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ORIGINAL ARTICLE

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TACE: a new target in epidermal growth factor receptor dependent tumors

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Abstract Soluble proteins play vital roles in mediating intercellular communication. Many of these proteins are secreted as freely soluble molecules, but an important class of signaling proteins are first synthesized and presented at the cell surface as transmembrane precursor proteins. Unlike classically secreted proteins, many of these molecules are regulated at an additional level, requiring proteolytic cleavage for activity. This review focuses on a subset of these proteins, which are cleaved by tumor necrosis factor alpha-converting enzyme (TACE)/ADAM17, and on their role in cancer.

Key words breast cancer · TACE · EGFR · metalloproteinases · cancer therapy

Introduction

Metalloproteinases comprise 186 of the 561 proteases encoded by the human genome (Puente and Lopez-Otin, 2004) and they play diverse roles in development, homeostasis and disease. Prominent families of metalloproteinases include the matrix metalloproteinases (Egeblad and Werb, 2002; Page-McCaw et al., 2007), the ADAMs (White, 2003), the ADAMTSs (Jones and Riley, 2005) and the metallocarboxypeptidases (Reznik and Fricker, 2001). This review describes one of these metalloproteinases, tumor necrosis factor alpha-converting enzyme (TACE)/ADAM17, which plays a key role in epidermal growth factor receptor (EGFR) signaling and has recently emerged as a new therapeutic target in several tumor types.

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ADAM family proteins

ADAM family members share many common structural features, notably an N-terminal signal peptide, followed by a pro-domain, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain and transmembrane and cytosolic domains (Fig. 1). The cytoplasmic tail of ADAMs frequently contains signaling motifs, such as binding sites for SH3-domain containing proteins. The pro-domain of ADAM proteins plays an important role in regulating the catalytic activity. It acts as an intermolecular chaperone, which prevents enzyme activity. Activation of ADAMs typically requires cleavage by a pro-protein convertase enzyme, which releases the pro-domain and exposing the active site. The first ADAM proteins (ADAM 1 and 2) were identified in Judith White's laboratory as the two subunits of the sperm protein, Fertilin (Blobel et al., 1990, 1992; Wolfsberg et al., 1993). ADAMs were then found to be widely expressed in eukaryotes, and there are 23 ADAM family members in humans (reviewed in Blobel, 2005).

Identification of TACE

The quest to discover the proteinase responsible for the shedding of the important pro-inflammatory cytokine, tumor necrosis factor α (TNFα), led to the identification of TACE/ADAM17 in 1997 (Black et al., 1997; Moss et al., 1997). Mice carrying targeted deletions of the zinc-binding domain of TACE died perinatally and had a significantly wider range of defects that was expected from previous analyses of mice null for TNFα or its receptors (Peschon et al., 1998). These mice had curly vibrissae, a failure in eyelid fusion and defects in the morphogenesis of several epithelial organs, a phenotype reminiscent of that of mice null for the EGFR ligand, TGFα (Luetteke et al., 1993). TACE was demonstrated to be the TGFα sheddase (Peschon et al., 1998) and has

