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p-Benzoquinone initiates non-invasive urothelial cancer through aberrant tyrosine phosphorylation of EGFR, MAP kinase activation and cell cycle deregulation: Prevention by vitamin C

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Abbreviations:
Bax
BCL2-associated X protein
Bcl-2
B-cell lymphoma 2
CS
cigarette smoke
DNPH
2 4-dinitrophenylhydrazine
GAPDH
glyceraldehyde 3-phosphate dehydrogenase
IARC
International Agency for Research on Cancer
p-BQ
p-benzoquinone
p-BSQ
p-benzosemiquinone
PAHs
polycyclic aromatic hydrocarbons
PBS
phosphate buffered saline
ROS
reactive oxygen species
SDS PAGE
sodium dodecyl sulfate polyacrylamide gel electrophoresis
TUNEL
terminal deoxynucleotidyl transferase dUTP nick end labelling
WHO
World Health Organization
UC
urothelial carcinoma
CIS
carcinoma in situ
EGFR
epidermal growth factor receptor
MAPK
mitogen activated protein kinase

Keywords:
p-Benzoquinone

ABSTRACT

According to WHO classification system, non-invasive urothelial carcinoma represents urothelial carcinoma in situ (CIS) and dysplasia. Dysplastic urothelium often progresses to CIS that further advances to urothelial carcinoma (UC). The strongest risk factor for UC is cigarette smoking. However, the pathogenesis of cigarette smoke (CS)-induced UC is poorly understood. Earlier we had shown that p-benzoquinone (p-BQ), a major toxic quinone derived from p-benzosemiquinone of CS in vivo, is a causative factor for various CS-induced diseases. Here, using a guinea pig model we showed that prolonged treatment with p-BQ led to non-invasive UC, specifically carcinoma in situ (CIS) of the renal pelvis and dysplasia in the ureter and bladder. The mechanisms of carcinogenesis were p-BQ-induced oxidative damage and apoptosis that were later suppressed and followed by activation of epidermal growth factor receptor, aberrant phosphorylation of intracellular tyrosine residues, activation of MAP kinase pathway and persistent growth signaling. This was accompanied by deregulation of cell cycle as shown by marked decrease in the expression of $p21^{waf1/cip1}$ and cyclin D1 proteins as well as hyperphosphorylation of pRb. UC has been characterised by histopathology and immunohistochemistry showing aberrant CK20, increased Ki-67, and marked p53 nuclear immunopositivity with uniformly negative labelling of CD44. Oral supplementation of vitamin C (30 mg/kg body weight/day) prevented CIS of the renal pelvis and dysplasia in the ureter and bladder. Since majority of non-invasive UC progresses to invasive cancer with increased risk of mortality, our preclinical study might help to devise effective strategies for early intervention of the disease.

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Carcinoma in situ
Dysplasia
Aberrant EGFR activation
Cell cycle deregulation
Vitamin C

1. Introduction

Urothelial carcinoma (UC), the fourth most common malignancy in men, is a disease characterised by multiplicity, recurrence and multifocality [1]. The disease is located in the upper (renal pelvis and ureter) or lower (urethra and bladder) urinary tract. UC is either invasive: the tumor has penetrated the basement membrane and invaded into the lamina propria and deeper or non-invasive: the tumor lies flat on the surface e.g. carcinoma in situ (CIS) [2,3]. According to the 2004 and 2015 WHO classification system for urothelial cancer, urothelial CIS and dysplasia have been identified as premalignant lesion and included under non-invasive urothelial carcinoma [4]. Urothelial CIS is a flat high grade non-invasive neoplasm, 60%-80% of which becomes invasive in 5 years [5]. Urothelial dysplasia is low grade non-invasive neoplasm with distinct cytologic and architectural changes in the urothelium. There is a very fine line of difference between CIS and dysplasia which often renders the pathologist bewildered. Dysplastic urothelium often progresses to CIS which further progresses to invasive carcinoma [3,6].

It is reported that toxic agents in the urine can transform urothelial cells and induce tumorigenesis [7]. A small percentage of UC arises following exposure to toxic chemicals including aristolochic acid, and abuse of the drug phenacetin [8,9]. However, the most important risk factor for urothelial cancer is cigarette smoking [10]. Previously we had shown that p-benzoquinone (p-BQ), derived from CS in vivo, is a causative factor for various CS-induced diseases [11–14]. It is known that quinones represent a class of toxicological intermediates which can create a variety of hazardous effects in vivo, including oxidative damage and carcinogenesis [15]. p-BQ is not present in CS. It is produced from p-benzosemiquinone (p-BSQ), a long-lived semiquinone of cigarette smoke (CS) in vivo, by disproportionation and oxidation [11]. p-BSQ is present in substantial amounts (100–200 μg) in commercial cigarettes [16]. We have observed that in the lung, p-BQ closely mimics emphysema in a guinea pig model [14]. In the blood, p-BQ forms Michael adducts with serum albumin as well as haemoglobin resulting in alteration of structure and function of the proteins [17,68]. In the systemic circulation, p-BQ reaches distant organs and causes myocardial damage and myelodysplastic syndromes (a type of cancer) [12,13]. Literature review also highlights the relationship between benzoquinone and renal disease [18].

Previously we had shown that p-BQ acts like a mitogen in vitro. It induces proliferation of cultured human lung cells through activation of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) pathway [16]. EGFR signaling is known to play a key role in UC [19,20]. The prognostic value of EGFR in UC was established by various studies that found a significant association between its expression and the presence of aggressive phenotype, advanced stage, likelihood of tumor recurrence, and survival [21–24]. The EGFR pathway modulates GTPase Ras, which further regulates MAPK signaling cascade that tightly regulates cell proliferation, survival and differentiation [25]. Persistent EGFR signaling often leads to deregulation of the Ras-MAPK pathway that ultimately leads to oncogenesis [26]. MAPK pathway is directly coupled with cell cycle regulation [27]. Thus it is conceivable that a portion of circulatory p-BQ excreted from the kidney may activate EGFR in the renal pelvis and cause persistent proliferation of the urothelial cells and subsequent tumorigenesis through activation of MAPK pathway and deregulation of cell cycle.

