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Frequent overexpression of *ETS*-related gene-1 *(ERG1)* in prostate cancer transcriptome

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Transcription factors encoded by the *ETS* family of genes are central in integrating signals that regulate cell growth and differentiation, stress responses, and tumorigenesis. This study, analysing laser microdissected paired benign and malignant prostate epithelial cells from prostate cancer (CaP) patients ($n = 114$; 228 specimen) by GeneChip and quantitative real-time RT–PCR, identifies *ETS*-related gene (*ERG*), a member of the *ETS* transcription factor family, as the most frequently overexpressed proto-oncogene in the transcriptome of malignant prostate epithelial cells. Combined quantitative expression analysis of *ERG* with two other genes commonly overexpressed in CaP, *AMACR* and *DD3*, revealed overexpression of at least one of these three genes in virtually all CaP specimen (54 of 55). Comprehensive evaluation of quantitative *ERG1* expression with clinicopathological features also suggested that *ERG1* expression level in prostate tumor cells relative to benign epithelial cells is indicator of disease-free survival after radical prostatectomy.
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Prostate cancer (CaP) is the most common malignancy and the second leading cause of cancer mortality in American men (Nelson *et al*., 2003; Srikantan and Srivastava, 2003). High-throughput gene expression analyses strategies are being widely applied for the identification of genes with aberrant expression in cancer by comparing tumor and normal areas of the particular organ or tissue (Nelson *et al*., 2003; Srikantan and Srivastava, 2003). Recent studies on CaP-associated gene expression profiling revealed consistent overexpression of *HEPSIN* (Dhanasekaran *et al*., 2001), *AMACR* (Rubin *et al*., 2002), and *DD3* (Bussemakers *et al*., 1999) in the majority CaP cells; however, functions of these genes in CaP biology remains to be defined. Decreased or absent expression of *GSTP1* has been noted as one of the earliest expression alterations in the majority CaP cells (Nelson *et al*., 2003). Despite intensive search, alterations of oncogenes or tumor suppressor genes that are prevalent in CaP remain to be defined (Isaacs and Kainu, 2001; Gelmann, 2003; Nelson *et al*., 2003; Srikantan and Srivastava, 2003). Alterations of oncogenes *BCL2* and *C-MYC*, tumor suppressor genes *p53* and *PTEN,* and androgen receptor (*AR*) associate with only a subset of primary CaP cells, and show more frequent association with advanced or metastatic CaP (Nelson *et al*., 2003; Srikantan and Srivastava, 2003).

Prostate tumor is a highly heterogeneous mixture of different cell types where both epithelial and stromal cells have been shown to play roles in the process of prostate tumorigenesis (Nelson *et al*., 2003; Srikantan and Srivastava, 2003). Therefore, monitoring gene expression changes in specific cell types, for example, the epithelial or stromal cells, may hold the key to defining gene alterations that contribute to CaP development. Consistent with this concept, our laboratory has been evaluating cell-specific gene expression signatures in CaP by laser microdissection (LCM) of epithelial cells from benign and malignant glands in radical prostatectomy specimens of patients with primary CaP using Affymetrix GeneChip platform. One of our major goals was to identify oncogenes common in primary CaP. Two patient groups (total $n = 18$) were selected from over 300 CaP patients undergoing radical prostatectomy: one with aggressive cancer (PSA recurrence, Gleason score 8–9, seminal vesicle invasion, poor tumor differentiation), the other with nonaggressive cancer (no PSA recurrence, Gleason score 6–7, no seminal vesicle invasion, well or moderate tumor

