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14. ABSTRACT

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein that complexes with activated factor VII (FVIIa) to initiate blood coagulation. Breast cancer tumors and cell lines that have high expression of TF appear to be aggressive and have high metastatic potentia. Formation of the TF-FVIIa complex induces signaling that leads to activation of p44/42 mitogen-activated protein kinase and protein kinase B (Akt) pathways and inhibition of apoptosis in breast cancer cells. The Akt-mammalian target of rapamycin (mTOR) pathway regulates cell growth and survival and plays a major role in the pathogenesis of breast cancer. Inhibition of mTOR has been shown to increase TF expression in some cell types which might increase tumor TF expression leading to enhanced TF-mediated signaling as well as an increased hypercoagulable state. Inhibition of mTOR, downstream of Akt, is a recent, emerging strategy in the treatment of breast cancer. In this proposal we test the hypothesis that the TF-VIIa signaling pathway interacts with the mTOR pathway to play a critical role in promoting dysregulated proliferation of breast cancer cells. In the present study, we show that formation of TF-FVIIa-FXa complex induces phosphorylation of mammalian target of rapamycin (mTOR) and p70 S6 kinase in a human breast cancer cell line, Adr-MCF-7. Activation of the mTOR pathway, which is probably mediated by PAR1 and/or PAR2, was associated with enhanced cell migration, a key step in the metastatic cascade. Inhibition of this pathway with the specific mTOR inhibitor, rapamycin, markedly decreased cell migration induced by formation of TF-FVIIa-FXa complex and modestly increased tumor cell TF expression. Targeting the TF-mediated cell signaling pathway along with mTOR inhibition might represent a novel strategy for the treatment of breast cancer.

15. SUBJECT TERMS
Tissue Factor; Factor VIIa; mTOR; Breast Cancer; Cellular Signaling

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Table of Contents

Page

Introduction

Tissue factor (TF) is a 47 kDa transmembrane glycoprotein that complexes with activated FVII (FVIIa) to initiate blood coagulation. Formation of TF-FVIIa complex leads to generation of downstream coagulation proteases that include activated factor X (FXa) and thrombin [1]. In a variety of nonmalignant cell types, TF-FVIIa complex induces phosphorylation of p44/42 mitogen-activated protein kinase (MAPK), p38 MAPK, Akt/protein kinase B (Akt/PKB) [2-4]. Also, TF-FVIIa mediated signaling upregulates expression of multiple genes including: early growth response-1, interleukin-8, cysteine-rich 61 (CCN1), and connective tissue growth factor [5-8]. Recently, studies from our laboratory [9] and others [2,10] demonstrated that formation of either TF-FVIIa-FXa complex or TF-FVIIa complex induces p44/42 MAPK phosphorylation via activation of protease-activated receptors (PARs) in human breast cancer cells. Phosphorylation of p44/42 MAPK was associated with enhanced cell migration, a key step in metastasis [9]. Subsequently, we showed that TF-FVIIa-mediated signaling prevents serum starvation-induced apoptosis of breast cancer cells via activation of both p44/42 MAPK and phosphatidylinositol 3-kinase (PI3K)-Akt/PKB pathways [11]. These findings implicate TF-FVIIa signaling in breast cancer growth and metastasis.

TF-FVIIa signaling involves the PI3K-Akt/PKB pathway, which is linked to cell growth, proliferation, and survival [3,12]. Formation of TF-FVIIa complex in fibroblasts leads to activation of Src family members, c-Src, Lyn, and Yes, followed by activation of PI3K and Akt/PKB [3]. In TF-transfected baby hamster kidney cells and human keratinocytes, formation of TF-FVIIa complex induces phosphorylation of p70/p85 ribosomal protein S6 kinase and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which in turn promotes protein synthesis [12]. That TF-FVIIa complex formation and subsequent PAR activation results in activation of the PI3K-Akt/PKB pathway in several cell types [13], suggests an important role for this pathway in TF-mediated signaling events.

Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that belongs to the PI3K-related kinase family with a central position in multiple cellular signaling pathways that are responsible for regulating cell survival, proliferation, and cytoskeletal organization [14-15]. Two structurally and functionally distinct mTOR complexes have been identified (mTORC1 and mTORC2) [16-18]. mTORC1 consists of mTOR, mLST8 (also termed G-protein β subunit-like protein) and a regulatory-associated protein of mTOR (raptor), whereas mTORC2 consists of mTOR, mLST8, Sin1, and a rapamycin-insensitive companion of mTOR (rictor). The mTORC1 is rapamycin sensitive and functions to control cell growth through regulation of the 40s ribosomal protein, S6 kinase (p70S6K1), and 4E-BP1 [15]. In contrast, the mTORC2 complex, which is not directly inhibited by rapamycin, regulates the activity of Akt/PKB, protein kinase C α and the actin cytoskeleton [19]. Thus, the PI3K-Akt/PKB pathway is upstream of the mTOR pathway and regulates mTOR signaling, but mTOR can also regulate Akt/PKB activation [20]. Because of it central position in many signaling pathways, dysregulation of the mTOR pathway is thought to play an important role in tumorigenesis and tumor progression [21-22]. Moreover, inhibition of mTOR has been shown to increase TF expression in some cell types which might increase tumor TF expression leading to enhanced TF-mediated signaling as well as an increased hypercoagulable state [23].

Inhibition of mTOR, downstream of Akt/PKB, is a recent, emerging strategy in the treatment of breast cancer. However, a phase II study involving temsirolimus showed only modest activity in heavily pretreated breast cancer patients indicating that combination therapy and/or biomarkers that might predict response to mTOR inhibitors are needed [24]. Inhibition of the TF-VIIa pathway along with mTOR pathway represents a novel therapeutic approach which could be synergistic in promoting breast cancer cell apoptosis.

