

## EGFR pathway polymorphisms and bladder cancer susceptibility and prognosis

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**The epidermal growth factor receptor (EGFR) pathway has recently been appreciated as a central mediator of tumorigenesis and an important drug target; however, the influence of genetic variation in this pathway on bladder cancer is not understood. Pathway activation leads to cell proliferation, angiogenesis and is antiapoptotic. We sought to test the hypothesis that bladder cancer susceptibility and survival are modified by inherited variations in the sequence of the EGFR and its pathway members. We tested associations using a population-based study of 857 bladder cancer cases and 1191 controls from New Hampshire. Multifactor dimensionality reduction software was used to predict gene–gene interactions. We detected an increased risk of bladder cancer associated with variant genotypes for the single nucleotide polymorphisms EGFR\_03 [adjusted odds ratio (OR) 1.7 (95% confidence interval (CI) 1.0–2.8)] and EGFR\_05 [adjusted OR 1.5 (95% CI 1.0–2.1)] compared with wild-type. EGFR variants experienced longer survival than those with wild-type alleles [e.g. adjusted hazard ratio EGFR\_1808 0.3 (95% CI 0.1–0.9)]. In contrast, the variant form of the ligand, EGF\_04, had worse survival [adjusted hazard ratio 1.5 (95% CI 1.0–2.3)] compared with wild-type. Our findings suggest modified bladder cancer risk and survival associated with genetic variation in the EGFR pathway. Understanding these genetic influences on increased bladder cancer susceptibility and survival may help in cancer prevention, drug development and choice of therapeutic regimen.**

### Introduction

In western countries, bladder cancer is the fourth most common malignancy in men and the eighth most common in women (1). The majority of this disease is attributed to cigarette smoking; bladder cancer risk is up to 4-fold higher among cigarette smokers compared with non-smokers (1). A hereditary component is likely since a family history of bladder cancer and variations in genes that detoxify aromatic amines are associated with increased risk (2,3).

Bladder cancer generally carries a favorable prognosis. However, in 2008, ~14 100 deaths occurred in the USA from bladder cancer. According to the USA Statistics Epidemiology and End Results program (1996–2002), the five-year survival rate for localized disease is 94%, whereas for regional staged cancers the survival rate is only 47%. Those with distant metastasis at the time of diagnosis carry the lowest survival rate of 6% (4). Established prognostic factors include multiplicity, tumor size and degree of invasion or carcinoma

**Abbreviations:** CCND1, cyclin D1; CI, confidence interval; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; LD, linkage disequilibrium; MDR, multifactor dimensionality reduction; OR, odds ratio; SNP, single nucleotide polymorphism; VEGF, vascular endothelial growth factor.

*in situ* (5). Although these histopathologic factors can be used to predict prognosis, the behavior of each group is heterogeneous, and the ability to predict which of the initially indolent non-invasive tumors will eventually become invasive is not yet achieved, highlighting the need for new prognostic markers (6).

The epidermal growth factor receptor (EGFR) is a tyrosine kinase transmembrane receptor in the ErbB family of receptors expressed on the surface of epithelial cells (7). EGFR regulates important processes in carcinogenesis, including cell survival, cell cycle progression, tumor invasion and angiogenesis. Ligands including epidermal growth factor (EGF) bind to EGFR activating signal transduction pathways that upregulate transcription factors leading to growth stimulation (8). A number of *EGFR* mutations have been recently characterized in tumors (9). Some of these mutations constitutively activate the receptor, sending pro-cancerous signals to genes downstream. EGFR overexpression is frequently observed in tumors and pre-cancerous lesions and induces tumor formation in animal studies. EGFR expression in bladder cancer independently predicts stage progression and mortality (10–13).

