Cardiac glycosides display selective efficacy for STK11 mutant lung cancer

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Although STK11 (LKB1) mutation is a major mediator of lung cancer progression, targeted therapy has not been implemented due to STK11 mutations being loss-of-function. Here, we report that targeting the Na+/K+-ATPase (ATP1A1) is synthetic lethal with STK11 mutations in lung cancer. The cardiac glycosides (CGs) digoxin, digitoxin and ouabain, which directly inhibit ATP1A1 function, exhibited selective anticancer effects on STK11 mutant lung cancer cell lines. Restoring STK11 function reduced the efficacy of CGs. Clinically relevant doses of digoxin decreased the growth of STK11 mutant xenografts compared to wild type STK11 xenografts. Increased cellular stress was associated with the STK11-specific efficacy of CGs. Inhibiting ROS production attenuated the efficacy of CGs, and STK11-AMPK signaling was important in overcoming the stress induced by CGs. Taken together, these results show that STK11 mutation is a novel biomarker for responsiveness to CGs. Inhibition of ATP1A1 using CGs warrants exploration as a targeted therapy for STK11 mutant lung cancer.

The tumor suppressor STK11 is inactivated in 30% of lung cancer cases (~20% of lung adenocarcinoma (LUAD) cases)1. Despite the importance of STK11 in lung cancer1–3 and the observation that therapeutic approaches for other types of cancer harboring genetic alterations have impacted patient care (Supplementary Table S1), targeted therapeutic approaches have not yet been implemented for lung cancer harboring STK11 mutations, a loss-of-function aberration. It remains challenging to identify effective targets with a clear mechanistic explanation for the concerted role of a target and a loss-of-function mutation.

NCI60 screening datasets include a set of GI50 data consisting of ~50,000 chemicals screened in 59 diverse cancer cell lines4,5. This dataset provides a unique opportunity to identify the association of chemical responses with lineages and mutations of cancer cells. Because several cell lines in the NCI60 panel harbored a loss-of-function mutation in STK11, we were able to investigate the specific and sensitive responses of STK11 mutant cell lines to chemicals in comparison with other cell lines. Interestingly, STK11 mutant lung cell lines were found to exhibit specific sensitivity to a group of cardiac glycosides (CGs).

CGs are a family of compounds (including digoxin6, digitoxin7, and ouabain8) that have been widely used for the treatment of congestive heart failure and arrhythmia9. CGs regulate the intracellular sodium and potassium ion concentrations by inhibiting the enzyme Na+/K+-ATPase (ATP1A1)10,11. Anticancer effects of CGs have been reported, but these compounds have not entered patient care in part due to a lack of selective markers of their bioactivity12.

In the present study, we demonstrated the association of CG efficacy with the mutational status of STK11 by collectively analyzing NCI60 chemical screen data and Achilles13 loss-of-function screen data. In vitro validation confirmed that restoring STK11 function decreased the cellular response to CGs in a wide variety of STK11 mutant lung cancer cell lines. We further investigated the in vivo efficacy of CGs using mouse xenograft models and demonstrated that clinically relevant doses of CGs exerted selective effects on STK11 mutant tumors.

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Proteomic analysis using reverse phase protein arrays (RPPAs) and molecular mechanism studies provided insights into the synthetic lethality of CGs (with or without chemotherapeutic agents) with STK11 mutant cells.

**Results and Discussion**

**Association of the effect of cardiac glycosides with STK11 mutation.** The characterization of genetic alterations in LUAD has enabled the discovery of new molecular targets and therapeutic applications. The presence of mutations or rearrangements in genes such as ALK, BRAF, EGFR, KRAS, MET, PTEN, RET, and ROS1 predicts sensitivity and clinical treatment outcome for agents targeting aberrations in LUAD. Targeted agents against oncogenic mutations such as ALK, EGFR, and KRAS have been widely reported as successful therapeutic approaches in non-small cell lung cancer. STK11 loss-of-function mutation is mutually exclusive from these oncogenic mutations in The Cancer Genome Atlas (TCGA)-derived LUAD samples, except for a subset of patient samples harboring concurrent mutations in KRAS and STK11 (Fig. 1a). The mutation rate of STK11 increases from the early to late stages of LUAD, consistent with the observation that STK11 is a modulator of lung cancer metastasis.

