Modulation of Gene Expression and Cell-Cycle Signaling Pathways by the EGFR Inhibitor Gefitinib (Iressa) in Rat Urinary Bladder Cancer

Yan Lu1,2, Pengyuan Liu1,2, Francoise Van den Bergh3, Victoria Zellmer3, Michael James3, Weidong Wen1, Clinton J. Grubbs4, Ronald A. Lubet5, and Ming You1,3

Abstract

The epidermal growth factor receptor inhibitor Iressa has shown strong preventive efficacy in the N-butyl-N-(4-hydroxybutyl)-nitrosamine (OH-BBN) model of bladder cancer in the rat. To explore its antitumor mechanism, we implemented a systems biology approach to characterize gene expression and signaling pathways in rat urinary bladder cancers treated with Iressa. Eleven bladder tumors from control rats, seven tumors from rats treated with Iressa, and seven normal bladder epithelia were profiled by the Affymetrix Rat Exon 1.0 ST Arrays. We identified 713 downregulated and 641 upregulated genes in comparing bladder tumors versus normal bladder epithelia. In addition, 178 genes were downregulated and 96 genes were upregulated when comparing control tumors versus Iressa-treated tumors. Two coexpression modules that were significantly correlated with tumor status and treatment status were identified [r = 0.70, P = 2.80 × 10^{-15} (bladder tumor vs. normal bladder epithelium) and r = 0.63, P = 2.00 × 10^{-42} (Iressa-treated tumor vs. control tumor), respectively]. Both tumor module and treatment module were enriched for genes involved in cell-cycle processes. Twenty-four and twenty-one highly connected hub genes likely to be key drivers in cell cycle were identified in the tumor module and treatment module, respectively. Analysis of microRNA genes on the array chips showed that tumor module and treatment module were significantly associated with expression levels of let-7c (r = 0.54, P = 3.70 × 10^{-8} and r = 0.73, P = 1.50 × 10^{-65}, respectively). These results suggest that let-7c downregulation and its regulated cell-cycle pathway may play an integral role in governing bladder tumor suppression or collaborative oncogenesis and that Iressa exhibits its preventive efficacy on bladder tumorigenesis by upregulating let-7 and inhibiting the cell cycle. Cell culture study confirmed that the increased expression of let-7c decreases Iressa-treated bladder tumor cell growth. The identified hub genes may also serve as pharmacodynamic or efficacy biomarkers in clinical trials of chemoprevention in human bladder cancer. Cancer Prev Res; 5(2); 1–12. ©2011 AACR.

Introduction

Bladder cancer is one of the most common cancers in the United States and occurs predominantly in men. In 2010, there were an estimated 70,530 new cases of bladder cancer, with 52,760 cases in males (1). It is also the most expensive form of cancer to treat. Approximately 15% of bladder tumors become invasive tumors after infiltration through the basement membrane. Patients with muscle invasive disease are at high risk for recurrence, progression, and metastasis.

Treatment of mice or rats with N-butyl-N-(4-hydroxybutyl)-nitrosamine (OH-BBN) results in transitional and squamous cell urinary bladder cancers that bear significant histopathologic similarities to human bladder cancer and tends to be muscle invasive (2–4). This rodent model has been used extensively to characterize the tumorigenic process and to assess the efficacy of potential chemopreventive agents to inhibit the development of carcinogen-induced bladder cancers (4–6). OH-BBN–induced bladder tumors have increased production of survivin and glutathione S-transferase pi (GST-Pi), and decreased production of fragile histidine triad (FHIT) due to promoter methylation by immunohistochemical analysis (7, 8). Similar molecular changes are observed in human bladder tumors (9). Microarray analysis of OH-BBN–induced bladder tumors revealed a wide variety of induced genes, including cyclin...
D1, Pena, Cox-2, the calcium-binding proteins S100a4, S100a8, S100a10, and Annexin1 (10). Tumors which form in the rat bladder tumor model are similar at the level of gene expression to muscle invasive bladder tumors found in humans (11).