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differentiation). The two patient groups were matched for known risk factors: age, race, and family history of CaP. Amplified RNA from the microdissected tumor and benign epithelial cells was assayed on the HG U133A high-density oligonucleotide GeneChip (Affymetrix, Santa Clara, CA, USA). The expression data from the paired benign and tumor cells of 18 CaP patients (36 GeneChips) was analysed by multidimensional scaling (MDS) using the MATLAB package (http://arrayanalysis.nih.gov/marray.html). Comparison of expression between matched tumor and benign prostate epithelial cells has identified the *ERG* oncogene, a member of the *ETS* transcription factor family (Reddy *et al*., 1987; Hart *et al*., 1995; Sementchenko *et al*., 1998; Oikawa and Yamada 2003; Hsu *et al*., 2004), as the most consistently overexpressed oncogene in malignant epithelial cells of the prostate. A probe set (213541_s_at) on the HG U133A chip for an EST (AI351043), which represents the three prime region of the *ERG* mRNA, indicated *ERG* overexpression (over twofold) in tumor cells of 14 of 18 CaP patients (78%) (Supplementary Figure 1).

Initial validation of the GeneChip data by TaqMan real-time quantitative RT–PCR (QRT–PCR) assay in microdissected tumor and benign prostate epithelial cells of 20 CaP patients (including all 18 patients analysed by GeneChip) confirmed a consistent, significant tumor-associated *ERG* overexpression in 85% of patients (17 of 20) (Figure 1). As a quality test of the LCM-RNA specimens used in this study, expression of *AMACR*, a recently identified frequent CaP-associated overexpression (Rubin *et al*., 2002), and expression of *GSTP1*, a gene known to be commonly absent in CaP (Nelson *et al*., 2003), were also determined (Figure 1). As expected, overexpression of *AMACR* was detected in CaP cells of 95% of the patients. Also consistent with the literature, *GSTP1* expression was significantly decreased in the tumor cells of each CaP patient (100%), confirming the high quality of the LCM-derived tumor and benign specimens and the reliability of the GeneChip as well as the presented QRT–PCR data.

A detailed mapping study of the chromosomal region (21q22.2–q22.3) containing the *ERG* gene has recently described its complete exon–intron structure with nine alternative transcripts (Owczarek *et al*., 2004). The Affymetrix GeneChip probe set (213541_s_at) that indicated *ERG* overexpression in CaP (Supplementary Figure 1), as well as the TaqMan probe designed for the validation experiment (Figure 1), recognize a region specific for both *ERG1* and *ERG2* isoforms (Figure 2a), but exclude isoforms 3–9 (Owczarek *et al*., 2004). Therefore, to further distinguish between these two isoforms, the expression of the *ERG1* and *ERG2* splice forms were tested in PC3 cells and in normal prostate tissue (pooled prostate RNA from 20 men, Clontech), as well as in microdissected tumor and normal prostate epithelial cells from five CaP patients (data not shown). Since only *ERG1* was expressed in the prostate and in