In this paper we demonstrate that prolonged p-BQ treatment produces non-invasive UC, particularly carcinoma in situ (CIS) in the renal pelvis and dysplasia in the ureter and bladder in a guinea pig model. The reasons for use of the guinea pig model are described in details elsewhere [12,14]. The early events after p-BQ treatment were oxidative protein damage and apoptosis that were later suppressed and followed by aberrant tyrosine phosphorylation of EGFR, activation of MAPK pathway and cell cycle deregulation. We further showed that oral supplementation of vitamin C (30 mg/kg body weight/day), a strong antagonist of p-BQ, prevented non-invasive UC. Our preclinical study might reveal insights into the underlying molecular mechanisms how *p*-BQ, a major toxic quinone derived from CS, initiates UC and to formulate effective strategies for early intervention of the disease.

2. Materials and methods

2.1. Materials

p-BQ was procured from Sigma-Aldrich (B10358) and freshly crystallized from n-hexane (Merck) before use. Polyclonal antibody to p-BQ raised in rabbit after immunization with p-BQ-bovine serum conjugate was supplied by Abexome Biosciences, Bangalore, India.

2.2. Guinea pigs and p-BQ treatment

Male guinea pigs weighing 450–550 g (Dunkin Hartley albino pure bred) were procured from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. All methods (detailed below) were approved by the Institutional Animal Ethics Committee, University of Calcutta, and were in strict conformity with NIH guidelines. The guinea pigs were divided into the following weight-matched experimental groups (n = 22/group). (i) p-BQ-treated (injected intramuscularly) marginal vitamin C-restricted group, given oral supplementation of 1 mg vitamin C/animal/day; (ii) p-BQ-treated vitamin C-sufficient group, given oral supplementation of 30 mg vitamin C/kg body weight/animal/day; (iii) sham control; injected water instead of p-BQ and fed 1 mg vitamin C/animal/day. Another control group of animals that were injected water instead of p-BQ and fed 30 mg vitamin C/kg body weight/day was excluded because previous results revealed similarities between this group and sham control [12,14]. p-BQ dissolved in 100 μl milli Q water was injected intramuscularly in the lumbar region. The amount of p-BQ injected in the guinea pig (25 μg/day) was based on the comparative calculation of the approximate amount of p-BQ produced in a moderate human smoker/day [14]. All animals were pair-fed with respect to p-BQ injected vitamin C-restricted group. The reasons for use of vitamin C-restricted guinea pigs were described before in details [12,14]. Briefly, guinea pigs, like humans, are unable of vitamin C synthesis and are exclusively dependent on dietary means [28]. The anatomy and CS-induced pathophysiology of the guinea pig have similarities with that of human smokers [11]. The vitamin C-restricted guinea pigs were used to minimize the vitamin C level in the tissues, because adequate vitamin C, an antagonist of p-BQ, would neutralize the toxic effect of p-BQ. Vitamin C-restricted guinea pigs reflect human smokers whose tissue vitamin C levels are low irrespective of dietary intake [11]. After each experimental time points, guinea pigs were euthanized under deep anesthesia using i.p. injection of ketamine hydrochloride (100 mg/kg body weight) and tissues were collected for further experiments.

