4E-Binding Protein 1: A Key Molecular "Funnel Factor" in Human Cancer with Clinical Implications

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Abstract

In an attempt to identify molecules that clearly reflect the oncogenic role of cell signaling pathways in human tumors, we propose a concept we term "funnel factor", a factor where several oncogenic signals converge and drive the proliferative signal downstream. In studies done in various tumor types, the expression of key cell signaling factors, including Her1 and Her2 growth factor receptors, as well as the RAS-RAF-mitogen-activated protein kinase and the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathways was correlated with the associated clinicopathologic characteristics of these tumors. The downstream factors p70, S6, 4E-binding protein 1 (4E-BP1), and eukaryotic translation initiation factor 4E, which play a critical role in the control of protein synthesis, survival, and cell growth, were also analyzed. We found that phosphorylated 4E-BP1 (p-4E-BP1) expression in breast, ovary, and prostate tumors is associated with malignant progression and an adverse prognosis regardless of the upstream oncogenic alterations. Thus, p-4E-BP1 seems to act as a funnel factor for an essential oncogenic capability of tumor cells, self-sufficiency in growth signals, and could be a highly relevant molecular marker of malignant potential. Further investigation into this concept may identify additional funnel factors in the oncogenic pathways and provide potential therapeutic targets. [Cancer Res 2007;67(16):7551–5]

Background

More than 250 types of malignant human tumors with distinctive clinical and pathologic characteristics and thousands of morphologic and pathologic tumor subtypes have been described. Moreover, several dozen tumors have an unknown or uncertain histogenesis, and others show divergent differentiation and/or an undefined neoplastic nature. Recently, new tumor entities with specific clinicotherapeutic features have been described based on their gene expression profile. Nevertheless, the diagnosis of most tumors is currently based on clinicopathologic criteria, such as tumor type, grade of differentiation, tumor size, and clinical stage, which still provide the most relevant prognostic and therapeutic information for patient management.

Since the description of human oncogenes, molecular characterization of tumors has been the main goal to understand the mechanisms of tumor formation and identify prognostic factors and therapeutic targets. However, molecular study of human tumors is an enormous job, and the huge puzzle that will integrate the continuous flow of new information is still under "construction." Earlier reports showed that oncogenic alterations are mediated by mutations, deletions, translocations, and amplifications of genes. Up to 300 mutated genes implicated in oncogenesis have been identified as human cancer genes (1). Now we know that gene methylation and the fascinating new world of microRNAs (2) can also orchestrate gene expression and play an important role in malignant transformation. Finally, taking into account that the real biochemical effectors are proteins, it is well established that increased activation or phosphorylation of proteins in signaling pathways mediated by various mechanisms is crucial in cancer. Therefore, the genetic landscape of human cancers, which has been recently elucidated in colon and breast carcinomas, with up to 189 mutated genes identified at significant frequency (3), should be incorporated to the functional information provided by expression arrays and proteomics. We know that there is not always a linear correlation among DNA alterations, RNA levels, and protein expression and that complex cross-talk between molecules and pathways may dictate the final effectors and the functional effect.

This intricate molecular background and its biochemical consequences must be responsible for inducing and mediating the malignancy of tumors, but how can we integrate this information? In their comprehensive review, Hanahan and Weinberg (4) proposed that six acquired capabilities are needed for malignant cellular growth: self-sufficiency in growth signals, insensitivity to anti–growth signals, limitless replicative potential, resistance to apoptosis, sustained angiogenesis, and, finally, the ability to infiltrate the surrounding tissue and metastasize. Each of these changes in cellular physiology can be brought about through dozens of signaling pathways or cascades, each implicating various genes or proteins. This cell transformation approach can help us to understand the great heterogeneity observed in tumors, where many different oncogenic alterations may be involved in each biochemical route.