Recently, we examined the preventive effects of the epidermal growth factor receptor (EGFR) inhibitor Iressa on the induction of bladder tumors by OH-BBN (12). Treatment with Iressa (10 mg/kg body weight per day), beginning either 2 weeks or 12 weeks after the last dose of OH-BBN (when microinvasive carcinomas exist), decreased the incidence of large palpable bladder cancer by more than 90% at 6 months after the last dose of OH-BBN. To explore the mechanism of the antitumor action of Iressa in the treatment of bladder tumors, we implemented a systems biology approach to characterize gene expression and pathway modulation in rat bladder tumors treated with Iressa.

Materials and Methods

Rat bladder tumors

Female Fischer-344 rats were obtained from Harlan Sprague-Dawley, Inc. (virus-free colony 202) at 28 days of age and were housed in polycarbonate cages (5 per cage). The animals were kept in a lighted room 12 hours each day and maintained at 22°C ± 0.5°C. Teklad 4% mash diet (Harlan Teklad) and tap water were provided ad libitum. The carcinogen, OH-BBN, (TCI America; 150 mg/gavage, 2×/wk) was started when the rats were 49 days of age and continued for 8 weeks. The carcinogen vehicle was ethanol:water (20:80) in 0.5 mL. All animals (unless sacrificed early because of palpable tumor burden greater than 2 cm, weight loss greater than 20% of initial body weight, or because animals became moribund), were sacrificed 8 months following the initial OH-BBN treatment. Starting 6 months after the initial dose of OH-BBN, animals were palpated twice per week for the development of lesions. When an animal had developed a small palpable tumor (150–300 mg), rats were treated daily with Iressa at a dose level of 10 mg/kg body weight and sacrificed 5 days later. At the time of sacrifice, the urinary bladder from each rat was tied off, and samples of control bladder tumors from rats treated with Iressa were excised, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent RNA isolation and protein extraction. Additional bladder tissue from the same rat was fixed with 10% neutral formalin. For fixation, the bladders were examined under a high-intensity light dissecting microscope for lesions. Each lesion was rinsed with Tris-buffered saline (Fisher Cat ID E0032005101) phenol/chloroform extraction, and then the biotin-labeled cRNA was transcribed in vitro from cDNA by a BioArray High Yield RNA Transcription Labeling Kit (ENZO Biochem) and further purified by the RNeasy Mini Kit.

Affymetrix rat exon arrays

The labeled cRNA was applied to the Affymetrix Rat Exon 1.0 ST Array (Affymetrix) according to the manufacturer’s recommendations at the Vanderbilt Gene Microarray Core. Routine quality assessment was conducted on each exon array. The study used 10 arrays from bladder tumors, 7 arrays from normal bladder epithelia, and 7 arrays from Iressa-treated bladder tumors that had passed quality control criterion, as recommended by the manufactures. Gene level and individual exon signal estimates for the CEL files from the platform Rat Exon 1.0 ST Array were derived by the Robust Multi-array Average (RMA) algorithm, as implemented with Expression Console v1.1.1 (http://www. affymetrix.com/products_services/software/specific/ expression_console_software.affs).

Identifying differentially expressed genes

Two-sample student t test was used to identify differentially expressed genes (DEG) between 2 groups. To adjust for multiple testing in the study of high-dimensional microarray data, the local false discovery rate (LFDR) was estimated (13), which was implemented in the R package fdrtool (http://www. r-project.org/). The DEGs were defined as genes with LFDR less than 0.05 and fold change more than 1.5 between 2 groups.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was conducted to analyze the pattern of differential gene expression. GSEA is a computational method that determines whether a set of genes shows statistically significant differences in expression between 2 biological states and has proved successful in discovering molecular pathways involved in human diseases (http://www.broad.mit.edu/gsea). Using the Kolmogorov–Smirnov statistic, GSEA assesses the degree of “enrichment” of a set of genes (e.g., a pathway) in the entire range of the strength of associations with the phenotype of interest. GSEA was used to identify a priori defined sets of genes that were differentially expressed (14, 15). We used curated gene sets (c2) which contain genes in certain molecular pathways and gene ontology (GO) gene sets.