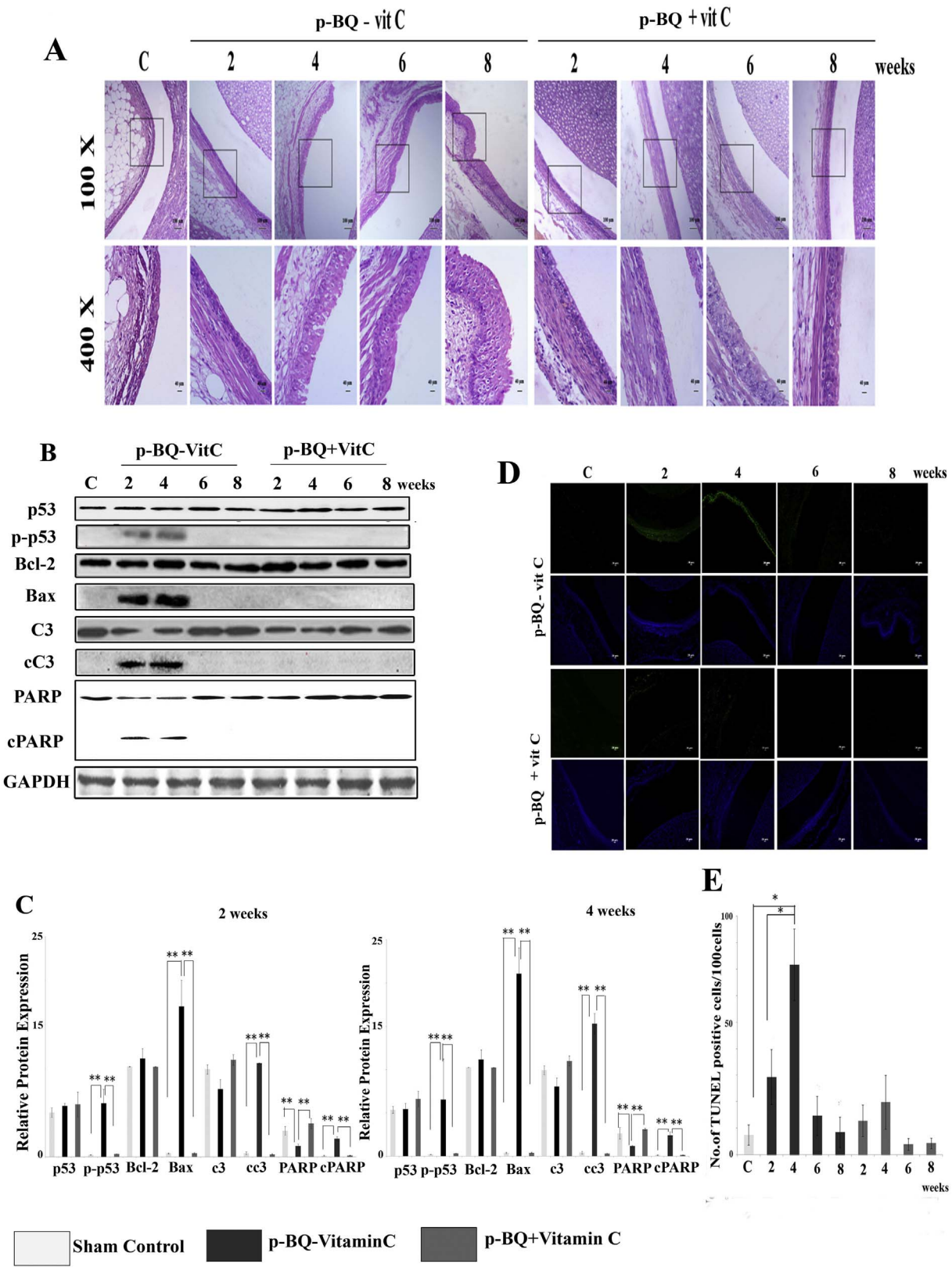

Fig 1. p-BQ-induced histological changes and apoptosis of urothelial cells of the renal pelvis (A) H & E stained histological image of renal pelvis showing p-BQ-induced cell death followed by hyperplasia. Upper panel magnification 100X; the inset of upper panel is shown in the lower panel at magnification 400X. (B) Immunoblots showing total p53 level, activation of p53 (formation of phospho–p53), expression of B-cell Lymphoma 2 (Bcl-2), overexpression of Bcl2 associated X protein (Bax), expression of caspase 3(c3), cleaved caspase 3(cc3) expression of total PARP and cleaved PARP (cPARP). (C) Quantitation of the apoptotic protein expression after 2 and 4 weeks of p-BQ treatment. (D) Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling; first and third row, (TUNEL)-positive cells; second and fourth row stained with DAPI (magnification, 200X). (E) Quantitation of TUNEL-positive cells. C; sham control; − vit C, vitamin C-restricted guinea pigs; + vit C, vitamin C-supplemented guinea pigs. Data represented as mean ± SD (n = 4). Significance is shown as *P ≤ 0.05, **P ≤ 0.005.

2.3. Tissue preparation and histological analysis

Both left and right kidneys, ureters and bladder were surgically excised and fixed in phosphate buffered saline (PBS) containing 10% formaldehyde and sections were stained with Hematoxylin & Eosin (H & E) (Sigma-Aldrich) by standard protocols as described earlier [13,14]. At least three sections per animal were analyzed by a histopathologist.

2.4. Tissue lysate preparation

This was done as before [14]. Tissue samples were lysed in lysis buffer in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% TritonX-100, 30% glycerol, 0.1%NP40, 0.1% protease inhibitor cocktail, 0.1% phosphatase inhibitor cocktail. The lysate was centrifuged at 13,000*g* at 4 °C for 20 min. The supernatant collected was stored at-80 °C as protein lysate for further experiment.

2.5. Protein carbonyl formation

Protein oxidation was assessed by I Protein carbonyl formation using Oxyblot™ protein oxidation detection kit (Intergen, NY). Tissue lysate containing 40 µg protein was derivatized with 10 µl of 1 × 2, 4-dinitropheny-hydrazine (DNPH) solution in 2 M HCl and incubated for 15 min at room temperature in the dark. The reaction was stopped by neutralization buffer and the samples were subjected to SDS-PAGE and further immunoblotting using antibody to DNPH.

2.6. Immunoblotting

Tissue lysate containing 30 µg protein was separated by SDS–PAGE and transferred to Immun-Blott PVDF membrane (GE Healthcare). The membrane was blocked in 5% milk for 1 h and then incubated with primary antibody (1:1000 dilution) of p53, phospho p53, Bax, Bcl-2, caspase 3, cleaved caspase 3, PARP, p44/p42 MAPK, phospho p44/p42 MAPK, c-Myc, phospho (serine 62) c-Myc, p21 $^{waf1/cip1}$, cyclin D1, phospho Rb (807/811) (Cell Signalling Technologies), anti-GTPase Hras (Abcam), respectively, as needed. GAPDH (Biobharati, India) was used as a loading control. After washing three times with TBS-T (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5), the membrane was incubated with HRP-conjugated secondary antibody (1:3000 dilution) for 1 h at room temperature. The membrane was further washed with TBS-T and developed with Lumiglo reagent (Cell Signaling Technologies).

2.7. Immunoprecipitation

Protein A sepharose beads (2 mg) (GE Healthcare) were coated with anti EGFR antibody (1:100) in PBS at room temperature for 4hr. 300 µg protein containing tissue lysate was added to the beads and incubated overnight at 4 °C. Sepharose beads harboring the immunoprecipitates were washed with PBS containing 0.2% Triton X-100 and subjected to immunoblotting. The membrane was blocked in 5% milk for 1 h and then incubated with 1:1000 dilution of primary anti-p-BQ antibody (Abexome, India), anti-phospho-Tyr845, anti-phospho-Tyr 1045, anti-phospho-Tyr1068, anti-phospho-Tyr 1173, anti-c-Cbl and anti-ubiquitin antibody (Cell signaling Technologies) overnight at 4 °C, and processed similarly like immunoblots.

2.8. TUNEL assay

Apoptosis was also measured by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay as described before [11].

2.9. Immunohistochemistry

Five micron thick deparaffinized tissue sections were processed for antigen retrieval in10 mmol/L citrate buffer (pH 6.0) for 15 min for permeabilisation The slides were then blocked with 10% BSA and incubated overnight with relevant primary antibodies as follows: Ki-67(Abcam), 1:100, overnight at 4 °C; p53(CST),1:100,overnight at 4 °C, cytokeratin 20 (Abcam), 1:100, 1 h at room temperature; CD44 (Abcam) 1:100, 1 h at room temperature. After washing with PBS-T, the sections were incubated with HRP conjugated secondary antibody for 1 h at room temperature. Images were acquired using digital Digieye 330/210 camera. Ki-67-positive and p53-positive cell counts were measured from six images per section per animal using Dewinter Biowizard 4.1 imaging software.

2.9.1. Quantitation of immunofluorescence and immunoblots

Quantitation of immunofluorescence and immunoblots were done using NIH Image J software.

2.9.2. Statistical analysis

Statistical analysis was performed using one-way ANOVA and Fisher's *t*-tests. The P values were calculated using appropriate F statistics for ANOVAs and t statistics for *t*-tests. P values < 0.05 were considered to be significant. The statistical analyses were performed using MINITAB software.