Many oncologists and pathologists ask whether all this information is really important for the management of individual cancer patients. The answer is unknown because only a few molecular targets have been identified in a few tumor types. For example, ERBB2 amplification is seen in 25% to 30% of breast carcinomas, epidermal growth factor receptor (EGFR) mutations in <10% of lung carcinomas, and c-KIT in the rare gastrointestinal stromal tumors; but in most carcinomas, there is no distinctive oncogenic target. In the near future, technological advances will allow us to study the complete genetic background, mRNA profile, and protein expression of individual tumors and identify a myriad of genetic and biochemical alterations. But even then, attempts to inhibit or counteract single genetic alterations with the use of multiple specific agents would probably be chaotic. Nevertheless, dissection of the biochemical pathways is progressing. We now

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know which factors are the final growth signaling effectors that can control transcription and protein synthesis. Then, it is logical to hypothesize that the level of expression of these final effectors, which channel the proliferation signal, can be associated with the real oncogenic role of a pathway in individual tumors.

In Search of "Funnel Factors"

Based on these facts and in an attempt to find factors that reflect the molecular information and transformation potential for each tumor, we have focused on the study of what we call funnel factors through which the transforming signal inexorably passes and is canalized. These funnel factors would provide a clear reflection of the transforming potential of the tumor regardless of the triggering genetic alteration upstream. If this hypothesis were correct, the level of expression of these factors should correlate with the degree of malignancy of the tumor and the most relevant clinical variables, such as metastasis and survival.

We chose to investigate the funnel factor corresponding to the acquired capability of growth factor self-sufficiency, one of the most extensively studied characteristics of tumor cells and one that is constitutively activated in nearly all tumors. The process of converting extracellular signals into cellular responses, in this case cell growth and division, is called signal transduction. The growth signal transduction pathway is composed of growth factors, growth factor receptors, factors transmitting the growth signal, and the final effector factors, some of which are located in the nucleus to activate transcription factors and some in the ribosomes to activate protein synthesis. The neoplastic cell, however, may be able to generate signals for survival or proliferation through various mechanisms without depending on exogenous signals. These mechanisms include alterations in the growth factors or receptors, or in the signaling pathways, themselves. Among the latter, the most highly recognized and important are the RAS-RAF-mitogen-activated protein kinase [extracellular signal-regulated kinase 1/2 (ERK1/2)] and phosphatidylinositol 3-kinase (PI3K)-AKT pathways, which regulate mammalian target of rapamycin (mTOR). Specific molecular alterations are detected in these signaling cascades in the majority of tumors. Usually, these are single alterations with an oncogenic effect, such as growth factor mutations or RAS mutations; other concomitant genetic alterations are not usually found in these biochemical pathways.

Studies in Breast, Ovary, Prostate, and Colon Carcinomas

We studied the expression of some of the most relevant factors implicated in growth signaling pathways [EGFR, HER2/neu, ERK1/2, AKT, 4E-binding protein 1 (4E-BP1), p70S6K1, and S6] in a large series of solid tumors of the breast (5), ovary (6), prostate,[3] and colon[4] by Western blot and immunohistochemistry. The phosphoantibodies used were validated on a panel of cell lines and by the correlation