To investigate the association of STK11 mutations with cellular responses to a wide variety of drugs, we used publically available high-throughput drug screening data from the NCI60 cancer cell line dataset. Strikingly, digoxin, digoxin, and ouabain demonstrated selective activity on STK11 mutant lung cancer cells based on the response profile of these drugs across NCI60 cell lines (Fig. 1b,c). In contrast, BRAF mutations were associated with marked resistance to CG treatment. Although KRAS mutations in the absence of STK11 mutations were weakly associated with resistance to CG treatment, KRAS mutations did not alter the sensitivity of STK11 mutant cell lines to CGs.
Figure 2. In vitro validation of the efficacy of CGs on STK11 mutant cancer cells. (a) Comparison of the change in cell viability induced by CG treatment between STK11 mutant and wild type cell lines. A total of 8 different lung cell lines were treated with a CG at 25, 50, or 100 nM for 3 days. (b) Comparison of digoxin sensitivity between STK11 mutant lung cell lines and their STK11-restored derivatives. Each cell line was treated with 25, 50, or 100 nM digoxin for 3 days. The percentage growth was calculated based on time zero and control growth data (See details in Methods section). Percentage growth of 0 means no net growth and −100 was indicated when all cells are killed. (c) Long-term effect of digoxin treatment on the cell viability of STK11 mutant cell lines (A549 and H460) and their STK11-restored derivatives (A549-STK11 and H460-STK11). The viability of each STK11 mutant cell line relative to its respective STK11-restored derivative was monitored following treatment with 50 nM digoxin for 3, 7 or 14 days. (d) Changes in cell migration after 100 nM digoxin treatment (24 hours) in the STK11 mutant (A549 and H460) and STK11-restored (A549-STK11 and H460-...
**STK11-dependent anticancer effect of CGs.** Confirming the NCI60 screening results, we demonstrated that digoxin, digitoxin, and ouabain exerted a significant dose-dependent selective inhibition of the viability of STK11 mutant cells compared to STK11 wild type cells in a collection of lung cancer cell lines (Fig. 2a). To determine the selectivity of CGs to cells harboring mutant STK11, we introduced wild type STK11 into A549, H23, H1993 LUAD and H460 large-cell lung carcinoma (LCLC) cells lacking wild type STK11. Introducing wild type STK11 into these cells increased cell viability following treatment with each CG (Fig. 2b and Supplementary Fig. S3a,b). This result indicated that STK11 function was directly linked to the sensitivity of cancer cells to CGs. In addition, the effects of CGs on STK11 mutant cells increased in a time-dependent manner (Fig. 2c and Supplementary Fig. S3c,d). CGs not only decreased cell viability but also decreased BrdU incorporation and wound closure in STK11 mutant cells; these results indicated the effects of CGs on both cell proliferation and migration (Fig. 2d and Supplementary Fig. S3e,f). Their effects on cell proliferation and migration were coordinately decreased by the introduction of wild type STK11. Furthermore, we performed 3D in vitro assays to mimic the in vivo tumor environment. Treatment with digoxin selectively inhibited anchorage-independent growth of STK11 mutant cells compared to STK11-restored cells, regardless of the presence (Fig. 2e) or absence (Fig. 2f) of serum. In summary, the ability of exogenous wild type STK11 to reverse the effects of CGs suggests that the activity of CGs depends on STK11 mutations, but not KRAS mutations, which frequently occur concurrently with STK11 mutations (Fig. 1a).