3. Results

3.1. p-BQ-induced histopathological changes in the epithelium of renal pelvis

Reports indicate that cancer of renal pelvis (CRP) develops after continued exposure to environmental toxic chemicals [29]. We therefore subjected the guinea pigs to prolonged treatment with p-BQ. We observed that daily intramuscular injection of p-BQ (25 µg/animal) for 4 weeks in vitamin C- restricted guinea pigs caused death of some epithelial cells of the renal pelvis as evidenced by hollowing of cells and missing nuclei (Fig. 1A). However, after treatment for 8 weeks, there was no apparent cell death. Rather, the transitional epithelium showed hyperplasia (Fig. 1A). Hyperplasia was characterised by markedly thickened mucosa with an increase in the number of cell layers without apparent change in the cytology of cells. The thickened epithelium also showed cytoplasmic clearing which was distinctive of hyperplastic cells [4]. No such histopathological changes were seen in either sham controls or vitamin C-supplemented animals (Fig. 1A).

3.2. p-BQ-induced oxidative protein damage and apoptosis were followed by hyperplasia in the epithelium of renal pelvis

p-BQ is a redox cycling agent that generates reactive oxygen species resulting in oxidative damage and apoptosis [14]. Here, we showed that after 2 and 4 weeks of treatment, p-BQ caused protein oxidation, as evidenced by significant formation of protein carbonyl ($p < 0.005$) after 2 and 4 weeks. (Supplementary Fig. S1A, B). On prolonging the treatment for 6 and 8 weeks the protein oxidation decreased ($p < 0.005$) after 2 and after 4 weeks. (Supplementary Fig. S1A, B). There was no protein carbonyl formation in sham control and vitamin C-supplemented guinea pigs (Supplementary Fig. S1A, B).

Compared to sham control and vitamin C-supplemented animals, p-BQ also caused significant apoptosis after 2 and 4 weeks of treatment (Fig. 1B, C), as evidenced by immunoblotting using antibodies against proapoptotic markers phospho-p53 ($p < 0.005$), Bax ($p < 0.005$), cleaved caspase 3 ($p < 0.005$), and cleaved PARP ($p < 0.005$). There was no significant change in total p53 and total PARP protein levels ($p > 0.05$). Apoptosis was also supported by TUNEL assay. There was no apparent change in protein expression level of the anti apoptotic Bcl-

2 with respect to sham controls and vitamin C-sufficient animals (Fig. 1B, C). This indicated an increase in the ratio of Bax/Bcl-2, supporting apoptosis. After continuation of the p-BQ treatment for 6 and 8 weeks, apoptosis subsided as indicated by lack of expression of the proapoptotic markers (Fig. 1 B, C) and decrease in TUNEL positive cells (Fig. 1D, E). Suppression of apoptosis was accompanied by hyperplasia as evidenced by increased expression of phospho-p44/p42 MAPK after 8 weeks of p-BQ injections ($p < 0.005$) (Supplementary Fig. S1C, D). Phosphorylation of p44/p42 MAPK is indicative of increased cell growth [30]. There was no hyperplasia in sham controls and vitamin C-supplemented animals (Supplementary Fig. S1C, D).

3.3. Prolonged treatment with p-BQ produced carcinoma in situ in the renal pelvis: prevention by vitamin C

Hyperplasia has been reported to be a precursor for CIS in several cancers including bladder cancer which later progresses to invasive cancer [31]. Since p-BQ treatment caused hyperplasia after 8 weeks, we continued the treatment for 24 weeks until the vitamin C-restricted animals showed a marked decrease in food intake and body weight (Supplementary Fig. S2). At that stage, the animals were sacrificed. H & E staining of the renal pelvis showed CIS in four out of six animals. There was no CIS in sham controls and vitamin C-sufficient guinea pigs. CIS was evidenced by hyperchromasia (increased eosinophilia), altered nuclear: cytoplasmic ratio, absence of polarity of cells without cytoplasmic clearing (Fig. 2). Accurate diagnosis of CIS in humans is done using immunostains for cytokeratin 20 (CK20) (a cytoplasmic protein), p53 (nuclear antigen), standard isoform of CD44 (expressed on the cellular membrane) and Ki67 (nuclear antigen). We confirmed CIS in guinea pigs by the above immunostains. In normal urothelium, CD44 remains confined to the basal cells of the transitional epithelium. We observed that CD44 was absent in p-BQ-treated vitamin C-restricted guinea pigs (Fig. 2), which was indicative of CIS. Normally CK20 is present in the superficial/umbrella cells of the transitional lining. We found that it was expressed in full thickness of the transitional lining of p-BQ-treated vitamin C-restricted animals (Fig. 2). p53 is generally absent in normal urothelium but is overexpressed in a large number of malignancies that is indicative of mutated p53 status [32]. Here, we showed that p53 nuclear immunoexpression increased about 34% ± 5.15 SD in vitamin C-restricted p-BQ-treated animals (Fig. 2). This indicated neoplasia [33]. Ki67, a marker for proliferation, is reported to be aberrantly overexpressed in CIS [34]. We also observed that the nuclear reactivity of Ki67 increased in 48% ± 5.72 SD of cells of the p-BQ-treated vitamin C-restricted animals (Fig. 2).

3.4. p-BQ-induced atypical phosphorylation of intracellular EGFR Tyr residues and persistent growth signaling

Previous reports from our laboratory had indicated that p-BQ derived from cigarette smoke binds to the extracellular domain of EGFR in cultured human lung cells and activates EGFR constitutively [16]. Here, we showed binding of p-BQ with EGFR in the renal pelvis of vitamin C-restricted guinea pigs those developed CIS. The binding was evidenced by immunoprecipitation using anti EGFR antibody followed by immunoblotting with anti p-BQ antibody (Fig. 3A, B). The amount of p-BQ bound to EGFR increased significantly on increasing the duration of p-BQ treatment from 8 weeks ($p < 0.005$) to 24 weeks ($p < 0.005$) (Fig. 3A, B). There was no such p-BQ binding in sham controls and vitamin C-sufficient animals (Fig. 3A, B).

The binding of p-BQ was accompanied by increased overall phosphorylation of the intracellular Tyr residues of EGFR as shown by immunoblotting the EGFR immunoprecipitates using anti PY20 antibody after 8 weeks ($p < 0.005$) and 24 weeks ($p < 0.005$) (Fig. 3C, D). Densitometric analysis of the phospho-tyrosine blots revealed significant decrease in phosphorylation of Tyr 1045 after 8 weeks of p-BQ treatment ($p < 0.005$). However, there was no significant change in phosphorylation pattern of Tyr 845 ($p > 0.05$), Tyr 1068 ($p > 0.05$) and Tyr 1173 ($p > 0.05$), compared to that of sham control and vitamin C-sufficient animals (Fig. 3C, D). On prolonging the p-BQ treatment up to 24 weeks, the phosphorylation of Tyr 845 significantly increased ($p < 0.005$) along with phosphorylation of Tyr 1068 ($p < 0.005$). On the contrary phosphorylation of Tyr 1045 significantly decreased compared to sham control and vitamin C-supplemented animals ($p < 0.005$) (Fig. 3C, D). There was no change in phosphorylation of Tyr1173 ($p > 0.05$) (Fig. 3C, D).

