

SHORT COMMUNICATION

Differential alterations of positive and negative regulators of beta catenin enhance endogenous expression and activity of beta catenin in A549 non small cell lung cancer (NSCLC) cells

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Abstract Beta catenin has been well documented in previous studies to be involved in non small cell lung cancer (NSCLC). Beta catenin abundance and transcriptional activity are significantly regulated by several factors. Though it is well known that Akt and Gsk3 beta are respective positive and negative regulators of beta catenin, however, no single study has so far documented how the expression and activity of both positive as well as negative regulators play favorable role on beta catenin expression and activity in NSCLC. In this study, we compared expression and activity of beta catenin and its regulators in normal lung cell WI38 and NSCLC cell A549 by western blot, qRT-PCR and luciferase assay. We observed that beta catenin positive regulators (Akt and Hsp90) and negative regulators (Gsk3 beta and microRNA-214) have differential expression and/or activity in NSCLC cell A549. However the differentially altered statuses of both the positive and negative regulators rendered cumulative positive effect on beta catenin expression and activity in A549. Our study thus suggests that chemotherapeutic modulations of regulating factors are crucial when abrogation and/or inhibition of key oncogenic proteins are necessary for cancer chemotherapy.

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Abbreviations: NSCLC, non small cell lung cancer; SCLC, small cell lung cancer; miRNA, microRNA; snRNA, small nuclear RNA.

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Introduction

Non small cell lung cancer (NSCLC) is a group of lung cancers that are named for the types of cells affected and their morphology under the microscope. NSCLCs account for 85% of all the cases of lung cancers.¹ NSCLCs are relatively chemo-resistant, with respect to small cell lung cancer (SCLC).

Beta catenin is an oncogenic protein that is pivotal for the expression of Wnt responsive genes^{2–6} which regulate proliferation, cell division, anti-apoptosis, angiogenesis, metastasis and is consequently necessary for carcinogenesis and cancer progression. In NSCLC, beta catenin mutations are extremely uncommon.^{7,8} But, the relationship between aberrant beta catenin expression and NSCLC poor prognosis is well documented.^{9,10} Beta catenin was previously shown to be important for proliferation, migration and clonogenic ability of A549 NSCLC cell.¹¹ Hence, beta catenin status in terms of its expression and activity is of immense importance in NSCLC.

Cellular abundance and transcriptional activity of beta catenin are regulated by multiple positive and negative regulators. Since, beta catenin has been demonstrated to be involved in NSCLC, the expressions and activities of key beta catenin regulators may have some crucial contribution in NSCLC. So far no single study has shown how the expression and activity of positive as well as negative regulators of beta catenin are altered in NSCLC and how they have overall effect on beta catenin. Hence, we studied whether the expression and activity of known regulators of beta catenin are modulated in NSCLC cell that could be beneficial for beta catenin abundance and transcriptional activity. We observed that, besides beta catenin upregulation, well known positive regulators of beta catenin like Akt & Hsp90 and negative regulators like Gsk3 beta and microRNA-214 (miR-214) are differentially modulated in A549 NSCLC cell that had overall positive effect on beta catenin expression and transcriptional activity.

Materials and methods

Antibodies and chemicals

A list of antibodies and other chemicals used in this study is given in [Supplementary Table S1](#).

Cell culture, drug treatment and transfection

Normal lung cell WI38 (Gifted by Dr. Mahadeb Pal, Bose Institute, India) and NSCLC lung cancer cell A549 (obtained from National Centre For Cell Science, India) were cultured in DMEM supplemented with 10% Fetal Bovine Serum and incubated at 37 °C in a humidified incubator supplied with 5% CO₂. Cells were treated with 500 nM 17AAG or vehicle (DMSO) and incubated for 48 h. For transfected cells to be used in western blot or real time qRT-PCR, cells were split in 60 mm dishes and were transfected with 3 µg pRNA-U61/Hygro empty vector (empty U61) control or 3 µg pRNA-U61-pre-miR-214 (miR-214-U61) and incubated for 72 h. 10 µl Lipofectamine 2000 was used per dish as transfection

reagent. Throughout all the experiments passage number varied between 5th to 26th for WI38 cells and between 3rd to 18th for A549 cells. For every experiment it was ensured that cells had been passaged at least thrice after thawing.

Sub-cellular extraction

Both cytosolic and nuclear extractions were performed as described.¹² Cells were fractionated after 48 h of plating when they reached ~90% confluency.