EGFR pathway inhibitors are clinically active in epithelial cancers (reviewed in ref. 14). More than nine trials are under way to evaluate the effectiveness of this strategy in the bladder (<http://www.clinicaltrials.gov/>) (15). Ionizing radiation in combination with the EGFR inhibitor gefitinib blocked bladder cancer cell colony formation in nude mice significantly better than radiation alone ( $P = 0.04$ ). While the initial results of the clinical trials of EGFR-tyrosine kinase inhibitors for other epithelial cancers are very encouraging, it is clear that there is dramatic interpersonal variation in drug response (15). A recent study in preclinical models of bladder cancer indicated dramatic variations in the level of EGFR expression and the ability of the EGFR TK inhibitor gefitinib to inhibit the EGFR pathway in different bladder cancer cell lines (10). Gefitinib treatment induced regression of bladder tumors induced by some, but not all these cell lines, suggesting interindividual variations in the efficacy (10). These inhibitors block expression of another polymorphic pathway member critical to angiogenesis, vascular endothelial growth factor (*VEGF*), and there is evidence that EGFR regulates the cell cycle control gene, cyclin D1 (*CCND1*) (16,17).

Polymorphisms in EGFR have previously been investigated in relation to the following cancers: lung (18–20), breast (21,22), oral (23), colorectal (24–27), gastrointestinal (28), brain (29,30), endometrial (31) and liver (32). These studies suggest that genetic variation in the *EGFR* gene may be related to increased cancer risk and is associated with increased EGFR protein levels and/or activity (20,21,23).

The influence of polymorphisms in EGFR and its pathway members, *EGF*, *VEGF* and *CCND1*, on bladder cancer risk and prognosis have not been investigated extensively. The amplification and overexpression of the EGFR dimerization partner human epidermal growth factor receptor 2 gene have been shown to be associated with bladder cancer and its progression. This study also suggested that the human epidermal growth factor receptor 2 gene polymorphism at Ile/Ile genotype for codon 655 might be related to an increased risk of disease progression (33). *EGFR* single nucleotide polymorphisms (SNPs), with the exception of *EGFR\_1808*, have not been examined functionally (19).

Our study's aim was to test whether inherited variations in EGFR and genes that EGFR regulates (*CCND1* and *VEGF*) modified bladder cancer susceptibility and survival. This project utilized 857 cases and 1191 controls from a population-based study of incident bladder cancer to assess EGFR pathway variations and their relationship to this cancer.

### Materials and methods

#### Study group

Detailed methods have been described previously (34). Briefly, we identified all cases of bladder cancer diagnosed among New Hampshire residents, ages

25–74 years, from 1 July 1994 to 31 December 2001 from the State Cancer Registry and interviewed a total of 857 bladder cancer cases, which was 85% of the cases confirmed to be eligible for the study. Controls <65 years of age were selected using population lists obtained from the New Hampshire Department of Transportation. Controls 65 years of age and older were chosen from data files provided by the Centers for Medicare & Medicaid Services of New Hampshire. For efficiency, we shared a control group with a study of non-melanoma skin cancer covering an overlapping diagnostic period of 1 July 1993 to 30 June 1995 (34). We selected additional controls for bladder cancer cases diagnosed from 1 July 1995 to 30 June 1998 and matched to these cases on age (25–34, 35–44, 45–54, 55–64, 65–69 and 70–74 years) and gender. Most (>95%) of the subjects in this study are of Caucasian origin, so our analyses were not appreciably altered by restricting to Caucasians. We interviewed a total of 1191 controls (the total shared control group  $n = 1063$  and additional controls  $n = 128$ ), which was 70% of the controls confirmed to be eligible for the study.

#### Personal interview

Informed consent was obtained from each participant and all procedures and study materials were approved by the Committee for the Protection of Human Subjects at Dartmouth College. Consenting participants underwent a detailed in-person interview, usually at their home. Questions covered, but were not limited to sociodemographic information (including level of education), lifestyle factors such as use of tobacco (including frequency, duration and intensity of smoking), family history of cancer and medical history prior to the diagnosis date of the bladder cancer cases or reference date assigned to controls.