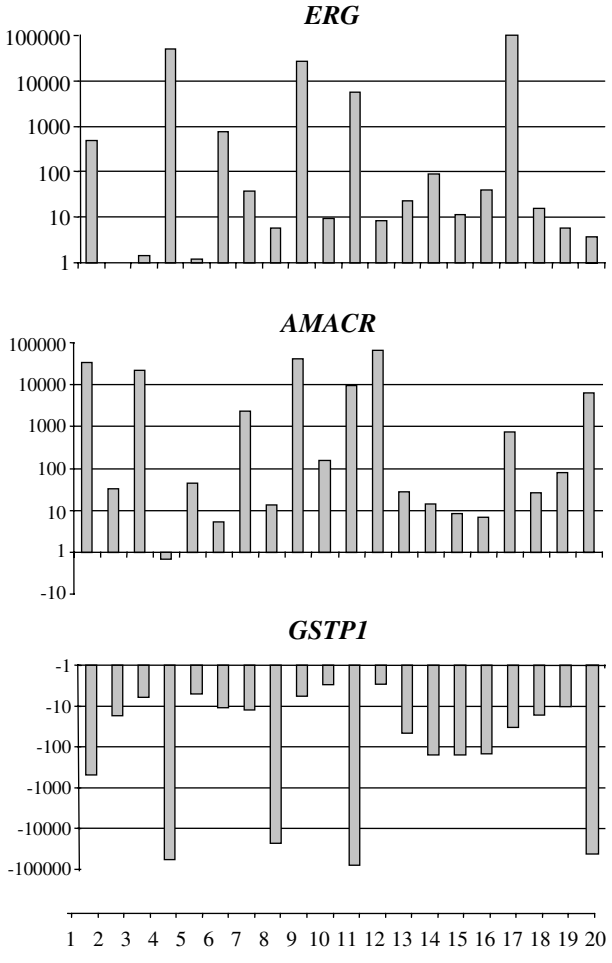

Figure 1 Relative expression levels of *ERG*, *AMACR*, and *GSTP1* genes in matched tumor and benign prostate epithelial cells. *Y*-axis: Gene expression ratios (log scale) measured in tumor versus matched benign LCM sample pairs by TaqMan-based QRT–PCR. The relative gene expression level is presented as fold change = $2^{(\Delta C_T \text{ benign} - \Delta C_T \text{ tumor})}$ of tumor versus matched benign cells, where ΔC_T means normalized C_T (threshold cycle) value of target genes to *GAPDH*; *X*-axis: CaP patients analysed (1–10: aggressive CaP, 11–20: nonaggressive CaP). Normal and cancer cells were laser-capture microdissected (LCM) by a pathologist from OCT-embedded and H&E-stained frozen prostate sections of radical prostatectomy specimens (2000 laser shots for one sample). Total RNA was isolated from the LCM samples with the MicroRNA kit (Stratagene, La Jolla, CA, USA) and quantified using RiboGreen dye (Molecular Probes, Eugene, OR, USA) and VersaFluor fluorimeter (BioRad, Hercules, CA, USA). Real time QRT–PCR (TaqMan) was essentially performed as described (Petrovics *et al*., 2004). Total RNA isolated from paired tumor and normal LCM epithelium specimens was converted to cDNA (Sensiscript, Qiagen, Valencia, CA, USA). Quantitative gene expression analysis was performed by TaqMan-based QRT–PCR on ABI 7700 (PE Applied Biosystems, Foster City, CA, USA). The TaqMan primers and probe recognizing both *ERG1* and *ERG2*, but not other *ERG* isoforms (Owczarek *et al*., 2004), were: forward primer: 5′-AGAGAAACATTCAGGACCTCATCATTATG -3'; reverse primer: 5′-GCAGCCAAGAAGGCCATCT-3′; and Taqman probe: FAM-TTGTTCTCCACAGGGT – TAMRA (see location of TaqMan probes in Figure 2a). The expression of *GAPDH* was simultaneously analysed as endogenous control, and the target gene expression in each sample was normalized to *GAPDH*. Thermal cycling conditions: 95°C for 10 min, 50 cycles at 95°C for 15 s, and 60°C for 1 min. RNA samples without reverse transcription were included as the negative control in each assay

PC3 cells, but *ERG2* expression was not detectable, a TaqMan QRT–PCR probe and primers were designed, which specifically recognize only the *ERG1* splice form (Figure 2a). *ERG1* expression was determined in 228 RNA specimens from microdissected matched tumor and benign prostate epithelial cells of 114 CaP patients. *ERG1* expression data, normalized to *GAPDH,* is summarized in Figure 2b. Overall, 62.4% of the 114 CaP patients analysed had significant overexpression of *ERG1* isoform in their tumor cells (Figure 2b), while 16.6% of CaP patients had no detectable *ERG1* expression. In all, 82 CaP patients who were analysed for both *ERG1* and *ERG* expressions (splice forms 1 and 2 together), as defined by specific TaqMan QRT–PCR probes (Figure 2a), revealed tumor-associated overexpression frequencies of 63.4 and 72.0%, respectively (Supplementary Figure 2). Therefore, *ERG1* isoform-specific expression may actually reflect an underestimate of the overall *ERG* expression in CaP.