Body

We showed previously that treatment with the combination of FVIIa (10 nM) and FX (150 nM) leads to formation of TF-FVIIa-FXa complex and induces Akt/PKB phosphorylation in Adr-MCF-7 cells [11]. Moreover, phosphorylation of Akt/PKB induced by the combination of FVIIa and FX could be blocked by using either the specific FXa inhibitor, TAP, or the PI3 kinase inhibitor, LY294002 [11]. Because Akt/PKB is linked with mTOR signaling [14,15,18], we first further characterized the role of FVIIa in leading to Akt/PKB phosphorylation. FVIIa (10 nM) or FX (150 nM) alone did not induce significant Akt/PKB phosphorylation compared with control, untreated cells. In contrast, treatment of the cells with the combination of FVIIa (10 nM) and FX (150 nM) resulted in a nearly 2-fold increase in Akt/PKB phosphorylation. Taken together, these data show that at low concentrations of FVIIa, formation of TF-FVII-FXa complex is required for efficient induction of phosphorylation of Akt/PKB in Adr-MCF-7 cells.

We next investigated whether mTOR phosphorylation occurs due to formation of TF-FVIIa-FXa complex in Adr-MCF-7 cells. Treatment of cells with the combination of FVIIa and FX resulted in a nearly 2-fold increase of mTOR phosphorylation compared with untreated, control cells **(**Figure 1**)**. By contrast, treatment with either FVIIa (10 nM) or FX (150 nM) alone did not induce significant phosphorylation of mTOR. To determine whether FXa generation was required for induction of mTOR phosphorylation, cells were treated with either anti-TF antibody or TAP prior to stimulation with the combination of FVIIa and FX. Treatment with anti-TF antibody led to an 84% decrease of mTOR phosphorylation, whereas, treatment with TAP inhibited 90% of mTOR phosphorylation **(**Figure 2**)**. Furthermore, treatment of the cells with FXa alone (150 nM) induced a 50% increase of mTOR phosphorylation which could be blocked by pretreatment with TAP. Taken together, these data indicate that TF-FVIIa-mediated signaling leads to induction of phosphorylation of mTOR and that generation of FXa is required for efficient signaling at low concentrations of FVIIa.

P70S6K1 is one of the downstream kinases in the mTOR pathway and phosphorylation of this protein reflects activation of the mTOR pathway [14, 24]. To determine whether p70S6K1 phosphorylation is induced by formation of TF-FVIIa-FXa complex in the Adr-MCF-7 cells, quiescent cells were treated either with FVIIa (10nM) or with the combination of FVIIa and FX. Treatment of the cells with the combination of FVIIa and FX led to a nearly 2.5-fold increase of p70S6K1 phosphorylation compared with untreated, control cells. In contrast, treatment of cells with either FVIIa or FX alone did not significantly increase p70S6K1 phosphorylation. To test whether p70S6K1 phosphorylation induced by the combination of FVIIa and FX was dependent on TF and FXa generation,

cells were pretreated with anti-TF antibody and TAP, respectively, and p70S6K1 phosphorylation determined. Treatment of the cells with either anti-TF antibody or TAP led to complete inhibition of p70S6K1 phosphorylation compared with untreated cells. Furthermore, treatment of the cells with FXa resulted in a 3-fold increase of p70S6K1 phosphorylation compared with untreated cells, which was similar to that observed with the combination of FVIIa and FX. These results indicate that activation of mTOR pathway by TF-mediated signaling requires formation of TF-FVIIa-FXa at low concentrations of FVIIa **(**Figure 3**).**

Adr-MCF-7 cells express both PAR1 and PAR2 [9] and these receptors are linked to TF-FVIIa-mediated cellular signaling in several cell types including breast cancer cells [6, 9, 10, 13]. To test whether these receptors mediate phosphorylation of Akt/PKB, mTOR and p70S6K1, quiescent cells were stimulated with either PAR1AP (100 μM) or PAR2AP (200 μM) and phosphorylation of Akt/PKB, mTOR, and p70S6K determined. Treatment of the Adr-MCF-7 cells with PAR1AP led to a nearly 3-fold increase of Akt/PKB phosphorylation, 3.5-fold increase of mTOR phosphorylation, and 2.5-fold increase of p70S6K phosphorylation compared with untreated cells. Similarly, treatment with PAR2AP induced a 2.6-fold increase of Akt/PKB phosphorylation, 4.2-fold increase of mTOR phosphorylation, and 2.3-fold increase of p70S6K phosphorylation. These data indicate that activation of either PAR1 or PAR2 induces phosphorylation of Akt/PKB, mTOR, and p70S6K1. Moreover, these results suggest that PAR1 and/or PAR2 probably mediate mTOR pathway activation induced by formation of TF-FVIIa-FXa complex in Adr-MCF-7 cells.

