Prostatic intraepithelial neoplasia and adenocarcinoma in mice expressing a probasin-Neu oncogenic transgene

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NEU (ERBB2) and other members of the epidermal growth factor receptor (EGFR) family have been implicated in human prostate cancer (CAP) development and progression to an androgen-independent state, but the extent of involvement and precise role of this signaling pathway remain unclear. To begin addressing such open questions in an animal model, we have developed a transgenic line in which an oncogenic Neu cDNA (Neu*) driven by the probasin gene promoter is overexpressed in the mouse prostate and causes development of prostatic intraepithelial neoplasia (PIN) that progresses to invasive carcinoma. Expression profiling using microarrays, which was selectively validated and extended by immunophenotyping of Neu*-induced PIN and CAP, led to the identification of some novel biomarkers and also revealed increased expression of Egr, Erbb3 and phosphorylated androgen receptor. In view of this information from our mouse model, which can be used to analyze further the role of Erbb signaling in prostatic tumorigenesis, we examined human prostate cancer tissue arrays by immunohistochemistry. Based on statistical analyses of the results, we propose the testable hypothesis that ERBB3, shown to be expressed in 86% of the human CAP cases that we examined, is the pivotal element of the Erbb pathway promoting tumorigenesis by heterodimerization with NEU or EGFR, while a NEU/EGFR dimer does not appear to play a significant role in CAP.

Introduction

According to current estimates (1), prostate cancer (CAP) is the most frequently diagnosed non-cutaneous malignancy in American males (33%; ~230,000 new cases per year) and the second leading cause of cancer mortality (10%; ~30,000 deaths, almost exclusively occurring in men older than 60 years). It is thought that prostate tumors progress from precursor lesions (prostatic intraepithelial neoplasia; PIN) to locally invasive carcinomas and finally to metastatic disease (2,3).

Abbreviations: AI, androgen-independent; Akt1, thymoma viral proto-oncogene 1; AR, androgen receptor; BPH, benign prostatic hyperplasia; BrdU, 5-bromo-2′-deoxy-uridine; CAP, prostate cancer; Neu*, activated Neu (Erbb2) receptor; IHC, immunohistochemistry; PAH, prostatic atypical hyperplasia; PIN, prostatic intraepithelial neoplasia; TANT, tumor-adjacent normal tissue.

The development of mouse models to analyze the molecular mechanisms underlying these stages of prostate tumor progression is crucial, despite some important concerns. Although the human and rodent prostates are embryologically related, their exact anatomical homologies are unclear (the human prostate consists of different zones and lacks lobular organization, whereas distinct dorsolateral, ventral and anterior lobes are present in rodents). Moreover, in animals and especially in rodents, the spontaneous occurrence of prostate cancer is uncommon. Nevertheless, the generation of genetically modified mice simulating to some extent the human disease could provide answers to key questions pertaining to prostate cancer biology. Unfortunately, the lesions in the majority of transgenic mouse models do not progress beyond a PIN stage, while the prostatic adenocarcinoma induced by transgenic expression of the SV40 T antigen (4) is a neuroendocrine cell tumor that resembles only a rare human variant (<0.1% of prostate carcinomas). On the other hand, the invasive adenocarcinomas that eventually develop in mice by prostate-specific ablation of Pten (5) or overexpression of Myc (6) mimic human CAP quite successfully.

Here we describe an additional mouse model in which PIN evolves into invasive CAP as a result of transgenic expression of activated Neu (Erbb2) receptor (henceforth referred to as Neu*) driven by the mouse probasin gene promoter. Development of such a transgenic line was warranted, considering that there are open questions about the potential involvement of NEU-mediated signaling in prostate tumor progression. Moreover, the EGF (ERBB) family of receptors has been implicated in androgen-independent prostate tumor growth (7,8).

Materials and methods

Generation of transgenic mice

To drive prostate-specific expression of an oncogenic Neu transgene, we used the promoter/enhancer elements of the mouse Pbsn (probasin) gene (9) that we isolated from a cosmid clone. Previously, analogous transgenes have been constructed using either a short (~0.4 kb) or a long (LPB; 11.5 kb) fragment of the rat Pbsn promoter (10). Considering the stronger activity of LPB (11), we have used in our construct a 4.5 kb DNA fragment that includes, in addition to the mouse Pbsn 5′ flanking sequence, the 5′ non-coding region of exon 1 (40 bp upstream from the initiator ATG, assuming that the cap site is the same as that of the homologous rat gene) (12). The proximal 3 kb of this mouse fragment exhibit a high level of sequence homology (86%) with the corresponding region of the rat Pbsn locus.