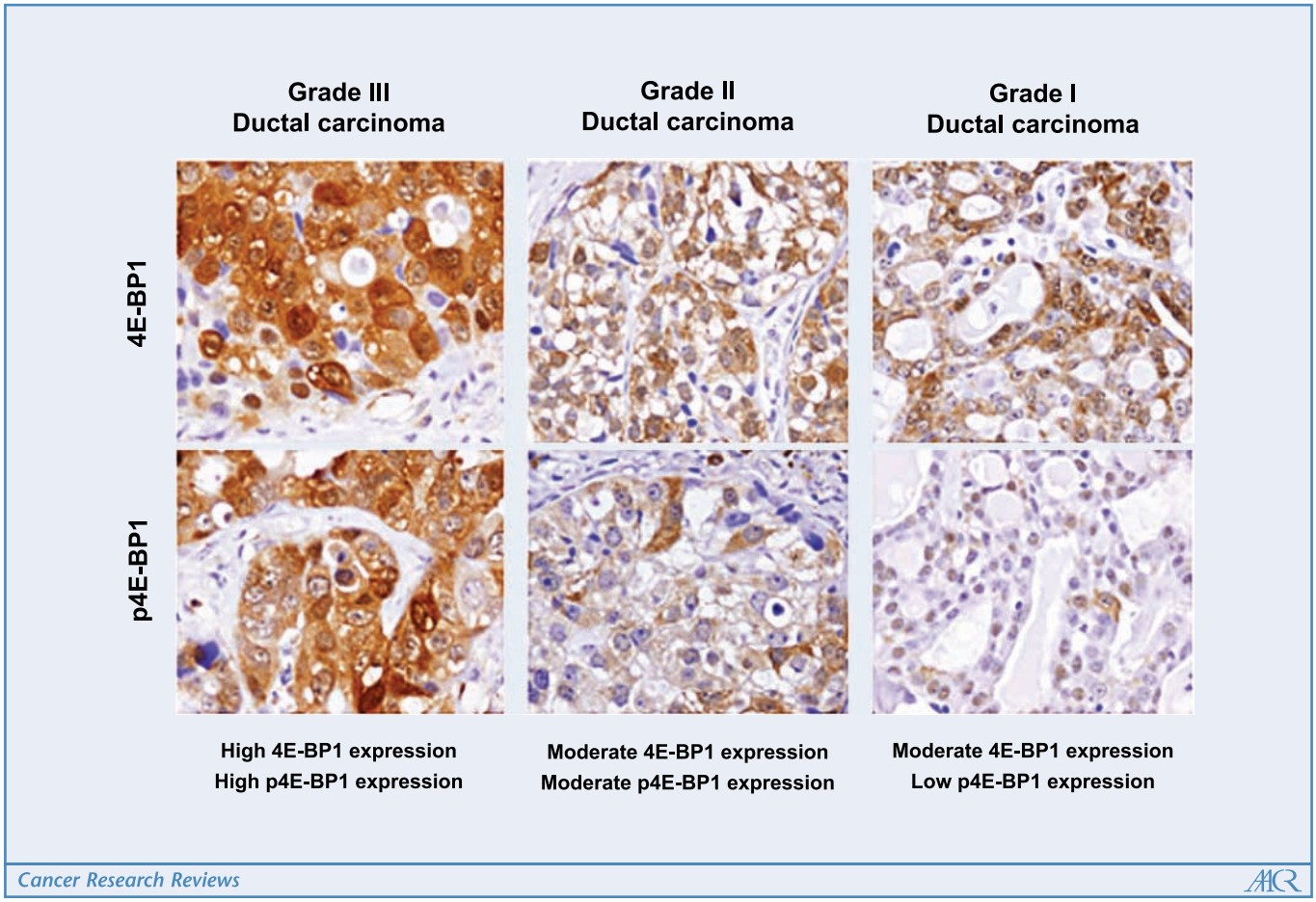

Figure 1. Levels of expression of total 4E-BP1 and p-4E-BP1 in infiltrating breast carcinomas shown by immunohistochemistry using anti-4E-BP1 and anti-p-4E-BP1 (Thr^{70}) antibodies (Cell Signaling Technology). 4E-BP1 and p-4E-BP1 levels were highest in high-grade carcinomas.