**STK11-dependent anticancer effect of ATP1A1 gene knockdown.** All of the tested CGs, digoxin, digitoxin and ouabain, exhibit highly selective, nano-molar-scale activity on ATP1A1. To examine whether the selectivity of CGs to STK11 mutant cells is dependent on ATP1A1 activity, we first checked the small RNA screening data for 216 human cell lines; this dataset was derived from Project Achilles. shATP1A1 significantly inhibited the growth of five different STK11 mutant NSCLC cell lines compared to the STK11 wild type cell lines (Fig. 3a). Furthermore, STK11 mutation markedly increased the inhibitory effect of shATP1A1 on a wide range of cancer cell lines, and this result indicated the dependence of ATP1A1 inhibition on the loss of STK11 function (Fig. 3b). For experimental validation, we silenced ATP1A1 expression in both STK11 mutant (A549 or H460) and STK11 wild type (H226 or H322M) cells. ATP1A1 knockdown significantly decreased cell viability in the STK11 mutant cell lines but not in the STK11 wild type cell lines (Fig. 3c), suggesting that CG activity is dependent on ATP1A1 inhibition. In addition, silencing both STK11 and ATP1A1 expression in H226 and H322M cells, which harbor wild type STK11, decreased cell viability to a greater extent than either STK11 or ATP1A1 knockdown alone (Fig. 3d). Moreover, introducing wild type STK11 decreased the effect of ATP1A1 knockdown on the A549 and H460 cell lines, which harbor KRAS mutation, further supporting the dependence of STK11 mutant cells on ATP1A1 activity (Fig. 3e).

**Effect of combination of CGs with other therapeutic agents on STK11 mutant cells.** We subsequently investigated the combined effects of digoxin with cisplatin and carboplatin, which are FDA-approved chemotherapy agents for lung cancers. Particularly, STK11 mutant cell lines were slightly more sensitive to cisplatin (Fig. 1d). Importantly, cisplatin, but not carboplatin, markedly increased the activity of digoxin in STK11 mutant cells (Fig. 4a,b). The combination of vemurafenib (BRAF inhibitor) with digoxin has been tested in metastatic melanoma patients harboring BRAF mutations (Supplementary Table S3). In the present study, we found that BRAF-activating mutation was associated with resistance to CGs (Fig. 1b,c). Proteomic profiling showed increased expression and phosphorylation of proteins in the MAPK signaling pathway after digoxin treatment (Fig. 4c and Supplementary Fig. S5a). We confirmed that digoxin or ouabain treatment induced ERK activation in both the STK11 mutant cell lines and their STK11-restored derivatives (Fig. 4d and Supplementary Fig. S5b–d). CGs have been proposed to induce cell apoptosis or autophagy by altering ERK signaling. However, we found that the anti-cancer effect of digoxin treatment was decreased by inhibiting ERK signaling using AZD6244 (MEK1 inhibitor), GSK1120212 (MEK1/2 inhibitor) or SCH772984 (ERK inhibitor) (Fig. 4e, Supplementary Table S4 and Supplementary Fig. S5e). Thus, it appears that continued MEK-ERK signaling is important for digoxin-induced anti-cancer activity and that a combination strategy using digoxin is not promising as a MEK- or ERK-targeted cancer therapy.
To determine the in vivo efficacy of CGs on STK11 mutant cancer, we utilized a mouse tumor xenograft model using A549 cells (STK11 mutant) and their STK11-restored derivatives. Digoxin at achievable doses in mice (3 mg/kg) significantly time-dependently reduced tumor growth in mice injected with A549 cells (Fig. 5a,b) but not in mice injected with A549 cells transfected with wild type STK11 (STK11-restored) (Fig. 5a,c). To investigate the clinical relevance of CG treatment in STK11 mutant lung cancers, we further determined the dose required to maintain human-equivalent blood levels of digoxin in mice. This therapeutic range of CG concentrations is very narrow (0.5 ~ 2.0 ng/ml) for clinical applications. To achieve this blood concentration in mice, the minimal dose of digoxin was 0.03 mg/kg and 0.3 mg/kg for administration at 24- and 48-hour intervals, respectively (Fig. 5d). At these doses and administration intervals, the plasma level of digoxin was maintained within (or below) the target range for ~70% of time period between