Founders of transgenic lines were generated by standard procedures, after injecting into fertilized eggs (derived from C57BL/6JxCBA crosses) a 12.9 kb Sall–NotI (cloning sites) DNA fragment (Figure 1A) excised from the pBSK plasmid vector. In the injected DNA, the 4.5 kb mouse Pbsn fragment was linked to a sequence excised from cloned genomic DNA recovered from an MMTV-Neu* transgenic animal (13), which consisted (in the 5′ to 3′ direction) of four segments: a rat VL30 element (0.65 bp; a member of a small family of retroposons); a transforming (activated) rat Neu cDNA (Neu*; 4.95 kb, including 0.2 kb of vector sequence); two portions of SV40 sequence (GenBank accession no. J02400; complements of coordinates 4100–4713 and 1782–2774; total of 1.6 kb) providing the intron of the small t antigen and the early mRNA polyadenylation signal; and 1.2 kb of mouse genomic DNA (3′ flank of the inserted MMTV-Neu* transgene in mouse
Chromosome 10, which was retained in the Phsn-Neu transgene as a cloning by-product during the construction process. Although it remains unknown whether the presence of the VL30 sequence (adventitiously acquired at an intermediate cloning step by the original MMTV-Neu* transgene) affects the transformability of the activated oncogene (13,15), we decided to leave the original transgene unaltered in our construct. A transgenic line was established for expression profiling, total RNA was extracted from the prostates (ventral and dorsolateral lobes only) of transgenic mice bearing PIN or carcinoma and CAP lesions was compared with that of apical epithelial cells in nonneoplastic controls. A list of the primary antibodies used for IHC and some comments on their performance can be found in Supplementary Table 1. The results of immunostaining were scored semiquantitatively from 0 to 3, by assessing both the intensity of signal and the percentage of positive cells (only apical epithelial cells of the dorsolateral lobe were scored in mice). No staining or weak focal staining in <15% of the cells was considered as negative (0 or minus). Scores of 1, 2 or 3 were assigned, in order, to diffuse strong staining in >15% of the cells; focal strong staining in <15% of the cells; and diffuse strong staining in >15% of the cells. When positive versus negative cases were considered, the numbers of specimens exhibiting positive signal for a particular marker were lumped, regardless of score. To simplify the statistical analysis of positive cases, the specimens were considered as exhibiting either ‘low’ (score 1) or ‘high’ (scores 2 and 3) level of immunostaining.

For BrdU (5-Bromo-2′-deoxy-uridine) labeling, animals were injected intraperitoneally with BrdU (200 μg per gram of body weight) and killed 2 h later. Dissected tissue samples were processed for histopathology and BrdU incorporation was visualized by immunostaining. Values were expressed as percentages of the total number of cells that were BrdU positive (labeling was evaluated by scoring a total of ~250 cells present in several optical fields). The average (± standard error) was calculated from measurements of three specimens per group. The level of apoptosis was assayed by activated caspase 3 IHC (16).

Microarray analysis
For expression profiling, total RNA was extracted from the prostates (ventral and dorsolateral lobes only) of transgenic mice bearing PIN or carcinoma and
from normal (control) littermates or age-matched animals. The samples were profiled individually. For each assay, 5–8 µg of total RNA were used to generate biotinylated cRNA, as described previously (17). Fragmented cRNA (15 µg) was then hybridized to MGI74Av2 DNA Chips (Affymetrix, Santa Clara, CA) with ~12,000 gene entries. The microarrays were scanned (Affymetrix Scanner) and expression values for the genes were determined using Affymetrix GeneChip Operating Software 1.2. These raw data were filtered prior to any other analysis as follows: if the averages of specimen expression values for a particular Chip entry were <1000 for all sample groups, this entry was eliminated. Although arbitrary, this cut-off point was used on the basis of our experience that hybridization signal intensities with readings <1000 tend to fluctuate and are unreliable. At this point, an unsupervised clustering dendrogram (generated by using Cygwin software) showed that the samples could be readily classified into groups (normal, PIN and carcinoma; see Results). In an attempt to pursue a gene-by-gene analysis, rather than a ‘pattern discovery’ procedure, the data remaining after the described first round of filtering were analyzed by using GeneCluster2 software (18), to determine the statistical significance of the expression differences observed. We then accepted only those entries that met a criterion of significance at the P < 0.05 level in the permutation analysis of the GeneCluster2 program and had a t-test score >2. Using then the average values of corresponding experimental and control entries that had passed both of these tests, we filtered the data further by applying the following rules: An entry was considered as ‘upregulated’ if it had an absolute expression value >1000 and an experimental/control ratio >2. The opposite rule was applied for entries considered as ‘downregulated’ (only control values >1000 and experimental/ratio >2 were accepted). Although arbitrary, a ratio of ≥2, used as a cut-off point indicative of a significant difference in transcript levels, is a widely used conventional setting in the vast majority of microarray analyses. The entries remaining on the final list of differentially expressed genes exhibiting a fold difference of ≥2 corresponded to 345 upregulated and 443 downregulated transcripts of which 297 and 339, respectively, were finally tabulated, after filtering-out unknown expressed sequence tags (ESTs) and studied in detail through extensive literature searches.

Statistical analysis

In each case, to test the null hypothesis that there is no difference in marker expression between CAP cases and controls, we generated an appropriate 2 x 2 contingency table and then computed a likelihood ratio test statistic. However, because some of the sample sizes were small, we estimated the P-values for a given statistic by a randomization test. Thus, for each contingency table, we generated 100,000 random tables under the null hypothesis that the analyzed markers were independent and estimated the frequency with which random datasets showed statistic values greater than or equal to the value seen in the actual dataset. These estimated P-values are the ones presented (see Tables II–V), where estimated P-values of 0 are presented as <10−5.

For each marker, we first tested whether there were differences between cancer cases and controls in the frequencies of negative (−) versus positive (+) expression (first test). Focusing then specifically on positive expression, we tested whether there were analogous differences in the frequencies of low versus high expression (second test; our results of 3 x 2 tests comparing simultaneously specimens in which expression was low, high or absent and not presented because they are less informative, despite their high significance for all markers). We note that the two separately performed tests are statistically independent because in the second test, which is based on the condition that expression is positive, the specimens not expressing a marker were ignored. Using the same approach, we performed similar tests for low and high Gleason scores and then extended our analyses to examine the co-expression of gene pairs in cancers and controls.

Results

Transgenic expression of activated Neu in the mouse prostate

Using standard methodology, we have generated a transgenic mouse strain expressing a constitutively active rat Neu receptor (Neu*) specifically in the prostate. This oncogenic variant originally arose by a point mutation of the corresponding proto-oncogene causing a valine to glutamic acid replacement (V664E) in the transmembrane domain of the protein product. In the transgene, the oncogenic Neu* cDNA was driven by the androgen receptor-dependent probasin (Pbsn) gene promoter (Figure 1A; for details, see Materials and methods).