The *ERG1* overexpression in tumor cells identified by GeneChip analysis and verified by real-time QRT–PCR assays was further validated by *in situ* hybridization. Based on the real-time QRT–PCR data, six patients with high *ERG1* overexpression in their tumor cells (and as a control one patient with no *ERG1* overexpression) were selected for *in situ* hybridization and quantitative image analysis in a blinded fashion. As expected, in each case, the *in situ* expression data confirmed the over-

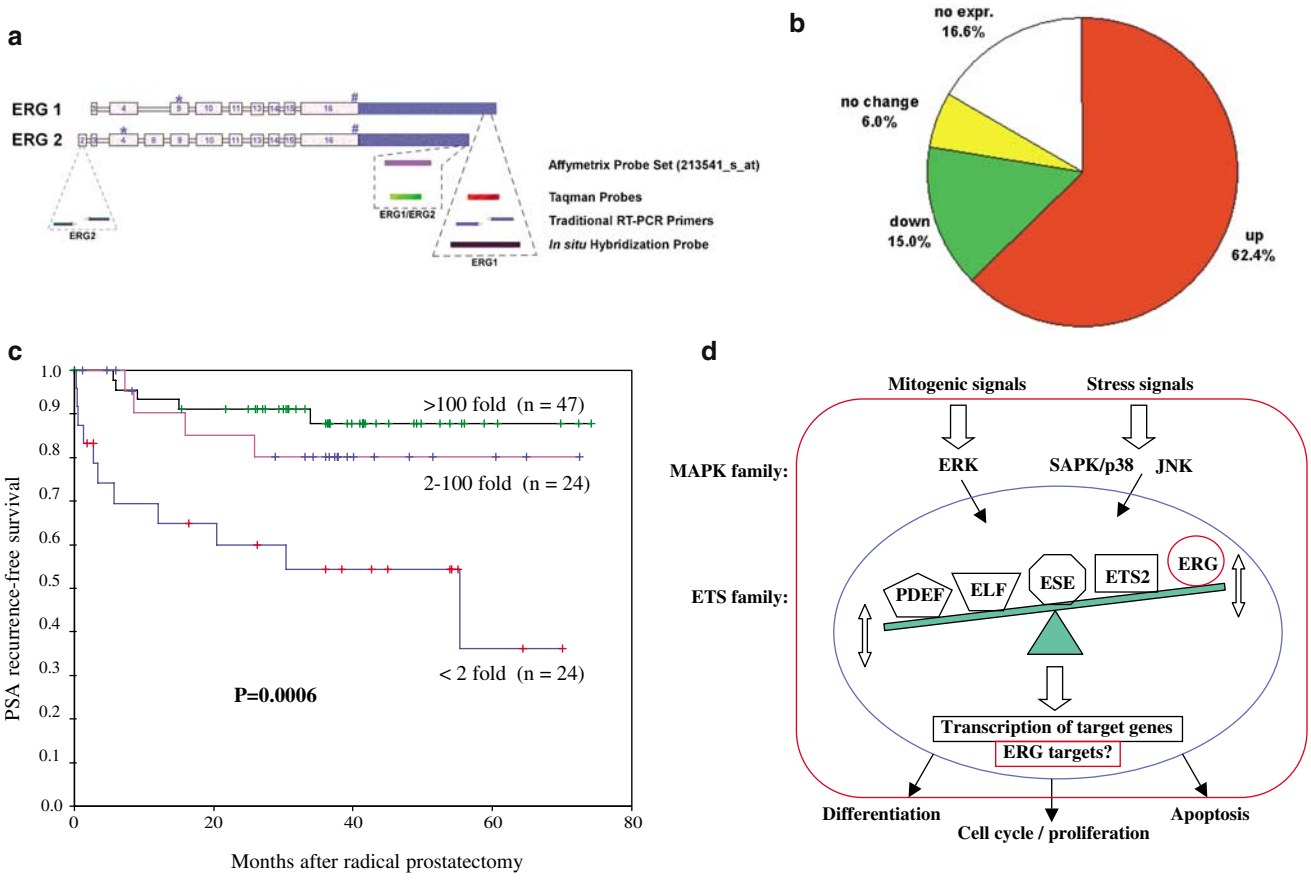

Figure 2 (**a**) Map of *ERG1* and *ERG2* isoforms with probe and primer locations. The light boxes represent exons, the blue boxes are the three prime noncoding exon regions (Owczarek *et al*., 2004). Translational start and stop codons are indicated by star and pound signs, respectively. The location of the Affymetrix probe set (213541_s_at), the TaqMan probes, the traditional RT–PCR primers, and the *in situ* hybridization probe is indicated. (**b**) *ERG1* expression in tumor and benign prostate epithelial cells of 114 CaP patients. The pie chart illustrates patient distribution by *ERG1* expression as measured by real time QRT–PCR (TaqMan). TaqMan primers and probe for the *ERG1* splice form were: forward primer: 5′-CAGGTCCTTCTTGCCTCCC-3′; reverse primer: 5′-TATGGAGGCTC-CAATTGAAACC-3′; and Taqman probe: FAM-TGTCTTTTATTTCTAGCCCCTTTTGGAACAGGA – TAMRA. Patients were sorted in four categories based on fold change of *ERG1* expression in tumor versus benign cells: 1. overexpression in tumor (>2-fold); 2. underexpression in tumor (<0.5-fold); 3. no significant difference (0.5–2-fold); 4. no detectable *ERG1* expression. (**c**) Correlation of *ERG1* expression and PSA recurrence-free survival. Kaplan–Meier analysis of correlation with postprostatectomy PSA recurrence-free survival was performed on 95 CaP patients that have detectable levels of *ERG1* mRNA by real-time QRT–PCR (TaqMan). Kaplan–Meier survival curves were stratified by the following *ERG1* expression categories: >100-fold overexpression; 2–100-fold overexpression; <2-fold overexpression or underexpression of *ERG1* in the prostate tumor cells. The *P*-value ($P = 0.0006$) is indicated in bold face. (**d**) Working hypothesis for potential *ERG* functions in CaP. On the basis of the observations in this report we hypothesize that in prostate epithelium, *ERG*, as a member of the *ETS* family, may respond to mitogenic and/or stress signals transduced by various MAP kinases, and modulate transcription of target genes favoring tumorigenesis. Changes in *ERG* expression level may influence these key pathways during CaP development/progression

