

## Gene Expression Profile of Hedgehog Signaling Pathway Inhibition in Human Carcinoma Cells

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**Abstract:** Background: Aberrant activation of the Hedgehog (Hh) signaling pathway frequency occurs in human cancers, and increased evidence implicates the considerable role of GANT61 (Gli-ANTagonist, a kind of Hh signaling inhibitor) in anticancer therapy. However, it is still lacking the systematic scanning for the common mechanism of various cancer cells in respond to Hh-inhibition. Methodology/Principal Findings: Gene expression profiling of Hh inhibited HT-29 and MKN45 cells was determined by Illumina® Sentrix® BeadChip arrays. From the 17,329 expressed genes across genome, we identified 668 and 269 differentially expressed genes (DEGs,  $p < 0.01$ ) in comparison pairs of HT-29 and MKN45, respectively. Interesting, large number of common DEGs (77 genes) were seen in both two comparison pairs, which was clearly more than the predicted number (10,  $p < 10^{-4}$ ). Further interpretation of gene ontology was based on over-representation analysis. Two nested categories of GO biological processes (“cell death”, and “response to stimulus”) were detected as candidates with the enrichments of DEGs in both two cell lines. In addition, cDNA microarray profiling of extra cancer cells (ES2 and H4) verified the change of expression in six common DEGs related to “cell death” (CDKN1A, DDIT3, IER3, IL8, MFGE8, and PPP1R15A) following GANT61 treatment. Conclusions/Significance: Our research indicates that inhibition of Hh has considerable effect on genes from pathway of “cell death” in various carcinoma cells. And, the aberrant of “response to DNA damage” related genes (such as DDIT3) may play a critical role in GANT61-induced cell death. In summary, this dataset provide insight into the molecular mechanisms of GANT61-induced antitumor activity, and the list of novel GLI-targets in cancer cells.

**Key words:** Hedgehog signaling pathways; GANT61; Differentially Expressed Gene; anticancer therapy

## 0 Introduction

The Hedgehog (Hh) signaling pathway plays many vital roles in development of stem cell, and regeneration of adult [tissues](#)<sup>[1]</sup>. In mammalian cells, activation of the canonical Hh signaling is characterized by the binding of ligands to the membrane receptor Patched (*PTCH1*), the PTC-mediated release of Smoothened (*SMO*), the Smo-dependent activation of GLI transcription factors, and subsequent Hh target gene trans-activation (for details see the reviews<sup>[2]</sup>). As the downstream targets of Hh signaling are controlled by the dynamic of GLI transcription factors, a number of researches have pointed out the central role of Gli proteins in Hh signal transduction processes<sup>[3]</sup>. Molecular genetic analyses in mice and other model systems had indicated that GLI proteins induces the transcription of Hh downstream genes (such as *BCL2*, *CCND2*, *MYC*, *SNAI1*, *SOX2 et al.*) involved in many critical cellular processes, including cell proliferation, survival, differentiation, self-renewal ability, and migration<sup>[4-8]</sup>.

On the other hand, the potential role of Hh pathway in carcinogenesis has been emphasized by more and more evidences in recent years. First of all, the genetic dysfunction of Hh-related genes

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has been reported in different types of cancers including basal cell carcinomas (BCC), medulloblastomas (MB), glioblastomas (GB), rhabdomyosarcomas (RMS), and rhabdomyomas (RM)<sup>[9]</sup>. In these cases, the high frequency of activating mutations in *Gli1*, *Gli2*, *SMO*, as well as inactivating mutations in *PTCH1*, *REN*, and *SUFU* suggested that an aberrantly activated Hh signaling is absolutely required for tumor development<sup>[10]</sup>. And, the etiologic role of these genetic alterations in tumorigenesis has been confirmed in the corresponding genetic mouse models<sup>[11-14]</sup>. In addition to the cancers mentioned above, the activation of Hh signaling, as revealed by upregulation of *Gli1* expression, often been reported in tumor tissues of breast, hematological, pancreatic and prostate<sup>[15]</sup>. Although the oncogenic mutations of Hh-related genes still need to be demonstrated, numbers of studies had linked Hh pathway activity to tumor initiation and/or progression in corresponding cancer cell models<sup>[4]</sup>.

Furthermore, data from different types of cancer cell models also suggested the clinical-translational advances of targeting the Hh pathway in anticancer therapy. For example, the malignant phenotype of human colon carcinoma cells was significantly reduced by genetic inhibition of the GLI function (such as RNAi)<sup>[16, 17]</sup>. With regards to this, inhibitors of Hh signaling (such as Hh ligand antagonists, Smo inhibitors, and GLI transcriptional activity inhibitors) had been developed as anticancer drugs<sup>[18, 19]</sup>. Indeed, treatment with these small molecules did block the cell growth on tumor cell lines grown *in vitro* and on xenografts *in vivo*<sup>[10]</sup>. Among these Hh-related inhibitors, GANT61 (Gli-ANTagonist 61) was identified from a cell-based screen for inhibitors of GLI1-mediated transcription<sup>[20]</sup>. This Gli-ANTagonist shows a high degree of selectivity for Hh/GLI signaling, and could inhibit all types of aberrant Hh pathway activity, regardless of the upstream activating event. Compare to other kinds of small-molecule inhibitors, increasing studies had confirmed the most considerable effect of GANT61 on the “cell proliferation” in a wide variety of cancers<sup>[17, 21-29]</sup>.