The exogenous Neu* sequence was expressed in three of four transgenic lines established from founder mice, but we chose to perform our analysis using progeny from the line expressing the highest level of transcripts. In these mice, ~15 copies of the transgene were integrated in a head-to-tail configuration, as shown by Southern analysis (Figure 1B, panel 1). As expected, northern analysis indicated that transgene expression was not observed in examined tissues other than the prostate, while prostatic transcription occurred in all lobes (Figure 1B, panel 2), in agreement with previous observations (9,11,19). Quantitation of the data showed that, regardless of lobe, the transgenic expression was ~9.6-fold higher than that of the endogenous Neu gene (Figure 1B, panel 2). This ratio, however, is an underestimate because in, comparison with wild-type animals, the expression of the endogenous Neu locus was increased ~3-fold in the presence of the Neu* transgene (Neu expression in wild-type prostates was barely detectable by northern analysis even after overexposure of autoradiograms; data not shown). The over-expression of Neu* protein in the prostate glands of the transgenic animals was confirmed by western analysis (Figure 1B, panel 3).

Oncogenic Neu* induces PIN and prostatic adenocarcinoma

Because there are no external signs indicative of prostatic tumor growth in mice, we killed animals at various times from ~5 months of age onward during a 20-month period of observation and evaluated histopathologically the prostate glands of 60 Pbsn-Neu* transgenic males and 19 wild-type controls.

In contrast to the control mice, all of the examined transgenic males exhibited histological abnormalities that were classified in order of severity into three groups: prostatic atypical hyperplasia (PAH; 30%, 18/60 transgenic specimens), PIN (58.3%, 35/60) and CAP (11.7%, 7/60).

In PAH cases, there was mild thickening (up to three cellular layers) of the lining of the prostatic glands that was populated by atypical cells (Figure 2A), whereas PIN was characterized by intraglandular growth of atypical secretory cells in a tufting or cribriform pattern, the latter appearing by coalescence of tufts in more progressed cases. Morphologically, we discriminated between an initial neoplastic stage exhibiting papillary projections filling partially and to various degrees the lumen of the ducts (‘early PIN’; 26/35 cases; Figure 2A) and an advanced stage of growth with distorted glandular profiles and neoplastic expansion obliterating the lumens of ducts that was commonly accompanied by central necrosis (‘late PIN’; 9/35 cases; Figure 2B).

Whereas early PIN was mostly located in the periphery of the dorsal and lateral prostatic lobes, late PIN involved in addition the ventral lobe of the gland (the extent of PIN observed in the anterior lobe was very small and barely exceeded AH levels). Typically, the neoplastic acinar cells were tall with large irregular nuclei that showed condensation of chromatin along the nuclear border and contained conspicuous nucleoli, while the cytoplasm was either clear or basophilic. The latter often stained deep rose with mucicarmine (Figure 2B, inset) indicating the presence of acidic mucin (the basophilic cells could acquire a goblet-cell shape of the intestinal type). This staining property was absent from normal prostatic tissue.

In two of seven CAP cases, the tumors were only microinvasive consisting of a few clusters of malignant cells found in a
mildly desmoplastic stroma adjacent to acini with late PIN. In the other five animals, however, extensive tumors resulting in considerable enlargement of the prostate were observed. These carcinomas (Figure 2C) were associated with a significant stromal response, including recruitment of fibroblasts, neoangiogenesis, presence of a myxoid matrix and inflammation. The malignant cells, which were cytologically similar to those observed in PIN lesions and possessed large irregular nuclei and conspicuous nucleoli, were arranged either singly or in small solid nests typical of a poorly differentiated carcinoma and rarely exhibited features of residual glands. Neither lymphovascular invasion nor disruption of the prostatic capsule were apparent.

The invasive character of the examined carcinomas was confirmed by immunohistochemical analysis for p63 (a p53 homolog), which is a marker of basal cells of epithelial organs, including the prostate (20). In wild-type glands and PIN, positive p63 immunostaining of the basal cell nuclei was readily evident, as expected (Figure 2D, arrows). In contrast, the cells of invasive carcinomas lacked a basal cell layer and were p63-negative (Figure 2D). Further immunostaining showed that PIN and invasive carcinomas were negative for synaptophysin, a marker of neuroendocrine differentiation (Figure 2E). This confirmed that, in contrast to the TRAMP model (21), the Neu-induced tumors were not neuroendocrine in origin, consistent with the detected strong expression of androgen receptor, as described below (neuroendocrine tumors lack this marker of secretory prostatic epithelium).

PIN or invasive carcinoma were not detected in any of the examined wild-type animals. In fact, only 2/19 control mice showed at a progressed age (~17–19 months) very mild stromal hyperplasia with rare lymphoid aggregates, while some scattered atypical cells appeared in a third animal.

A comparison of the incorporation of BrdU into DNA showed a significant degree of hyperproliferation in the neoplastic over the control prostates. Whereas only a small percentage of BrdU-positive nuclei was detected in the controls (0.3 ± 0.08%), an 18 and 34-fold increase of this score was observed in PIN (5.5 ± 0.17%) and invasive cancer (10.3 ± 1.0%), respectively (n = 3 specimens per class). Neither neoplastic nor control prostates exhibited signs of apoptosis, as demonstrated by the absence of immunostaining for activated caspase 3 (not shown).

After specimen classification by histopathology, we calculated separately for each category the elapsed time for manifestation of the corresponding phenotype in 50% of the specimens of a particular group (median time; T<sub>50</sub>). In this regard, to present cumulative incidences, we either considered all of the experimental animals (n = 60) as belonging to a single population (Figure 3, inset A) or, for better display, the number of animals in each class was separately normalized to a level of 100% (Figure 3).