Recruitment procedures for both the shared controls from the non-melanoma skin cancer and additional controls were identical and ongoing concomitantly with the case interviews. Case-control status and the main objectives of the study were not disclosed to the interviewers. To ensure consistent quality of the study interviewer, interviews were tape-recorded with the consent of the participants and routinely monitored by the interviewer supervisor. To assess comparability of cases and controls, we asked subjects if they currently held a driver's license or a Medicare enrollment card. Subjects were asked to provide a blood sample (buccal sample is requested in the case of a refusal). Samples were maintained at 4°C and sent via courier to the study laboratory at Dartmouth within 24 h for processing and analysis. To examine potential genotype-phenotype relationships, we stained 12 paraffin-embedded tumors with antibodies to EGFR (clone EGFR.113; Novocastra Laboratories, Newcastle, UK), phospho-EGFR and CCND1 (CP236A; Biocare Medical, Walnut Creek, CA), as described previously, and included both positive and negative controls (35). The intensity of positively stained tumor cells was scored by the study pathologist on a scale of 0–4. We then graphed the mean intensity of positively stained cells for each genotype.

#### Genotyping

DNA was isolated from peripheral circulating blood lymphocyte specimens harvested at the time of interview using Qiagen genomic DNA extraction kits (QIAGEN, Valencia, CA). DNA sufficient for genotyping was obtained on 658 cases and 932 controls. Genotyping was performed using the GoldenGate Assay system through Illumina's Custom Genetic Analysis service (Illumina, San Diego, CA) supplemented by Taqman assays (Applied Biosystems, Foster City, CA). We analyzed SNPs in the EGFR, its ligand EGF and pathway members CCND1 and VEGF that were included in the Illumina Cancer Panel because they were hypothesized to modify cancer risk plus additional haplotype tagging SNPs selected for major regions of the EGFR. Samples repeated on multiple plates yielded the same call for 99.9% of SNPs and 99.5% of samples submitted were successfully genotyped. Genotype calls were 99% concordant between genotyping platforms. We applied the PHASE 2.1 software to infer haplotypes from the analyzed SNPs. Linkage disequilibrium (LD) between SNPs was assessed using Haploview software (36).

#### Statistical analysis

A multifactor dimensionality reduction (MDR) interaction dendrogram was constructed from 14 EGFR pathway SNPs. The non-parametric MDR approach is described in detail elsewhere (37–40) and reviewed by Moore *et al.* (40). MDR is a data reduction (i.e. constructive induction) approach that seeks to identify combinations of multilocus genotypes and discrete environmental factors that are associated with either high risk or low risk of disease. Thus, MDR defines a single variable that incorporates information from several loci and/or environmental factors that can be divided into high-risk and low-risk combinations. This new variable can be evaluated for its ability to classify and predict outcome risk status using cross-validation and permutation testing. Here, we selected the best MDR model as the one with the lowest average prediction error. An error rate of 50% is expected under the null hypothesis. Statistical significance is determined using permutation testing. Here, the case-control labels are randomized 1000 times and the entire MDR model fitting

procedure repeated on each randomized data set to determine the expected distribution of testing accuracies under the null hypothesis. It is the combination of cross-validation and permutation testing that reduces the chances of making a type I error due to multiple testing (41,42). In this study, we used 10-fold cross-validation and 1000-fold permutation testing. MDR results were considered statistically significant at the 0.05 level. The MDR software is open-source and freely available from <http://www.epistasis.org/>.

Odds ratios (ORs) and their 95% confidence intervals (CIs) were estimated by multivariate logistic regression modeling using Intercooled STATA 9.0 (StataCorp LP, College Station, TX). The main goal was to assess the individual effects of each SNP on bladder cancer risk by comparing individuals with one or two variant alleles to homozygous wild-type alleles. All analyses were adjusted for age (<64 or ≥64), gender and smoking status (never, former, current). We also assessed bladder cancer risk within categories of toenail arsenic levels (<0.28, ≥0.28 µg/g, the 90th percentile), smoking status (never, ever) and genotype (wild-type, any variant). Haploview v.4.0 was used to check SNP LD and calculate Hardy-Weinberg equilibrium (36,43).