To reveal the mechanism by which Gli-ANTagonist works as an anticancer agent, Shi *et al.*, preformed the genomic scanning for the downstream targets of the *Gli* genes in colon cancer models with and without GANT61-treated [30]. This cDNA microarray analysis identified numbers of *DEGs* (Differentially Expressed Genes, such as *CDKN1A*, *CDKN2B* *et al.*), which might control the Hh-dependent cellular proliferation and survival. Most importance, consisted with the GANT61-induced cell death and G1 arrest [17, 21-23, 25-27], this dataset did point out a large proportion of common *DEGs* including in pathways of “cell cycle checkpoint control”, “DNA damage response”, “DNA replication” “DNA repair”, and “stress response” in both colon cancer cells (HT29 and GC3/c1). These results suggested that the gene expression profiling could aid our understanding of the anticancer role of Hh-related antagonists.

As the genome-wide scanning for the common mechanisms of different cancer cells in respond to Hh signaling inhibited is still lacking, we measured the gene transcript alterations in colon and gastric carcinoma cells with GANT61-treated, and identified large number of common *DEGs* in both comparison pairs. Then, gene enrichment analyses further demonstrated that alteration of “cell death” related pathways may be the shared molecular mechanisms of the inhibition of Hh signaling in various cancers. Furthermore, profiling of transcripts from ovarian and neuroglioma cancer cells confirmed the considerable change of “cell death” related genes (*CDKN1A*, *DDIT3*, *IER3*, *IL8*, *MFGE8*, and *PPP1R15A*) in respond to Hh signaling inhibited.

## 1 Materials and Methods

### 1.1 Reagents and cell lines

90 GANT61 (G9048) was purchased from Sigma-Aldrich (St. Louis, MO). Colon cancer cell line HT-29, stomach cancer cell line MKN45, ovarian cancer cell line ES2, and neuroglioma cancer cells H4 were purchased from the American Type Culture Collection (ATCC, Manassas). Cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) and were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% humidity. In all experiments, the medium was replaced daily.

### 1.2 RNA isolation

Cancer cells (HT-29, MKN45, ES2, and H4 cell lines) were treated with and without GANT61 (20 µM for 48 hr), and three repeated samples for each state were performed to reduce experimental error. Total RNA from each sample was isolated by Trizol reagent (Invitrogen) and purified using RNeasy Protect Mini Kit (QIAGEN). RNA quality was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

### 1.3 Hybridization to Illumina gene expression arrays

Double-stranded cDNA and biotin-labelled cRNA were synthesized from RNA (300ng) using the Illumina® TotalPrep RNA amplification kit (Ambion Inc., Austin, TX). Each biotinylated cRNA (750 ng) was hybridized to prototype Sentrix Expression BeadChip (Illumina Inc., San Diego, CA). For twelve samples from four comparison pairs, individual hybridizations were performed. Following washing steps and staining with Streptavidin-Cy3, arrays were scanned on the Illumina® BeadArray Reader.

### 1.4 Microarray analysis of differential gene expression profile

110 The Illumina transcription datasets were used to carry out statistical data analyses of gene expression changes between samples with and without GANT61-treated by BeadStudio Data Analysis Software (Illumina, Inc). After scanned microarray images of Sentrix® BeadChips collected from the Illumina BeadArray Reader, image data files are directly downloaded into BeadStudio for data visualization and analysis. BeadStudio executes differential analysis, which aims to detect the change of gene expression levels between comparison pairs. The combination of DiffScore and P value was used to assess differences of gene expression between the two groups. A P value of less than 0.01 in any groups (DiffScore less than -20 or more than 20 of the GANT61-treated group) were selected to considered the values of signal statistically significant for pairwise comparisons.

### 120 1.5 DEGs Enrichment analyses of Gene Ontology terms and KEGG pathways

We performed enrichment analyses based on the databases GO (Biological Process) and KEGG pathways<sup>[31]</sup>. For enrichment analyses, the input gene set consisted of all differentially expressed genes list. The tools were used with the following options: significance threshold of less than 0.0005 for adjusted P value, at least four genes from the input list in the enriched category, and the list of 31,333 transcripts represented in the BeadChip as the reference background.

### 1.6 Quantitative real-time PCR

The expression levels of selected genes identified by cDNA microarray expression profiling at 48 hr following GANT61 treatment, were validated by real-time PCR. Total RNA (1 µg) was

employed to prepare cDNA via reverse transcription using cDNA synthesis kit (Invitrogen) according to manufacturer's instructions and analyzed using an Applied Biosystems 7500 PCR Detection System (Applied Biosystems Inc.). Real-time PCR reaction conditions were as follows: activation at 95°C for 10 min with 40 cycles of denaturation at 95°C for 15 s, primer annealing and extension at 60°C for 1 min and ramping back to 95°C. Melt curve analysis of all samples was routinely performed to ascertain that only the expected products had been generated. A fluorescence reading determined the extent of amplification at the end of each cycle. Expression levels of target genes were normalized to the expression of GAPDH and quantified using the comparative CT method. Real-time PCR for each gene was determined in triplicate, and each experiment was repeated at least twice to ensure quantitative accuracy. The primers used for real-time PCR are listed in Table 1.