Immunoblotting using anti c-Cbl antibody and anti ubiquitin antibody on EGFR immunoprecipitates showed that after 24 weeks of p-BQ treatment, c-Cbl ($p < 0.005$) and ubiquitin binding ($p < 0.005$) to EGFR decreased markedly (Fig. 3E, F). Lack of c-Cbl binding results in lack of ubiquitylation and EGFR degradation leading to persistent growth signaling [35].

3.5. Persistent growth signaling leads to activation of MAPK (erk1/2) and c-Myc

Increased kinase activity of EGFR activates Hras by changing the GDP-bound Hras to GTP- bound Hras [35,70,36]. We showed that compared to sham control and vitamin C-sufficient animals, protein expression level of GTPase Hras increased significantly after 8 weeks of p-BQ treatment in vitamin C-restricted animals ($p < 0.005$) (Fig. 4A, B). On prolonging the treatment for 24 weeks, the expression level of GTPase Hras increased further ($p < 0.005$) (Fig. 4A, B). The active Hras ultimately activates p44/p42 MAPK by phosphorylation [37]. We showed that in the p-BQ-treated guinea pigs protein expression level of

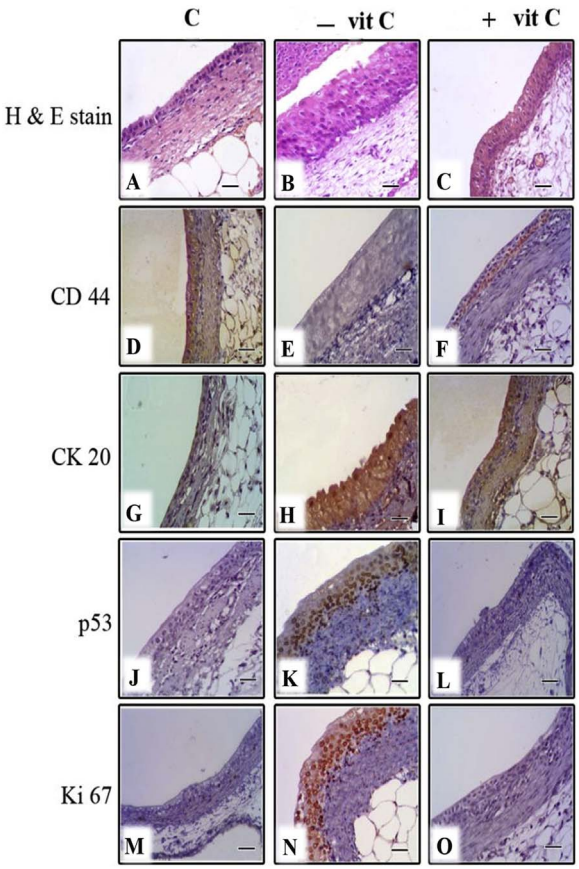

Fig. 2. p-BQ-induced carcinoma in situ (CIS) in the transitional epithelium of renal pelvis. Left column: C; sham control; middle column: – vit C, vitamin C-restricted guinea pigs; right column: + vit C, vitamin C-supplemented guinea pigs. (A, B, C), H & E stained sections of renal pelvis; (D, E, F), CD44 immunostained sections; (G, H, I), CK20 stained sections. (J, K, L), p53 stained sections; (M, N, O), Ki67 stained sections. Scale bar represents 40 μm. Magnification 400X.