expression of *ERG1* in the tumor epithelial cells (Supplementary Figure 3). Representative *in situ* hybridization photographs of tumor and benign epithelium from the same areas of the prostates that were previously used for the LCM–QRT–PCR quantitation are presented in Supplementary Figure 4.

The quantitative features of *ERG1* expression in benign and tumor epithelial cells of prostate were analysed for any association with clinicopathological parameters. Since the tumor versus benign expression ratios of *ERG1* did not have normal distribution, the Wilcoxon Rank Sum Test was used to analyse its relationship with various clinicopathologic features (Supplementary Table 1). Intriguingly, *ERG1* expression in prostate tumor tissue showed highly significant association with longer PSA recurrence-free survival ($P=0.0042$), well and moderately differentiated grade ($P=0.0020$), lower pathologic T stage ($P=0.0136$), and negative surgical margin status ($P=0.0209$), suggesting that *ERG1* overexpression in tumor cells is generally higher in less aggressive CaP than in more aggressive CaP. We also found a significant correlation of high *ERG1* overexpression with Caucasian versus African American ethnicity ($P=0.0086$) (Supplementary Table 1). To further explore the correlation with PSA recurrence, Kaplan–Meier survival analysis was performed based on three patient groups: CaP patients with tumor versus benign *ERG1* expression ratio of <2-fold, 2–100-fold, and >100-fold (Figure 2c). The results showed that patients with higher *ERG1* overexpression in their prostate tumor tissue had significantly longer PSA recurrence-free survival (log rank test, $P=0.0006$) (Figure 2c). The 36-months PSA recurrence-free survival for patients with <2-fold *ERG1* expression ratio ($n=24$) was 54.4%, while for patients with >100-fold *ERG1* expression ratio (n = 47) it was 87.7%. From a univariate COX proportional hazard ratio regression analysis for PSA recurrence-free time using *ERG1* tumor/benign cells expression ratio, race, diagnostic PSA, Gleason sum, pathologic T stage, margin status, and seminal vesicle invasion status, we found that five of these variables (*ERG1* tumor/benign cells expression ratio, Gleason sum, pathologic T stage, margin status, seminal vesicle invasion) had a significant *P*-value (Supplementary Table 2). The multivariate COX proportional hazard ratio regression analysis of the significant variables from the univariate analysis shows that *ERG1* overexpression (>100-fold versus <2-fold: $P=0.0239$, RR = 0.274, overall *P*-value 0.0369) and Gleason sum (Gleason 8–10 versus Gleason 2–6: $P=0.0478$, RR = 4.078, overall *P*-value 0.0148) are independent predictors of PSA recurrence after radical prostatectomy (Table 1). These results strongly suggest that some features of *ERG1* expression (tumor versus benign ratios) in radical prostatectomy specimens carry a predictive value for patient prognosis.