## 2 Results

### 2.1 Differentially expressed genes detected from comparison pairs

To describe the changes of gene expression in response to GANT61-treated, the expression profile of 31,333 transcripts in HT29 and MKN45 cell lines was displayed (detail shown in Dataset S1). As a result, 17,329 transcripts showed significant signals of expression compared to background (Detected  $P < 0.01$ ), and 860 candidates present considerable change of expression ( $P < 0.01$ , Dataset S2) in at least one comparison pair. In colon carcinoma cell, 4.3% of expressed genes (668/15,539) were defined as DEGs, of which 294 genes were up-regulated, and 374 genes were down-regulated (Figure 1A). And, 269 DEGs (1.8% of 14,757 expressed genes) were identified in gastric carcinoma cells, including 208 up-regulated and 61 down-regulated genes. In agree with the considerable effect of GANT61 on the cell growth, the *altered* expression of many genes related to "cell proliferation" was detected in our scanning. As shown in Figure 1B, 75 DEGs from "cell proliferation" pathway were detected, including 51 candidates in HT29, 34 in MKN45, as well as 12 common DEGs in both two comparisons.

On genome-wide scale, substantial proportion of DEGs (9.0%, 77/860) shared by both colon and gastric carcinoma models, and this number is over seven times more than the expectation of each individual pairwise comparing (10 genes,  $\chi^2$  test,  $df = 1$ ,  $P < 10^{-4}$ ). Of these common DEGs, 61 were up-regulated (79.2% of 77 common DEGs), which were also significantly larger than the corresponding numbers by independent assumption ( $\chi^2$  test,  $df = 1$ ,  $P < 10^{-2}$ ). Among above differentially expressed genes, interestingly, there was a prevalence of common DEGs (12 genes, ADM, CDKN1A, EMP1, IL8, ISG20, MFGE8, ODC1, PIM1, PTGS2, SOD2, TNFRSF12A, WARS) enriched in "cell proliferation" processes, consisted with the extensive growth arrest of both cancer cells caused by GANT61 (data not shown). These results suggested that HT29 and MKN45 cell lines may share the molecular mechanism in response to GANT61-treated.

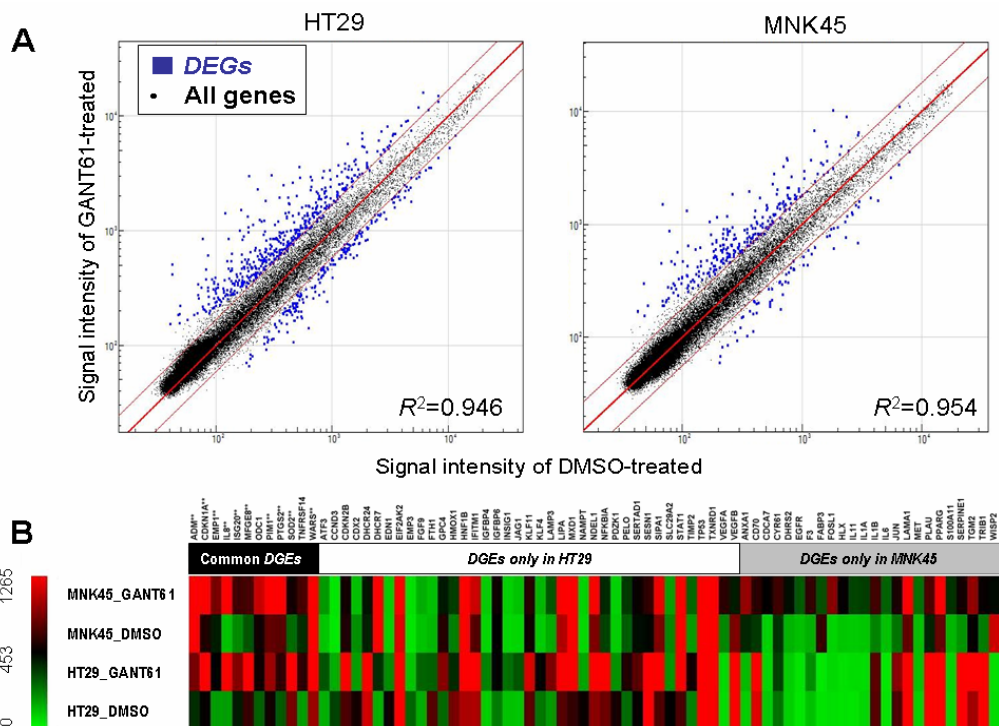


Fig. 1 Differential expression genes (DEGs) in two cancer cells after GANT61-treated. A) The scatter plot showing the gene expression level of DEGs (blue crosses) against the background of all 31,333 transcripts for the DMSO (x-axis) and the GANT61 group (y-axis) from HT29 and MKN45 cell lines. B) Heat map showing gene expression patterns of DEGs involved in “cell proliferation”. Genes denoted with asterisks define common DEGs shared by two comparison pairs.