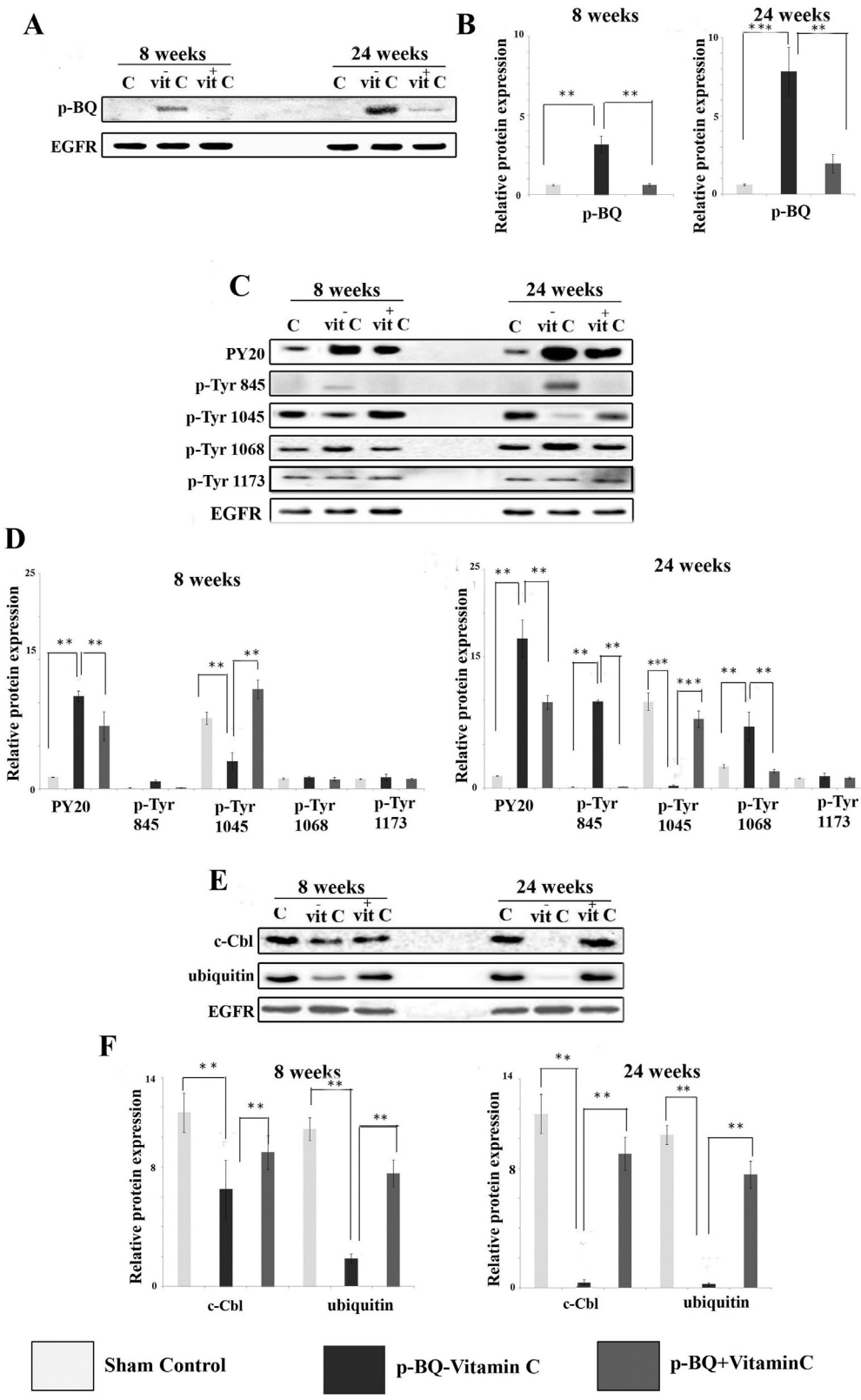

Fig. 3. p-BQ-induced aberrant activation of EGFR and phosphorylation of intracellular tyrosine residues. (**A**) Immunoblots showing binding of p-BQ to EGFR. (**B**) Quantitation of p-BQ binding to EGFR after 8 and 24 weeks of treatment. (**C**) Immunoblots showing phosphorylation pattern of tyrosine (Tyr) residues of the intracellular domain of EGFR (p-Tyr). (**D**) Quantitation of phosphorylation of Tyr residues after 8 and 24 weeks of p-BQ treatment. (**E**) Immunoblot showing binding of c-cbl and ubiquitin to EGFR. (**F**) Quantitation of c-Cbl and ubiquitin binding to EGFR after 8 and 24 weeks of treatment. C, sham control; − vit C, vitamin C-restricted guinea pigs; + vit C, vitamin C-supplemented guinea pigs. Data represented as mean ± SD (n = 4). Significance is shown as *P ≤ 0.05, **P ≤ 0.005.

phosphorylated (activated) p44/p42 MAPK increased significantly after 8 weeks ($p < 0.005$) and 24 weeks ($p < 0.005$) (Supplementary Fig. S1C, D, Fig. 4A, B). Activated MAPK activates various transcription factors including c-Myc [34]. c-Myc is overexpressed in a number of tumors [38] and phosphorylation at ser62 upon mitogen stimulation stabilizes c-Myc [39]. Our results indicated significant overexpression of c-Myc protein after 24 weeks of p-BQ treatment ($p < 0.05$) (Fig. 4A, B). c-Myc was significantly phosphorylated at ser62 after 8 and 24 weeks of treatment ($p < 0.005$).

3.6. Deregulation of cell cycle caused by prolonged p-BQ treatment

Cyclin D1 has been reported to be overexpressed in several types of human cancer [40]. However, a number of reports indicate that in