We showed previously that formation of TF-FVIIa-FXa complex promotes migration of the Adr-MCF-7 cells [9]. Furthermore, activation of PAR2, but not PAR1, stimulated migration of Adr-MCF-7 cells and migration was also dependent upon phosphorylation of p44/p42 MAPK [9]. The mechanisms, however, by which TF-FVIIa signaling enhances cell migration are not fully known. mTOR has been recently shown to mediate cell motility [25,26]. To test whether mTOR pathway activation is involved in migration induced by formation of TF-FVIIa-FXa complex, we first determined the effect of LY294002 and rapamycin on phosphorylation of mTOR and p70S6K1. Phosphorylation of mTOR, and p70S6K1 was increased 1.8-fold, and 2-fold, respectively, by treatment of the cells with the combination of FVIIa and FX compared with untreated, control cells. By contrast, pretreatment with LY294002 (25 μM) led to complete abrogation of p70S6K1 phosphorylation, and an 80% decrease in mTOR phosphorylation compared with cells treated only with the combination of FVIIa and FX (Figure 5). Similarly, pretreatment of the cells with rapamycin (200 nM) led to complete inhibition of mTOR and p70S6K1 phosphorylation (Figure 5). To test whether activation of the mTOR pathway is involved in migration induced by formation of TF-FVIIa-FXa complex, we determined the effect of LY294002 and rapamycin on cell migration using a modified Boyden chamber chemotaxis assay (Figure 6). Treatment of the cells with LY294002 (25 μM) for 30 min, led to complete inhibition of cell migration induced by the combination of FVIIa and FX. Similarly, LY 294002 inhibited migration induced by either FXa or PAR2AP. Inhibition of migration induced by the combination of FVIIa and FX with Ly294002 was dose dependent with an IC_{50} of 8.6 nM . Treatment of cells with rapamycin (25-400 nM) for 30 min did not inhibit migration compared with untreated cells. However, prolonged incubation for 24 hrs with rapamycin (200 nM) markedly decreased migration of the cells induced by the combination of FVIIa and FX, FXa, and PAR2AP. In contrast to the complete inhibition of migration observed with LY294002, treatment of cells with rapamycin maximally inhibited only 68% of migration with an IC_{50} of 147 nM (Fig. 6D). Cell viability was $\geq$ 90 % for the cells treated with rapamycin for 24 hrs. Treatment of the cells with rapamycin (200 nM for 16 hrs) also increased TF expression modestly on Western blot analysis.

The receptor for epidermal growth factor 1 (EGFR1) is overexpressed in many cancers. One important signaling pathway regulated by EGFR1 is the phosphatidylinositol 3'-kinase (PI3K)-phosphoinositide-dependent kinase 1-Akt pathway. Activation of Akt leads to the stimulation of antiapoptotic pathways, promoting cell survival. Akt regulates the mTOR-S6K-S6 pathway to control cell growth in response to growth factors and nutrients. In breast cancer, there is an increasing recognition of the pivotal role played by the EGFR1 and HER2/EGFR2 together with the various downstream signal transduction pathways, in particular the Ras/Raf/Mek/erk1/2 pathway that regulates cell proliferation together with the phosphatidylinositol-3-OH kinase (PI3K)/Akt/mTOR pathway that is implicated in cell survival. We therefore investigated the role of TF and EGFR1 in triple-negative breast cancer (TNBC), this subtype of breast cancer accounts for 15-20% of all breast cancers and is defined as tumors that lack estrogen and progesterone receptors as well as lack HER2/EGFR2 overexpression. TNBC is more biologically aggressive than other breast cancer subtypes and has a poor prognosis. We identified 45 biopsy specimens of TNBC at Temple University Hospital from 2003 to 2008. Eighty per cent of these biopsies (36/45) expressed TF and 49 % (22/45) expressed EGFR1. Western blotting analysis also revealed that TF and EGFR1 overexpressed in three TNBC cell lines (Figure 7). A high level of TF expression was found in the MDA-MB-231 and HCC-1806 cell lines. In contrast, the highest level of EGFR1 expression was in the MDA-MB-468 cell line. These data suggest that expression of TF and EGFR1 is inversely correlated. Therefore, we used an siNRA knock-down approach to probe the relationship of TF and EGFR1 expression in TNBC. SiRNA knock-down experiments revealed that down-regulation of TF expression did not affect EGFR1 expression, vice versa, indicating that expression of TF and EGFR1 in TNBC was not tightly coupled (Figure 8). Several studies have also suggested that intracellular signaling triggered by the TF-FVIIa complex involves FVIIa-mediated cleavage and activation of protease-activated receptor 2 (PAR-2), a member of the PAR family of G-protein-coupled seven-transmembrane receptors consisting of four members (PAR-1–4), thereby leading to subsequent activation of small GTPase pathways. Because TF-FVIIa complex formation leads to FXa generation, which activates PAR1 and PAR2, we tested whether PAR1 AP, PAR2 AP and FXa treatment of serum starved MDA-MB-468 cells would lead to activation of EGFR1. As shown in Figure 9, PAR1 AP, PAR2 AP and FXa (100nM) transactivated EGFR.

To evaluate the possibilities for inhibition of cell proliferation by suppression of TF-FVIIa signaling as well as EGFR1, we performed a MTT assay in MDA-MB-468 cell line. As shown in Figure 10: we found that FVIIa (50nM) enhanced cell proliferation by 46.37% ($P<0.005$) compared with the control (saline). Cetuximab (200ug/ml) and anti-TF antibody (10ug/ml) decreased FVIIa-induced cell proliferation by 57% ($P<0.001$) and 45% ($P<0.001$), respectively. Compared with the control, anti-TF antibody and cetuximab reduced cell proliferation by 11.25% ($P>0.05$) and 32.79% ($P<0.01$), respectively. In the presence of FVIIa, the combination of anti-TF antibody and cetuximab reduced cell proliferation by 68% ($P<0.001$). Compared with cetuximab and anti-TF antibody alone, the combination of two antibodies inhibited cell proliferation by 15.69% ($P<0.001$) and 32.78% ($P<0.001$),

respectively. These data suggest that inhibition of either TF signaling or EGFR1 signaling will suppress tumor cell proliferation, the additive inhibitory effects of cell proliferation were also observed when cell were treated with the combination of inhibitors of TF signaling and EGFR1 signaling. We currently plan to test if inhibition of EGFR1 by Cetuximab would result in suppression of mTOR phosphorylation in TNBC. We will also determine whether activation of mTOR by TF-FVIIa-FX complex is through the transactivation of EGFR1.

Key Research Accomplishments

In summary, we have shown that formation of TF-FVIIa-FXa complex in human breast cancer cells induces activation of the mTOR pathway, which includes activation of the mTORC1 and possibly mTORC2. Activation of the mTOR pathway, which is probably mediated by PAR1 and/or PAR2, was associated with enhanced cell migration, a key step in the metastatic cascade. Expression of TF in TNBC is not correlated with EGFR expression. PAR2 AP can transactivate EGFR and in turn enhance cell proliferation.