Western blot

All western blotting experiments were done in this study as per our previous work.¹³ Untreated/untransfected cells were harvested at 48 h after plating when they reached ~90% confluency. 17-AAG treated and empty-U61 or miR-214-U61 transfected cells were harvested after 48 h of incubation.

RNA isolation, cDNA preparation and real-time quantitative PCR

Cells were harvested at ~48 h after plating when they reached ~90% confluency or harvested at 48 h after transfection with empty U61 and miR-214-U61. To study the expression of mRNAs viz. CTNNB1, GSK3B, AKT1, MYC, CCND1, JUN, FOSL1, BIRC5, ACTB (reference gene), the same reagents and same RNA isolation, cDNA preparation, real time qPCR techniques were used as per our previous work.¹³ For miRNA expression study, the use of U6 snRNA as reference gene has been well validated.¹⁴ 100 ng of RNA was reverse transcribed into cDNA with MuLV-Reverse Transcriptase and stem-loop primer for miR-214 and U6 snRNA and then subjected to real time qPCR using SYBR green mastermix on Mastercycler Ep Realplex (Eppendorf, Germany) using the forward primers of miR-214, U6 snRNA and universal reverse primer. Ct value of target (miR-214) was normalized to Ct value of reference (U6 snRNA). $2^{-\Delta\Delta C_t}$ method was used to calculate the fold change in miR-214 expression. A list of sequences of the primers used in this study is added in [Supplementary Table S2](#).

Luciferase assay

Luciferase assays were performed with same principle and method as described previously by us.¹³ Briefly, 200 ng TOPFLASH TCF-reporter plasmid was transfected in cells grown in 24 well plates and then incubated for 24 h and 48 h respectively and then harvested for experiments. 5 µg lysates from incubated samples were subjected to luciferase reporter assay as described.¹³

Statistical analysis

All values were expressed as mean $\pm$ SD. Significant difference between experimental groups were determined by two tailed Student's t-test using GraphPad QuickCals, an online tool.

Results & discussion

Beta catenin endogenous expression and transcriptional activity

We measured the relative endogenous levels of beta catenin mRNA CTNNB1 that was significantly higher in A549 NSCLC cell compared to WI38 normal lung cell (Fig. 1A). Elevated mRNA expression resulted in significantly higher level of beta catenin protein in A549 cell (Fig. 1B, C). Similarly, beta catenin protein levels were also higher in cytosol and nucleus of A549 (Fig. 1D).

Accumulation of beta catenin in the nucleus is a directly related to its transcriptional activity. We measured transcriptional activity of beta catenin in terms of luciferase activity of TOPFLASH TCF reporter plasmid. Transcriptional co-activator beta catenin combines with TCF transcription factors in the nucleus to turn on Wnt responsive genes. Hence, increased relative luciferase activity of TOPFLASH is related to enhanced Wnt/beta catenin signaling in terms of enhanced transcriptional activity of beta catenin in a cell. We observed that beta catenin transcriptional activity was significantly higher in both 24 h and 48 h post-transfection A549 cell than that of WI38 cell (Fig. 1E). We selected some of the targets of Wnt/beta catenin to validate elevated

Wnt/beta catenin signaling in A549 cell. We found that all the target genes tested in this study (viz; MYC, CCND1, BIRC5, JUN and FOSL1) were significantly upregulated in A549 NSCLC cell (Fig. 1F). These genes are involved in cell proliferation, division, survival, growth and target gene regulation. Hence, upregulation of these genes in A549 cancer cell was justified.

Beta catenin level can be reduced if it is phosphorylated by Gsk3 beta, because phospho-beta catenin is a target of ubiquitin-proteasomal pathway of protein degradation.¹⁵ However, we documented that overall phospho-beta catenin (Serine 33) level in A549 was much lower than WI38 (Fig. 1G). Again, undegraded phosphorylated (Serine 33) beta catenin is transcriptionally inactive as the complex of phospho-beta catenin (Serine 33) and LEF/TCF cannot form ternary complex with DNA in the nucleus and hence, it cannot switch on Wnt responsive genes.¹⁶ Hence, reduced phospho (serine 33) beta catenin in A549 NSCLC cell indicates higher overall beta catenin transcriptional activity.