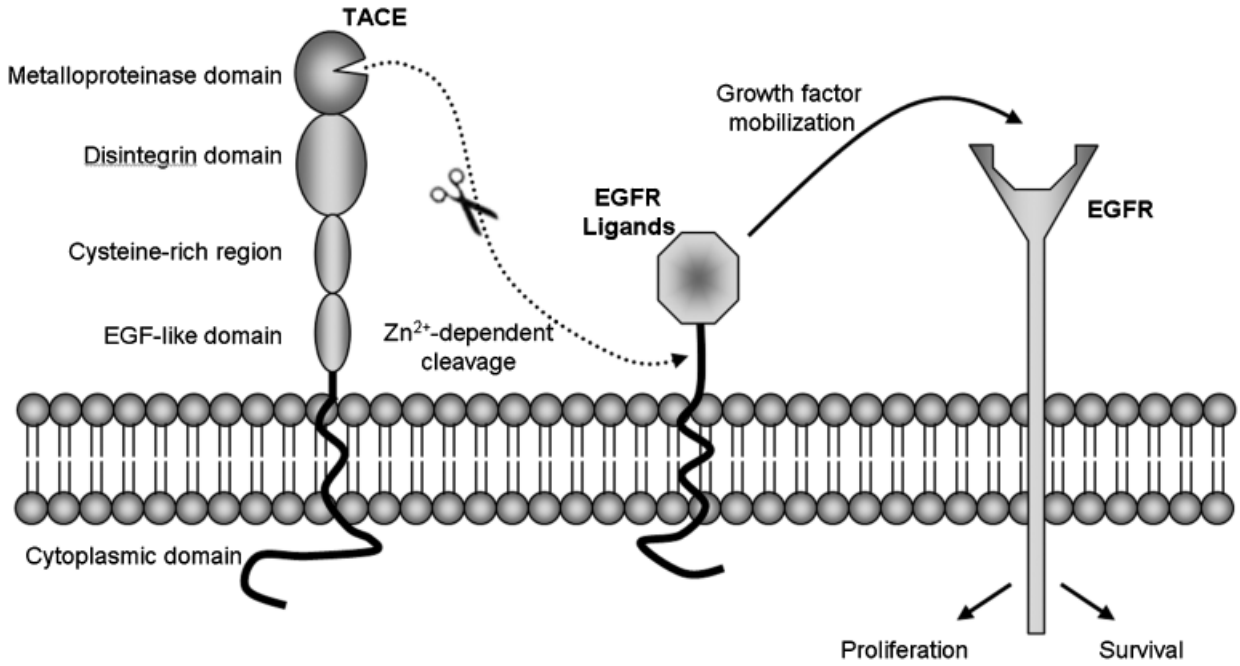

Fig. 1 Tumor necrosis factor alpha-converting enzyme (TACE)-dependent ligand cleavage and activation of epidermal growth factor receptor (EGFR). A schematic representation of catalytically active TACE is shown (the pro-domain has been removed). TACE cleaves several EGFR ligands in a zinc-dependent manner, releasing them from the cell surface so that they can interact with their receptor.

since been reported to shed several additional important proteins from the cell surface, including the other EGFR ligands Amphiregulin and HB-EGF (Sunnarborg et al., 2002), L-selectin and TNFRII (Peschon et al., 1998), β-APP (Buxbaum et al., 1998), collagen XVII (Franzke et al., 2002), growth hormone receptor (Zhang et al., 2000), TrkA (Diaz-Rodriguez et al., 2002), ErbB4 (Rio et al., 2000) and GPIbα (Bergmeier et al., 2004). Normal mammary gland development is an EGFR-dependent process and the ligand, Amphiregulin, plays a key role (Luetteke et al., 1999). Tissue recombination experiments have shown that Amphiregulin and TACE are co-expressed in the epithelium and that activation of stromally expressed EGFR is crucial for mammary development (Sternlicht et al., 2005).

TACE-dependent EGFR ligand shedding in cancer

EGFR was the first receptor tyrosine kinase to be identified (Ullrich et al., 1984) and it has been shown to be amplified and overexpressed in tumors of many tissues. EGFR and its downstream signaling pathway is a key regulator of cell proliferation and it is frequently deregulated in cancer. Depending on the tissue of origin, the mechanism by which the pathway is activated varies. Mutations in the small GTPase Ras proteins are particularly common in pancreatic, thyroid and colorectal tumors (Downward, 2003) while mutations in B-Raf are prevalent in melanoma (Davies et al., 2002). Activating mutations in the receptor itself have been reported in tumors of several tissues, especially non-small cell lung cancer (NSCLC, Lynch et al., 2004; Paez et al., 2004; Perez-Soler et al., 2004) and glioblastomas (Frederick et al., 2000), while receptor amplification occurs frequently in NSCLC (Hirsch et al., 2003), glioblastomas (Frederick et al., 2000) and, at lower frequency, in breast tumors (Bhargava et al., 2005) and squamous cell carcinomas of the head and neck (Chung et al., 2006). Each of these mechanisms of activation requires genomic changes. The activity of the pathway can also be increased by overproduction of ligands, resulting in hyperstimulation of the receptor. EGFR can be activated by several ligands: Amphiregulin, Betacellulin, EGF, Epigen, Epiregulin, HB-EGF and TGFα (reviewed in Holbro and Hynes, 2004). Of these, Amphiregulin HB-EGF, Epigen, Epiregulin and TGFα have been reported to be cleaved by ADAM17/TACE, while EGF and Betacellulin are substrates of ADAM10 (Sahin et al., 2004; Sahin and Blobel, 2007). Overexpression of these ligands is a common event in many tumors and frequently correlates with poor prognosis (Nicholson et al., 2001).