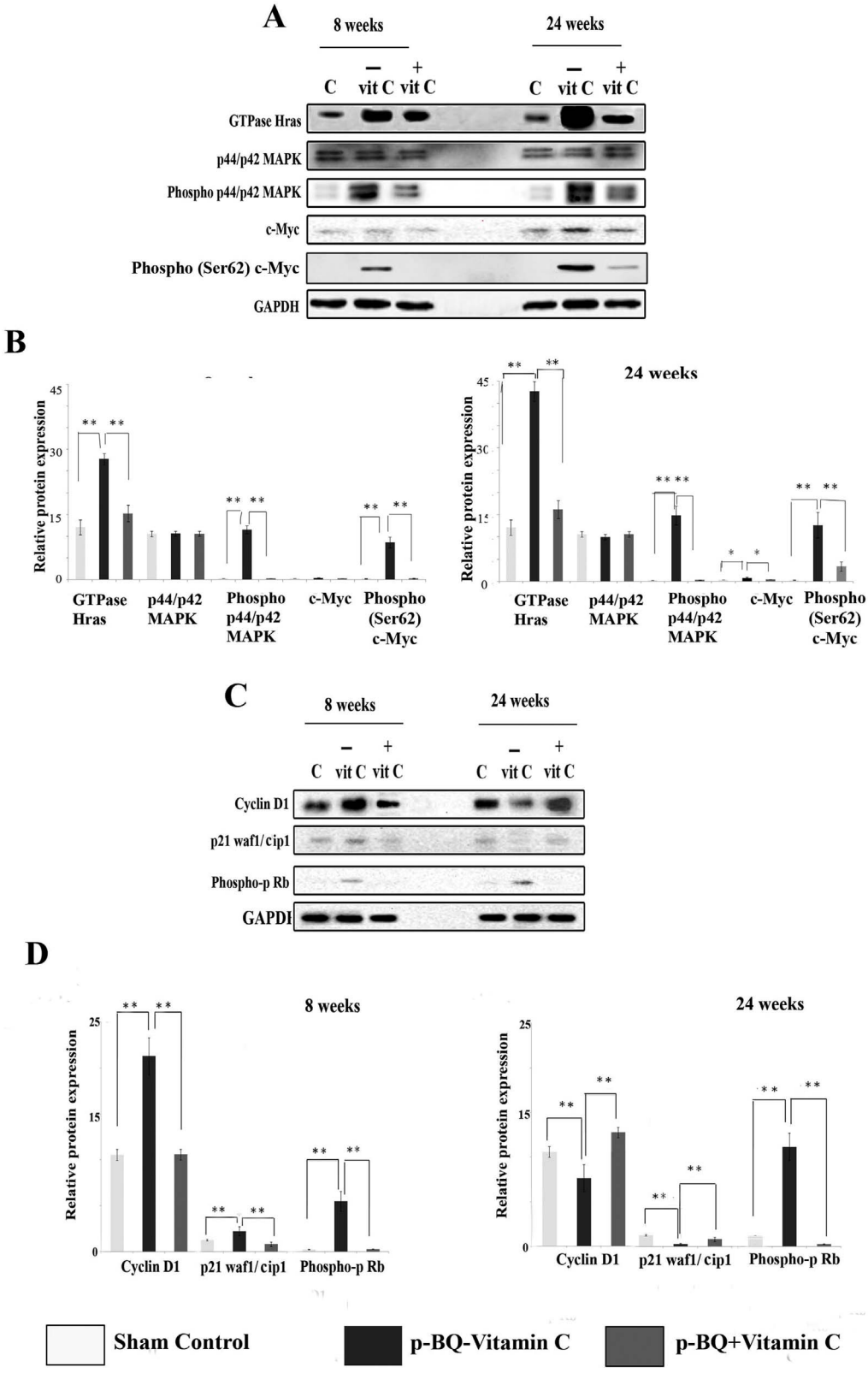

Fig. 4. p-BQ-induced activation of MAPK, c-Myc and cell cycle deregulation. (**A**) Immunoblots showing expression of GTPase Hras, p44/p42 MAPK, phospho-p44/p42 MAPK, c-Myc and phospho(ser62)c-Myc. (**B**) Quantitation of expression of GTPase Hras, p44/p42 MAPK, phospho-p44/p42 MAPK, c-Myc and phospho(ser62)c-Myc after 8 and 24 weeks of p-BQ treatment. (**C**) Immunoblots showing protein expression of Cyclin D1, $p21^{waf1/Cip1}$ and hyperphosphorylated Retinoblastoma (pRb) at Ser 807/811. (**D**) Quantitation of protein expression of cell cycle regulatory proteins after 8 and 24 weeks of treatment. C, sham control; − vit C, vitamin C-restricted guinea pigs; + vit C, vitamin C-supplemented guinea pigs. Data represented as mean ± SD (n = 4). Significance is shown as *P ≤ 0.05, **P ≤ 0.005.

urothelial cancer cyclin D1 expression level decreases with p53 mutations and invasiveness of tumor [33]. Here, we showed that although cyclin D1 protein expression increased after 8 weeks of p-BQ treatment in vitamin C-restricted guinea pigs ($p < 0.005$) (Fig. 4C, D), the expression level of cyclin D1 markedly decreased on prolonging the p-BQ treatment for 24 weeks, when CIS developed ($p < 0.005$) (Fig. 4C, D). Cell cycle progresses through the formation of cyclin D1-cdk4/6 complex [41]. The stability of this complex is regulated by $p21^{waf1/cip1}$ [42]. Down regulation of $p21^{waf1/cip1}$ causes inactivation of retinoblastoma (pRb) protein through hyperphosphorylation at serine 807/811 resulting in cell cycle progression [42]. We also observed that $p21^{waf1/cip1}$ expression level decreased significantly after 24 weeks of p-BQ treatment ($p < 0.005$) that was accompanied by hyperphosphorylation of pRb at serine 807/811 ($p < 0.005$), rendering it

inactive (Fig. 4C, D). Taken together our results indicated that prolonged treatment with p-BQ caused cell cycle progression through deregulation of G1/S checkpoint, the most important checkpoint in the mammalian cell cycle.

3.7. p-BQ-induced dysplasia in the ureter and bladder

Only about 25–30% of upper tract UC involves the ureters [43,44], and involvement of the bladder is seen up to 50% of the patients [1]. Here, we showed that three out of four animals that developed CIS show dysplastic lesions in the ureter and two animals showed dysplasia in the bladder. Details are given in Supplementary information (Supplementary information; supplementary Figs. 3 and 4).

4. Discussion

The aim of the study was to delineate the mechanism by which p-BQ, a major toxic quinone derived from CS, initiated non-invasive UC that was prevented by oral supplementation of vitamin C. Except several murine models used to study bladder cancer, till date there is little information about investigations on non-invasive UC in animal model [45,5]. Using a guinea pig model, here we showed that after prolonged treatment p-BQ produced CIS in the renal pelvis and dysplasia in the ureter and bladder. The mechanisms of carcinogenesis were oxidative damage and apoptosis that were later suppressed and followed by aberrant EGFR activation, persistent growth signaling, marked increase in immunoexpression of nuclear p53 indicating p53 mutation and cell cycle deregulation. Loss of apoptosis can eventually lead to an expansion of a population of preneoplastic or neoplastic cells [46]. It is reported that oxidative damage plays a pivotal role in apoptosis [54]. It is also reported that cells that escape apoptosis produce persistent signaling that usually contributes to the formation of cancer [71]. Our finding supports the hypothesis of Hanahan and Weinberg that suppression of apoptosis and persistent proliferation are hallmarks of most types of cancer [47]. It is also reported that deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression [46].

Quinones are reported to be implicated in carcinogenesis. Quinones produced from polycyclic aromatic hydrocarbons (PAHs) as well as estrogens have been reported to produce cancers of the lung and endometrium, respectively [48,49]. p-BQ is known to induce leukemia [50,51]. However, the mechanism of p-BQ-induced carcinogenesis is not clear. p-BQ is a Michael acceptors and known to form a DNA adduct [15]. p-BQ is also mutagenic [52]. Moreover, p-BQ is an inhibitor of topoisomerase II, which has implications for the formation of deleterious translocation leading to leukemogenesis [53] Furthermore, p-BQ is a redox cycling agent that leads to formation of reactive oxygen species (ROS) that may lead to single-strand breaks as well as oxidation of DNA bases. Moreover, ROS causes oxidative stress within cells through the formation of oxidized cellular macromolecules, including proteins. Studies also indicate that ROS and the resulting oxidative stress play a pivotal role in apoptosis [54]. Here, we showed that p-BQ initiated protein oxidation, which was accompanied by apoptosis. Besides oxidative stress, ROS can activate a number of signaling pathways, including protein kinase C and RAS [15].

Majority of CIS in the urothelium is known to progress to invasive cancer with a higher risk of cancer-specific mortality [5,55]. We have confirmed CIS by the immunohistochemical profile of CK20 positive staining in full thickness of the urothelium, marked nuclear positivity of Ki67 and p53 and also negative staining of CD44. In case of urothelial cancer, over expression of Ki67 is known to be coupled with undesirable pathological features [27,56]. p53 has a short half-life and nuclear staining for p53 is absent in normal urothelium, but present in many flat urothelial lesions [33]. Increased nuclear accumulation of p53 protein indicates mutations of the TP53 gene, which is a common event in the development of urothelial CIS and invasive neoplasms [27].