All genotypes were sorted by  $P$  for trend; the top three ranked SNPs were chosen for analysis by Cox regression. Survival analysis for bladder cancer cases was performed using Kaplan-Meier plots. To adjust for additional factors related to patient survival, Cox proportional hazards regression analysis was performed with age, gender, smoking status (never, former, current) as well as tumor stage/grade (non-invasive low grade, non-invasive high grade, invasive, carcinoma *in situ*) and treatment (surgery, chemotherapy, radiation, immunotherapy) in the model.  $P$  values represent two-sided statistical tests with statistical significance at  $P < 0.05$ .

## Results

Table 1 shows characteristics of the genotyped population. There were a higher proportion of men among cases than controls and more cases than controls reported that they were current smokers. As our study is population based, the majority of the tumors are non-invasive. We tested the 14 SNPs in the EGFR pathway for agreement with Hardy-Weinberg equilibrium and found all chi-square  $P > 0.05$  among cases or controls. An internal quality assurance sample was also utilized to check all genotyping; the control repeated on each plate was 100% concordant for each SNP.

In logistic regression models with adjustment for age, gender and smoking status (Table II), we found that EGFR\_03 variants had an increased risk of bladder cancer [OR 1.7 (95% CI 1.0–2.8)]. EGFR\_05 variants were also at increased risk [OR 1.5 (95% CI 1.0–2.1)]. As shown in the Haploview diagram (Figure 1), these two SNPs only showed 20% LD. This plot shows that the SNPs were not measuring redundant loci due to linkage disequilibrium. The EGFR\_529 SNP variant was rare and heterozygotes did not have a statistically significant OR compared with wild-type individuals. Haplotype analysis supported the increased risk associated with EGFR\_03 variant [haplotype 00010, OR 1.2 (95% CI 1.0–1.5)] and EGFR\_05 variant [haplotype 01100, OR 1.2 (1.0–1.4)] genotype compared with individuals who were wild-type at all loci tested (Table III). There was also a suggestion of elevated risk for haplotypes 01101 and 01110, although the prevalence was low and the estimates imprecise. Risk estimates did not differ significantly by gender.

There were no significant independent effects of the SNPs tested in VEGF, EGF or CCND1. Compared with wild-type, heterozygote and variant ORs with 95% CIs were VEGF\_04 (rs3025039, \*236C>T) 1.0 (0.8–1.2), VEGF\_05 (rs25648, Ex1-73C>T) 1.0 (0.8–1.3) and VEGF\_19 (rs1005230, -2487C>T) 0.9 (0.7–1.2), EGF\_02 (rs2237051, Ex14+71G>A) 1.0 (0.8–1.2), EGF\_04 (rs971696, IVS22-1443T>A) 1.0 (0.8–1.5), EGF\_08 (rs4444903, Ex1+61A>G), CCND1\_01 (rs678653, Ex5+852C>G) 0.9 (0.7–1.2), CCND1\_02 (rs603965, Ex4-1G>A) 0.9 (0.7–1.1) and CCND1\_03 (rs7177, Ex5+230C>A) 1.0 (0.8–1.2).

Using MDR software, we identified five potentially interacting SNPs in these EGFR pathway genes. We tested for two-way interactions among these five SNPs using logistic regression with adjustment for age, gender and smoking status. We observed a statistically significant increase in bladder cancer risk for individuals with at least one variant allele for both CCND1\_02 and VEGF\_05, OR 1.8 (95% CI 1.1–3.1), interaction  $P = 0.03$ .

**Table I.** Overall characteristics of genotyped population

	Controls, n (%)	Cases, n (%)
Age		
<64 years	427 (46)	297 (45)
≥64 years	505 (54)	361 (55)
Gender		
Female	350(37)	157 (24)
Male	582 (63)	501 (76)
Smoking status <sup>a</sup>		
Never	316 (34)	118 (18)
Former	469(50)	321 (49)
Current	147 (16)	218 (33)
Tumor stage/grade		
CIS	—	24 (4)
Non-invasive, low grade	—	358 (54)
Non-invasive, high grade	—	44 (7)
Invasive	—	162 (25)

CIS, carcinoma *in situ*.

<sup>a</sup>Former smoker defined as smoking stopped >1 year prior to the diagnosis date. One case was missing in smoking data, 70 cases were missing in stage/grade data.