The conventional approach for targeting this pathway has been to develop specific small molecular inhibitors of the kinases (e.g., Iressa, Tarceva, BAY43-9006) or to generate blocking antibodies (e.g., Cetuximab and Trastuzumab) which bind to epitopes on the extracellular domain of the receptors and interfere with functions such as ligand binding or dimerization. Recent data from a number of laboratories suggest that using specific protease inhibitors to limit ligand mobilization

is a viable alternative approach which may prove particularly useful in those tumors dependent on stimulation of the receptor by ligands (as opposed to those tumors in which activation of the pathway has been achieved, e.g., via Ras mutations).

Ullrich and colleagues examined the role of G-protein coupled receptor induced TACE activation in the cleavage of Amphiregulin in head and neck squamous carcinoma cells (HNSCC). Using both broad spectrum inhibitors and siRNA-mediated suppression of TACE, they showed that TACE-dependent Amphiregulin shedding played an important role in activation of the EGFR pathway and cell proliferation (Gschwind et al., 2003). Grandis and co-workers implicated Src-mediated phosphorylation of TACE as a key intermediate step between GPCR stimulation and TACE activation (Zhang et al., 2006).

Lee and co-workers have reported that TGFα upregulation is a frequent event in renal carcinoma cells deficient in the Von Hippel Lindau tumor suppressor. Inhibition or suppression of TACE in this model attenuated TGFα shedding, EGFR phosphorylation and cell migration and proliferation (Franovic et al., 2006). Importantly, these workers showed that stable suppression of TACE using siRNAs significantly attenuated the growth of these tumor cells in xenografts.

The role of TACE in the shedding of ErbB ligands has been further explored in non-small cell lung cancer by scientists from Incyte Corporation which has a number of promising TACE inhibitors in the pipeline. In NSCLC cell line models, Zhou and colleagues reported that TACE expression correlated with shedding of both Heregulin and TGFα, which played key roles in NSCLC proliferation and response to ErbB inhibitors. These workers used potent and selective TACE inhibitors to demonstrate efficacy of this approach in an *in vivo* xenograft model both as a single agent and in combination with Paclitaxel (Zhou et al., 2006). Similar data have been reported in breast cancer xenograft models, and these inhibitors were well tolerated even at doses far greater than required for anti-tumor efficacy (Fridman et al., 2007).

Our interest in TACE arose from our investigations into the mechanisms by which a breast cancer cell line, the T4-2 subline of HMT3522, acquired the ability to grow without exogenous EGF. HMT3522 was derived from a reduction mammoplasty and several sublines have been propagated. S1 cells are absolutely dependent on exogenous EGF for proliferation (Briand et al., 1987) and form small, spherical, polarized colonies with a central lumen when grown in a 3D Matrigel culture assay (Petersen et al., 1992). A subline derived from S1, termed S2, was selected for the ability to grow in the absence of exogenous EGF (Madsen et al., 1992). These cells form disorganized colonies in 3D Matrigel culture, but do not form tumors in nude mice. After 120 passages of S2, a new variant emerged, termed T4-2, which had acquired the ability to form tumors in nude mice (Briand et al., 1996). Thus, the HMT-3522 series represents a progression from non-malignant to malignant breast epithelial cells from the same patient. A considerable body of work form the Bissell laboratory has explored the specific signaling defects upon which the malignant phenotype of these cells depends. This work has shown that the malignant phenotype of these cells can be ''reverted'' in a 3D culture model by inhibition of key molecules such as the β1-integrin ECM receptor (Weaver et al., 1997), and, as one might expect from the way in which the cells were selected, upon inhibition of EGFR (Wang et al., 1998) and downstream signaling intermediates such as PI3-kinase (Liu et al., 2004) and MAPK (Wang et al., 1998). Because of the strong dependence on EGFR signaling, this system represents an excellent model to study the contribution of this pathway to malignancy.