It has been shown that both *ERG* and other members of the *ETS* family, such as *ETS2*, are proto-oncogenes with mitogenic and transforming activities (Reddy *et al*., 1987; Hart *et al*., 1995; Sementchenko *et al*., 1998; Oikawa and Yamada 2003; Hsu *et al*., 2004). Chromosomal translocations involving *ERG* is linked to Ewing sarcoma, myeloid leukemia, and cervical carcinoma (Oikawa and Yamada, 2003). *ERG* overexpression, without amplification of DNA copy number, was recently reported in acute myeloid leukemia (Baldus *et al*., 2004). Other oncogenes, such as *C-MYC, N-MYC*, *and L-MYC*, *HER2, BCL-2* (Srikantan and Srivastava, 2003), *CYCLIN D1* (Nelson *et al*., 2003; Srikantan and Srivastava, 2003), and *C-MAF* (Hurt *et al*., 2004) are frequently overexpressed in various cancers, the latter one often without DNA amplification. The *ETS* family of proteins shows a wide variety of expression patterns in human tissues. *ERG* is expressed in endothelial tissues, hematopoietic cells, kidney, and in the urogenital track (Oikawa and Yamada, 2003). *ERG* expression has been detected in endothelial cells (microvessels) of the stroma in a small proportion of CaPs (Gavrilov *et al*., 2001). Our results establish *ERG1* as one of the most frequently overexpressed proto-oncogenes described thus far in the transcriptome of malignant prostate epithelial cells. The *ETS*-related transcription factors play a central role in mediating mitogenic signals transmitted by major cellular pathways including the MAPK pathway (Oikawa and Yamada, 2003). *ETS*2 has been implicated in CaP, but it is overexpressed only in a small proportion of CaP specimens (Liu *et al*., 1997). *ERG*, similarly to *ETS2*, is a transcription factor with oncogenic activity, but its role in CaP remains to be determined. On the basis of the observations in this report we hypothesize that in prostate epithelium, *ERG*, as a member of the *ETS* family, may respond to mitogenic and/or stress signals transduced by various MAP kinases, and modulate transcription of target genes favoring tumorigenesis (Figure 2d). Changes in *ERG* expression level may

Table 1 Multivariate COX proportional hazard ratio analysis of PSA recurrence-free time

Factors	*Crude hazard ratio (95% CI)*	*P*
ERG1 fold changes		**0.0369**
2–100-fold versus <2-fold	0.320 (0.097–1.059)	0.0620
>100-fold versus <2-fold	0.274 (0.089–0.843)	**0.0239**
Gleason sum		**0.0148**
7 versus 2–6	0.948 (0.223–4.033)	0.9424
8–10 versus 2–6	4.078 (1.014–16.401)	**0.0478**
Pathologic T stage		
PT3/4 versus pT2	3.306 (0.636–17.177)	0.1550
Margin status		
Positive versus negative	1.116 (0.421–2.959)	0.8254
Seminal vesicle		
Positive versus negative	1.308 (0.466–3.670)	0.6098

Statistical analysis was performed with the SAS software package (SAS Institute Inc., Cary, NC, USA). Crude hazard ratios with 95% confidence interval are shown for tumor versus benign *ERG1* expression ratios, and for four clinical parameter categories, in a multivariate COX proportional hazard ratio analysis. Significant *P*-values (<0.05) are in bold face

influence these key pathways during CaP development/progression. The reason for the significantly reduced overexpression of *ERG1* in aggressive CaP is not clear at this time, but this type of expression profile during tumor development is not unprecedented. Similar biphasic expression profile of *HEPSIN* (Dhanasekaran *et al*., 2001) and *AMACR* (Rubin *et al*., 2002) was also observed in prostate cancer during tumor development. Further studies will include assessment of ERG1 protein expression and expression patterns of ERG1 target genes.