## 2.2 Enriched gene categories identified using pathway analysis

To better understand the molecular mechanisms underlying transcriptional change, we obtained significantly over-represented DEGs categories (KEGG pathway or GO biological-process) by gene-set enrichment analyses. As a result, eight and thirteen KEGG pathways were significant overrepresented with DEGs from HT29 and MKN45, respectively (Figure 2A). In these candidates, “pathways in cancer” and “metabolic pathways” from KEGG database were common categories shared by both cell lines. Furthermore, from the 46 candidate biological-processes from GO database, four nested GO-categories (“anti-apoptosis”, “response to chemical stimulus”, “response to organic substance”, and “response to stress”) from “cell death”, and “response to stimulus” were detected as common gene-sets (Figure 2B).

In additional, gene-set annotation implied the functional relationship among “cell death” (anti-apoptosis) and the other DEGs enriched gene-sets. For example, lots of DEGs (23 genes) were shared by anti-apoptosis pathway and “response to stimulus”. As shown in Figure 3, four of common DEGs (CDKN1A, SOD2, ADM, and SQSTM1) in the anti-apoptosis pathway were involved in the biological-processes either of “response to stress”, “response to chemical stimulus”, or “response to organic substance”. On the other hand, advance interpretation of gene ontology indicated that another three common DEGs in the “response to stimulus” were related to “cell death” pathway (DDIT3, PTGS2, PPP1R15A), and two were related to “cell proliferation” (IL8, MFGE8). In summary, large number of common DEGs related to “cell death” (11 genes, ADM, BIRC3, CDKN1A, DDIT3, IER3, IL8, MFGE8, PPP1R15A, PTGS2, SOD2, SQSTM1) consisted with the extensive GANT61-induced cell death in both colon and gastric carcinoma models, suggested that aberrant of “cell death” played a central role in Hh-dependent cancer cell



proliferation and survival. These results further supported the hypothesis that HT29 and MKN45 share the similar molecular mechanism after blocking Hh signaling at the level of the GLI genes.

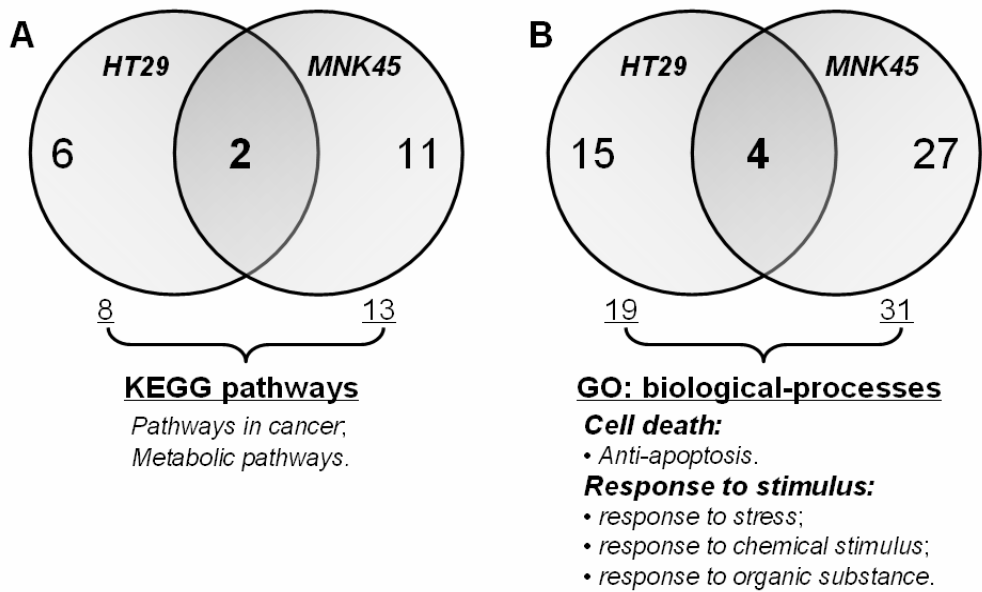


Fig. 2 Summary of gene-set enrichment analyses. The categories with DEGs Over-represented were detected based on gene-sets from KEGG pathways A) and GO biological-process B). For certain comparison pair, the counts of category with over-represented DEGs were listed. The numbers in the circles indicate the existence of categories detected in at least one comparison. The numbers in the intersection regions are those of enrichment categories shared across comparisons. The consistency categories common in both two comparison pairs were presented below.