RNA isolation and amplification

Total RNA from normal bladder epithelia, bladder tumors, and bladder tumors treated with Iressa were isolated by TRIzol (Invitrogen) and purified by the RNeasy Mini Kit and RNase-free DNase Set (Qiagen) according to the manufacturer’s protocols. In vitro transcription–based RNA amplification was carried out on each sample. cDNA for each sample was synthesized by a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-(dT)24 primer as follows: 5'-GGCCAGTGAATTGTAATACGACT-CACTATAGG-GAGCCGG- (dT)24-3'. The cDNA was purified by phase-lock gel (Fisher Cat ID E0032005101) phenol/chloroform extraction, and then the biotin-labeled cRNA was transcribed in vitro from cDNA by a BioArray High Yield RNA Transcription Labeling Kit (ENZO Biochem) and further purified by the RNeasy Mini Kit.

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(c5) which consist of genes annotated by the same GO terms in the Molecular Signature Database (MSigDB; http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html). Because of a small sample size, the GSEA with the gene set permutation option was conducted. Selected gene sets identified from GSEA were then visualized with MetaCore software (http://www.genego.com/).

Hierarchical clustering analysis
Hierarchical clustering was carried out as follows. For the selected genes, expression indexes were transformed across samples to an N (0, 1) distribution with a standard statistical Z-Transform. These values were input to the GeneCluster program of Eisen and colleagues (16), and genes were clustered using average linkage and correlation dissimilarity.

Coexpression network analysis
To characterize gene expression and pathways in rat bladder tumors treated with Iressa, we applied a systems biology approach using a weighted gene coexpression network analysis (WGCNA; ref. 17). The WGCNA converts gene coexpression measures into connection weights as topology overlap measure (TOM). We chose expression profiles of 4,500 genes in the coexpression network analysis. These genes were either significantly differentially expressed between tumors with and without Iressa treatment (LFDR < 0.05 and fold change > 1.5 between 2 groups) or showed a large variability in expression. In weighted gene coexpression networks, modules correspond to clusters of highly correlated genes. We defined modules using a static method by hierarchically clustering the genes using 1-TOM as the distance measure with a height cutoff value of 0.95 and a minimum size (gene number) cutoff value of 40 for the resulting dendrogram.

To identify which module is correlated with treatment, we first calculated the module eigengene (i.e., first principal component of the expression values across subjects) using all genes in each module. We then correlated the module eigengenes to treatment using the Spearman Correlation. We determined intramodular connectivity for each gene by summing the connectivity of that gene with every other gene in that module. All network analyses were implemented in the statistical package WGCNA in R environment (18). The VisANT program was used to construct gene–gene interaction (connection) networks (19). Genes within modules were analyzed for GO term enrichment by the program DAVID (20).

Exon splicing analysis
A number of splicing-related mutations have been reported to be associated with malignancies, and some of these are within splice sites or splicing enhancers/silencers of cancer-related genes (21–23). To identify exon splicing, we first calculated the splicing index for each exon. The splicing index gives a measure of the difference in expression level for each probe set in a gene between 2 sets of arrays, relative to the gene level average in each set. This is calculated only for those probe sets that are defined as exon targeting and nonmultitargeted. Then, MIDAS (Microarray Detection of Alternative Splicing) algorithm was used for the detection of alternative splicing events. MIDAS P value was calculated by the “splanova” function. For genes containing at least one significant exon targeting probe set, we also calculated the maximum splicing index and retrieved the average fold change for each gene. The average fold changes were used to partition the data set into (on average) up- and downregulated genes. All exon splicing analyses were implemented in the statistical package exonmap (http://bioinformatics.picr.man.ac.uk).

Cell culture and treatment
The Iressa sensitive UMUC-5 cell line (human squamous cell carcinoma of the bladder; Sigma) were cultured in Eagle’s Minimal Essential Medium [EMEM; Earle’s balanced salt solution (EBSS)] supplemented with 2 mmol/L glutamine, 1% nonessential amino acids (NEAA), 1% penicillin/streptomycin, and 10% FBS (Invitrogen). The Iressa (Santa Cruz Biotechnology) was reconstituted in dimethyl sulfoxide (DMSO) at a stock concentration of 70 mmol/L and stored at −80°C. This stock was diluted in medium just before use so that the concentration of DMSO never exceeded 0.1%. For quantitative PCR (qPCR), the cells were grown in 10-cm dishes and allowed to reach 50% to 70% confluency; at that point the cells were treated with the indicated concentration of Iressa for 24 hours. Following the incubation, the cells were collected by trypsinization, washed in cold PBS, counted, and frozen as cell pellets. For MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega), 10,000 cells per well were plated in 24-well plates. Twenty-four hours later, the medium was changed, and the indicated amount of Iressa was added. The MTS assay was conducted following manufacturer’s protocol on that day (as day 0) and the following days.