The strikingly high frequency of *ERG* overexpression in CaP cells prompted us to compare *ERG* expression with two other genes, *AMACR* and *DD3*, that are commonly overexpressed in CaP cells. We have evaluated quantitative gene expression features of *AMACR* and *DD3*, along with the *ERG* gene, in laser microdissected matched tumor and benign prostate epithelial cells from 55 CaP patients. As expected, *AMACR* and *DD3* showed upregulation in tumor cells of 78.2 and 87.3% of CaP patients, respectively (Figure 3). *ERG* overexpression in tumor cells was detected in 78.2% of the same group of CaP patients (Figure 3). Comparative expression analysis revealed that when the *AMACR* and *ERG* expression data are combined, 96.4% of the CaP patients showed upregulation of either of the two genes in tumor cells (Figure 3). Similarly, the combination of the *ERG* and *DD3* expression data improved the cancer detection power of either of the genes to 96.4% (Figure 3). When combining the expression data from all the three genes, 98.2% of the CaP patients showed upregulation of at least one of the three genes in tumor cells (Figure 3).

Our finding presented here, that *ERG1* is overexpressed in the majority of CaP specimens, suggest for a role of this *ETS*-related transcription factor in prostate tumorigenesis. Combined gene expression analysis of *ERG* with *AMACR* and *DD3,* exhibiting CaP association in virtually all patients, shows a promising potential of *ERG* along with *AMACR* and *DD3* as a gene panel in CaP diagnosis. Our results also strongly suggest that certain features of *ERG1* expression are valuable prognostic indicators of pathologic stage and disease-free survival after radical prostatectomy. In addition, this study provides rationale for future investigations of *ERG1* functions in CaP cells, and for the exploration of potential therapeutic applications of the *ERG1* transcription factor in CaP treatment.

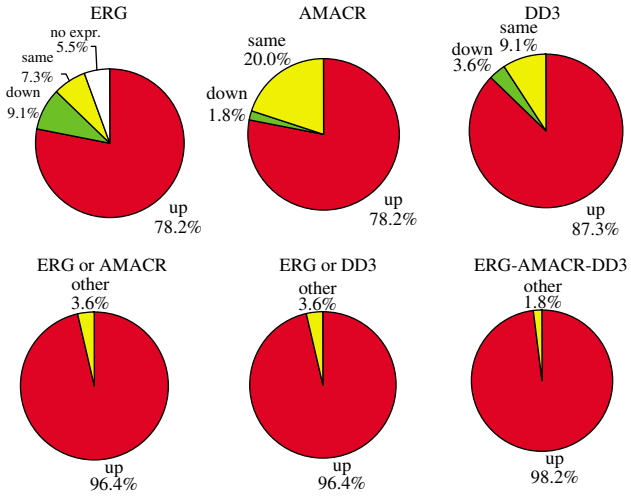

Figure 3 Combined gene expression analysis of *ERG*, *AMACR*, and *DD3* in tumor and benign prostate epithelial cells of 55 CaP patients. The pie charts illustrate patient distribution by tumor versus benign gene expression ratios. Four gene expression categories are indicated: 1. 'Up': overexpression in tumor compared to benign (>2-fold); 2. 'Down': underexpression in tumor compared to benign (<0.5 fold); 3. 'Same': no significant difference (0.5–2 fold); 4. 'No expr.': no detectable gene expression. 'Other' (other than expression category 1.) collectively defines patients with expression categories 2, 3, and 4 for the indicated genes

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