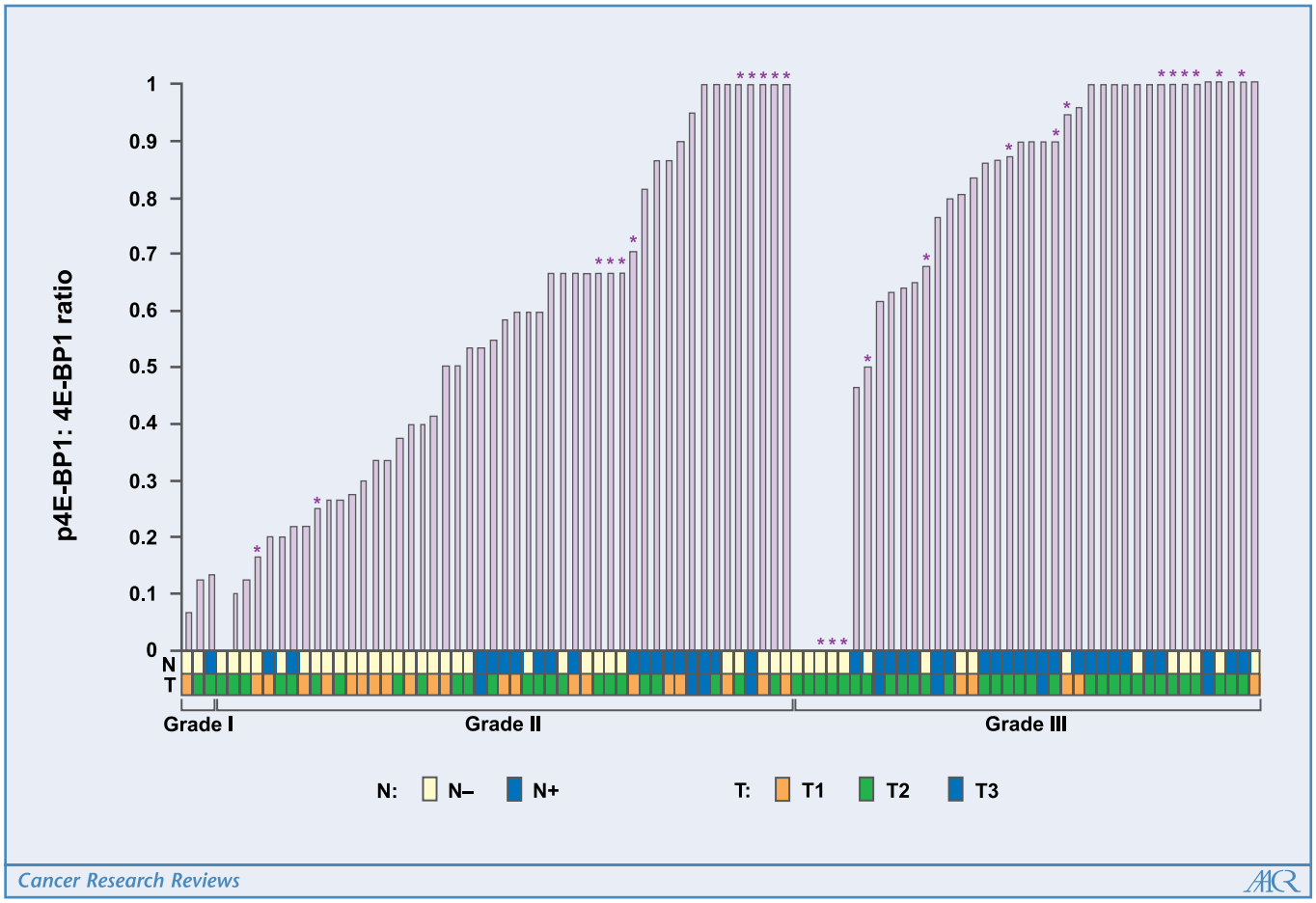

Figure 2. Correlation between the p-4E-BP1 to total 4E-BP1 ratio and histologic grade, tumor size (*T*), and presence of lymph node metastases (*N*) in infiltrating breast carcinomas after immunohistochemistry using the same antibodies as in (*A*). Note that the percentage of p-4E-BP1 was higher in the more aggressive tumors (high histopathologic grade) with lymph node metastasis or large in size. *Asterisks,* cases that showed a higher amount of EIF4E than 4E-BP1 (EIF4E to 4E-BP1 ratio, >1).

between Western blot and immunohistochemistry results. The best correlation for anti-4E-BP1 antibodies was obtained with the Thr^{70} phosphorylated 4E-BP1 (p-4E-BP1) antibody. As is known, the Ser^{65} and Thr^{70} phosphorylation sites lie close to the eukaryotic translation initiation factor 4E (EIF4E)-binding site and are considered the phosphorylation sites for EIF4E release (7).

In addition, we investigated correlations between the expression of all the proteins studied to detect possible cross-activations and activations by pathways or mechanisms that have not as yet been well defined. Most importantly, we examined the clinical characteristics associated with the tumors to correlate the factors studied with the tumor stage and patient survival.