Status of important negative regulators of beta catenin: Gsk3 beta and miR-214

Gsk3 beta is a known negative regulator of cytosolic beta catenin level and its nuclear activity. In absence of Wnt

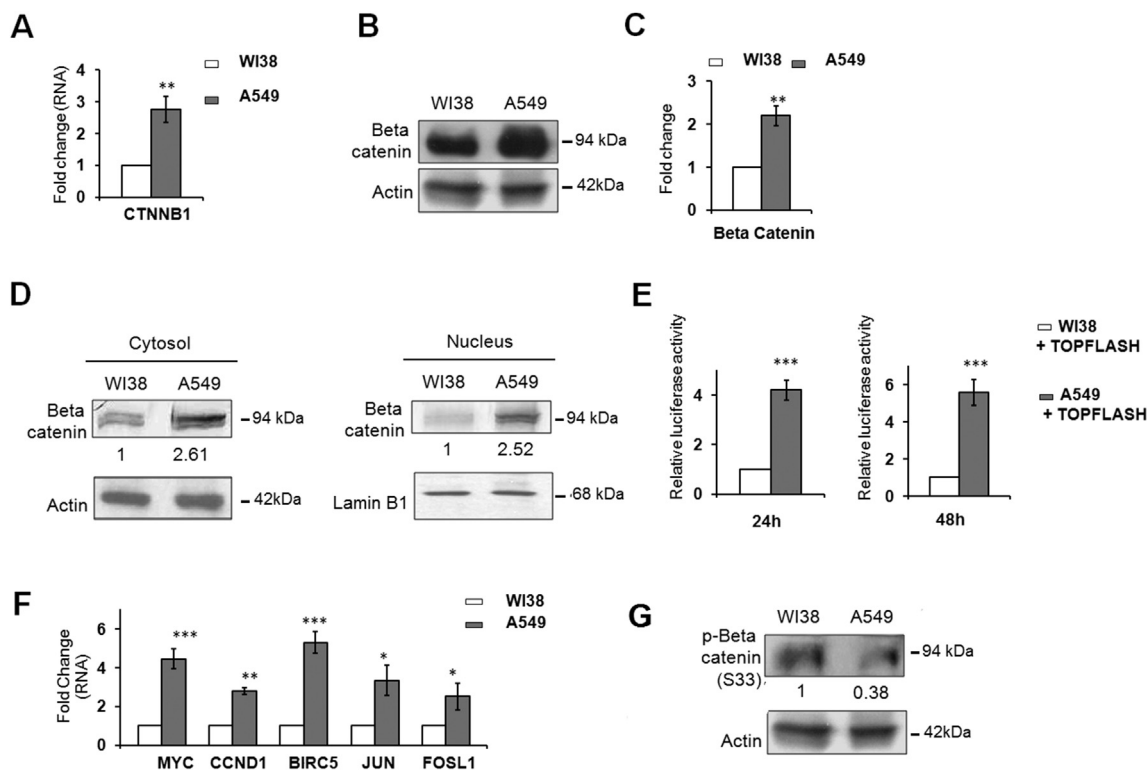


Fig. 1 Beta catenin endogenous expression and transcriptional activity. (A) Normalized fold change of CTNNB1 mRNA level in A549 with respect to WI38, ACTB (Actin) was the internal control ($n = 3$). (B) Western blot shows beta catenin levels in WI38 and A549 cells. Intensity analysis is normalized to Actin and displayed in bar diagram ($n = 3$). (C) Bar graph shows fold change of beta catenin protein level in A549 with respect to WI38. (D) Western blots (representative of two independent experiments) show cytosolic and nuclear beta catenin levels in WI38 and A549 cells. (E) TCF reporter activities (at 24 h and 48 h) in WI38 and A549 cells shown in terms of relative luciferase activity ($n = 3$). (F) Normalized fold changes of MYC, CCND1, BIRC5, JUN and FOSL1 mRNA levels in A549 with respect to WI38, ACTB (Actin) was the internal control ($n = 3$). (G) Western blot (representative of two independent experiments) shows phospho-beta catenin (Serine 33) levels in WI38 and A549 cells. Error bar represents standard deviation. Statistical significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

signaling, Gsk3 beta sequentially phosphorylates at Threonine 41, Serine 37 and Serine 33 residues of beta catenin.¹⁵ Reduced phospho-beta catenin (Serine 33) level in A549, prompted us to study the status of Gsk3 beta. The expression of GSK3B mRNA was found to be significantly lower in A549 compared to WI38 (Fig. 2A). This was reflected in reduced amount of Gsk3 beta protein (Fig. 2B, C). However, we noticed that A549 cell had rather elevated level of overall phospho-Gsk3 beta (Serine 9) (inactive population of Gsk3 beta) (Fig. 2B, C). We measured the ratio of phospho-Gsk3 beta to total Gsk3 beta in WI38 and A549 cell and the ratio was significantly higher in A549 (Fig. 2D). All these imply that negative regulator Gsk3 beta is less active and less abundant in A549 NSCLC cell which is beneficial for beta catenin abundance and activity.