Reportable Outcomes

TF-FVIIa signaling induces phosphorylation of mTOR in human breast cancer cells via PAR2 and PAR1 activation. Activation of mTOR by TF-FVIIa complex might be through EGFR transactivation.

1. Zhang XM, Schneider CM, Padmanabhan A, Sterling R, Bromberg ME. Tissue factor expression in triple-negative breast carcinomas. Mod Pathol. 2010; 23 (supplement 1): 80a.
2. Liu, Y and Bromberg ME. Tissue factor and epidermal growth factor receptor 1 expression are not coupled, but linked in tissue factor-factor VIIa-induced cell proliferation in triple negative breast cancer. In prepararti on.

Conclusion

Formation of TF-FVIIa-FXa complex induces phosphorylation of mammalian target of rapamycin (mTOR) and p70 S6 kinase in a human breast cancer cell line, Adr-MCF-7. Activation of the mTOR pathway, which is probably mediated by PAR1 and/or PAR2, was associated with enhanced cell migration, a key step in the metastatic cascade. In addition, transactivation of EGFR1 is also mediated by PAR1 and PAR2 and therefore enhances cell proliferation. The EGFR-PI3 kinase/AKT-mTOR signaling pathway might play a critical role in tumor metastasis and growth.

References

1. Nemerson Y. Tissue factor and hemostasis. Blood 1988;71:1-8.

2. Poulsen LK, Jacobsen N, Sorensen BB, et al. Signal transduction via the mitogen-activated protein kinase pathway induced by binding of coagulation factor VIIa to tissue factor. J Biol Chem 1998; 273: 6228-6232.

3. Verteeg HH, Hoedemaeker I, Diks SH et al. Factor VIIa/tissue factor-induced signaling via Src-like kinases, phosphatidylinositol 3-kinase, and Rac. J Biol Chem 2000; 275: 28750-6.

4. Sorensen BB, Rao LV, Tornehave D, el al. Antiapoptotic effect of coagulation factor VIIa. Blood 2003; 102: 1708-1715.

5. Rao LV, Pendurthi UR. Tissue factor-factor VIIa signaling. Arterioscler Thromb Vasc Biol. 2005; 25: 47-56.

6. Hjortoe GM, Petersen LC, Albrektsen T, et al. Tissue factor-factor VIIa-specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated by PAR-2 and results in increased cell migration. Blood. 2004;103:3029-3037.

7. Camerer E, Rottingen JA, Gjernes E, et al. Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the egr-1 gene. J Biol Chem. 1999;274:32225-32233.

8. Pendurthi UR, Allen KE, Ezban M, Rao LV. Factor VIIa and thrombin induce the expression of Cyr61 and connective tissue growth factor, extracellular matrix signaling proteins that could act as possible downstream mediators in factor VIIa·tissue factor-induced signal transduction. J Biol Chem. 2000;275:14632-14641.

9. Jiang X, Bailly MA, Panetti TS, Cappello M, Konigsberg WH, Bromberg ME. Formation of tissue factor-factor VIIa-factor Xa complex promotes cellular signaling and migration of human breast cancer cells. J Thromb Haemost. 2004;2:93-101.

10. Morris DR, Ding Y, Ricks TK, et al. Protease-Activated Receptor-2 Is Essential for Factor VIIa and Xa-Induced Signaling, Migration, and Invasion of Breast Cancer Cells. Cancer Res. 2006;66:307-314.

11. Jiang X, Guo YL, Bromberg ME. Formation of tissue factor: Factor VIIa-factor Xa complex prevents apoptosis in human breast cancer cells. Thromb Haemost. 2006; 96: 196-201.

12. Versteeg HH, Sorensen BB, Slofstra SH, et al. VIIa/tissue factor interaction results in a tissue factor cytoplasmic domain-independent activation of protein synthesis, p70, and p90 S6 kinase phosphorylation. J Biol Chem. 2002;277:27065-27072.

13. Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci USA 2000; 97: 5255-5260.

14. Guertin DA and Sabatini DM. An expanding role for mTOR in cancer. Trends Mol Med. 2005;11:353-361.

15. Sarbassov dos D, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 2005;17:596-603.

16. Loewith R, Jacinto E, Wullschleger S, et al. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell. 2002;10:457-468.

17. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004;14:1296-1302.

18. Yang Q, Guan KL. Expanding mTOR signaling. Cell Research 2007; 17: 666-81.

19. Jacinto E, Loewith R, Schmidt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6:1122-8.

20. Sarbassov DD, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assemby and Akt/PKB Mol Cell 2006; 22: 159-68.

21. Bjornsti MA and Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer. 2004;4: 335-48.

22. Hynes NE, Boulay, A. The mTOR Pathway in Breast Cancer. J Mammary Gland Biol Neoplasia 2006; 11: 53-61.

23. Guba M, Yezhelyev, Eichhorn ME, Schmid G, Ischenko, Papyan A, Graeb C, Seeliger, H, Geissler EK, Jauch KW, Burns CJ. Rapamycin induces tumor-specific thrombosis via tissue factor in the presence of VEGF. Blood 2005; 105: 4463-9.

24. Chan S, Scheulen ME, Johnson S, Mross K, Cardoso F, Dittrich C, Eiermann W, Hess D, Morant R, Semiglazov V, Borner M, Salzberg M, Ostapenko V, Illiger HJ, Behringer D, Bardy-Bouxin N, Boni J, Kong S, Cincotta M, and Moore L. Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer. J Clin Oncol 2005; 23: 5314-22.

