forms of deregulation and aberrations of this pathway have been implicated not only in breast cancer development and progression [21], but also in resistance to targeted therapies directed to tyrosine kinase receptors and hormone receptors [22–25]. As a result, multiple drugs targeting the PI3 K pathway are in early clinical trials as mono or combination therapies in breast cancer including TNBC [26].

Our study had limitations, the small sample size only allowed us to conduct leave-one-out cross-validation rather than formal validation on an independent set. Nevertheless, the RS model of five proteins that predicted outcomes in patients with residual TNBC warrants further testing to evaluate its prognostic potential and clinical applicability. In conclusion, we found a five-protein model that independently predicted RFS risk in patients with residual TNBC disease after NCT. The RS may have value in stratifying patients based on their risk of relapse, providing potential targets for novel agents to treat this resistant disease.

funding

disclosure

The authors have declared no conflicts of interest.

references

1. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010; 363: 1938–1948.
2. Kaufmann M, Hortobagyi GN, Goldhirsch A et al. Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: an update. J Clin Oncol 2006; 24: 1940–1949.
3. Liedtke C, Mazouni C, Hess KR et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 2008; 26: 1275–1281.
4. Symmans WF, Peintinger F, Hatzis C et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. J Clin Oncol 2007; 25: 4414–4422.
5. Gonzalez-Angulo AM, Hennessy BT, Meric-Bernstam F et al. Functional proteomics can define prognosis and predict pathologic complete response in patients with breast cancer. Clin Proteomics 2011; 8: 11.
6. So AI, Levitt RJ, Eigl B et al. Insulin-like growth factor binding protein-2 is a novel therapeutic target associated with breast cancer. Clin Cancer Res 2008; 14: 6944–6954.
7. Golouh R, Cufer T, Sadikov A et al. The prognostic value of stathmin-1, S100A2, and SYK proteins in ER-positive primary breast cancer patients treated with adjuvant tamoxifen monotherapy: an immunohistochemical study. Breast Cancer Res Treat 2008; 110: 317–326.
8. Jeon TY, Han ME, Lee YW et al. Overexpression of stathmin1 in the diffuse type of gastric cancer and its roles in proliferation and migration of gastric cancer cells. Br J Cancer 2010; 102: 710–718.
9. Balasubramani M, Nakao C, Uechi GT et al. Characterization and detection of cellular and proteomic alterations in stable stathmin-overexpressing, taxol-resistant BT549 breast cancer cells using offgel IEF/PAGE difference gel electrophoresis. Mutat Res 2011; 722: 154–164.
10. Curmi PA, Noguès C, Lachkar S et al. Overexpression of stathmin in breast carcinomas points out to highly proliferative tumours. Br J Cancer 2000; 82: 142–150.
11. Saal LH, Johansson P, Holm K et al. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci USA 2007; 104: 7564–7569.
12. Hoeflich A, Reisinger R, Lahm H et al. Insulin-like growth factor-binding protein 2 in tumorigenesis: protector or promoter? Cancer Res 2001; 61: 8601–8610.
13. Mehrian-Shai R, Chen CD, Shi T et al. Insulin growth factor-binding protein 2 is a candidate biomarker for PTEN status and PI3K/Akt pathway activation in glioblastoma and prostate cancer. Proc Natl Acad Sci USA 2007; 104: 5563–5568.
14. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. Biochem J 2012; 441: 1–21.
15. O'Reilly KE, Rojo F, She QB et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006; 66: 1500–1508.
16. Sun SY, Rosenberg LM, Wang X et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res 2005; 65: 7052–7058.
17. Wan X, Harkavy B, Shen N et al. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene 2007; 26: 1932–1940.
18. Wang X, Yue P, Kim YA et al. Enhancing mammalian target of rapamycin (mTOR)-targeted cancer therapy by preventing mTOR/raptor inhibition-initiated, mTOR/rictor-independent Akt activation. Cancer Res 2008; 68: 7409–7418.
19. Zhang J, Gao Z, Yin J et al. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. J Biol Chem 2008; 283: 35375–35382.
20. Ollila S, Makela TP. The tumor suppressor kinase LKB1: lessons from mouse models. J Mol Cell Biol 2011; 3: 330–340.
21. Agarwal R, Carey M, Hennessy B et al. PI3K pathway-directed therapeutic strategies in cancer. Curr Opin Investig Drugs 2010; 11: 615–628.
22. Perez-Tenorio G, Stal O. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer 2002; 86: 540–545.
23. Miller TW, Perez-Torres M, Narasanna A et al. Loss of phosphatase and tensin homologue deleted on chromosome 10 engages ErbB3 and insulin-like growth factor-I receptor signaling to promote antiestrogen resistance in breast cancer. Cancer Res 2009; 69: 4192–4201.
24. Nagata Y, Lan KH, Zhou X et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004; 6: 117–127.
25. Berns K, Horlings HM, Hennessy BT et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell 2007; 12: 395–402.
26. McAuliffe PF, Meric-Bernstam F, Mills GB et al. Deciphering the role of PI3K/Akt/mTOR pathway in breast cancer biology and pathogenesis. Clin Breast Cancer 2010; 10(Suppl 3): S59–S65.