**STK11-specific in vivo efficacy of digoxin.** To determine the in vivo efficacy of CGs on STK11 mutant cancer, we utilized a mouse tumor xenograft model using A549 cells (STK11 mutant) and their STK11-restored derivatives. Digoxin at achievable doses in mice (3 mg/kg) significantly time-dependently reduced tumor growth in mice injected with A549 cells (Fig. 5a,b) but not in mice injected with A549 cells transfected with wild type STK11 (STK11-restored) (Fig. 5a,c). To investigate the clinical relevance of CG treatment in STK11 mutant lung cancers, we further determined the dose required to maintain human-equivalent blood levels of digoxin in mice. This therapeutic range of CG concentrations is very narrow (0.5 ~ 2.0 ng/ml) for clinical applications. To achieve this blood concentration in mice, the minimal dose of digoxin was 0.03 mg/kg and 0.3 mg/kg for administration at 24- and 48-hour intervals, respectively (Fig. 5d). At these doses and administration intervals, the plasma level of digoxin was maintained within (or below) the target range for ~70% of time period between
administrations. In our second mouse xenograft experiment, 0.03 mg/kg digoxin at 24-hour intervals and 0.3 mg/kg digoxin at 48-hour intervals similarly resulted in a 30 ~ 40% difference in the tumor size between the A549 and A549 -restored tumors (Fig. 5e,f). These results show that CGs exert their effects on tumors harboring an STK11 mutation in a clinically relevant dose range.