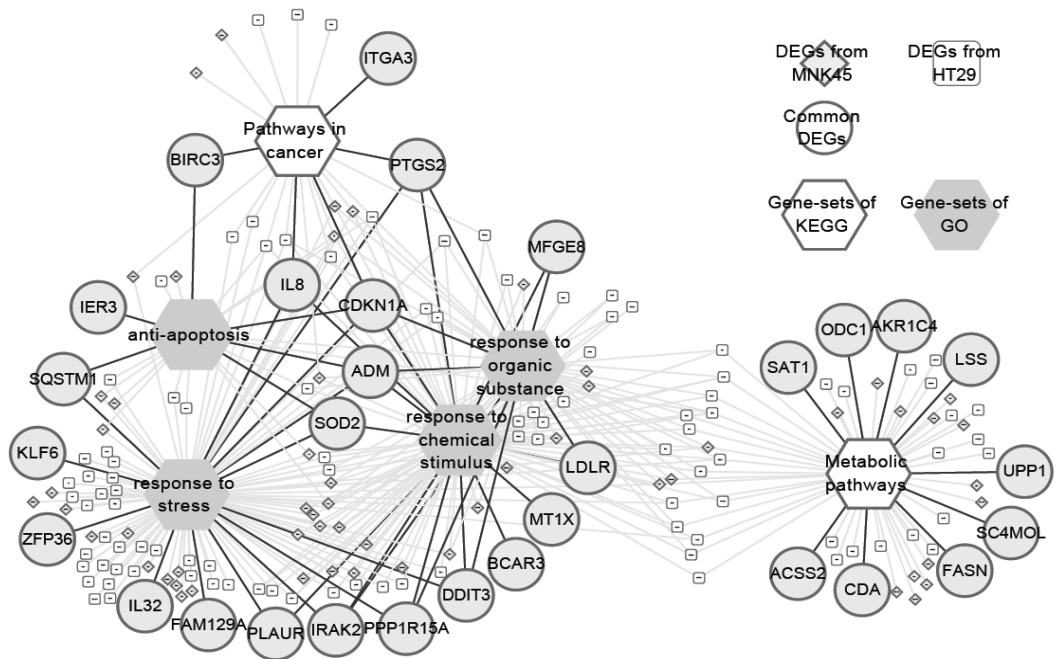


Fig. 3 Enrichment for DEGs from two comparison pairs. The network was constructed with Cytoscape, and displayed using an Edge-Weighted Spring Embedded layout. DEGs from comparison of HT29 are shown as rectangle shape, DEGs from comparison of MKN45 are shown as diamond shape, and the common DEGs are shown as ellipse shape. The DEGs enriched gene-sets are indicated by hexagon shape.

### 2.3 Change of common DEGs related to “cell death” in various cancer cells

To further check for the biological significance of common DEGs in GANT61-induced cytotoxicity, we described the expression pattern of 11 common DEGs related to “cell death” in ES2 and H4 cell lines using cDNA arrays (Table 2, full dataset not shown). Almost all of these candidates (except the SOD gene in comparison of ES2 cell) showed the same direction of changes after GANT61-treated. As a result, seven and ten candidates were detected as differentially expressed genes ( $P < 0.01$ ) in comparison pairs of ES2 and H4, respectively. Six “core DEGs” (CDKN1A, DDIT3, IER3, IL8, MFGE8, and PPP1R15A) were detected in all four comparison pairs, which was clearly more than the predicted number (less than one gene in whole dataset, Fisher's exact test,  $P < 10^{-4}$ ).

Furthermore, QRT-PCR was performed to confirm the transcript alteration of six “core DEGs” (Table 1). All candidates showed the same direction of change by QRT-PCR and by the expression arrays. And all genes measured by QRT-PCR showed statistically significant up-regulation following GANT61 treatment (Figure 4). In a word, our profiling of gene expression indeed suggested the considerable role of “cell death” related genes in response to GANT61-treated.

Tab.1 Primers used for real-time PCR amplification

Genes	Forward primer ( 5' to 3' )	Reverse primer ( 5' to 3' )
Gli1	5'-TCCTACCAGAGTCCCAAGTT-3'	5'-CCCTATGTGAAGCCCTATTT-3'
Gli2	5'-CCTGGCATGACTACCACTATGAG-3'	5'-GGCTTGGCTGGCATGTTG-3'
CDKN1A	5'-TGTCCTTGGGCTGCCTGTT-3'	5'-TTGCTGCCGCATGGGT-3'
DDIT3	5'-CCAGGGAAGTAGAGGCG-3'	5'-TGCATGTGGGATTGAG-3'
IER3	5'-GCTCTGGACCTCAGCACTTTC-3'	5'-GGTACGCCTGGTGTTCCTTTG-3'
IL8	5'-TGAAGATGCCAGTGAAA-3'	5'-AACCCCTACAACAGACCC-3'
MFGE8	5'-CCAGCGTGCCATTCCA-3'	5'-GGCGGCGATCTGTGAGTT-3'
PPP1R15A	5'-CTAAAGGCCAGAAAGGTGCG-3'	5'-TGATCGGCGTGCAG-3'
GAPDH	5'-CAGGGCTGCTTTAACTCTGGT-3'	5'-GATTTTGGAGGGATCTCGCT-3'

Tab.2 Expression change of common DEGs related to “cell death” in different cancer cell lines