The calculated median times reflected the progression of abnormalities from PAH to PIN and CAP. The T<sub>50</sub> for detection of PAH was ~8 months (Figure 3) and there was a statistically significant difference between this time and the T<sub>50</sub> for...
early PIN (12 months, \( P < 0.003 \); Figure 3). Also significantly different were the median times for detection of early versus late PIN (12 versus 16 months, respectively, \( P < 0.01 \); Figure 3). The manifestation of invasive carcinoma was a late event but, consistent with the observed development of CAP on a late PIN background, the \( T_{50} \) for CAP (~18 months; Figure 3) could not be discriminated statistically from the \( T_{50} \) for late PIN. Interestingly, when the extent of PIN (defined as the percentage of prostatic ducts exhibiting PIN in each specimen) was averaged for cases of early PIN, late PIN and PIN associated with invasive carcinoma, the means (%) (24.9 ± 3.0 SEM, 59.6 ± 4.5 and 73.9 ± 6.7, respectively) were found to be proportional to the median times (coefficient of correlation \( r = 0.997 \)) and fitted a regression line intercepting the time axis almost exactly at the \( T_{50} \) for the appearance of PAH (no PIN; Figure 3, inset B).

Erbb receptors and signaling effectors in Neu*-induced prostate neoplasia

Neu does not bind any of the known ligands of Erbb receptors, but serves as the preferred heterodimeric partner of other family members because its conformation favors dimerization (22). In fact, Neu and Erbb3 are active and have the potential to function as an oncogenic unit, only when they form a heterodimer, as the former receptor is ligandless, while the latter lacks intrinsic tyrosine kinase activity (23). In addition to the Neu/Erbb3 combination, the Egfr/Neu and Egfr/Erbb3 heterodimers are the ones most often associated with malignancies (ref. 24 and other references therein).

In contrast to wild-type Neu, the constitutively active oncogenic Neu* does not need a partner and can act predominantly as a homodimer. Nevertheless, Erbb heterodimers may enhance the Neu* oncogenicity or become involved in the progression of Neu*-induced tumors. This hypothesis is consistent with the overexpression of endogenous Erbb3 (10–15-fold over normal) occurring at a post-transcriptional level in mouse mammary tumors induced by oncogenic Neu activated by microdeletion (25). Egfr is also overexpressed in the same tumors, but the increase is less dramatic than that of Erbb3 (25). Considering this information, we sought to compare the increased expression of Neu* with the expression status of Egfr and Erbb3 in the PIN and CAP of the transgenic animals by performing immunohistochemistry (Figure 4).

Immunostaining for Neu using a monoclonal antibody (3B5; see Supplementary Table 1) showed moderate to strong signal in subpopulations of neoplastic cells both in PIN and in invasive carcinoma, while the immunoreactivity, if any, of some scattered cells in normal prostates was very weak (Figure 4). These observations were consistent with the significantly higher expression of Neu in neoplastic over normal tissue, as detected both by northern and western analyses (Figure 1B, panels 2 and 3).

The Egfr immunostaining was weak in wild-type glands, but had become focally strong in the tumor-adjacent normal tissue (TANT) of transgenic animals and exhibited further uniform increase in PIN and cancer (Figure 4). This confirmed expression profiling data (see next section) showing that in the prostatic neoplasms the expression of Egfr was increased 2-fold (Table I; Neu and Erbb3 were not microarray entries). Even higher expression relative to wild-type (average of 2.8-fold in PIN, \( n = 3 \); and 5.3-fold in CAP, \( n = 4 \)) was detected by northern analysis (not shown). Thus, in the presence of oncogenic Neu*, the expression of Egfr was elevated even prior to the manifestation of neoplastic changes.

Finally, Erbb3 immunostaining was undetectable both in control prostate and TANT, but a strong uniform signal was detected in PIN and cancer (Figure 4). It remains to be seen whether this increased Erbb3 expression occurs predominantly at a post-transcriptional level (in CAP, but not in PIN, northern analysis showed that there was only a marginal 1.6-fold increase of Erbb3 transcripts over the normal level; data not shown). An increase of ERBB3 expression in human CAP was...
previously demonstrated by IHC (23/29 adenocarcinomas exhibited moderate to strong immunoreactivity) (26) and more recently by microarray analysis (the level of ERBB3 transcripts was 2-fold higher than normal) (27).

Considering that Erbb-mediated signaling promotes cell proliferation by regulating mitogen-activated protein kinases (Mapk) p44 and p42 (Mapk3 and Mapk1 or extracellular signal-regulated protein kinase (Erk) 1/2) and cell survival through activation of Akt1 (thymoma viral proto-oncogene 1), we performed comparative immunostaining to assess the status of these effectors in tumors and controls. For this purpose, we used antibodies (see Supplementary Table 1) specifically recognizing the active (phosphorylated) forms of Erk1/2 and Akt1 (p-Erk1/2 and p-Akt1). We observed that PIN and cancer exhibited strong, diffuse immunostaining for nuclear p-Akt1 (Figure 4), while the signal for nuclear p-Erk1/2 had a patchy pattern in the neoplastic prostate tissue with continuous clusters of strongly positive neoplastic cells alternating with cells that remained non-reactive to the antibody (Figure 4). Interestingly, there was no visible difference in staining patterns between PIN and invasive lesions for p-Akt1 or for p-Erk1/2, while the immunoreactivity for these markers was consistently negative in wild-type prostates and TANT (Figure 4).

Although there is a presumptive functional relationship between Erbb signaling and androgen receptor (AR) activation in hormone-refractory human prostate cancer (see below), we did not have an opportunity to examine directly a response to castration using our model because of the long latency period before the appearance of invasive cancer. We note, however, that western analysis demonstrated a significant increase in the phosphorylated form of AR (at Ser81; p-AR) in neoplastic prostate relative to normal (Figure 1B, panel 3). This observation was consistent with the results of IHC examining the staining pattern of AR and p-AR in tumor and control samples. In wild-type prostates and TANT, positive staining was detected for AR, but not for p-AR, while PIN and carcinomas were strongly positive for both AR and the p-AR (Figure 4).