We went on to investigate whether the impact of EGFR pathway polymorphisms was modified by exposure to the bladder carcinogens in cigarette smoke. We did not observe any significant differences in bladder cancer risk associated with smoking by EGFR pathway polymorphisms (data not shown).

As shown in Figure 2, we observed longer survival time among bladder cancer cases with the EGFR\_05 variant genotype compared with wild-type. These results are consistent with the phenotypic difference in phospho-EGFR (pEGFR) staining, which was decreased in homozygous variants compared with heterozygotes (Figure 3). Cox proportional hazards modeling with adjustment for age, gender, smoking status, stage/grade and treatment supported these results with an estimated hazard ratio of 0.6 (95% CI 0.3–1.0). Likewise, EGFR\_1808 variant cases also experienced better survival than wild-type adjusted hazard ratio 0.3 (95% CI 0.1–0.9). EGF\_04 heterozygosity was associated with reduced survival [hazard ratio 1.5 (95% CI 1.0–2.3)], while other EGFR pathway SNP variants were unrelated to bladder cancer survival rates.

## Discussion

EGFR pathway activation leads to proliferation, angiogenesis and is antiapoptotic. Activation by EGF involves receptor dimerization and asymmetric auto-phosphorylation of the tyrosine kinase region. This phosphorylation event activates signal transduction pathways that up-regulate transcription factors and control expression of downstream genes (8). Somatic mutations in the tyrosine kinase region can lead to constitutive activation and are associated with cancer. Likewise, certain EGFR genetic variations have also been shown to change EGFR protein levels in non-small cell lung cancers (20) and in our study and others may modify cancer risk (19).

We specifically found an increased risk of bladder cancer in relation to EGFR\_03 and EGFR\_05, whereas EGFR\_05 and EGFR\_1808 variants experienced longer survival. The EGFR\_03 SNP is located in exon 25 in a regulatory domain of the gene (44). This portion of the gene is a tyrosine kinase region, involved in phosphorylation and EGFR pathway signaling (28). Thus, it is conceivable that a SNP in this region could increase receptor activation.

The EGFR\_05 SNP is located in the intron preceding exon 21. Exon 21 is another tyrosine kinase region known for a number of activating mutations in multiple cancers including but not limited to lung, ovarian and other metastatic cancers (45–47). This SNP could be acting through other polymorphisms in LD either by increasing the propensity for this region to be somatically mutated or by introducing a splice variant. A variant allele associated with increased receptor

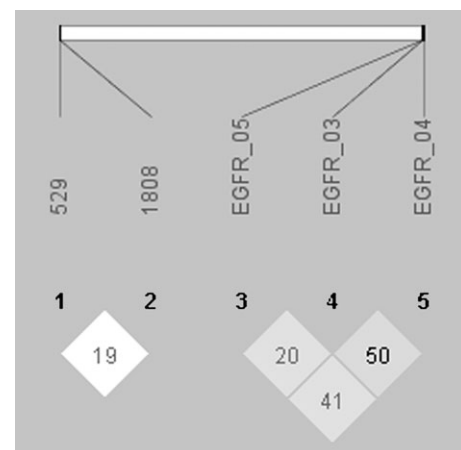
**Table II.** EGFR SNPs and risk of bladder cancer