Figure 4. Investigation of the clinical potential of digoxin for the treatment of STK11 mutant cancers. Synergistic effect of digoxin in combination (72 hours) with (a) cisplatin or (b) carboplatin on cell viability in STK11 mutant and STK11-restored cell lines. (c) Proteomic analysis of the STK11 mutant-specific effect of digoxin treatment. The median normalized log2 phosphorylation levels of 38 proteins (40 phosphorylation sites) based on the results of the RPPA experiment, were compared between A549 cells and their STK11-restored derivatives after digoxin treatment. Red color represents an increased phosphorylation level, and green represents a decreased phosphorylation level. The listed proteins satisfied the cutoff value of $p < 0.01$ (Student's t-test) for the difference in phosphorylation between digoxin-treated and DMSO-treated cells (4 replicates each group). (d) CG induced ERK activation in A549 cells and STK11-restored A549 cells. ERK phosphorylation was measured after 3, 6, and 12 hours of treatment with 100 nM digoxin, and the quantified ERK phosphorylation level is shown as a numeric value. (e) Inhibiting ERK signaling reduces the effects of CGs. Cells were treated with inhibitors of the ERK pathway - AZD6244, GSK212 or SCH772984 in the presence or absence of digoxin for 72 hours. The rate of cell viability was calculated using DMSO as a control for each inhibitor. Additionally, the rate of protein phosphorylation was calculated using GAPDH as a control. *$p < 0.05$ and **$p < 0.01$ (Student's t-test) between the compared groups.
Mechanism for digoxin sensitivity of STK11 mutant cancer cells. We confirmed that digoxin treatment induced G2/M arrest more efficiently in STK11 mutant A549 cell than in STK11-restored cells, but did not induce detectable apoptosis (Fig. 6a,b). Digoxin and other CGs block tumor growth by diverse mechanisms associated with altering ERK23,24 or HIF-125,28 signaling. CGs induce cellular stress by increasing intracellular Ca2+ levels and blocking ATP hydrolysis29,30. CG-mediated generation of reactive oxygen species (ROS) is also responsible for the cytotoxicity of CG treatment31. We thus compared the changes in ROS levels between A549 and STK11 restored A549 cell lines after digoxin treatment. Digoxin significantly induced the generation of ROS in STK11 mutant cells but not in STK11-restored cells (Fig. 6c). Furthermore, the ROS inhibitor NAC significantly reduced digoxin-mediated G2/M arrest in STK11 mutant A549 cells (Fig. 6d). Inhibition of ROS also attenuated the efficacy of digoxin in STK11 mutant A549 cells (Fig. 6e), implying that the CG-induced increase in cellular stress was associated with the STK11-sensitive efficacy of CGs. Functional STK11-AMPK signaling is important for cancer cells to overcome stress responses induced by anti-cancer agents32,33. Particularly, AMPK activation inhibits the formation of ROS34. However, the phosphorylated AMPK levels were consistently lower in STK11 mutant cells than in STK11-restored cells, regardless of digoxin treatment (Fig. 6f and Supplementary Fig. S6a). Applying siAMPK to STK11 restored cells significantly increased their sensitivity to digoxin treatment (Fig. 6g and Supplementary Fig. S6b), and this observation implies that STK11-AMPK signaling is important for overcoming cellular stress induced by CGs. Furthermore, growth inhibition by digoxin treatment was attenuated
Figure 6. Mechanistic validation of the CG-mediated effect on STK11 mutant cancer. (a) G2/M arrest was significantly induced in STK11 mutant A549 cells by digoxin treatment. A549 and STK11-restored A549 cells were treated with 50 nM and 100 nM digoxin for 72 and 48 hours, respectively. (b) Apoptosis was not induced by digoxin treatment. Caspase3 cleavage was detected after digoxin treatment (72 hours). Total proteins were immunoblotted with anti-caspase-3, anti-cleaved caspase-3 and anti-STK11 antibodies. (c) The ROS level was increased by digoxin treatment (48 hours) in STK11 mutant A549 cells. The change in the level of ROS (green dots) was observed using a fluorescence microscope. (d) ROS inhibition attenuated digoxin-mediated G2/M arrest in STK11 mutant A549 cells. Cells were incubated in digoxin (100 nM) for 48 hours combined with or without pretreatment of the ROS inhibitor NAC (1 mM) for 1 hour. (e) The influence of ROS inhibition on the efficacy of CGs. Cells were incubated in digoxin (50 nM) for 72 hours combined with or without pretreatment of the ROS inhibitor NAC (1 mM) for 1 hour. (f) AMPK signaling was sustained in STK11-restored A549 cells regardless of CG treatment. AMPK phosphorylation was measured after 6 hours of treatment with 100 nM digoxin, and the quantified AMPK phosphorylation level is shown on the right. (g) The influence of AMPK regulation on the efficacy of CGs. Cells were incubated in AMPK-targeted siRNA and A769662 (AMPK
in STK11 mutant cells by activating AMPK using the direct AMPK agonist, A769662 (Fig. 6g and Supplementary Fig. S6c). The AMPK activation seems to provide a resistant mechanism against anti-cancer actions of CGs through induction of oxidative cell damage. Taken together, the results of the present study showed that STK11 mutations sensitize cancer cells to CG treatment via dysregulation of cellular stress responses (Fig. 6h).

Despite the anticancer activities of CGs via ATP1A1 inhibition, CGs have not been used clinically due to a lack of biomarkers for the identification of patients who would be most likely to benefit from CG treatment (Supplementary Table S3). Our data mining and experimental validation results suggest that clinically relevant doses of CGs exert selective effects on STK11 mutant cancer samples. STK11 loss-of-function mutation is specifically observed in LUAD tissue samples and is mutually exclusive from alterations of known drug targets such as ALK, BRAF, and EGFR. These findings provide a novel selection approach to predict the utility of CGs for lung cancer patients with STK11 mutant LUAD, who are most likely to benefit from CGs either alone or in concert with other therapies.