Microarray analysis of Neu*-induced PIN and prostate cancer
To obtain some clues on prostatic tumorigenic pathways, we examined comparatively the expression profiles of normal glands (n = 6; excluding the anterior lobe) and Neu*-induced neoplastic specimens bearing either early PIN (n = 5) or CAP developing on a late PIN background (n = 4). In the latter samples (referred to simply as ‘carcinomas’, for brevity), the PIN component predominated (PIN:CAP was ~5:1 in terms of surface area: 3.0 ± 0.6 versus 0.6 ± 0.3 mm², respectively). We thought, however, that it was not warranted at this stage to attempt difficult microdissection procedures prior to microarray profiling, because unavoidable cross-contamination,
Table I. Genes differentially expressed between *Neu*^−/-^ induced PIN/CAP and normal prostate

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>PIN</th>
<th>CAP</th>
<th>Pten</th>
<th>Akt1</th>
<th>hCAP</th>
</tr>
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### A. Upregulated genes

#### Transcription

- **Bcl3**: B-cell leukemia/lymphoma 3 6.2 5.5 + 1 4
- **Klf3**: Krüppel-like factor 3 4.4 2.5
- **Klf5**: Krüppel-like factor 5 2.1 2.1
- **Nfix**: Nuclear factor I/X 2.5 6.5 2
- **Sof2**: SRY-box containing gene 2 2.4 2.3 2
- **Ezh2**: Enhancer of zeste homolog 2 2.3 2.1 3
- **Sox2**: SRY-box containing gene 2 2.1 2.1
- **Trif**: Early growth response 1 2.0 2.0 + 1
- **Egr2**: Early growth response 2 1.5 4.0 1
- **Foxp1**: Forkhead box P1 1.9 2.5 3
- **Elf3**: E74-like factor 3 2.1 2.2 1
- **Egr1**: Early growth response 1 2.0 2.0 + 1
- **Aebp1**: AE binding protein 1 0.6 2.3

#### Antiapoptosis

- **Clu**: Clusterin 3.6 5.3 + 1
- **Traf4**: Tnf receptor associated factor 4 2.8 2.0 1
- **Tpt1**: Tumor protein, translationally-controlled 1 1.2 2.0 1
- **Pin1**: Pin1 1.3 2.1 3

#### Signaling

- **Egfr**: Epidermal growth factor receptor 2.0 2.0
- **Rhou**: Ras homolog gene family, member U 2.4 2.5 3
- **Arha2**: Ras homolog gene family, member A2 1.9 2.4 1
- **Sfrp1**: Secreted frizzled-related sequence protein 1 0.8 3.1 1
- **Sfrp4**: Secreted frizzled-related sequence protein 4 2.2 2.8 1
- **Fzd4**: Frizzled homolog 4 (*Drosophila*) 2.6 2.6 1
- **Homer2**: Homer, neuronal immediate early gene, 2 2.4 3.5 2
- **Osmr**: Oncostatin receptor 1.7 2.2 1

#### Adhesion, ECM, extracellular space

- **Reg3g**: Regenerating islet-derived 3 gamma 40.2 16.8 1
- **Pap**: Pancreatitis-associated protein 19.4 7.4 1
- **Cd44**: CD44 antigen 17.2 6.0 1
- **Spp1**: Secreted phosphoprotein 1 1.9 4.8 1
- **Thbs1**: Thrombospondin 1 1.8 2.9 1
- **Ltg6**: Integrin alpha 6 1.7 2.0 2
- **Mmp7**: Matrix metalloproteinase 7 1.7 2.4 1
- **Mmp15**: Matrix metalloproteinase 15 3.0 2.4 2
- **Timp1**: Tissue inhibitor of metalloproteinase 1 1.7 3.7 1
- **Sparc**: Secreted acidic cysteine rich glycoprotein 1.0 3.4 1
- **Lam**: Laminin, alpha 5 0.8 2.0 1
- **Lama5**: Laminin, alpha 5 2.2 2.0 1
- **Ppat**: Pheronectin 2.2 2.7 1
- **Col1a1**: Procollagen, type I, alpha 1 0.4 2.9 1
- **Col1a2**: Procollagen, type I, alpha 2 0.5 2.8 1
- **Col3a1**: Procollagen, type III, alpha 1 0.8 3.4 1
- **Col5a2**: Procollagen, type V, alpha 2 0.4 2.8 1
- **Col8a1**: Procollagen, type VIII, alpha 1 0.7 3.7 1
- **Col25a1**: Procollagen, type XXV, alpha 1 1.7 3.7 1

#### Other

- **Tff3**: Trefoil factor 3, intestinal 37.7 4.7 5
- **Dmdr1**: Deleted in malignant brain tumors 1 28.9 29.0 1

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especially including stromal elements, would not have improved the results to a great extent.

Although microarray analyses are useful for tumor classification by identifying ‘molecular signatures’ (‘clusters’ of discriminatory genes), the data cannot establish causal relationships between aberrant events without supporting experimental evidence. In fact, relative levels of transcripts can only serve as surrogate (and not always accurate) assays of protein amounts, while other processes, such as abnormal protein interactions, remain invisible by this profiling approach. Nevertheless, within such limitations, a gene-by-gene evaluation of the data through literature-based annotations has the potential to provide hypothesis-generating information for addressing mechanistic questions.