Symbol	HT29		MNK45		ES2		H4		Pathway related to “cell death”
	FC	P-val	FC	P-val	FC	P-val	FC	P-val	
ADM	1.18	<0.000	1.79	<0.000	0.89	-	1.08	<0.000	anti-apoptosis
BIRC3	1.87	<0.000	1.74	<0.000	0.75	-	1.32	0.004	anti-apoptosis
CDKN1A <sup>#</sup>	2.43	<0.000	2.03	<0.000	2.03	<0.000	1.93	<0.000	anti-apoptosis
DDIT3 <sup>#</sup>	2.11	<0.000	1.87	<0.000	1.62	<0.000	0.84	0.006	cell death
IER3 <sup>#</sup>	1.27	<0.000	2.45	<0.000	1.49	<0.000	2.12	<0.000	anti-apoptosis
IL8 <sup>#</sup>	3.09	<0.000	3.60	<0.000	1.03	0.001	3.32	<0.000	cell proliferation
MFGE8 <sup>#</sup>	1.01	<0.000	1.18	0.005	1.42	<0.000	1.97	<0.000	cell proliferation
PPP1R15A <sup>#</sup>	1.77	<0.000	1.70	<0.000	0.97	0.003	1.36	<0.000	cell death
PTGS2	0.91	0.001	1.46	<0.000	0.48	-	1.23	<0.000	cell death
SOD2	1.05	0.006	2.39	<0.000	-0.09	-	1.29	<0.000	anti-apoptosis
SQSTM1	1.30	<0.000	0.78	0.007	1.60	<0.000	0.47	-	anti-apoptosis

Log<sub>2</sub> (Signal of GANT61-treated /Signal of DMSO-treated); # indicated the “core DEGs”; - indicated  $P$  value > 0.01.

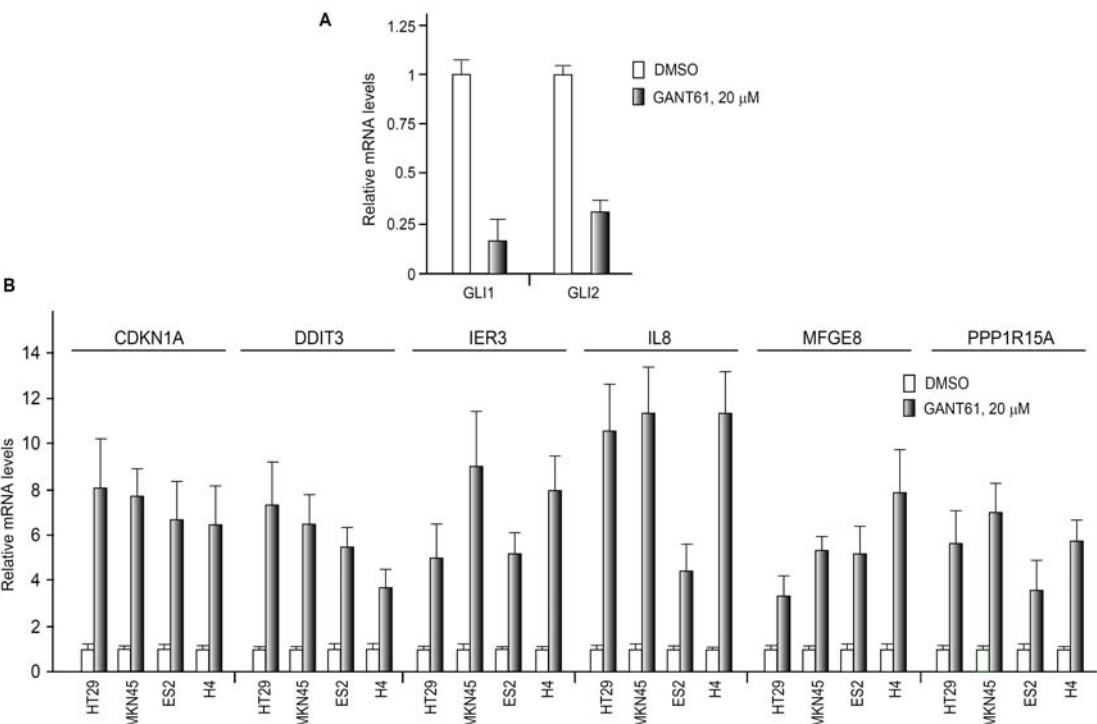


Fig. 4 Verification of differentially expressed genes (DEGs) in GANT61-treated human carcinoma cells. (A) Expression of both Gli1 and Gli2 mRNA was decreased following treatment with GANT61 (20 μM; 48 hr), determined by real-time PCR. Data are shown as mean ± SD of three independent experiments. (B) Selected DEGs from cDNA array gene expression profiling analyzed by real-time PCR in human carcinoma cells. Human carcinoma cells were treated with vehicle alone (0.2% DMSO) or GANT61 (20 μM) for the indicated times, total RNA was extracted and real-time PCR was performed as described in Materials and Methods using the primer sets in Table 1. Data represent the mean ± SD of three determinations, and GAPDH was used to normalize the relative mRNA levels.