Sequencing of commonly mutated proto-oncogenes in T4-2 cells (H-Ras, K-Ras, N-Ras, EGFR and PI-K3CA) revealed that all were wild type. The lack of proto-oncogene mutations and the ability to revert the malignant phenotype of the cells using EGFR blocking antibodies suggested that the cells had acquired the ability to produce their own EGFR ligands. Screening for all ErbB interacting ligands revealed that two, Amphiregulin and TGFα, are upregulated in the malignant T4-2 cells (Kenny and Bissell, 2007). Because they were both reported TACE substrates (Peschon et al., 1998; Sunnarborg et al., 2002), we sought to determine whether the malignant phenotype of these cells could be reverted by TACE inhibition. Initially, we showed that a metalloproteinase inhibitor, TAPI-2, reverted the malignant phenotype of these cells, restoring the normal mammary acinar organization (Figs. 2A–2D) and suppressed ligand shedding. Cells treated with the TACE inhibitor formed small, smooth colonies (Fig. 2B) in which mammary epithelial polarization has been restored (Fig. 2D), an identical phenotype to EGFR inhibition in this model (Wang et al., 1998). This treatment reduced the activity of kinases in the EGFR pathway (Fig. 2E). Specific ablation of TACE using siRNA also reduced shedding of the ligands (Fig. 2F) and reverted the malignant phenotype of T4-2 cells in 3D culture, mimicking the effect of the more broad-spectrum protease inhibitor (Fig. 2G). This implicates TACE as the key target of the protease inhibitor in this system. Targeting TACE with specific siRNAs also resulted in the restoration of mammary epithelial polarity (Fig. 2G, right inset). No striking morphological effects were observed in similarly treated cells cultured on plastic substrata (Fig. 2G, left inset) which demonstrates the utility of the 3D culture system for evaluating tumor-relevant phenotypes which are not readily distinguished in cells cultured using more conventional methods. Because TACE is a promiscuous protease with many substrates, we made stable cell lines