EGFR SNPs	Controls, n (%)	Cases, n (%)	Bladder cancer OR (95% CI)
EGFR_03 (rs1140475, Ex25 + 8T>C) <sup>a</sup>			
Wild (TT)	662 (74)	453 (71)	1.0 Ref
Het (TC)	192 (22)	147 (23)	1.1 (0.9–1.5)
Var (CC)	36 (4)	37 (6)	1.7 (1.0–2.8)
Het/var (TC/CC)	228 (26)	184 (29)	1.2 (1.0–1.5)
EGFR_04 (rs2293347, Ex27 + 36C>T) <sup>a</sup>			
Wild (CC)	705 (81)	486 (80)	1.0 Ref
Het (CT)	152 (18)	117 (19)	1.1 (0.8–1.4)
Var (TT)	8 (1)	7 (1)	1.3 (0.5–3.8)
Het/var (CT/TT)	160 (18)	124 (20)	1.1 (0.8–1.4)
EGFR_05 (rs2017000, IVS21 + 96A>G) <sup>a</sup>			
Wild (AA)	387 (46)	254 (44)	1.0 Ref
Het (AG)	346 (42)	229 (40)	1.0 (0.8–1.3)
Var (GG)	98 (12)	94 (16)	1.5 (1.0–2.1)
Het/var (AG/GG)	444 (53)	323 (56)	1.1 (0.9–1.4)
EGFR_529 (rs35515689, Ex3 A>C T95P) <sup>b</sup>			
Wild (AA)	748 (99)	467 (97)	1.0 Ref
Het (AC)	8 (1)	11 (2)	1.7 (0.7–4.3)
Var (CC)	0 (0)	1 (1)	—
Het/var (AC/CC)	8 (1)	12 (2)	—
EGFR_1808 (rs11543848, Ex13 G>A R521K) <sup>b</sup>			
Wild (GG)	446 (56)	278 (54)	1.0 Ref
Het (GA)	303 (38)	212 (41)	1.1 (0.9–1.4)
Var (AA)	42 (5)	25 (5)	0.9 (0.5–1.5)
Het/var (GA/AA)	345 (44)	237 (46)	1.1 (0.9–1.4)

Analyses were adjusted for gender, age and smoking. Genotype data were unavailable for EGFR\_03 on 57 subjects, EGFR\_04 on 57 subjects, EGFR\_05 on 124 subjects, EGFR\_529 on 86 subjects and EGFR\_1808 on 15 subjects. Ex, exon; het, heterozygous; var, variant; Het/var, heterozygous and variant; IVS, intervening sequence; P, protein.

<sup>a</sup>Analyses are based on 639 cases and 893 controls included in the Illumina assays.

<sup>b</sup>Analyses are based on 520 cases and 801 controls included in the Taqman assays.



**Fig. 1.** EGFR haplotype LD plot. LD plot of EGFR SNPs showed 20% LD between significant SNPs EGFR\_05 and EGFR\_03. This plot shows that the tested SNPs were in tagged regions of the gene with low linkage and did not measure redundant loci.

activation, EGFR expression, or stability would increase cancer risk by promoting cell proliferation (48). EGFR heterodimerizes with other receptors, such as ErbB2/human epidermal growth factor

**Table III.** EGFR haplotypes and bladder cancer risk

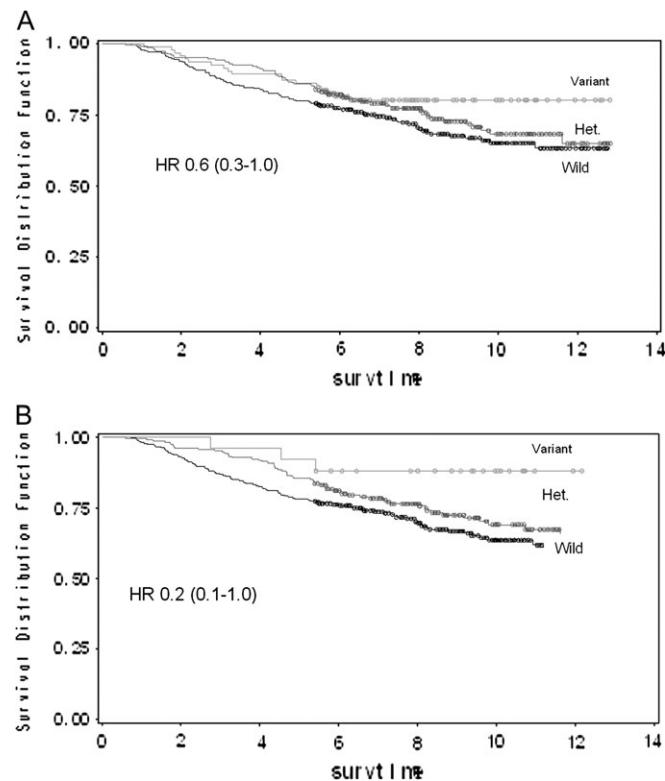
EGFR haplotypes <sup>a</sup>	(%) <sup>b</sup>	Overall OR (95% CI)
1 00000	(60)	1.0 Ref
2 00001	(2)	0.9 (0.6–1.4)
3 00010	(11)	1.2 (1.0–1.5)
5 00100	(3)	1.0 (0.7–1.4)
8 01000	(1)	1.1 (0.5–2.3)
10 01100	(16)	1.2 (1.0–1.4)
11 01101	(5)	1.3 (0.9–1.7)
12 01110	(1)	1.6 (0.9–3.0)
Other <sup>c</sup>	(1)	—