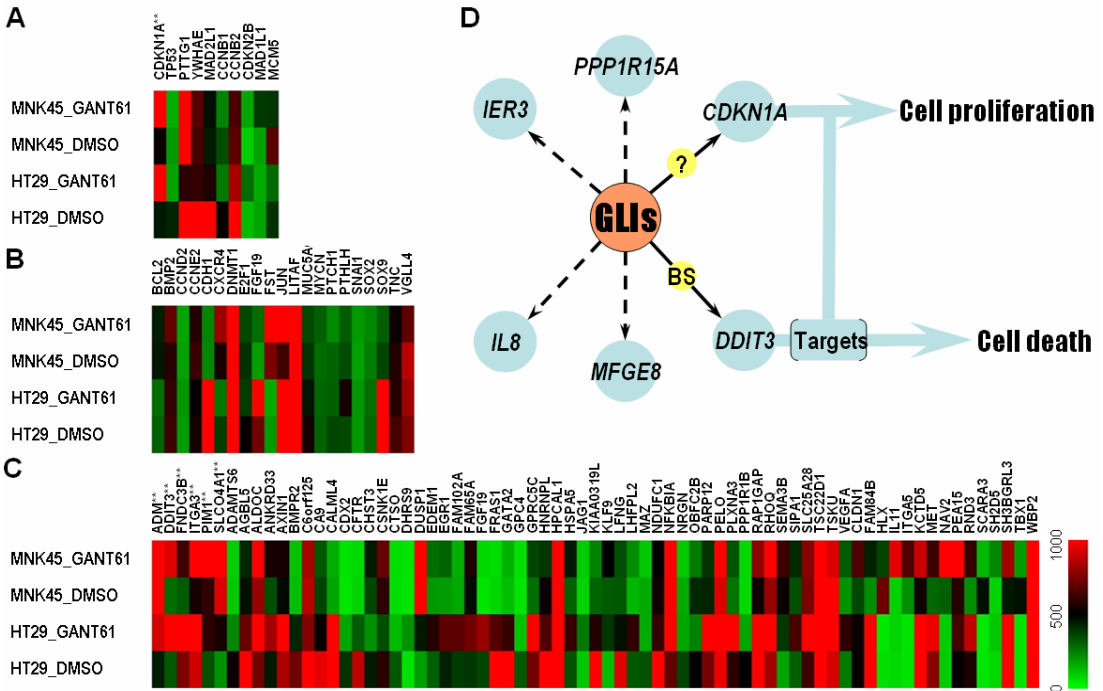




Fig. 5 Expression pattern and regulation model in cancer models with GLI-inhibition. A) Heat map showing gene expression patterns of DEGs involved in “cell cycle”. Genes denoted with asterisks define common DEGs shared by two comparison pairs. B) Heat map showing expression patterns of gene involved in “known GLI-target in cancer model”. The genes of CCND2, MYCN, PTCH1, SNAI1, and SOX2 were detected as “not expressed” (Detected  $P > 0.01$ ) in all states. C) Heat map showing gene expression patterns of DEGs involved in “predicted GLI-target with binding- site”. D) Common regulation model of GLI-inhibition in cancer cells.

### 3 Discussion

In pharmacological analyses, GANT61 had shown vigorous cytotoxicity against various cancers, such as human colon carcinoma cells (HT29 and GC3/c1), AML cells (Kasumi-1, K562, HL60 and U937), E-RMS cells (CCA, Rh36, and A673), and OSCC cells (CAL27, SCC4, SCC9, and SCC25)<sup>[17, 21-23, 25-27]</sup>. In current genome-wide scanning, the significant enrichment of DEGs in the “cell death” related gene-sets, such as “cell proliferation”, “anti-apoptosis”, and “cell death”, was consistent with the growth arrest and cell death in various cancers following GANT61 treatment. These results did imply that the dysfunction of “cell death” processes contributed to Hh-driven tumorigenesis in a wide range of human cancers. Furthermore, the enriched nested GO-categories of “response to stress” provided new insight into the molecular mechanisms of Hh-induced cancer cell survival in various cancers. In previous researches, Shi et al., did identify these pathways as candidates with DEGs over- representation in colon cancer cells<sup>[30]</sup>. As shown in Figure 3, the large number of cross connects among gene-sets related to “response to stress” and “cell death” implied that “cell death might induced by cell stress” with GANT61-treated. And the four of “core DEGs” (CDKN1A, DDIT3, IL8, and PPP1R15A) were involved in the pathway of “response to DNA damage” further suggested that the stress induced by DNA damage play a vital role in cancer cell death under the Hh- inhibition.

Among these six “core DEGs”, CDKN1A was a potential gene activated downstream of GLI<sup>[32]</sup>, which encodes a potent cyclin-dependent kinase inhibitor (p21 protein). The p21 binds to and inhibits the activity of cyclin-CDK2 or -CDK1 complexes, and thus functions as a regulator of cell cycle progression<sup>[33]</sup>. During the cell cycle, a series of events was regulated by different types of proteins, and p21 play a key role in the G1/S checkpoint. In this transcription map, numbers of cell cycle-related DEGs, including CDKN1A, TP53, PTTG1, YWHAE, MAD2L1, CCNB1, CCNB2, CDKN2B, in HT29 cell, and CDKN1A, MAD1L1, MCM5 in MNK45 (Figure 5A), indicated the changes of cell cycle progression causing by Hh-inhibition. Most important, the significant over-expression of CDKN1A in both GANT61-treated cancer cells was consisted with the substantial cellular accumulation at the G1/S boundary and in early S-phase. On the other hand, although previous studies suggested that the up-regulation of CDKN1A might associate with the DNA damage response (DDR)<sup>[34]</sup>, the stable p21 knockdown had no effect on GANT61-induced cell death in colon carcinoma cell model<sup>[17]</sup>. In summary, these researches suggested that CDKN1A does not induce cell death on its own directly, and the relationship between the increase of p21 mRNA and the Hh-mediated cellular stress, such as GANT61-induced DNA damage, is still need to further explore.