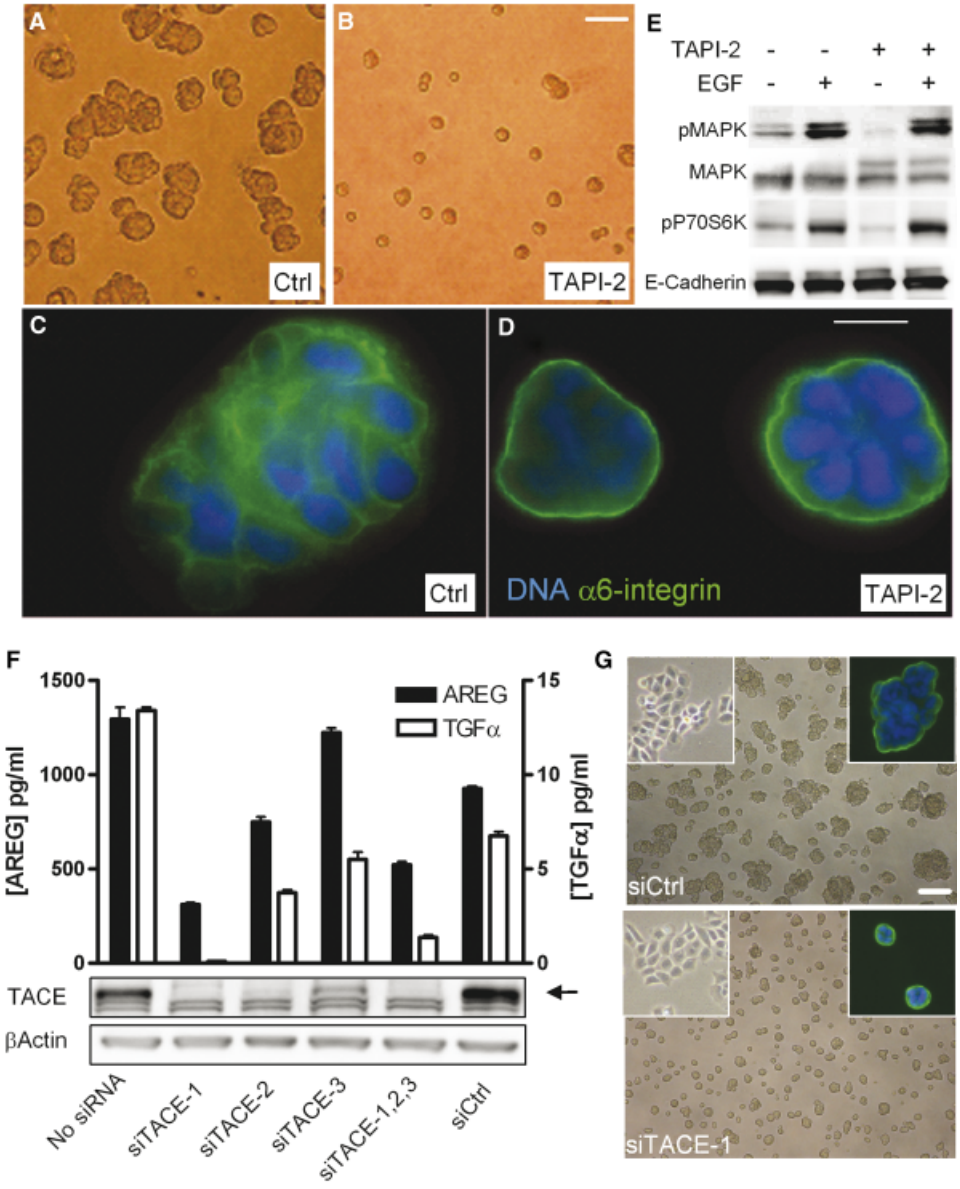

Fig. 2 Inhibition of tumor necrosis factor alpha-converting enzyme (TACE) activity "reverts" the malignant phenotype of T4-2 cells by suppressing mobilization of growth factors and down-regulating epidermal growth factor receptor (EGFR) pathway activity. (**A**) T4-2 cells grown in 3D lrECM cultures form continuously proliferating, disorganized, and apolar colonies. (**B**) T4-2 cells treated with a broad-spectrum MMP/ADAM inhibitor (20 μM TAPI-2) undergo a morphological reversion similar to that of EGFR inhibitor-treated cells. Bar = 100 μm. (**C**) α6-integrin staining of vehicle-treated T4-2 cells indicates absence of tissue polarity. (**D**) α6-integrin staining of TAPI-2-treated T4-2 cells shows restoration of tissue polarity. Bar = 10 μm. (**E**) TAPI-2 treatment (24 hr) reduces the basal activity of kinases downstream of EGFR, but cells remain competent to respond to exogenous EGF (860 pM, 5 min stimulation). (**F**) ELISA analysis of EGFR ligand shedding in T4-2 cells transfected with three siRNA oligos, either individually or as a pool. Ligand shedding is proportional to the level of TACE expression. (**G**) Reversion of the malignant phenotype of T4-2 cells in 3D lrECM culture following transfection of siRNA against TACE. The most efficient siRNA (si-TACE-1) was used. Left insets: phase contrast micrographs of transfected cells grown on plastic. Right insets: α6-integrin immunostaining of representative colonies (reproduced with permission from Kenny and Bissell, 2007).

expressing mutants of Amphiregulin and TGFα which did not require proteolytic activity for release from the cell surface. These cell lines were susceptible to the inhibitor of EGFR, but resistant to the TACE inhibitor. These latter data argue that, at least for this cell line, while the cleavage of all other TACE substrates is attenuated, it is the prevention of the cleavage of EGFR ligand which mediates the potent anti-tumor effect (Kenny and Bissell, 2007).

Collectively, these biochemical, cell biology and *in vivo* studies provide compelling evidence that TACE is a key player in the EGFR pathway which can be inhibited using existing drugs thereby preventing ligand mobilization and attenuating receptor activity. What then is the evidence that TACE plays a role in spontaneous human breast cancers? To address this question, we examined the expression levels of TACE and two key ligands, Amphiregulin and TGFα, in 295 human breast cancer patients (van de Vijver et al., 2002). In agreement with previous reports describing it as an ERα target gene (Martinez-Lacaci et al., 1995), Amphiregulin was most frequently expressed at high levels in ERα positive tumors, and high levels of Amphiregulin correlated with good outcome. The positive outcome in these patients is not surprising as, like the expression of another ERα target, progesterone receptor, Amphiregulin positivity may be taken as a hallmark of a functional estrogen receptor and so these patients are more likely to respond to existing drugs which effectively target this receptor and block transcription of its target genes. In contrast, TGFα expression was found primarily in ERα-negative tumors and was significantly over-represented in tumors of the