Previously we had shown that p-BQ derived from CS activated EGFR and acted like a mitogen in cultured human lung cells [16]. Here, we showed that p-BQ activated EGFR in the renal pelvis apparently by binding to cysteine or lysine residues of the extracellular domain and induced aberrant phosphorylation of cytoplasmic tyrosine residuesinsert reference [17]. EGFR is a trans-membrane glycoprotein with an extracellular epidermal growth factor binding domain and an intracellular tyrosine kinase domain that regulates signaling pathways to control cellular proliferation. EGFR binding to its ligand results in autophosphorylation by intrinsic tyrosine kinase activity that triggers several signal transduction cascades, such as activation of MAPK and transcription factors including c-myc. In normal cell processes EGFR signal is inactivated through endocytosis of the receptor-ligand complex. However, in persistent proliferation, mitogen activated EGFR evades degradation and is recycled to the cell surface for augmentation of growth factor signaling leading to aberrant cell proliferation and cancer development. EGFR degradation process involves a ubiquitin ligase known as c-Cbl. Tyrosine 1045 of the cytoplasmic domain of EGFR is essential for c-Cbl mediated degradation and downregulation of EGFR signaling [57]. Phosphorylation of Tyr 1045 acts as the docking site for E3 ubiquitin ligase c-Cbl. This ligase recruits ubiquitin, which results in EGFR ubiquitylation and degradation. Our results show that continued p-BQ treatment has resulted in unphosphorylation of Tyr 1045. Unphosphorylated Tyr 1045 is unable to dock c-Cbl and thereby prevents ubiquitylation and degradation of EGFR, which leads to persistent growth signaling.

EGFR is highly expressed in a variety of human tumors, including UC. UCs with metaplastic morphology has been shown to be more resistant to conventional radiotherapy or chemotherapy. The overexpression of EGFR has been observed in both premalignant lesions and malignant tumors. Constitutive or sustained activation of these sequences of downstream targets is thought to yield more aggressive tumor phenotypes [58,59,24].

We have shown that after continued p-BQ treatment, phosphorylation of Tyr 845 increases which is important for cell growth and migration [60]. Similarly increased phosphorylation at Tyr 1068 is seen that acts as the docking site for Grb2 which activates GTPase Hras as well as p44/p42 MAPK leading to proliferation [61]. Overexpression of Ras protein has been associated with various cancers including UC [62].

We have also shown that c-Myc protein is overexpressed in the CIS and dysplastic samples. Overexpression of c-Myc causes deregulation of the cell cycle, coordinating both cell growth and concomitant progression through the cell cycle [27]. c-Myc is a transcription factor that is associated with various proliferative signals and has been reported to be overexpressed in a variety of tumors [38]. Ras/Raf/MAPK pathway plays a key role in stabilizing c-Myc by phosphorylation at ser62. We showed that c-Myc was phosphorylated at ser62. The stable c-Myc is unable to undergo degradation and is pivotal for malignant transformation [39].

Cell cycle deregulation is one of the most frequent alterations during tumor development. P53, generally referred to as the ‘guardian of the genome’, acts as a key regulator by inhibiting cell-cycle progression at the G1-S transition. Normally p53 is not accumulated in the nucleus. Here we have shown intranuclear accumulation of p53, which is an indication of p53 mutation. Altered p53 directly perturb regulation of the cell cycle in urothelial cells that give rise to CIS. CIS is believed to represent the precursor lesion for invasive tumor [27]. p53 mediates cell cycle control by activating the transcription of p21 $^{waf1/cip1}$, a cyclin-dependent kinase inhibitor. We have also shown that p21 $^{waf1/cip1}$ is downregulated. It is reported that patients with p53 altered and p21 $^{waf1/cip1}$-negative tumors have a high probability of recurrence and poor survival [27]. Along with p53, retinoblastoma (pRb) protein has a central role in restricting the cycle progression at the G1/S check point [41]. pRb remains hypophosphorylated and bound with E2F family of transcription factors and prevents excessive cell growth in the early G1 and G0 stage. As the cells progress into early S phase, pRb becomes

hyperphosphorylated. This dissociates it from disrupts E2F resulting in excessive proliferation [33]. In majority of cancers, pRb is dysfunctional. We have shown that in p-BQ-induced CIS, pRb is hyperphosphorylated, which results in cell cycle progression evading G1/S checkpoint.

We showed that vitamin C at a dose of 30 mg/kg body weight/guinea pig/day prevented all the pathobiologies caused by p-BQ including CIS. Vitamin C is a strong reducing agent ($E_0 = +0.08$ V). It reduces p-BQ ($E_0 = +0.71$ V) to less toxic hydroquinone and thereby inactivates p-BQ. It was possible that being a strong antioxidant, vitamin C prevented carcinogenesis by preventing p-BQ-induced formation of ROS that would otherwise trigger oxidative damage and activate a number of signaling pathways, including Ras leading to persistent signaling and cancer [15,69]. Previous reports from our laboratory indicated that doses of vitamin C lower than 30 mg/kg/animal/day was insufficient to protect the animals from CS/p-BQ induced pathophysiologies [11].Vitamin C has a long and controversial history in the prevention and treatment of cancer. Some papers show that pharmacologic doses of vitamin C decrease the growth and weight of human, rat, and murine tumor xenografts in athymic nude mice [63,64]. A dose-response meta-analysis indicated that high intake of vitamin C might have a protective effect against lung cancer [65]. It has also been demonstrated that human patients have a remarkable tolerance to very high doses of vitamin C [66].

5. Conclusion

Cancer is a major cause of death and unless prevented it will continue to increase. Our present investigation provides the mechanism how p-BQ initiates non-invasive urothelial neoplasm. Since p-BQ is derived from CS in smokers, preventing smoking would prevent UC. However, this has been unachievable. Since majority of non-invasive UC later becomes invasive, we consider that our preclinical study on the preventive role of vitamin C might be useful to devise effective intervention for prevention CS-induced urothelial neoplasia by vitamin C.

Authors contribution

Conceived and designed the experiments: Indu B Chatterjee, Shinjini Ganguly.

Performed most of the experiments: Shinjini Ganguly. Analyzed most of the data: Indu B Chatterjee, Shinjini Ganguly, Dhrubo J Chattopadhyay.

Statistical analysis done: Ayan Chandra.

Wrote the paper: Indu B Chatterjee, Shinjini Ganguly.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2017.06.005.

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