ORs adjusted for gender, age and smoking.

<sup>a</sup>Order: EGFR529 EGFR1808 EGFR\_05 EGFR\_03 EGFR\_04.

<sup>b</sup>Percent of subject chromosomes by haplotype.

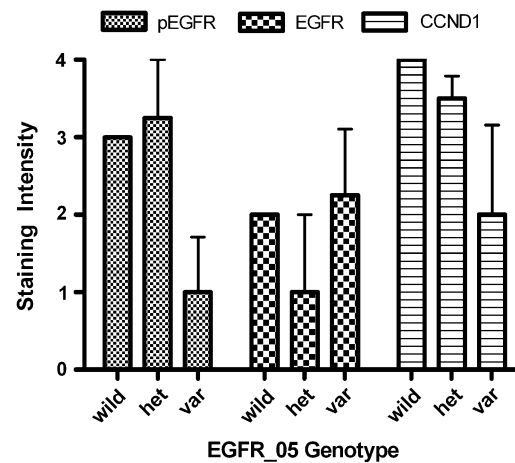
<sup>c</sup>Other: haplotypes where  $n \leq 10$ .



**Fig. 2.** Bladder cancer survival in relation to EGFR pathway SNPs. Kaplan-Meier plots show survival by (A) EGFR\_05 genotype, and (B) EGFR\_1808 genotype. Hazard ratios for heterozygotes and variants compared with wild-type were calculated using Cox proportional hazards modeling with adjustment for age, gender, smoking status, stage/grade and treatment. Black lines are homozygous wild-type, light grey are homozygous variant and dark grey are heterozygous.

receptor 2 gene to activate a downstream pathway that increases cell motility. For individuals who already have a tumor, if the receptors in variants had lower rates of heterodimerization than wild-type, they might have lower risk of metastasis and longer survival. Thus, the differential effects on risk versus survival could be due to modification of different downstream signaling cascades mediated by the receptor's dimerization options. Our risk and survival observations motivate further investigation into the molecular aspects modified by genetic variation in this pathway.

The EGFR\_1808 non-synonymous polymorphism is located in the extracellular ligand-binding domain 2 of the EGFR gene (18,28,44). As



**Fig. 3.** Level of pEGFR, EGFR and CCND1 protein in bladder tumors by genotype. Intensity of bladder tumor cells staining positively for pEGFR, EGFR and CCND1 is graphed by genotype for the EGFR\_05 SNP. Staining intensity was scored on a scale of 0–4 by a single pathologist.

in our study, bladder cancer risk was not associated with this exon 13 SNP at position 1808 in a previous gastrointestinal tract cancer study (28). Consistent with our observation of longer survival, this polymorphism decreases ligand-binding affinity and blocks tyrosine kinase activation, growth stimulatory signals and the induction of proto-oncogenes such as FOS, JUN, MYC in Chinese hamster ovary cells (49).

Other EGFR SNPs appeared unrelated to bladder cancer risk and survival. The very low frequency of variant EGFR\_529 resulted in wide CIs. The EGFR\_529 polymorphism occurs in exon 3 encoding the extracellular domain of the receptor. Thus, while the polymorphism could be functionally important, further studies of this polymorphism would be needed in even larger series than our own. The EGFR\_04 SNP is located in exon 25 near an intracellular internalization domain (28). Modification of this region would be hypothesized to affect the stability of the protein in the membrane. Although we did not observe significant differences in bladder risk by EGFR\_04 genotype, a study of lung cancer among Koreans detected an increased risk for EGFR\_04 variants that was statistically significant in ever-smokers, but not in never-smokers (19).