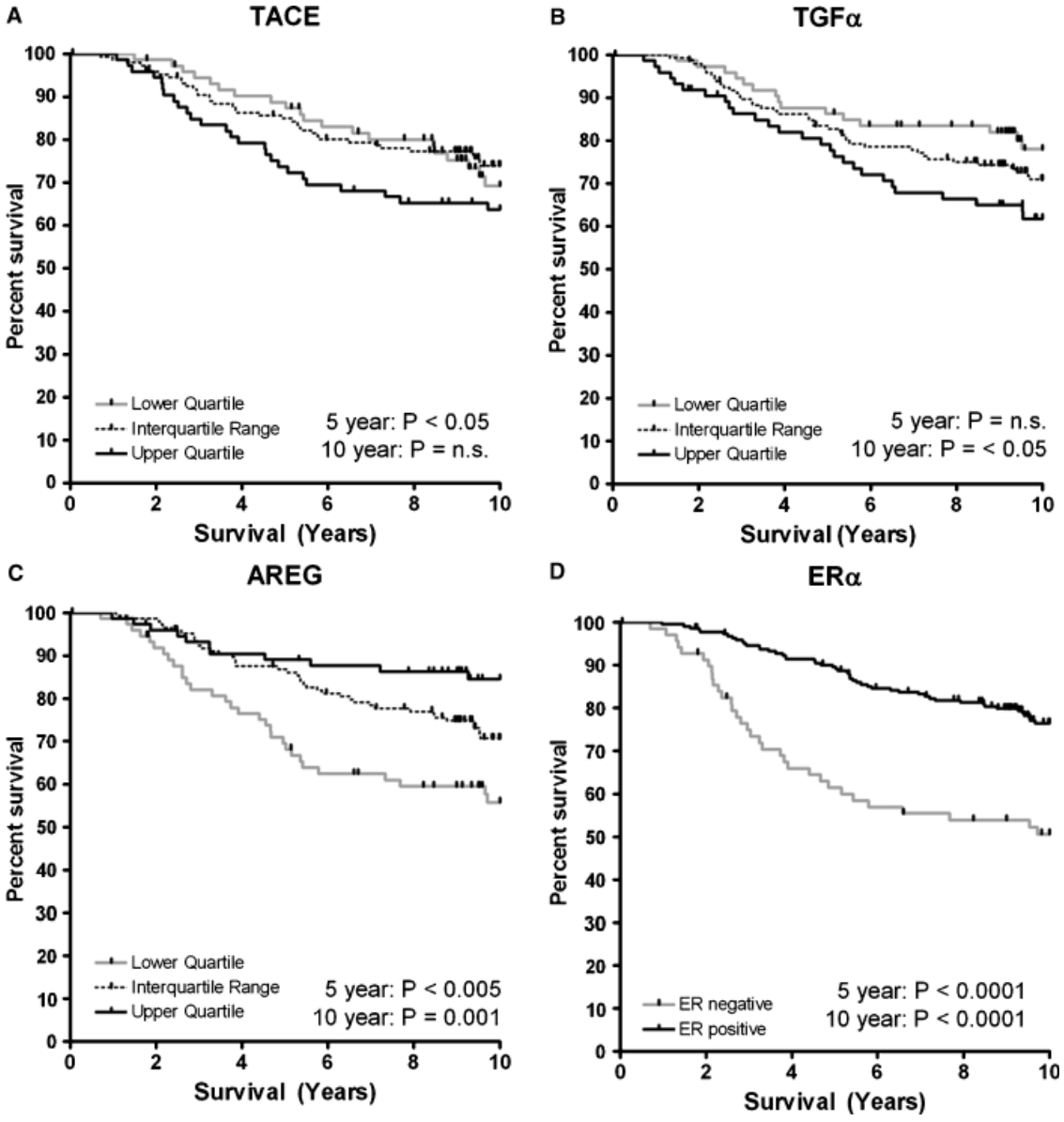

Fig. 3 Kaplan–Meier survival analysis of 295 human breast tumors stratified by marker expression level. High levels of (**A**) tumor necrosis factor alpha-converting enzyme (TACE) and (**B**) TGFα predict poor survival. High levels of (**C**) Amphiregulin or (**D**) ERα are correlate with good outcome (the latter two are related, see Discussion). p-values represent the log-rank comparison between the upper and lower quartiles of marker expression, evaluated at 5 and 10 years postsurgery (reproduced with permission from Kenny and Bissell, 2007).

basal subclass (Kenny and Bissell, 2007). These tumors also had the highest levels of TACE, and this patient group had the poorest outcome (Fig. 3). Studies of TACE protein expression have been reported in smaller patient cohorts. In a study of 36 patients, TACE was shown to be frequently expressed at elevated levels in breast tumors compared with matched normal samples (Borrell-Pages et al., 2003).