Breast and Ovary Tumors

In the study conducted in breast tumors (5), we found that p-4E-BP1 was expressed in a high percentage of tumors and was strongly and significantly associated with a poor prognosis independently of other factors. Thus, high levels of p-4E-BP1 expression were detected in an important proportion of breast tumors regardless of HER2/neu status or activation of the PI3K/AKT or ERK1/2 signaling pathways. p-4E-BP1 was mainly expressed in poorly differentiated tumors and correlated with tumor size, presence of lymph node metastasis, and locoregional recurrence, indicating that it could be a useful factor for establishing the prognosis in breast cancer (Fig. 1). Interestingly, the ratio p-4E-BP1 to total 4E-BP1 was associated with histologic grade and lymph node metastasis; that is, in tumors with a poor prognosis, most 4E-BP1 was phosphorylated (Fig. 1*B*). Moreover, ~30% of tumors showed a higher amount of EIF4E than 4E-BP1 (EIF4E to 4E-BP1 ratio >1). Assuming that 4E-BP1 acts by sequestering EIF4E at a 1:1 ratio, some EIF4E would be free in these cases regardless of the amount of p-4E-BP1. Interestingly, these tumors included three of the five grade III cases with a p-4E-BP1 to 4E-BP1 ratio of 0 (Fig. 2). Similarly, in ovarian tumors, we found that p-4E-BP1 expression correlated with tumor progression and an unfavorable prognosis regardless of the status of HER2/neu, EGFR, PTEN, or PI3K (6).

Prostate Tumors

Furthermore, quite interesting results were obtained in prostate tumors. As occurs with carcinomas developing at other sites, incipient prostate lesions may require more than 5 years to acquire invasive properties and metastasize. This progression goes from low-grade prostatic intraepithelial neoplasia (PIN) to high-grade PIN (HGPIN), which is currently one of the most controversial entities in the field of urology. Detection of histologic HGPIN,

[3] C. Iglesias et al. Overexpression of p4E-BP1 in high grade prostatic intraepithelial neoplasms associates with progression to prostatic cancer, submitted for publication.
[4] M. Cuatrecasas et al. P4E-BP1 in colorectal carcinomas. A surrogate of the real oncogenic role of the mTOR pathway, submitted for publication.

theoretically the precursor lesion to adenocarcinoma, can indicate the need for more aggressive treatment to prevent the development of this neoplasm. In fact, the short-term predictive value of HGPIN is high, >18% (8), and there are currently no morphologic variables able to distinguish HGPIN with a higher probability of progressing to cancer. Because some HGPINs evolve to carcinomas in a relatively short time and many others do not, it is reasonable to hypothesize that some HGPINs contain molecular alterations associated with tumor progression, whereas in others that remain stable over long periods, these alterations are absent.

Along this line, we designed a study in patients with HGPIN lesions and prostate carcinomas, analyzing signaling pathway factors in biopsy samples and surgical specimens from prostatectomies and cystoprostatectomies.[3] Once again, we attempted to identify funnel factors through which several oncogenic signals converge and determine their association with the disease prognosis. Activation of the AKT/mTOR cascade was detected in prostate carcinomas and in the HGPIN areas around them, with similar phosphorylated AKT and phosphorylated p70S6K expression. p-4E-BP1 tended to be high in HGPIN. Interestingly, p-4E-BP1 expression in HGPIN adjacent to prostate carcinomas was higher than in HGPIN without carcinoma ($P < 0.001$). With the aim of determining whether p-4E-BP1 overexpression could identify HGPIN with higher risk of progressing to carcinoma, we analyzed the immunohistochemical expression of p-4E-BP1 in 76 HGPIN needle biopsies of patients who later underwent repeat biopsies. We found that p-4E-BP1 expression was significantly higher in HGPIN areas corresponding to cases that progressed to prostate cancer than in those that did not ($P = 0.000$), suggesting that this factor may help to identify patients at high risk for developing the disease.

Colon Carcinomas

Lastly, p-4E-BP1 was also investigated in colon carcinomas.[4] In an analysis of 120 colon adenocarcinomas, high expression of p-4E-BP1 did not correlate with poorly differentiated tumors or survival, but it did associate with the presence of lymph node metastasis ($P = 0.005$). We believe that further studies are needed to determine the role of p-4E-BP1 and its other isoforms in colon carcinoma.