miRNA qPCR
The protocol for miRNA isolation by the miRNeasy Mini kit (Qiagen) was followed, including following Appendix A which details specifically with the preparation of a miRNA-enriched fraction by the Qiagen RNeasy MinElute Cleanup Kit. The protocol for RT2 miRNA first strand kit was followed to obtain the cDNA template for the qPCR reaction. The SsoFast EvaGreen Supermix (Bio-Rad) protocol was followed for the qPCR reactions—carried out in triplicate, a no- cDNA reaction and a primer set corresponding to a housekeeping miRNA was included. The cycling program included a melt curve. The primer sets for let-7c (MPH0003A-200) and RNU6-2 (MPH01653A-200) were purchased from Qiagen.

Results
Differentially expressed bladder cancer genes altered with Iressa treatment
We observed 713 downregulated and 641 upregulated genes in OH-BBN–induced rat bladder tumors (LFDR < 0.05 and fold change > 1.5 between 2 groups). Downregulated genes in bladder tumors are enriched in several
metabolic processes such as lipid metabolic processes and cellular ketone metabolic processes (Fig. 1A and Supplementary Table S1) and upregulated genes are enriched in cell proliferation and migration processes (Fig. 1B and Supplementary Table S2). Similarly, we compared gene expression in control bladder tumors and Iressa-treated tumors and identified 178 upregulated genes and 96 downregulated genes in Iressa-treated versus control tumors. Most of the Iressa upregulated genes are involved in metabolic processes (Fig. 1C and Supplementary Table S3). The 3 most significant GO terms found in the downregulated genes after Iressa treatment were: cell-cycle phase, cell-cycle processes, and M phase (Fig. 1D and Supplementary Table S3). DEGs were input into hierarchical clustering analysis of all the samples. Three distinct groups were identified in complete concordance with the sample status as normal bladder epithelia, bladder tumor, and bladder tumor treated with Iressa (Fig. 2A), implying that these DEGs capture biological differences of the 3 tissue groups. In addition, Iressa-treated tumors clustered more closely to normal bladder epithelia than control tumors (Fig. 2B).

Gene coexpression networks and biological pathways

Sets of genes with expression levels that are highly correlated may share common biological process and regulatory mechanisms. To examine this possibility, we analyzed global expression profiles and their interactions in the study samples. To accomplish this, we constructed weighted gene coexpression networks based on pairwise Pearson correlations between the expression profiles. As indicated previously, the identified DEGs captured biological significance of the 3 tissue groups and thus were used to construct the network. The analysis included an additional 2,872 genes with greatest variability which determined by their coefficient of variance from 13,855 remaining genes. We conducted coexpression network analyses in data sets of control tumors versus normal bladder epithelia and control bladder tumors versus bladder tumors treated with Iressa, separately.

The WGCNA analysis identified a number of modules with highly coexpressed genes in both data sets (Fig. 3). These modules are significantly enriched for biologically important processes that are relevant to cancer, including
those for the cell cycle, immune response, lymphocyte and T-cell activation, response to DNA damage, and RNA binding and processing. We then examined whether individual expression profiles within each of the identified modules are associated with clinically related traits by estimating the module eigengene. In the data sets of control tumors versus normal samples, we found significant correlation of tissue status (i.e., cancer vs. normal) with the salmon-colored module ($r = 0.70, P = 2.80 	imes 10^{-15}$; Fig. 4A). Interestingly, the salmon-colored module also showed a significant correlation with expression levels of let-7c which is a microRNA gene ($r = 0.54, P = 3.70 	imes 10^{-8}$; Fig. 4B). In the data set of control bladder tumors versus bladder tumors treated with Iressa samples, we found that the green-colored module is associated with both tumor treatment status (i.e., tumors with Iressa treatment vs. tumors without Iressa treatment; $r = 0.63, P = 2.00 	imes 10^{-42}$; Fig. 4C) and let-7c expression ($r = 0.73, P = 1.50 	imes 10^{-65}$; Fig. 4D). Both the salmon- and
green-colored modules are significantly enriched for cell-cycle processes ($P = 5.97 \times 10^{-9}$ and $P = 1.90 \times 10^{-16}$, respectively).