The co-expression of TACE and its ligand, TGFα, in the basal subclass of tumors (Kenny and Bissell, 2007) suggests the possibility of targeting TACE-dependent TGFα shedding in this disease. These tumors are often referred to as ''triple-negative'' as they typically lack expression of estrogen receptor, progesterone receptor and HER2 and are thus unresponsive to many of the targeted agents which have proved so useful in other breast cancer subtypes and often have a poor outcome (Sorlie et al., 2001; van de Rijn et al., 2002; Potemski et al., 2005; Carey et al., 2006). Basal-like tumors are frequently EGFR positive (Rakha et al., 2007), suggesting that this pathway may be a viable target to explore in this disease, either by inhibiting the receptor itself, or blocking the shedding of TGFα using TACE inhibitors, or a combination of the two strategies.

Although HER2 appears to be the predominant ErbB family member involved in breast cancer patho-

genesis, the advent of potent FDA-approved small molecule inhibitors of EGFR, and the fact that EGFR is expressed in as many as half of all breast cancers has resulted in a series of Phases I and II studies using either an EGFR inhibitor alone or in combination with an established cytotoxic chemotherapy. Most of these trials have been performed with Iressa, although there have been case reports and small studies using Erlotinib (Tan et al., 2004; Catania et al., 2006). No Phase III studies have been reported to date, and many of the studies have involved heavily pre-treated patients with advanced metastatic disease. Nevertheless, it is encouraging that partial responses and maintenance of stable disease have been reported for Iressa—either as monotherapy or in combination with other agents (Polychronis et al., 2005; Ciardiello et al., 2006), although other studies have reported no significant benefit (Baselga et al., 2005; von Minckwitz et al., 2005). The size of the studies to date have been too small to determine whether subsets of patients, for example those with "triple-negative" tumors, are more likely to be among the responders. Accordingly, prospective studies will be necessary to determine whether therapies targeted at EGFR pathway activity have a benefit in this patient group.

TACE inhibitors in the clinic

Because of its implication as the sheddase of TNFα, an important pro-inflammatory cytokine, several orally active TACE inhibitors have been developed with the aim of treating rheumatoid arthritis (Smolen and Steiner, 2003). These include Ro 32-7315 from Roche (Basel, Switzerland) (Beck et al., 2002), TMI-1 from Wyeth (Madison, NJ) (Zhang et al., 2004) and GW3333 from GlaxoSmithKline (London, UK) (Conway et al., 2001). The first three compounds performed well in animal models of arthritis, but efficacy in human clinical trials was disappointing. For example, Apratastat (closely related structurally to TMI-1) was well tolerated in Phase I studies and demonstrated efficacy by reducing endotoxin-induced TNFα shedding; however, Phase II studies failed to show efficacy in rheumatoid arthritis and further development was discontinued by Wyeth in October 2006 (Thabet and Huizinga, 2006).

While the failure of these drugs to achieve clinical efficacy in rheumatoid arthritis patients is disappointing, it must be remembered that many factors in addition to TNFα contribute to inflammation in this disease (Lee and Weinblatt, 2001), thus the failure of an agent targeting just one of these processes might not be unexpected. It is encouraging that, where biological markers of TACE inhibition (such as reduced levels of TNFα) were measured, the drugs were clearly pharmacologically active *in vivo*. Therefore, it seems reasonable to propose that the existing drugs are sufficient to inhibit TACE and, in principle, such a study could be carried out in cancer patients. A number of compounds have been developed by Incyte (Wilmington, DE), including INCB3619, which are potent, orally active TACE inhibitors (Zhou et al., 2006). These have shown efficacy as single agents and in combination with established chemotherapies in pre-clinical tumor xenograft models and clinical trials with these drugs are currently underway in human cancer patients.

Future prospects

The earlier clinical trials of broad-spectrum metalloproteinase inhibitors proved very disappointing (Coussens et al., 2002) and it has since become clear that the role of metalloproteinases in development and disease is considerably more complex than was appreciated when these trials were performed. The availability of more selective inhibitors should allow us to overcome the toxicities which limited the amount of drugs which could be administered in earlier studies while reducing "off-target" effects. Furthermore, the ability to target specific tumor-relevant processes, such as TACE-dependent EGFR ligand shedding, rather than aspiring to disrupt multifactorial processes such as tumor cell invasion, should allow the selection of patients in whom these strategies are likely to be beneficial.

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