Methods

Acquisition of drug screening data. The therapeutic anticancer drug screening data for NCI-60 human tumor cell lines were obtained from the NCI/NIH Developmental Therapeutics Program (DTP) (www.dtp.nci.nih.gov). This dataset, released in July 2012, provides the GI50 values characterizing the sensitivity of NCI-60 DTP human tumor cell lines to 50,839 compounds. The GI50 value is defined as the concentration of a compound required to inhibit cell growth by 50% at 48 hours after compound treatment compared with DMSO vehicle treatment. The DrugBank database has widely been used to facilitate drug discovery and in silico drug target discovery.

Acquisition and analysis of somatic mutation data. Analysis and visualization of genetic alterations in lung adenocarcinoma were performed using cBioPortal. The details of data mining are provided in the Supplementary Methods.

Cell line enrichment analysis. To quantify the association between the compound response (GI50) and a given mutation (e.g., STK11 or BRAF), cell line enrichment analysis was applied as a statistical method. The details of statistical methods are provided in the Supplementary Methods.

siRNA transfection. The details of the siRNAs and experimental procedures used for transfection are provided in the Supplementary Methods.

Real-time PCR. The details of the primers and experimental procedures used for real-time PCR are provided in the Supplementary Methods.

Acquisition and analysis of shRNA screening data. The genome-scale shRNA library screening data for 216 cancer cell lines was obtained from the data portal of Project Achilles. We used the gene-level shRNA scores in the Achilles version 2.4 dataset. The zysogity of STK11 mutations in cancer cell lines was determined using somatic mutation data derived from the data portal of the Cancer Cell Line Encyclopedia (CCLE). The shRNA scores of ATP1A1 were compared between homozygous (NCI-H23, HCC44, PSN1, NCI-H2122, A549, NCI-H838 and JHOM1) and heterozygous (CAOV3, 22RV1, KALS1, A2058 and RKN) STK11 mutant cell lines.

Expression plasmid construction and transient transfection. The pLenti-LKB1-puro mammalian expression vector, a generous gift from Dr. Zhijun Luo (Boston University School of Medicine, Boston, MA, USA) was manipulated to construct the pBabe-puro-LKB1 expression vector. pLenti-LKB1-puro or pLenti-LKB1-mock was transiently transfected into HEK-293T cells to produce viral particles using a Thermo Scientific Trans-Lentiviral Packaging Kit (Dharmacon Inc.) in accordance with the guidelines of the institutional biosafety committee. Viral particles were infected into A549, NCI-H460, NCI-H23 and NCI-H1993 cells. The infected cells were selected using 2 μg/ml puromycin, and ectopic LKB1 expression was confirmed via western blot.

Cell viability assay. The cells were seeded in a 96-well plate at a density of 2 × 10^3 cells per well for 3 days, 1 × 10^4 cells per well for 7 days or 200 cells per well for 14 days. After 24 hours in culture, the cells were treated with chemicals at the indicated concentrations. The cells were incubated for an additional 3, 7 or 14 days, and cell viability was measured using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA). The culture medium was replaced every 3–4 days. The rate of cell viability was calculated using DMSO as a control for each chemical. And the percentage growth is calculated as: [(Ti − Tz) / (C − Tz)].

Cell cycle analysis. A549 and STK11-restored A549 cells were treated with 100 nM digoxin for 48 hours. Cell distribution in the G1, S, and G2/M phases of the cell cycle was determined via fluorescence-activated cell sorting (FACS) analysis, and the results were analyzed using FlowJo software (FlowJo LLC, USA). Further details are provided in the Supplementary Methods.
Invasion assay. To measure the effect of digoxin on cancer cell invasion, transwell cell culture chambers (Costar 3422, Cambridge, MA, USA) containing 12-μm pore size filters were used for cell invasion assays. Further details are provided in the Supplementary Methods.

Soft agar colony formation assay. A549 and STK11-restored A549 cells were diluted to 5 × 10^4 cells/well in 0.35% Noble agar (Sigma) solution in RPMI-1640 medium containing 10% FBS. Further details are provided in the Supplementary Methods.