Highly connected “hub” genes are hypothesized to play an important role in organizing the behavior of biological modules (24). To identify hub genes in the salmon- and green-colored modules, we estimated intramodular connectivity for each gene based on its Pearson correlation with all of the other genes in the modules. The genes with high intramodular connectivity tended to have stronger correlation with clinically related traits (Fig. 4). We set the weighted cutoff value of 0.40 to identify hub genes with the strongest connections to other genes. As a result, we identified 24 hub genes with at least 40 connections in the data sets of control tumors versus normal samples including \textit{Cdca2}, \textit{Lmnb1}, \textit{Sgo2}, \textit{Tpx2}, \textit{Anln}, \textit{Nuf2}, \textit{Kif4}, \textit{Ect2}, \textit{Bub1}, \textit{Prc1}, \textit{Kif20a}, \textit{Cit}, \textit{Uhrf1}, \textit{Depdc1}, \textit{Racgap1}, \textit{RGD1310335}, \textit{Casc5}, \textit{Dil}, \textit{Cena2}, \textit{Bub1b}, \textit{Tacc3}, \textit{Igapp3}, \textit{Fancl2}, and \textit{Cenpf}; whereas 20 genes were identified in the data set of control bladder tumors versus bladder
tumors treated with Iressa samples (Fancd2, Cep55, LOC683179, Kif2c, Tih, Cenpf, Bub1, Ect2, Kif20a, Nuf2, Tpx2, Cdca2, Ccnb1, Depdc1, Ncapd2, Melk, RGD1562646, Mastl, Ccnb2, and Sgo1). To illustrate their relationship, these genes are visualized in Fig. 5, suggesting a complex regulatory gene network with varying topology.

To explore the effects of let-7c expression on Iressa-treated bladder tumor growth, we checked the expression of let-7c in an Iressa-sensitive human squamous cell carcinoma of the bladder cell line UMUC-5 in the presence of increasing amount of Iressa. We first confirmed that the UMUC-5 cell line was Iressa sensitive following their growth curve in 0.1, 0.2, and 0.5 mM Iressa compared with no Iressa added. As previously described (25) and as seen in Fig. 6A, Iressa affected the growth of UMUC-5 at concentration higher than 0.1 mM. This experiment was done in triplicate and was carried out twice with similar results. We then collected the miRNA from UMUC-5 exposed for 24 hours to 0.2 and 0.5 mM of Iressa in parallel with a no treatment condition. These 3 samples of miRNA were used in qPCR reactions using either let-7c or RNU6-2 primer set. The results from the RNU6-2 reactions were used to normalize let-7c expression. As observed in Fig. 6B, Iressa induced 3- and 8-fold upregulation of let-7c when added at 0.2 and 0.5 mM, respectively. This qPCR was carried out twice with similar results. These results confirmed the association of let-7c expression with Iressa treatment observed in our microarray analysis of rat model.