MicroRNAs are important regulators of gene expression and they have multiple targets in a cell. Hence, they can act as tumor promoters or tumor suppressor. Previously, we and also other groups showed the negative role of miR-214

on beta catenin in different diseases.^{13,17} As beta catenin level in A549 cell was higher than that of WI38, we measured matured miR-214 expressions in these two cells and expectedly observed significant downregulation of miR-214 in A549 NSCLC cell (Fig. 2E). When we overexpressed miR-214 in A549, we observed significant reduction in beta catenin protein expression (Fig. 2F, G). Overexpression of miR-214 in A549 cell also resulted in significant downregulation of beta catenin mediated Wnt responsive genes like MYC, CCND1, JUN, FOSL1 and BIRC5 (Fig. 2H). Hence, downregulation of miR-214 in A549 NSCLC cell supported elevated cellular level of beta catenin.

Status of important positive regulators of beta catenin: Akt and Hsp90

Akt is a well known crucial positive regulator of beta catenin as it can inhibit negative regulator Gsk3 beta by

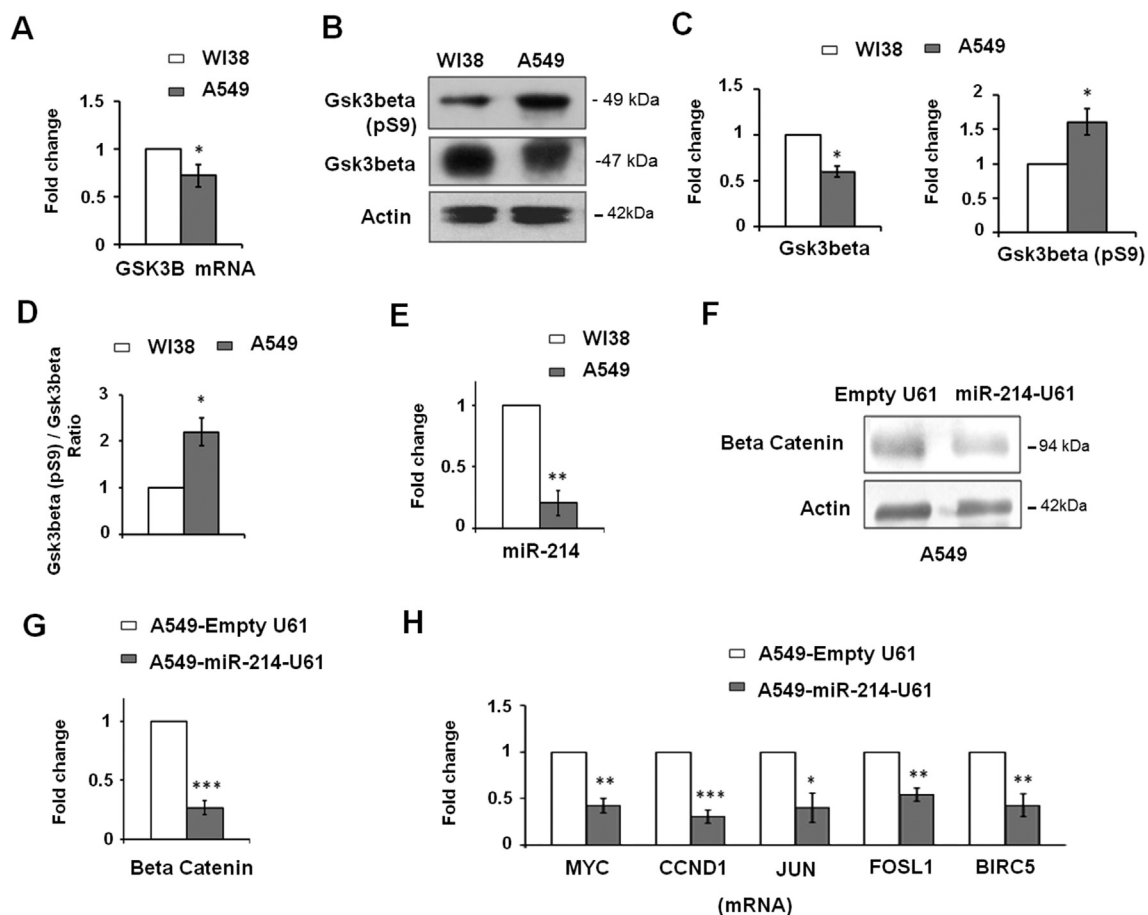


Fig. 2 Status of important negative regulators of beta catenin: Gsk3 beta and miR-214. (A) Normalized fold change of GSK3B mRNA level in A549 with respect to WI38, ACTB (Actin) was the internal control ($n = 3$). (B) Western blots show phospho-Gsk3 beta (Serine 9) and total Gsk3 beta levels in WI38 and A549 cells. Intensity analysis are normalized to Actin and displayed in bar diagram ($n = 3$) (C). (D) The ratio of normalized intensities (as obtained from (C)) of phospho-Gsk3 beta (Serine 9) to total Gsk3 beta ($n = 3$). (E) Normalized fold change of miR-214 level in A549 with respect to WI38, U6 snRNA was the internal control ($n = 3$). (F) Western blot shows beta catenin levels in empty U61 vector and miR-214-U61 plasmid transfected A549 cells. 72 hr post transfection samples were assayed. Intensity analysis is normalized to actin and displayed in bar diagram ($n = 3$) (G). (H) Normalized fold changes of MYC, CCND1, JUN, FOSL1, BIRC5 mRNA levels in A549 cells transfected with empty U61 vector and miR-214-U61 plasmid. ACTB (actin) taken as internal control ($n = 3$). Error bar represents standard deviation. Statistical significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