Supporting Data:

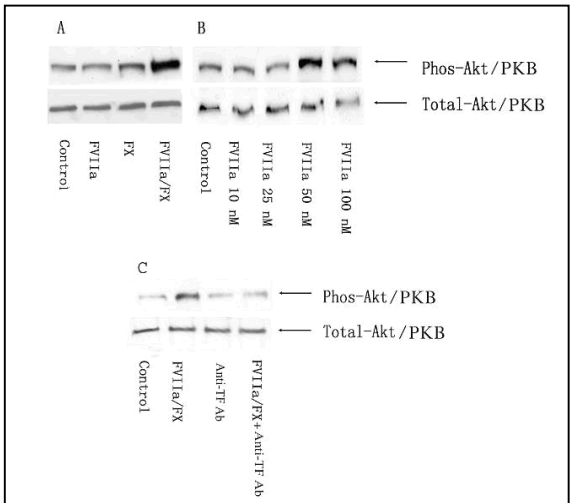

Figure 1. The combination of FVIIa and FX induces Akt/PKB phosphorylation in Adr-MCF-7 cells.

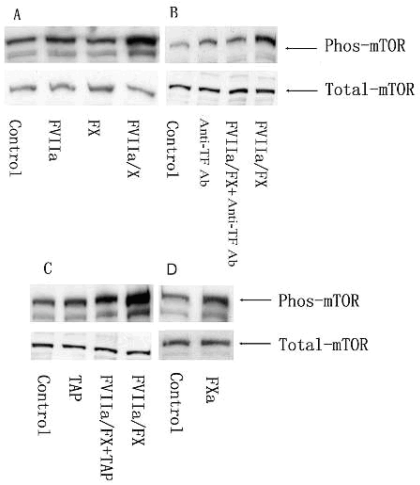

Figure 2: The combination of FVIIa and FX induces mTOR phosphorylation in Adr-MCF-7 cells.

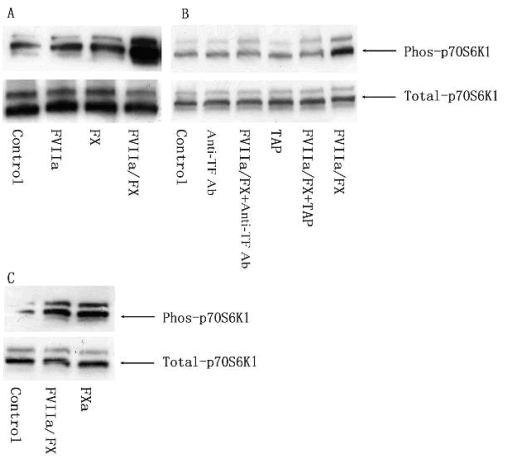

Figure 3: The combination of FVIIa and FX induces phosphorylation of p70S6K1 in Adr-MCF-7 cells.

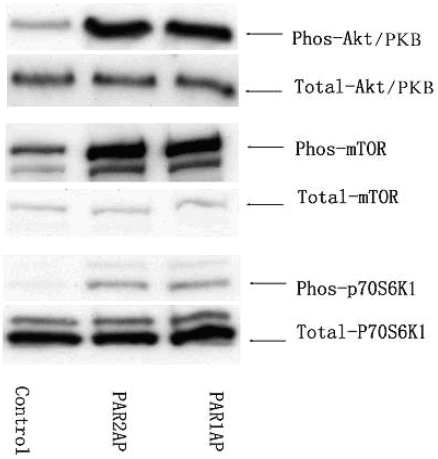

Figure 4: PAR1 agonist peptide (TFFLRN) and PAR2 agonist peptide (SLIGKV) induce phosphorylation of Akt/PKBt, mTOR, and p70S6K1 in Adr-MCF-7 cells.

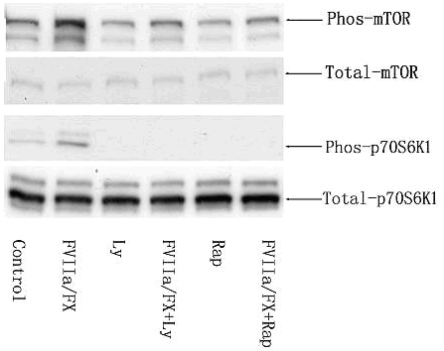

Figure 5: Ly294002 and rapamycin inhibit phosphorylation of mTOR and p70S6K1 induced by the combination of FVIIa and FX in Adr-MCF-7 cells.

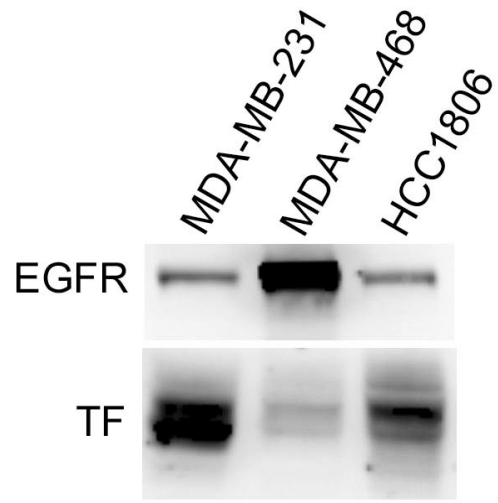

Figure 7: Expression of TF and EGFR in TNBCs.

