Galanin Receptor Subtype 2 Suppresses Cell Proliferation and Induces Apoptosis in *p53* Mutant Head and Neck Cancer Cells

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Abstract

Purpose—Galanin and its three receptors (GALR1-3) are expressed in many normal tissues, but silenced in some tumors. Contradictory roles for galanin and its receptors in various tumors have been reported. To understand their function, investigations of individual GALRs are necessary. In head and neck squamous carcinoma cells (HNSCC) with silenced GALR1 and GALR2, we showed that re-expressed GALR1 suppresses tumor cell proliferation via Erk1/2-mediated effects on cdk inhibitors and cyclin D1. Others showed that GALR2 could induce apoptosis in neuroblastoma cells with wild-type *p53*, whereas GALR2 stimulated proliferation in small cell lung cancer. In this study, we investigated the role of GALR2 in HNSCC cells that have mutant *p53* and do not express GALR1.

Experimental Design and Results—UM-SCC1, a human oral carcinoma cell line with a splice site mutation causing a 46-bp *p53* off frame deletion, was stably transfected to express GALR2 (UM-SCC-1-GALR2). Galanin treatment of UM-SCC-1-GALR2 caused morphological changes and a marked decrease in cell number that were not observed in UM-SCC-1-mock cells. Galanin and GALR2 resulted in decreased BrdU incorporation, p27^{Kip1} and p57^{Kip2} up-regulation, and decreased cyclin D1 expression. These effects were similar to GALR1 signaling in HNSCC, however, GALR2 also induced caspase-3-dependent apoptosis, which was confirmed by annexin-V staining and DNA fragmentation analysis. These were not observed with GALR1.

Conclusion—This study demonstrates that GALR2 re-expression can inhibit cell proliferation and induce apoptosis in HNSCC cells with mutant *p53*. GALR2 may be a feasible target for HNSCC therapy.

Keywords

Galanin; G-protein-coupled receptor; apoptosis; p53 independent pathway; head and neck neoplasm

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Introduction

Patients with advanced head and neck squamous cell carcinoma (HNSCC) have poor prognosis even when aggressive treatments are used (1). Current progress in molecular biology should make it possible to create new strategies for cancer such as molecular targeting therapy. Approximately 30% of all pharmaceuticals on the market today, exert their therapeutic effect by interacting with a G-protein coupled receptor (GPCR). GPCRs control a wide array of signaling pathways in all tissues of the body and we are only just beginning to understand the full range of their effects (2). Galanin, a 30-amino-acid peptide and its receptors, GALR1, GALR2 and GALR3, are members of the GPCR superfamily and are variably expressed in many normal tissues, including squamous epithelium, and some tumors, such as glioblastoma, neuroblastoma, small cell lung cancer and head and neck cancer (3–6). Reports of the effects of galanin signaling in tumors vary with tumor type. Seufferlein et al. (7) reported that galanin has mitogenic effects in small cell lung carcinoma, whereas, Ishii et al. (8) reported that galanin inhibits pancreatic carcinogenesis. One reason for these conflicting results is lack of knowledge about the effects of individual galanin receptors in cancer cells. To better understand galanin function, studies of individual galanin receptors are needed. Our previous findings indicated galanin and its receptors are likely to act as tumor suppressors in HNSCC because expression of the receptors is frequently silenced by LOH and promoter hypermethylation (9,10, Misawa et al. Clin Can Res. In Press). Using cells that lack endogenous GALR1 expression we demonstrated that in cells expressing exogenous GALR1, galanin stimulation suppressed cell proliferation, consequent to the extracellular signal regulated kinase pathway, suggesting that this receptor is a likely tumor suppressor (6). Berger et al. (4) reported that exogenous expression of GALR2 inhibited cell proliferation and induced apoptosis in neuroblastoma. Furthermore, like GALR1, GALR2 is frequently silenced by methylation and deacetylation in HNSCC (Misawa et al., unpublished data). To further explore how galanin and GALR2 function in HNSCC, we stably transfected HA tagged GALR2 into UM-SCC-1, an HNSCC cell line in which both GALR1 and GALR2 are silenced by methylation (Misawa et al. unpublished data). In the present study, we demonstrated that galanin and GALR2 induce apoptosis and inhibition of cell proliferation and incorporation of BrdU in HNSCC cells with mutant *p53*. These findings suggest that GALR2 is a potential target gene for molecular therapy in HNSCC.