Discussion

Our results showing that 4E-BP1 is associated with the prognosis in breast, ovary, and prostate tumors are supported by data from other authors. In a study on breast cancer, phosphorylation of AKT, mTOR, and 4E-BP1 was associated with tumor development and progression (9). Moreover, experimental studies have shown that 4E-BP1 is essential for cell transformation. Transfer of 4E-BP1 phosphorylation site mutants into breast carcinoma cells suppressed their tumorigenicity (10). Kremer et al. (11) recently investigated the expression patterns of several biomarkers of the

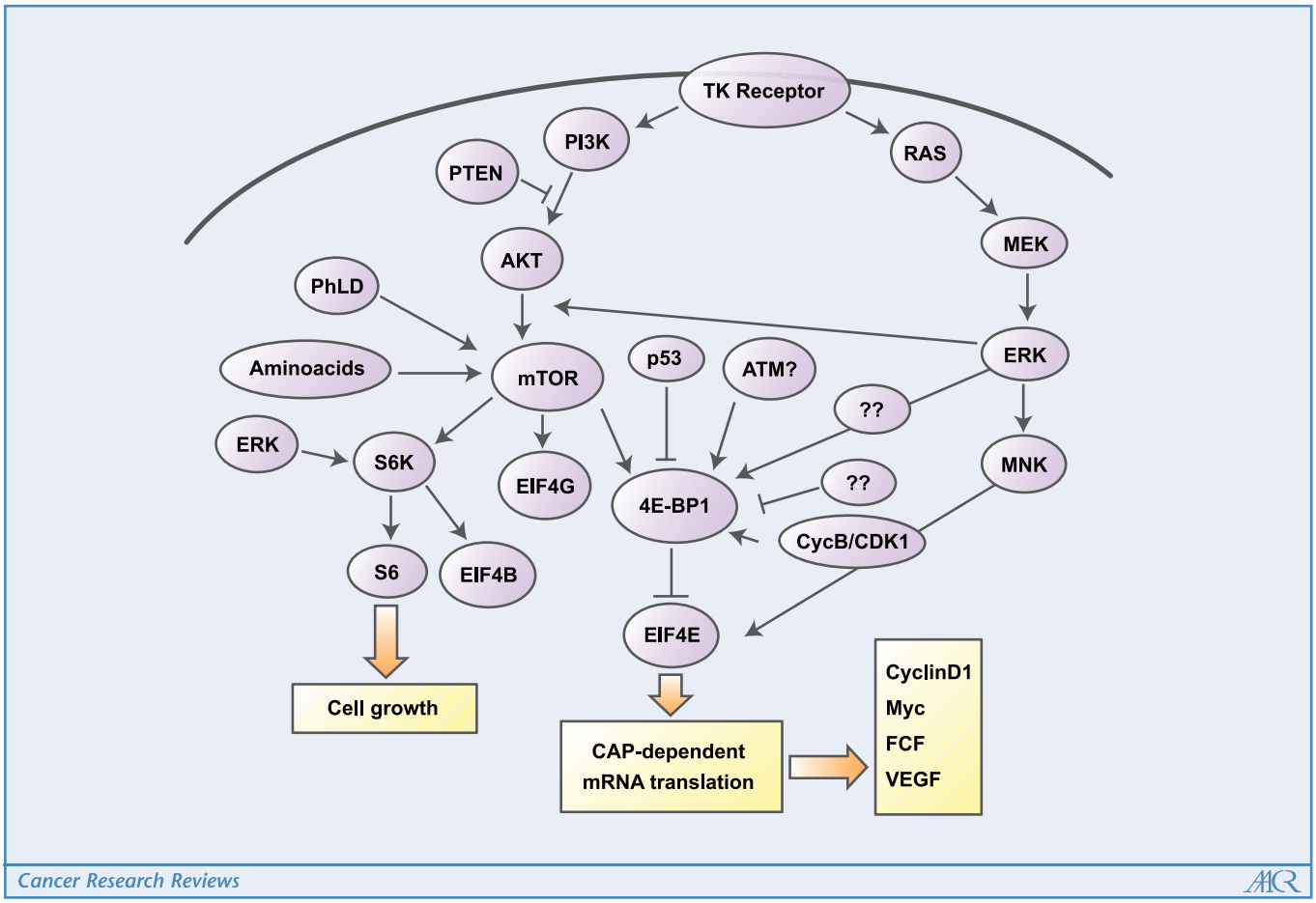

Figure 3. Schematic proposal of signaling pathways converging to 4E-BP1. Beyond the myriad of factors and cascades involved in cell signaling, there must be a few factors that channel the proliferation signal. One of these funnel factors could be 4E-BP1, which controls EIF4E and, therefore, translation of cap-dependent RNAs. 4E-BP1 phosphorylation can be the consequence of many different oncogenic events occurring in several biochemical pathways and involving some known and possibly some unknown kinases.

mTOR pathway in prostate cancer. The authors observed that 4E-BP1 was one of the best biomarkers because overexpression of this factor was highly associated with prostate cancer.