**Exon splicing**

Alternative processing of pre-mRNA transcripts is a major source of protein diversity in eukaryotes and has been implicated in several disease processes including cancer (26). To identify alternative splicing variants, we calculated the splicing index (SI) for each exon; a measure of how much exon-specific expression, with gene induction factored out, differs between 2 types of tissues (e.g., bladder tumors vs. normal bladder epithelia or bladder tumors with treatment vs. without Iressa treatment). Using the cutoff value of maximum SI more than 1 and P value less than 1.0 \times 10^{-2}, we identified 194 and 272 genes that have alternative splicing that are down- and upregulated in bladder tumors versus normal epithelia, respectively. Supplementary Figure S1 listed the top genes alternatively spliced between normal bladder epithelia and bladder tumors.
These alternatively spliced genes are enriched in organ morphogenesis, regulation of cell proliferation, cell migration, and immune system processes. Using the same criteria, we also identified 57 genes that have alternative splicing that are differentially expressed in control tumors and Iressa-treated tumors (Supplementary Table S4). Interestingly, 24 of the 57 genes overlapped with the list of genes that have alternative splicing in bladder tumors versus normal bladder epithelia, suggesting that Iressa treatment blocks aberrant splicing in these 24 genes in bladder tumors. For example, we identified exon splicing in Prkm2 when comparing exon-specific expression between bladder tumors and normal bladder tissues (Supplementary Fig. S2). The same exon splicing was observed between control tumors and treated tumors. The rationale behind these 2 observations is that Iressa treatment blocks aberrant splicing, and thus treated tumors have similar splicing status to normal samples.
effects of Iressa agrees with findings that genes and proteins associated with the EGFR pathway are overexpressed in bladder tumors as compared with normal bladder epithelia in both rodent model and humans (10, 30). Initial studies showed great promise for the use of EGFR inhibitors in the setting of advanced bladder cancer in humans (31, 32). As expected, Iressa treatment strikingly decreased levels of phosphorylated EGFR, AKT, and extracellular signal-regulated kinase (ERK; data not shown). The present study examined the effects of Iressa on gene expression in lesions with 2 objectives as follows: to (1) identify processes and pathways involved in the mechanism of Iressa efficacy and (2) identify biomarkers which might be useful in clinical trials with this class of antitumor agents. Studies suggest that highly centralized “hub” genes in the network architecture are more likely to be key drivers for cellular function (33). Characterization of these hub genes may help to give novel insight into bladder tumorigenesis and to develop additional novel drug targets for bladder cancer. Many of the hub genes we identified in the tumor and treatment modules are cell-cycle regulators such as
**Bub1, Casc5, Ccna2, Cenpf, and so on. Bub1**, which was identified as a highly connected gene in both modules, encodes a kinase involved in spindle checkpoint function. This kinase functions in part by phosphorylating a member of the mitotic checkpoint complex and activating the spindle checkpoint. p53 physically interacts with Bub1 at kinetochores in response to mitotic spindle damage, suggesting a direct role for hBub1 in the suppression of p53-mediated cell death (34). Altered expression of Bub1 is associated with therapy failure and death in patients with multiple types of cancer (35). Casc5, identified as another hub gene in both modules, is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1 (36). Ccna2 binds and activates CDC2 or cyclin-dependent kinase 2 (CDK2) kinases and thus promotes both cell cycle G1–S and G2–M transitions (37, 38). Cenpf is another cell-cycle regulator. Farne-sylation of Cenpf is required for G2–M progression and degradation after mitosis (40).

Several unique hub genes were identified in the module associated with efficacy of Iressa treatment, including Ccnb1, Cep55, Kif2c, LOC683179, Mastl, Melk, Ncapd2, and RGD1562646. Cep55 encodes a centrosomal associated protein involved in centrosome duplication, cell-cycle progression, and in the regulation of cytokinesis (41). Kif2c encodes a kinesin-like protein which has a role in bipolar spindle assembly (42). Melk is a potential regulator of G2–M progression and may antagonize the CDC25B phosphatase (43). Ncapd2 is an essential component of the human condensin complex required for mitotic chromosome condensation (44). These hub genes might be useful biomarkers in clinical trials with this class of agents.

Previous studies showed that several hub genes are associated with human bladder cancer. For example, the expression levels of NFI2 were increased in the majority of small cell lung cancer, cholangiocellular cancer, urinary bladder cancer, and renal cell cancers (45). The MPHOSPH1/PRC1 complex is likely to play a crucial role in bladder carcinogenesis and that inhibition of the MPHOSPH1/PRC1 expression or their interaction should be novel therapeutic targets for bladder cancers (46). KIF20A were upregulated in bladder tumors in both humans and rodents (47). An immunohistochemistry-based UHRF1 detection in urine sediment or surgical specimens can be a sensitive and cancer-specific diagnostic and/or prognosis method and may greatly improve the current diagnosis based on cytology (48). Immunocytochemical staining analysis detected strong staining of endogenous DEPDC1 protein in the nucleus of bladder cancer cells, and DEPDC1 expression was hardly detectable in any of 24 normal human tissues except the testis. Suppression of DEPDC1 expression with siRNA significantly inhibited growth of bladder cancer cells. DEPDC1 might play an essential role in the growth of bladder cancer cells and would be a promising molecular target for novel therapeutic drugs or cancer peptide vaccine to bladder cancers (49). RacGAP1 are expressed at high levels in superficial samples of bladder from patients with transitional cell carcinoma (TCC; ref. 50).