phosphorylating at Serine 9 residue.¹⁸ On the other hand, direct phosphorylation of beta catenin at Serine 552 position by Akt also promotes beta catenin transcriptional activity enhances tumor invasion.¹⁹ We studied the Akt status. We observed significant upregulation of AKT1 at mRNA level in A549 cell (Fig. 3A). We also found that total Akt protein and its active form phospho-Akt (Serine 473) were increased in A549 compared to WI38 (Fig. 3B, C).

Heat shock protein Hsp90 is known to inhibit apoptosis in cancer cells by stabilizing and modulating the activity of Akt.²⁰ It has been found that Akt forms a complex with Hsp90 beta and thereby becomes protected from being dephosphorylated (hence inactivated) by Protein Phosphatase 2 A (PP2A).²⁰ Hence Hsp90 indirectly acts as an important positive regulator of beta catenin. Hsp90 level was found to be in significantly higher in A549 NSCLC cell with respect to normal cell WI38 (Fig. 3D, E). To study the impact of Hsp90 on phospho-Akt levels in A549, we

inhibited Hsp90 by 500 nM 17-AAG (a Geldanamycin derivative) for 48 h in A549 and observed that Hsp90 inhibition drastically reduced phospho-Akt (Ser 473) level in A549 lung cancer cell (Fig. 3F, G). As active Akt (phosphorylated at Serine 473) is a positive regulator of beta catenin, we extended our study to find the role of Hsp90 on beta catenin level in A549. Hsp90 inhibition with 500 nM 17-AAG for 48 h in A549 cell significantly reduced beta catenin protein level (Fig. 3H, I).

All these data indicate that upregulation of Hsp90 and Akt (and enhanced Akt activation) were supportive to beta catenin abundance in A549.

Conclusion

We summarize that differential alterations of positive and negative regulators can simultaneously occur in cancer to