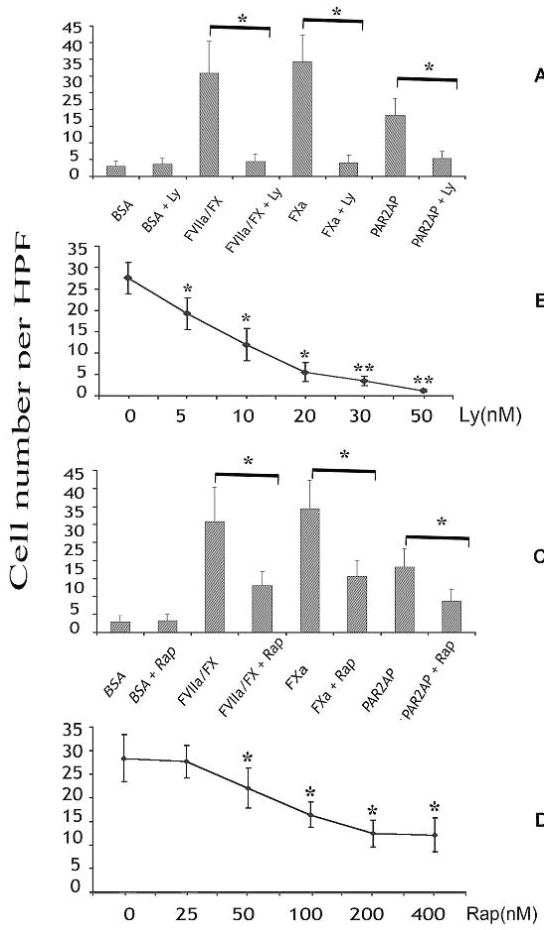

Figure 6: Ly294002 and rapamycin inhibit migration of Adr-MCF-7 cells induced by either the combination of FVIIa and FX, FXa, or PAR2AP.

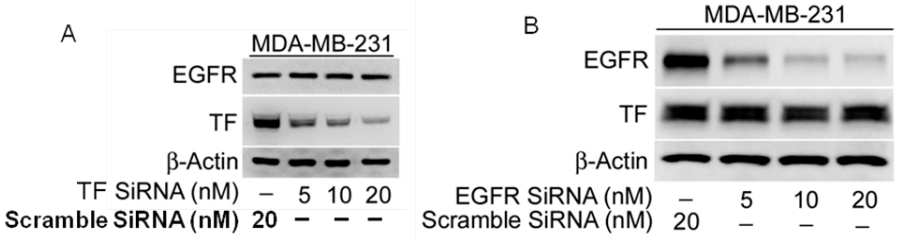

Figure 8: Neither TF or EGFR SiRNA affected expression of EGFR or TF.

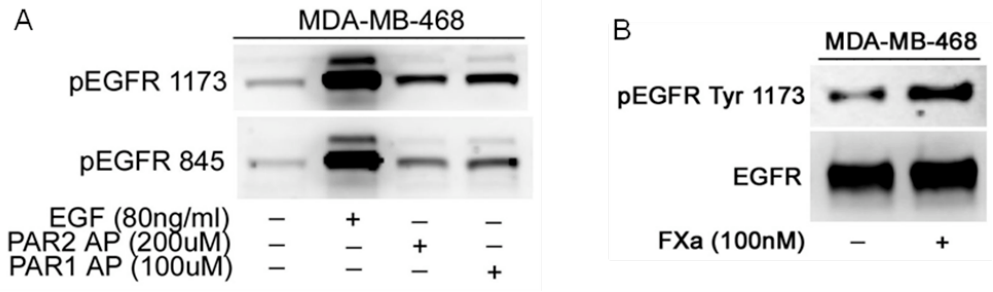

Figure 9: PAR1 AP and PAR2 AP (A), FXa (B) transactivated EGFR.

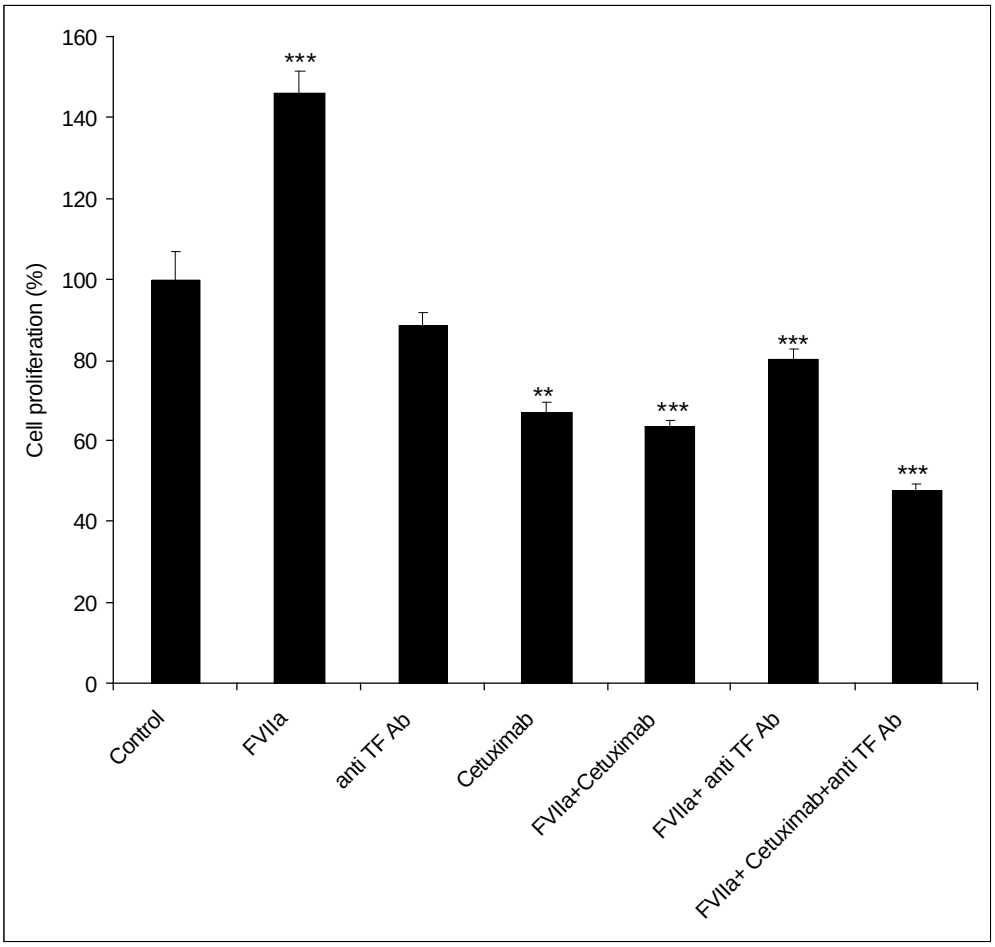

Figure 10: Anti-TF Ab and Cetuximab inhibited cell proliferation.