A clustering dendrogram derived without prior grouping of the data (unsupervised analysis) showed that the samples could be readily classified into three categories exactly corresponding to histopathologically assigned classes (normal prostates, PIN and carcinomas; Figure 5). However, although clearly different from wild-type, the PIN and carcinoma patterns, notwithstanding discernible alterations (see Figure 5, asterisk), were more similar between them than strikingly distinct. At least in part, this could be attributed to the co-existence of PIN and cancer in the same specimens, with the malignant portion being the lesser component (see above). On the other hand, the absence of dramatic changes in expression profiles would suggest that differences from wild-type eventually promoting invasive growth might already be present at a premalignant stage. This may turn out to be a general trend, as analogous observations were made for human PIN and CAP (28) and for human DCIS and invasive ductal breast carcinoma (29).

To avoid a tedious account of our entire set of ‘filtered’ data (see Materials and methods), we chose to present only some noteworthy results pertaining to transcription factors, antiapoptotic effectors, signaling molecules, cell-surface, extracellular and secreted proteins and potential tumor markers (Table I) and to comment briefly on a few interesting examples (Supplementary Table 2) paying special attention to genes relevant to human CAP or to cancer in general. In Table I, our data have been correlated with expression profiling results reported for human CAP cases (30–37) that were further analyzed in the Cancer Microarray Database Oncomine (68) are listed in column hCAP (human CAP). The numbers shown in column hCAP represent the frequency of occurrence of each Chip entry in the eight studies.

### Table I. Continued

<table>
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The listed genes are functionally categorized and some of them are annotated in Supplementary Table 2. For upregulated genes, the numbers in columns PIN and CAP are the fold differences of the average expression values for the neoplastic tissues over normal prostate, i.e. the ratios PIN:WT and CAP:WT (values are not listed in order of magnitude). For downregulated genes, the numbers in the corresponding PIN and CAP columns are the fold differences of the average expression values for the neoplastic tissues over normal prostate, i.e. the ratios PIN:WT and CAP:WT. Listed in column Akt1 are differentially expressed genes in mouse PIN induced by transgenic overexpression of myristoylated Akt1 (67). Corresponding results from eight expression profiling studies of human prostate cancer cases (30–37) that were further analyzed in the Cancer Microarray Database Oncomine (68) are listed in column hCAP (human CAP). The numbers shown in column hCAP represent the frequency of occurrence of each Chip entry in the eight studies.
influencing the expression of their target genes, including those encoding other transactivators and repressors. We sought, therefore, to identify in our set of profiling data transcription factors known to be linked to the Erbb/Erk signaling cascade. We observed (Table I and Supplementary Table 2) that both in PIN and CAP there was an increase of transcripts for Elf3, an Ets family transcription factor known to be upregulated by Neu signaling (38) and Egr1, a zinc-finger transactivator stimulated by Erk1/2 activation (39). Also upregulated was the expression of Egr2, which belongs to the same family as Egr1 and of Klf5, which is an Egr1 transcriptional target gene (40). The overexpression of EGR1 in human prostate cancer and its likely significance are already known (41), while our observations about Elf3, Egr2 and Klf5 are consistent with microarray data of human prostate cancer (27,31,33,35). However, with the exception of Elf3, there was no correspondence between mRNA levels and the transcription factor amounts assayed by IHC, as the signal intensity did not differ between normal and neoplastic tissue (not shown). The Elf3 signal, which was weak in wild-type glands, was increased in TANT and further increased in PIN and carcinoma (Figure 4). We note that in the examined prostatic tumors
we also found upregulated, at least at the mRNA level, the Ezh2 transcriptional repressor, which has been linked to androgen-refractory human CAP (42). Among other interesting cases of upregulated transcripts, particularly notable is the very high expression of Dmbt1. The corresponding locus encodes a putative receptor of Tff3 (also found increased), which was previously linked to human CAP (43,44). Consistent with the profiling results, IHC showed weak immunodetection of Dmbt1 in wild-type prostate, a slight increase of signal in TANT and strong immunoreactivity in PIN and cancer (Figure 4). Finally, we used IHC to examine Traf4 protein levels, considering that the mRNA for this apparently antiapoptotic molecule was found increased not only in our model, but also in 5/8 microarray datasets of human CAP (30–37). In contrast to other members of the Traf family, which are exclusively cytosolic, Traf4 has been found localized either to the cytoplasm or to the nucleus (45) (the significance of this observation is unclear). We found no difference in the intensity of positive nuclear Traf4 immunoreactivity between neoplastic tissues and controls. In contrast, the signal for cytoplasmic Traf4 was absent from normal prostate and TANT and strongly positive in PIN and cancer (Figure 4).

Table II. Immunohistochemical analyses of human prostate tissue arrays: comparison of marker expression levels between CAP and controls