We also investigated EGFR pathway-related genes including VEGF. In a mechanistic study investigating this polymorphism, VEGF plasma levels were significantly lower in carriers of the VEGF\_04, 936T allele ( $9.1 \pm 2.7$  pg/ml, mean  $\pm$  SEM) than in non-carriers ( $28.0 \pm 5.5$  pg/ml,  $P = 0.033$ ). This base-pair change also led to the loss of a potential binding site for transcription factor AP-4 (50). Better survival rates in non-small cell lung cancer patients ( $P = 0.07$ ) (51) and acute myeloid leukemia patients ( $P = 0.03$ ) (52) have also been published. Bladder cancer risk was not modified by this polymorphism in our study, which is consistent with a large hospital-based Spanish bladder cancer study (6), although we did not find the main effect they observed for VEGF\_05 [OR 5.11 (95% CI 2.33–11.0)] (6). This could be attributed to variation in minor allele frequency between the controls within the two studies (minor allele frequency of 0.19 in the Spanish study, minor allele frequency of 0.26 in our study), country-specific exposures and ethnic differences. Nevertheless, we did observe increased risk and a significant interaction associated with having a variant allele for VEGF\_05 combined with a variant for CCND1\_02 (interaction  $P$  value = 0.03). This risk modification is biologically plausible since VEGF is a critical factor in angiogenesis, which feeds tumor growth, while CCND1 regulates the rate of division of the cells in the tumor. VEGF expression in bladder tumors has been linked to tumor progression (6,53). VEGF\_05 has also been associated with the development of coronary artery lesions in Kawasaki disease ( $P = 0.0002$ ) (54). CCND1 requires co-operation with other transforming factors or is otherwise regarded as a weak

oncogene (55,56). The Cyclin D1 SNP CCND1\_02 causes a splice variant that modifies cell growth, specifically entry into and completion of the S phase. It is located on the splicing region of exon 4, which modulates the production of two types of transcripts (56). This SNP was associated with an increased risk of non-Hodgkin's lymphoma in a population-based case-control study ( $P$  trend = 0.021) (57) and bladder cancer in a Japanese hospital-based population case-control study of 222 subjects ( $P$  = 0.022) (58); however, there was no association in a larger non-Hispanic Californian population-based case-control study with 1679 subjects (59) or in a Texas hospital-based case-control study (60) and no main effect in our study of Caucasians.

In our study, variant forms of EGF, the ligand that binds to the receptor EGFR, were unrelated to bladder cancer susceptibility, but shortened survival in our population. The variant G allele for EGF\_08 was associated with increased EGF expression in malignant melanoma (61) and increased gallbladder cancer risk ( $P$  = 0.012) (62), but to our knowledge has not been investigated in bladder cancer. EGF\_08 variants were also at decreased risk of ovarian cancer ( $P$  = 0.01) (63). The ligand SNP EGF\_04 was associated with a higher death rate among variants than in wild-type cases in our study. Previous studies of other EGF variants have also shown worse survival rates for esophageal cancer (64), higher EGF protein levels and quicker relapse of prostate cancer (65).

It should be noted that EGFR-targeted drugs were not yet utilized in these patients. EGFR polymorphisms do influence response to treatment with tyrosine kinase inhibitors in breast cancer patients (22). They may also be potential indicators of radiosensitivity in patients with rectal cancer treated with chemoradiation (26,27). Our population-based study was largely composed of non-invasive tumors, and thus our findings may be attributed more to differences in tumor aggressiveness than to differential response to treatment. To our knowledge, these polymorphisms have not been studied previously for their effect on bladder cancer survival and further studies are needed. Nevertheless, our data suggest that EGFR pathway polymorphisms may modify both bladder cancer risk and survival. Further confirmation of these relationships could help ultimately guide cancer prevention efforts or modify clinical care.

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