between these two groups (p=0.2136). The mean ages were 57.43 for node negative tumors and 55.50 for node positive tumors (p=0.2181). There was a significant difference of tumor size between node negative (2.27cm) and node positive tumors (3.55cm), with a p-value less than 0.0001. While we did not observed a significant different for histologic grade between these two groups (p=0.4018); there was a significant difference for nuclear grade between these two groups (p=0.018). There was also difference in HER2 over-expression between these two groups (p=0.0595), but no difference was observed in the expression of ER (p=0.4379) and PR (p=0.6648) between these two groups of tumors.

Conclusions: Tumor size, nuclear grade, and HER2 positivity predict lymph node metastasis in breast carcinomas that have lymphovascular invasion. Tumor type, patient age, histologic grade, and expression of ER and PR are not predictive.

348 Fibroepithelial Lesion with Cellular Stroma: Topoisomerase 2 Is a Helpful Marker To Differentiate Fibroadenoma from Phyllodes Tumor on Needle Core Biopsy

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Background: The differential diagnosis of fibroepithelial lesions with cellular stroma (FELCS) in core needle biopsy (CNB) specimens ranges from fibroadenoma (FA) to phyllodes tumor (PT). The management of these two lesions is different. We intended to explore possible morphologic and immunohistochemical (IHC) parameters that may predict the final diagnosis on the excisional biopsy.

Design: A series of FELCS cases diagnosed on CNB with matching excisional biopsy were retrieved from our files between 2003 and 2009. The following histologic parameters were recorded: stromal cellularity (1, 2, or 3), stromal cell atypia (1, 2, or 3), stromal cell mitosis per ten high power fields, stroma overgrowth, infiltrative edge, stromal cellularity enhanced at epithelium and leaf-like pattern. Patients' age and tumor size were also recorded. The following IHC stains were performed on CNB: KI-67 (clone MIB-1), p53 (clone DO7) and Topoisomerase 2 (TOPO2) (clone EP1054Y). Percentage of positive cells was recorded. Fisher's exact test and Wilcoxon non-parametric test were used for statistical analyses.

Results: The table below illustrates all the findings.

Histologic, Clinical and IHC Finding

	FA (n=8)	PT (n=12)	p value
Age (y), median (range)	42 (35-48)	47 (30-82)	NS
Size (cm), median (range)	1.35 (0.5-2.5)	3.45 (0.5-15)	0.022
Stromal cellularity			
1 or 2	7 (87.5)*	8 (66.7)	NS
3	1 (12.5)	4 (33.3)	NS
Stromal cell atypia			
1	6 (75.0)	5 (41.7)	NS
2 or 3	2 (25.0)	7 (58.3)	NS
Stromal cell mitosis			
No	6 (75.0)	8 (66.7)	NS
Yes	2 (25.0)	4 (33.3)	NS
Stromal overgrowth			
No	5 (62.5)	6 (50.0)	NS
Yes	3 (37.5)	6 (50.0)	NS
Infiltrative edge**			
No	8 (100.0)	6 (66.7)	NS
Yes	0 (0.0)	3 (33.3)	NS
Stromal cellularity enhanced at epithelium			
No	8 (100.0)	11 (91.7)	NS
Yes	0 (0.0)	1 (8.3)	NS
Leaf-like pattern			
No	6 (75.0)	5 (41.7)	NS
Yes	2 (25.0)	7 (58.3)	NS
Median (range) Ki-67 (%)	5 (0-20)	10 (1-50)	NS
Median (range) p53 (%)	2.5 (0-90)	20 (0-80)	NS
Median (range) TOPO2 (%)	0 (0-2)	2 (0-10)	0.037

* No. (%), ** n=9 for PT

Conclusions: Larger clinically measured tumor size and presence of Topoisomerase 2 staining can predict PT on CNB.

349 Breast Hormonal Receptors Test Should Be Repeated on Excisional Biopsies after Negative Core Biopsy

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Background: Accurate estrogen receptor (ER) and progesterone receptor (PR) results are important for therapeutic decision making for patients with breast carcinoma. The purpose of this study was to assess the concordance of breast cancer immunohistochemical receptor assays on core biopsy and surgical specimens.

Design: We identified 176 patients whose core biopsy was performed either at Roswell Park Cancer Institute (RPCI) or at an outside facility between 2007 and 2009. Surgical specimens were processed in RPCI. ER and PR, for biopsies and excisions, were scored using Allred scoring system. While biopsies were processed in 12 different laboratories, stained in 5 different laboratories using 3 different vendors, the excisional biopsies were processed and stained in RPCI using one vendor (Dako). While the following antibodies were used for ER, 1D5, 6F11 and SP1, the following antibodies were used for PR, PgR636, 16 and 1E2 from Dako, Leica and Ventana respectively. Correlation of scores of biopsies with matching excision was analyzed using Spearman correlation coefficient test.

Results: Seventeen (9.7%) patients were biopsied in RPCI and 159 (90.3%) patients in an outside facility. While there were 141 (80.1%) cases positive for ER and 118 (67%) cases positive for PR for the core biopsy, there were 143 (81.3%) cases positive for ER and 130 (73.9%) cases positive for PR for the excision. Concordance for ER and PR was seen in 93% and 89.8% respectively. Table illustrates the concordance between biopsy and excision for both markers based on vendors. Spearman correlation coefficient between biopsy and excision was 0.75 for ER and 0.79 for PR (p<0.0001 each).