<table>
<thead>
<tr>
<th>Marker</th>
<th>Controls</th>
<th>CAP</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n &lt;versus +&gt;</td>
<td>n &lt;versus +&gt;</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>EGFR</td>
<td>33 22 9 2</td>
<td>127 41 30 56</td>
<td>0.00045</td>
<td>0.004</td>
</tr>
<tr>
<td>NEU</td>
<td>33 33 0 0</td>
<td>127 75 29 23</td>
<td>&lt;10-6</td>
<td>N/A</td>
</tr>
<tr>
<td>ERBB3</td>
<td>33 16 16 1</td>
<td>127 18 16 93</td>
<td>0.0001</td>
<td>&lt;10-6</td>
</tr>
<tr>
<td>ELF3</td>
<td>33 22 7 4</td>
<td>114 48 19 47</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>EGR1</td>
<td>33 22 9 2</td>
<td>114 54 21 39</td>
<td>0.05</td>
<td>0.005</td>
</tr>
<tr>
<td>EGR2</td>
<td>33 16 10 7</td>
<td>115 33 17 65</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>KLF5</td>
<td>33 25 3 5</td>
<td>114 41 19 54</td>
<td>0.00005</td>
<td>ns</td>
</tr>
<tr>
<td>DMBT1</td>
<td>33 25 8 0</td>
<td>97 30 16 51</td>
<td>0.000007</td>
<td>0.000004</td>
</tr>
<tr>
<td>TRAF4</td>
<td>30 30 0 0</td>
<td>98 32 21 45</td>
<td>&lt;10-6</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Examples of our immunohistochemical results are shown in Figure 6. In analyzing this set of data, we made two kinds of comparisons. First, we evaluated the absence versus the presence (− versus +) of immunostaining, regardless of signal intensity. Focusing then only on the positive cases, we evaluated low versus high (L versus H) level of expression (as defined in Materials and methods). In either type of comparison, the difference in EGFR, NEU and ERBB3 expression between CAP and controls was statistically highly significant (Table II). Interestingly, the member of this family of receptors that was expressed most frequently in CAP was ERBB3 (~86% of the cases; 109/127), with EGFR being second in order (~68%; 86/127) and NEU being third (~41%; 52/127). Moreover, in the L versus H comparison, the ERBB3 expression was significantly associated with a high Gleason score, as did the expression of EGFR in the (−) versus (+) comparison, while the expression of NEU was not related to this parameter (Table III). We think, however, that the most interesting fact revealed by our analysis was the high statistical significance in the co-expression of ERBB3 and EGFR.

Fig. 6. Immunophenotyping of human prostate cancer tissue arrays. Shown are examples of immunostaining of human specimens bearing either BPH or CAP using antibodies against the markers indicated to the left and right of two sets, each consisting of two columns. The BPH specimens were used as controls (results with normal prostates were indistinguishable to those for BPH). The increased staining in human CAP versus BPH is analogous to that observed for the same markers in mouse Neu−/−-induced prostatic carcinomas. Original magnification ×400 (all panels).
(− versus + comparison) and also ERBB3 and NEU (both comparisons), while a NEU/EGFR association was not observed (Table IV). Interestingly, the same co-expression relationships between these three ERBB receptors were observed in breast cancer cases (24).

During this IHC analysis of human CAP cases, we also examined the expression of other markers previously studied in our mouse model: the transcription factors ELF3, EGR1, EGR2 and KLF5; the putative DMBT1 receptor; and the anti-apoptotic TRAF4 molecule (Figure 6). Statistically significant differences in the expression or pairwise co-expression of these markers between cancer and control cases are shown in Tables II–V. A strong association in co-expression between DMBT1 and TRAF4, which might turn out to be useful molecular markers for CAP, is notable. TRAF4 was also associated with a high Gleason score and both markers were associated with NEU and ERBB3, but not with EGFR.

### Discussion

The mouse model that we have generated shows that prostate-specific expression of Neu* carrying an activating point mutation can induce the appearance of invasive CAP that is histopathologically analogous to the predominant type of human CAP (acinar adenocarcinoma, derived from the luminal epithelial cells of the gland). Apparently, the oncogenic Neu* acts in collaboration with secondary tumorigenic events, as implied by the long latency period preceding the manifestation of malignancy.

To our knowledge, somatic mutational activation of NEU has not been observed in human CAP. Thus, the presumptive participation of ERBB-mediated signaling in prostatic tumor progression in a subset of sporadic cases should be attributed to receptor overexpression. Our activated Neu* model does not simulate directly the human condition but, despite this limitation, it should have physiological relevance, especially in the context of dissecting Erbb-related tumorigenic signaling components. It is notable, in this regard, that transgenic overexpression in the mouse mammary glands of either activated or Neu overexpressor forms leads to somatic activation of NEU and an increasing incidence of invasive CAP in the mammary glands.

**Table III.** Immunohistochemical analyses of human prostate tissue arrays: comparison of marker expression levels between carcinomas with low (1–6) and high (7–10) Gleason scores.

<table>
<thead>
<tr>
<th>Low Gleason score</th>
<th>High Gleason score</th>
<th>P-value (− versus +) (L versus H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n− +</td>
<td>n− +</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Low</th>
<th>High</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>24</td>
<td>14</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>NEU</td>
<td>24</td>
<td>18</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>ERBB3</td>
<td>24</td>
<td>6</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>ELF3</td>
<td>20</td>
<td>13</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>EGR1</td>
<td>20</td>
<td>10</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>EGR2</td>
<td>21</td>
<td>11</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>KLF5</td>
<td>20</td>
<td>11</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>DMBT1</td>
<td>20</td>
<td>9</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>TRAF4</td>
<td>18</td>
<td>9</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

P-values (− versus + and L versus H; above and below the diagonal, respectively). The data are presented in Supplementary Table 3.

**Table IV.** Immunohistochemical analyses of human prostate tissue arrays: P-values of statistical analyses of marker co-expression levels in carcinomas.

<table>
<thead>
<tr>
<th>EGFR</th>
<th>NEU</th>
<th>ERBB3</th>
<th>ELF3</th>
<th>EGR1</th>
<th>EGR2</th>
<th>KLF5</th>
<th>DMBT1</th>
<th>TRAF4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>ns</td>
<td>0.008</td>
<td>0.0065</td>
<td>ns</td>
<td>0.045</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NEU</td>
<td></td>
<td>ns 0.0003</td>
<td>0.0005</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ERBB3</td>
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<td>ns 0.04</td>
<td>ns</td>
<td>ns</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ELF3</td>
<td>0.01</td>
<td>0.05</td>
<td>0.000001</td>
<td>&lt;10^-6</td>
<td>&lt;10^-6</td>
<td>0.000007</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>EGR1</td>
<td>0.035</td>
<td>0.01</td>
<td>0.005</td>
<td>0.04</td>
<td>&lt;10^-6</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGR2</td>
<td></td>
<td></td>
<td>ns 0.01</td>
<td>0.0004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLF5</td>
<td></td>
<td>ns 0.02</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMBT1</td>
<td></td>
<td>ns 0.01</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAF4</td>
<td></td>
<td>ns 0.03</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P-values (− versus + and L versus H; above and below the diagonal, respectively). The data are presented in Supplementary Table 3.