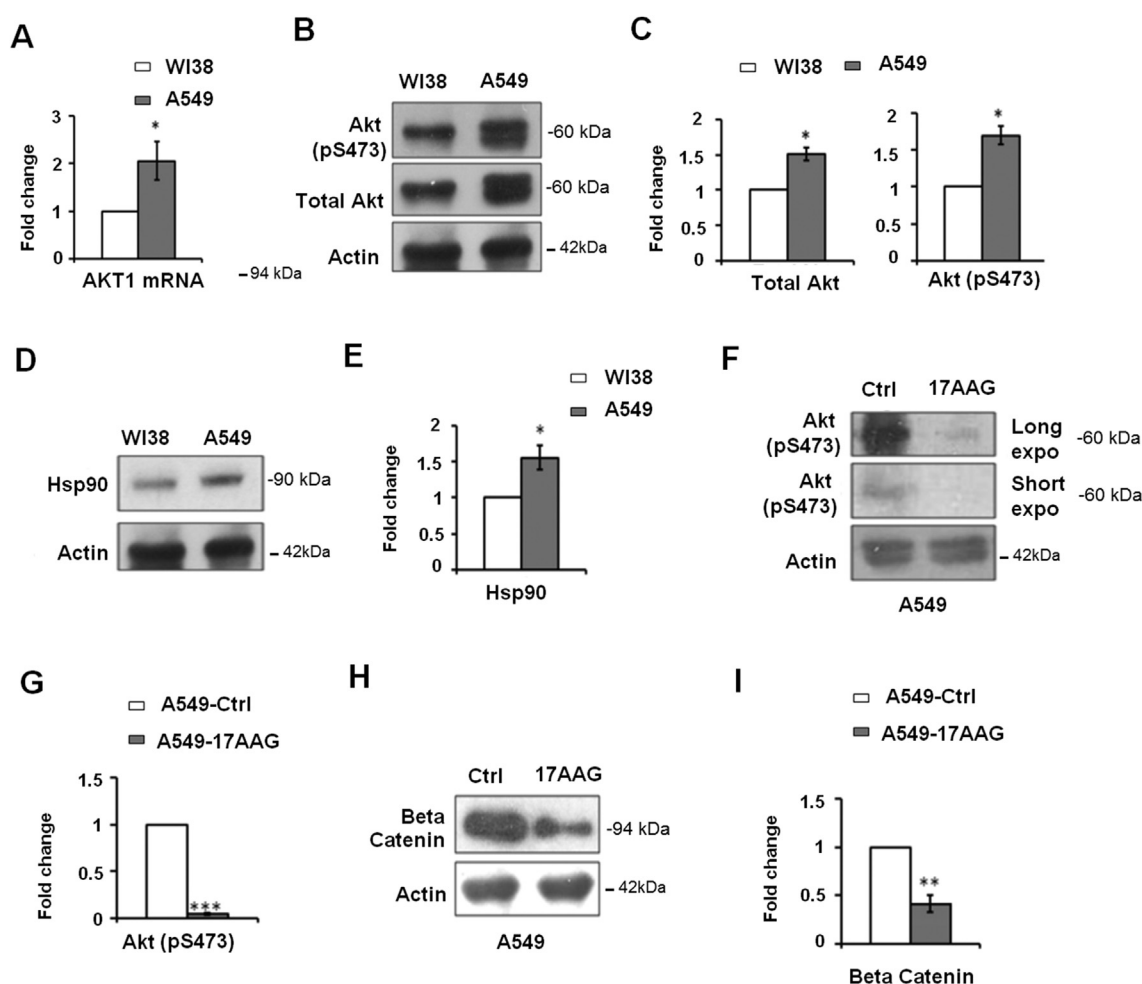


Fig. 3 Status of important positive regulators of beta catenin: Akt and Hsp90. (A) Normalized fold change of AKT1 mRNA level in A549 with respect to WI38, ACTB (Actin) was the internal control ($n = 3$). (B) Western blots show phospho-Akt (Serine 473) and total Akt levels in WI38 and A549 cells. Intensity analysis are normalized to Actin and displayed in bar diagram ($n = 3$) (C). (D) Western blot shows endogenous Hsp90 levels in WI38 and A549 cells. Intensity analysis is normalized to Actin and displayed in bar diagram ($n = 3$) (E). (F) Western blot shows phospho-Akt (Serine 473) levels in A549 cells treated with 500 nM of Hsp90 inhibitor 17-AAG or vehicle (control) DMSO for 48 h. Intensity analysis is normalized to Actin and displayed in bar diagram ($n = 3$) (G). (H) Western blot shows beta catenin levels in A549 cells treated with 500 nM of Hsp90 inhibitor 17-AAG or vehicle (control) DMSO for 48 h. Intensity analysis is normalized to Actin and displayed in bar diagram ($n = 3$) (I). Error bar represents standard deviation. Statistical significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