Serum-free 3D sphere formation assay. A549, NCI-H23, STK11-restored A549, H23 (2.5 × 10^5 cells/well), NCI-H1993, and STK11-restored H1993 cells (5 × 10^5 cells/well) was seeded in ultra-low attachment 96 well plates and cultured in CSC medium (20 ng/ml EGF, 20 ng/ml FGFβ, 2% b-27, 1% penicillin/streptomycin and 10% FBS). After 5 days, the cells were stained with DAPI and cell spheres were counted (≥100 μm).

BrdU incorporation assay. Details are provided in the Supplementary Methods.

Measurement of ROS. Intracellular ROS production was determined by incubating the cells in 10 μM H2DCFDA and 1 μg/mL Hoechst 33342 (Sigma-Aldrich, USA) for 30 min at 37 °C in the dark. The cells were washed twice in PBS and analyzed using a Cytation3 cell imager (BioTek, USA) or observed using a fluorescence microscope (Olympus, Japan).

RPPA. RPPA data for 223 pan- and 62 phosphorylation-specific antibodies (a total of 245 unique proteins) for DMSO and digoxin-treated A549 and STK11-restored cells were generated in the Functional Proteomics Core of the M.D. Anderson Cancer Center, University of Texas. Further details are provided in the Supplementary Methods.

Western blot. The details of the antibodies and experimental procedures used for western blot are provided in the Supplementary Methods.

Xenograft mouse model. Athymic nude mice were purchased from Oriental Bio Inc. (Guro-gu, Seoul, Korea) and were maintained under specific pathogen-free conditions in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at the Catholic University of Korea (Approval numbers: 2014–005 and 2015–006). In the first in vivo experiment, 3 × 10^5 A549 or STK11-restored A549 cells were subcutaneously injected into the right dorsal flank of athymic nude mice, which were housed until the tumor volume reached 50 mm^3. The mice were divided into the following 2 treatment groups: vehicle alone (n = 10) or 60 μg digoxin (n = 10). Digoxin and vehicle were orally administered 3 times per week for 5 weeks. In the second experiment, 3 × 10^5 A549 or STK11-restored A549 cells were subcutaneously injected into the right and left dorsal flanks of athymic nude mice, which were housed until the tumor volume reached 50 mm^3. The mice were divided into the following 3 treatment groups: vehicle alone (n = 19), 0.6 μg digoxin (n = 20) or 6 μg (n = 19) digoxin. Vehicle and 6 μg digoxin were orally administered 3 times per week and 0.6 μg digoxin was orally administered 7 times per week for 3 weeks. Further details are provided in the Supplementary Methods.

Pharmacokinetics of digoxin. Pharmacokinetic analysis of digoxin was conducted at the end of the in vivo pharmacodynamic experiment in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at the Catholic University of Korea (Approval number: 2015–006). Male athymic nude mice were administered 0.6 or 6 μg/ind. digoxin. To measure the level of digoxin, blood samples (approximately 45 μL) were collected before administration and at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours after administration for 0.6 μg/individuals or at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 30, and 48 hours after administration for 6 μg/individuals. Plasma samples were treated and prepared for LC-MS/MS analysis. Further details are provided in the Supplementary Methods.

A detailed description of Methods is available in Supplementary Methods.

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**Author Contributions**

N.K., H.Y.Y. and N.H. performed experimental design, data mining, data interpretation and the manuscript preparation. N.K., N.H. and E.J. conducted bioinformatic analyses. H.Y.Y., N.H., S.K. and M.S. performed in vitro assays and measured experiments. C.-J.L., J.H.K., J.-S.C. and H.S.L. performed in vivo experiments and interpreted associated experiments. S.Y., W.-Y.K., S.J., G.B.M. and Y.-Y.C. conducted scientific direction. N.K., N.H., Y.-Y.C. and S.Y. wrote the manuscript. S.Y. designed and coordinated the overall research and experiments. All authors discussed the results and commented on the manuscript.

**Additional Information**

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