**Table V.** Immunohistochemical analyses of human prostate tissue arrays: P-values of statistical analyses of marker co-expression levels in control samples.

<table>
<thead>
<tr>
<th>EGFR</th>
<th>NEU</th>
<th>ERBB3</th>
<th>ELF3</th>
<th>EGR1</th>
<th>EGR2</th>
<th>KLF5</th>
<th>DMBT1</th>
<th>TRAF4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NEU</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>B</td>
</tr>
<tr>
<td>ERBB3</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>B</td>
</tr>
<tr>
<td>ELF3</td>
<td></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGR2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLF5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMBT1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAF4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P-values (− versus + and L versus H; above and below the diagonal, respectively). The data are presented in Supplementary Table 3.

N/A (non-applicable) indicates that, although there is a significant difference, a P-value cannot be assigned because a marker is not expressed in one of the compared cases. Absence of a marker from both cases in a pairwise comparison is denoted as B (blank). Only the cytoplasmic Traf4 is taken into consideration in these comparisons.
wild-type Neu, which exhibit similar signaling features, results in the development of histopathologically indistinguishable carcinomas, although those induced by the mutated form of the receptor appear with significantly shorter latency (47–49). In the case of prostate, however, there is no alternative to our model. Considering the long latency and low incidence of prostatic cancer observed with the constitutively active Neu transgene, we think that generation of a more realistic mouse model by prostatic overexpression of wild-type Neu is not feasible, as tumor development within the mouse lifespan cannot be expected.

Consistent with our mouse data using wild-type prostate glands, definite membrane staining of NEU was not detected in control human prostate tissue (Figure 6), in agreement with previous reports (50,51). In addition, the staining pattern of NEU in human prostate cancer was analogous to that seen in our mouse model and was confirmed in 41% of the samples that we have examined (Figure 6 and Table II). However, such high frequency was not always observed in previous studies. In fact, for patients treated by surgery alone, the reported frequencies of positive cases varied widely in the range between 10 and 38% (50–60). This variability in IHC results, even when the same or comparable antibodies of high-specificity are used, can be attributed to variations in methodology and tissue selection, in conjunction with the intratumoral heterogeneity and multifocality that characterizes prostate cancer. Interestingly, NEU expression has also been detected by IHC in human PIN (61).

Whenever it is encountered, NEU overexpression is presumably caused by unknown transcriptional and/or post-translational mechanisms because, in contrast to breast cancer, NEU amplification (defined with the stringent criterion of ≥4 gene copies per nucleus in a significant fraction of malignant cells), if at all detectable, is a rare event in prostate tumors observed in no more than 2–3% of the NEU-positive cases (56,62,63).

Importantly, NEU overexpression appears to play a role in androgen-independent (AI) prostate tumor growth in a significant fraction of cases. The AI state corresponds to tumor recurrence after a period of successful androgen-deprivation treatment and is characterized by the appearance of cells that can proliferate even in the presence of very low levels of androgen.

One of the proposed mechanisms for this hormone-refractory condition is stimulation of the AR through a cross-talk between the NEU and AR pathways. It was shown, e.g., that xenograft-derived AI sublines expressed NEU at levels higher than those found in their androgen-dependent counterparts (64). Moreover, NEU overexpression in an hormone-dependent line allowed cell proliferation in androgen-deprived medium and resulted in transcriptional activation of androgen-dependent genes in the absence of ligand (64,65).

Although the mechanistic details of a functional link between NEU signaling and AR activation in human prostate cancer are not fully understood, progress was recently made by examining the effects of a dual EGFR/NEU small molecule inhibitor (PKI-166) using cell lines and prostate cancer xenografts (46). The results indicated that the active receptor complex for AR function was not EGFR/NEU, but NEU/ERBB3, which appeared to protect AR protein from degradation while stimulating its binding to regulatory regions of AR target genes.

Although AR appears to be a substrate for an unknown kinase regulated by NEU signaling, at least as evaluated by using a commercially available antibody recognizing phosphorylated AR at Ser81 (46), it continues to remain unclear whether AR phosphorylation correlates with its transcriptional activity and if it is relevant to tumorigenesis. In fact, when we used the same antibody against p-AR (Ser81) to study the previously examined prostate cancer tissue arrays (see above), we detected ubiquitous positive immunostaining that did not differ in intensity from that of control specimens (data not shown). This is in contrast with our data from Neu-induced mouse CAP, in which the presence of p-AR, lacking from controls, appears to be tumor-specific (Figure 1B, panel 3 and Figure 4). It should not escape attention, however, that Serine residues are phosphorylated in vivo in at least seven AR positions, including Ser81 (66).

Aside from the observed p-AR difference, the increased expression of both Egrf and Erbβ3 in Neu-induced mouse prostatic neoplasms detected by IHC prompted an analogous investigation of human CAP tissue arrays that revealed co-expression of the receptor pairs NEU/ERBB3 or EGF/ERBB3, but not NEU/EGFR, in apparently different tumor groups. Based on these relationships, considered at face value, we propose that the prevalent heterodimerization partner in CAP is ERBB3, which can functionally associate either with NEU or with EGFR and become involved in the process of androgen-independence in different subsets of prostate carcinomas. This hypothesis is experimentally testable and can accommodate previous observations about the importance of either NEU (46) or EGFR (60) in human CAP progression.

Supplementary material
Supplementary material is available on www.carcin.org/

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Conflict of Interest Statement: None declared.

References


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