4E-BP1 is a EIF4E-binding protein that plays a critical role in the control of protein synthesis, survival, and cell growth (12, 13). During cap-dependent translation, EIF4E binds to the mRNA cap structure and promotes formation of the eIF4F initiation complex and ribosome binding. When 4E-BP1 is active (non-p-4E-BP1), it binds to EIF4E and impedes formation of the initiation complex; translation is then blocked, favoring apoptosis. However, when 4E-BP1 is phosphorylated, the affinity for EIF4E binding is reduced, EIF4E is released, and cap-dependent translation can initiate.

It is important to point out that 4E-BP1 has seven phosphorylation sites (7). It is likely that mTOR is the main phosphorylation pathway of 4E-BP1 (14), although other kinases may be implicated, such as cyclin-dependent kinase 1 (12, 15), ataxia-telangiectasia mutated (ATM; ref. 16), PI3K-AKT (17, 18), ERK1/2 (19), and perhaps other, still unidentified, kinases. Therefore, 4E-BP1 phosphorylation can be the consequence of many different oncogenic events occurring in several biochemical pathways, including amplification or mutation of growth factor receptors, loss of function or mutations in PTEN, ATM, p53, PI3K, or RAS, or other collateral mechanisms of cellular oncogenic activation, such as activation of phospholipase D, which can activate the mTOR cascade, or other unknown kinases or phosphatases (Fig. 3). Because of the elevated number of genetic alterations that regulate 4E-BP1, we propose that the phosphorylated form of this protein can act as a "bottleneck" or funneling factor through which the transforming signals converge, channeling the oncogenic proliferative signal regardless of the upstream-specific oncogenic alteration. In fact, previous studies with a mutant 4E-BP1 in the phosphorylation sites showed a marked antitumor effect (10), and recently, we obtained similar results in some, but not all, breast and colon carcinoma cell lines.[5]

The role of other 4E-BP isoforms, such as 4E-BP2 and 4E-BP3, in human tumors is still unclear, and it is not known whether they can be up-regulated in 4E-BP1–negative tumors. Study of the EIF protein family will also be determinant when reliable antibodies allow us to analyze their expression in large series of tumors. Recent data have provided novel perspectives into the proliferative and oncogenic properties of EIF4E because it has been shown to have an effect on nearly every stage of cell cycle progression (20). Earlier studies have shown that EIF4E levels are substantially elevated in several types of cancers (reviewed in ref. 21). EIF4E can promote tumor formation and cooperate with CMYC in lymphomagenesis (22); small interfering RNA silencing of EIF4E inhibits the growth of head and neck squamous cell carcinomas (23).

Extending the concept we propose, it is possible that there might be several funnel factors where the final biochemical effect converges for each of the oncogenic capabilities of tumor cells (e.g., in the apoptosis pathways, where the expression of certain proteins that inhibit apoptosis, such as survivin and livin, might be associated with resistance to apoptosis regardless of the activation of other antiapoptotic or proapoptotic genes that might be present).

Study of the expression profiles of funnel factors from all the cell transformation pathways would allow us to obtain an individual functional molecular signature for each tumor. This signature, combined with clinical and pathologic data, would help us to establish the malignant potential of each individual tumor and deduce its potential resistance to conventional chemotherapy and radiotherapy. Obviously, in addition to molecular characterization of tumors for prognostic purposes, it is necessary to study factors that might be potential therapeutic targets, currently one of the most promising areas in the field of cancer treatment. With this functional approach, it seems worthwhile to investigate whether these funnel factors can be critical targets for cancer treatment.

[5] In preparation.

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