We identified 2 modules that are significantly associated with either cancer status (control cancer vs. normal bladder epithelium) or Iressa treatment status (Iressa-treated tumors vs. control tumors). An increase in proliferation-related genes was observed in the cancer versus normal bladder epithelium, whereas a decrease in proliferation-related genes was observed in Iressa-treated tumors versus control tumors. Interestingly, these 2 modules also showed significant association with let-7c expression. Let-7 is a microRNA and is a bona fide tumor suppressor. Let-7 inhibits the expression of multiple oncogenes, including RAS, MYC, and HMG2. Multiple important cell-cycle control genes are repressed by let-7, including cyclin D1, cyclin D3, cyclin A, CDK4 and CCNA2, CDC25A, CDK6, and CDK8 (51, 52). Decreased let-7 expression is linked to increased tumorigenesis and poor patient prognosis (53–56). This in agreement with our observation that a decrease in let-7c expression in tumors versus normal bladder epithelium and an increase in expression in Iressa-treated tumors versus control tumors. Cell-culture study also confirmed that the increased expression of let-7c decreases Iressa-treated bladder tumor cell growth. By searching miRNA target database, we found 2 hub genes differentially expressed with Iressa treatment, UHRF1 and Tacc3, which are target of let-7c.

TCC is by far the most common form of human bladder cancer. It accounts for overall more than 90% of bladder cancer cases. A total of 70% of TCC cases are characterized as superficial, meaning that the cancer is confined to the lining of the patient’s bladder, and therefore unlikely to spread into neighboring tissue (i.e., metastasize). The remaining 30% of TCC cases are characterized as muscle invasive, meaning that they have invaded through the lining into the muscular wall of the bladder, and therefore potentially into other nearby organs. Squamous cell carcinoma accounts for around 8% of bladder cancer cases. These cancers originate from the thin, flat cells that typically form as a result of bladder inflammation or irritation that has taken place for many months or years. Nearly all squamous cell carcinomas (SCC) are invasive. The comparison of gene expression profile of advanced squamous and TCC of the bladder showed that out of the 516 genes which were differentially expressed, only 30 showed significant differences between TCC and SCC (57). Another study showed that MHC class II, TNF, cytokines, and chemokines are among the upregulated genes in bladder SCC (58), which are similar to the findings of others who found that the invading cells of the bladder TCC stimulate the immune system. However, a group of immunology-related genes were downregulated in SCC compared with normal urothelium including interferon, different immunoglobulins, human leukocyte antigens protein, and interleukin-8 and interleukin-13. Cell adhesion molecules such as laminins, integrins, and 2 cadherins (E-cadherin and P-cadherin)
were downregulated in both SCC bladder cancer and invasive bladder TCC. These data indicated that the majority of gene expression profiles of bladder TCC and SCC are similar. In experiments conducted using male and female Fischer-344 rats, intragastric administration of OH-BBN has been shown to induce TCCs of the urinary bladder which were histologically similar to the human counterpart (4). This should be a good animal model to find potential biomarkers of chemoprevention agents.

In summary, we identified 2 coexpression modules that are significantly correlated with tumor status and Iressa treatment status. We further showed that these 2 modules are also significantly associated with expression levels of let-7c. Both tumor module and treatment module are enriched for genes involved in cell-cycle control. The tumor module and treatment module contain 24 and 21 highly connected hub genes that are likely to be key drivers of the cell cycle. These results suggest that let-7c and its regulated cell-cycle pathway might play a key role in governing bladder tumor suppression or collaborative oncogenesis and that Iressa exhibits its preventive efficacy on bladder tumorigenesis by regulating let-7 and its cell-cycle control. Cell-culture study confirmed that the increased expression of let-7c decreases Iressa-treated bladder tumor cell growth. The identified hub genes may also serve as pharmacodynamic or efficacy biomarkers in clinical trials of bladder cancer chemoprevention. Characterization of these hub genes may help to give novel insight into bladder tumorigenesis and develop novel drug targets. Finally, we showed that alternative exon usage correlates with bladder tumorigenesis and could be inhibited by the chemopreventive agent Iressa. The short-term Iressa treatment may operate through blocking of such effects.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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