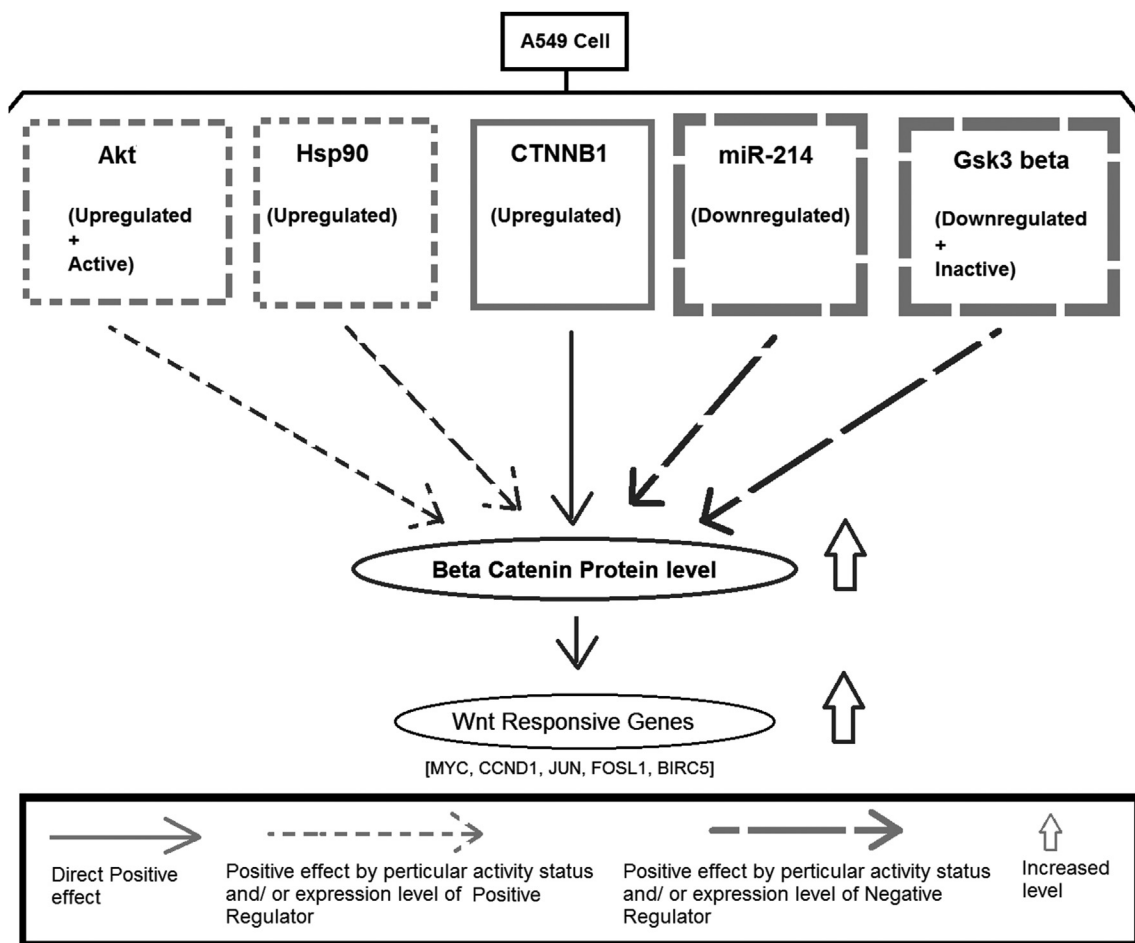


Fig. 4 Cumulative positive effect by positive and negative regulators on beta catenin level and activity in A549 NSCLC cells. Besides direct effect of CTNNB1 (beta catenin gene) upregulation, activation and upregulation of Akt (positive regulator), upregulation of Hsp90 (positive regulator) and downregulation of miR-214 (negative regulator), downregulation and inactivation of Gsk3 beta (negative regulator) had cumulative positive effect on beta catenin cellular abundance (increased level) which resulted in increased level of Wnt responsive genes.

increase abundance and activity of an oncogenic protein. In our study, downregulation and/or inhibition of negative regulators (Gsk3 beta and miR-214) and upregulation and/or activation of positive regulators (Akt and Hsp90) cumulatively increased beta catenin level and transcriptional activity (elevated expression of Wnt responsive genes) (Fig. 4). Our study thus suggests that consideration of regulators of oncogenic proteins might be useful in cancer chemotherapy.

Conflicts of interest

All authors have none to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.gendis.2016.10.004>.

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