As cytotoxicity of GANT61 was thought to occur through its inhibition of GLI-mediated gene trans-activation, the expression change of GLI downstream-targets could provide further insight into the molecular mechanisms of GANT61-induced antitumor activity. We summarized 21 known direct-target with GLI-binding site (GLI-BS) from cancer models based on Literature review (detail saw Supp Table 1). In this gene expression profile, most of these candidates were detect as “not expressed” (Detected  $P > 0.01$ ) in both GANT61-treated and untreated states (Figure 5B, such as BCL2, CCND2, MYCN, PTCH1, SNAI1, and SOX2), and none of these

candidates showed significantly transcript alterations in both comparison pairs. These results suggested there were new GLI-related regulatory elements in cancer cells. To detect the novel direct-targets of GLI in colon and gastric carcinoma models, we *in-silico* predicted the candidate GLI-BS in promoter region with a position weight matrix of known motif (“GACCACCCA”)<sup>[35]</sup>. Among 17,329 ‘expressed’ genes, there were 1,005 candidate direct-targets with at least one predicted GLI-BS (within 10 Kbp upstream and 1 Kbp downstream of TSS), and 71 candidates present considerable change of expression ( $P < 0.01$ , Figure 5C) in at least one comparison pair. This proportion (71/1005) is significant larger than the genomic background (860/17,329,  $\chi^2$  test,  $df = 1$ ,  $P < 10^{-4}$ ), suggested treatment with GANT61 may priority to promote the up- or down-regulation of GLI-targets.

In additional, the conserved GLI-binding site (BBS  $> 4$ ) in the DDIT3 promoter region (9,796 bp upstream) suggested this “core DEGs” might be a directed-target controlled by GLI transcription factor (Figure 5D). DNA-damage-inducible transcript 3 (DDIT3), also known as G1 arrest and DNA damage 153 (GADD153), encodes a member of the CCAAT/enhancer-binding protein (C/EBP) family, which is activated when DNA damage in cellular stress conditions<sup>[36]</sup>. As a transcription factor of the dimer forming C/EBP family, DDIT3 played an importance role in the processes of “G1 arrest” and “cell death” response to cellular stress<sup>[37]</sup>. Although the relationship between GANT61-induced cell death and DDIT3 up-regulation still unclear, RNA-microarray analyses indicated that nuclear DDIT3 could drastically affected the functional categories of “cell death”, and “cellular proliferation”<sup>[38]</sup>.

In summary, although the molecular mechanisms of GANT61-induced DNA damage and the direct downstream targets of the Gli family still need further studied, our analyses suggest that “cell death induced by DNA damage” is the common cell process following the Hh signaling inhibition in different cancer cell models.

## 4 Competing interests

The authors declare that they have no competing interests.

## 5 Funding

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## 抑制人肿瘤细胞 Hedgehog 信号通路的 基因表达谱分析

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摘要: 背景: 在人类肿瘤中发现 Hedgehog (Hh) 信号通路异常激活, 并且越来越多的证据表明 GANT61 (Gli 拮抗剂, 是一种 Hh 信号通路抑制剂), 在抗癌治疗中起着相当大的作用。然而, 目前仍然缺乏对 Hh 抑制剂作用各种癌细胞的共同机制的系统扫描。方法/主要发现: Illumina® Sentrix® Bead 芯片分析出了 Hh 抑制 HT-29 和 MKN45 的基因表达谱。17329 个表达基因中, 我们鉴定了在 HT-29 和 MKN45 细胞中分别有 668 和 269 个不同的基因表达, 这两者之间具有差异表达 ( $P < 0.01$ )。发现大量的常见差异表达基因 (77 个基因) 均出现在这两株细胞中, 这超出了预计数 (10,  $P < 0.0001$ )。基因本体论的进一步解释是基于过代表性分析。在这两株细胞中以基因本体生物过程的两个分类 (“细胞死亡” 和 “对刺激物的响应”) 筛选差异表达的基因。此外, 对 GANT61 处理的 ES2 和 H4 进行基因芯片分析, 证实六个 (CDKN1A, DDIT3, IER3, IL8, MFGE8 和 PPP1R15A) 与 “细胞死亡” 相关基因差异表达。结论/意义: 我们的研究表明, 在各种肿瘤细胞中, 抑制 Hh 信号通路后, “细胞死亡” 相关的基因有影响。并且, “DNA 损伤反应” 相关的基因 (如 DDIT3) 异常表达, 在 GANT61 诱导的细胞死亡中起着重要的作用。总之, 这些数据有助于理解 GANT61 抗癌治疗活性的分子机制, 以及在肿瘤细胞中发现 GLI 新的靶基因。

关键词: Hedgehog 信号通路; GANT61; 基因差异表达; 抗癌治疗  
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