Table: Comparison of ER and PR between biopsy and excision based on Vendors.

	Dako (No. 23)	Leica (No. 124)	Ventana (No. 29)	P value
ER: BX+EX+	16 (69.6)*	100 (81.3)	20 (66.7)	0.11
ER: BX+EX-	0 (0)	3 (2.4)	2 (6.7)	
ER: BX-EX+	3 (13)	3 (2.4)	1 (3.3)	
ER: BX-EX+	4 (17.4)	17 (13.8)	7 (23.3)	
PR: BX+EX+	13 (56.5)	85 (69.1)	17 (56.7)	0.48
PR: BX+EX-	0 (0)	2 (1.6)	1 (3.3)	
PR: BX-EX+	2 (8.7)	11 (8.9)	2 (6.7)	
PR: BX-EX+	8 (34.8)	25 (20.3)	10 (33.3)	

* Number (percentage); BX, biopsy; EX, excision

Conclusions: Although there was no uniformity in biopsies processing or staining, practically speaking, ER and PR should be repeated on the excisional biopsies for patients whose core biopsies have negative hormonal receptor.

350 Tissue Factor Expression in Triple-Negative Breast Carcinomas

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Background: Tissue factor (TF) is expressed in a variety of tumor cells and has been linked to cellular signaling, angiogenesis, and tumor progression. However, its role in human cancer is not fully known. Recently, upregulation of TF has been linked to expression of epidermal growth factor receptor (EGFR), a prognostic factor of breast cancer. Triple-negative (ER, PR and HER-2) breast carcinomas (TNBC) belong to a subgroup of breast cancer with aggressive clinical behavior and poor prognosis.

Design: Forty-five cases of TNBC diagnosed from 2003 to 2008 were retrieved from the archive of the Department of Pathology of Temple University. Adequate tissue was available in 41 cases that formed the basis of this study. All patients were female with an age range from 32 to 81. Immunohistochemistry for CK5/6, EGFR and TF was performed, and results were scored as positive (tumor cells stained) or negative (no tumor cell stained). Basal-like carcinoma (BLC) was defined as a TNBC positive for CK5/6 and/or EGFR.

Results: 20 (49%) of patients presented with regional lymph node metastasis, 9 (22%) demonstrated distal metastasis and 24 (59%) had advanced clinical stage (III/IV). All cases were invasive ductal carcinoma (IDC), except for one adenoid cystic carcinoma. 36 of the 40 IDC were histologically high grade and the remaining 4 were intermediate grade. BLC was identified in 36 of the 41 (88%) cases, among which 21 (58%) were positive for both CK5/6 and EGFR, 14 (39%) were positive for only CK5/6 and 1 (3%) was positive only for EGFR. The remaining 5 cases were negative for both markers (non basal-like carcinoma, NBLC). Overall, TF expression was found in 35 of the 41 (85%) cases. TF expression was detected in 31 of 36 (86%) BLC and in 4 of 5 (80%) NBLC. With respect to EGFR expression, 18 of the 22 (81%) cases positive for EGFR expressed TF, however TF expression was also found in 4 of 6 (80%) cases that lacked EGFR expression.

Conclusions: TF expression was found in the majority of cases of TNBC (88%), suggestive that TF expression is linked with the aggressive tumor behavior and poor prognosis of the patients with TNBC. Tumor TF expression was similar in BC (86%) and NBC (80%). However, a close association between TF and EGFR expression was not observed. Further study is warranted to explore the clinical significance of TF expression and its association with EGFR expression in this breast cancer subtype.

Cardiovascular

351 Causes-Mechanisms of Death Following Stage I Repair for Hypoplastic Left Heart Syndrome (HLHS)

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Background: Staged hybrid repair is a recent advancement in treatment of HLHS. Stage I (of 3 stages) includes balloon atrial septostomy and ductus arteriosus stenting (by catheterization) as well as surgical pulmonary artery banding. The causes-mechanisms of death between the stage I and II procedures have not been well-documented at autopsy.

Design: Autopsy reports and microscopic slides were reviewed from 13 consecutive patients (August 2002 - August 2009) who died after first stage repair for HLHS (6 males and 7 females). Causes-mechanisms of death as well as nonfatal complications were recorded.

Results: The mean age at death was 60 days (range 9 to 180 days). Six deaths occurred at < 30 days following stage I repair (group 1) and 7 deaths occurred at > 30 days (group 2). Eleven patients had the complete stage I repair, one had only atrial septostomy and one had only ductus arteriosus stenting. Autopsy permit included no restrictions (n=7), chest and abdomen only (n=2) or chest only (n=4). The causes-mechanisms of death in group 1 were ductus arteriosus closure (n=1, patient had only atrial septostomy), left atrial appendage tear (n=1, occurred during atrial septostomy), arrhythmias that developed during the procedure (n=3) and pneumonia (n=1). Two of the patients with arrhythmias had a myocardial substrate that may have predisposed to arrhythmia (fibrosis and myocyte disarray). The causes-mechanisms of death in group 2 were intestinal necrosis (n=1), myocardial necrosis due to insufficient retrograde flow into the aortic arch (n=1), pneumonia (n=2), pulmonary emboli (n=1), pulmonary vein stenosis (n=1) and sudden death without documented arrhythmia (n=1). Aorta and/or vena cava thrombosis or a significant thromboembolic event were found in four group 2 patients.

Conclusions: Intraprocedural events (arrhythmia, left atrial tear) accounted for 67% of deaths in group 1 patients. Pneumonia and ischemic necrosis (heart, intestines) accounted for the majority of deaths (57%) in group 2. Thrombotic / thromboembolic