Materials and Methods

Cell Culture and Proliferation Assay

UM-SCC-1 was established from a human oral carcinoma of the retromolar trigone (Krause, et al. Arch Otolaryngol 107:703–710, 1981). UM-SCC-10B and UM-SCC-74B were established respectively from a lymph node metastasis of a laryngeal cancer and from a recurrent tongue cancer (Bradford et al. Head Neck 25:654–661, 2003). Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Irvine Scientific Santa Ana, CA) at 37°C in 5% CO_2. The C-terminal HA-tagged GALR2 sequence was obtained from human cDNA library (Invitrogen, Carlsbad, CA), subcloned into the pcDNA3 (Invitrogen) containing the internal ribosomal entry site (Ires) and Green Fluorescent Protein (GFP) sequence. The pCMVIresGFP was used as a negative control. The UM-SCC-1-GALR2 and UM-SCC-1-mock cells were established by transfecting pCMVGALR2IresGFP or pCMVIresGFP into UM-SCC-1 using lipofectamine reagents (Invitrogen). GFP positive cells were selected by Flow Cytometry using FACS Vantage SE (BD Biosciences, San Diego, CA). To examine cell proliferation

and morphology, 24 h after the cells were plated, the cells were cultured with serum free media containing with 0.1% BSA (SFM) for 24 h to induce quiescence. Then various concentrations of galanin (ANASPEC, San Jose, CA) were added. A caspase-3 inhibitor, 50 µM Ac-DEVD-CHO (DEVD; Biomol, Plymouth Meeting, PA) was added 2 h prior to galanin treatment. Cell proliferation was determined by counting the cells with a Coulter counter model Z1 (Beckman Coulter Inc., Hialeah, FL). To observe the changes in cell morphology, photographs were taken using the Olympus IX71 inverted microscope (Olympus Corporation, Tokyo, Japan).

p53 Gene Mutation Analysis

The status of the *p53* gene in UM-SCC-1 was analyzed by sequencing of cDNA. Total RNA was isolated from UM-SCC-1-GALR2, UM-SCC-74B, and UM-SCC-1-mock cells using the RNAeasy mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized by the reverse transcription of 2 µg of extracted RNA with 200 units of SuperScript II (Invitrogen) in the presence of random primers and deoxynucleotide triphoshates (Invitrogen). The *p53* gene transcripts were amplified using FastStart Taq DNA Polymerase (Roche Diagnostics GmbH). Three overlapping primer sets were used: p53-1 forward 5'-gcgtgctttccacgacg; reverse 5'-ccttccactcggataagatg, p53-2 forward 5'-ttgcattctgggacagccaa; reverse 5'-ggcatccttgagttccaagg, p53-3 forward 5'-caccatcatcacactggaag; reverse 5'-ctgacgcacacctattgcaa. The cycler was programmed with the following conditions: (a) initial denaturation at 94°C for 5 min, followed by (b) 40 cycles of 95°C for 40 s, (c) annealing of the primer template at 58°C for 50 s, (d) extension at 72°C for 50 s, and (e) an additional extension at 72°C for 7 min. The PCR products were separated by electrophoresis through a 1.5% agarose gel containing ethidium bromide and were extracted from the gel with the QIA quick Gel Extraction Kit (Qiagen Inc.). Purified PCR products were sequenced at the University of Michigan DNA Squencing Core on an ABI 3700 (Applied Biosystems, Foster City, CA). *p53* sequences and mutations were confirmed using data from the National Center for Biotechnology Information website.

Immunoblotting

The cells were lysed with 1% Nonidet-P 40 lysis buffer containing protease inhibitors (Calbiochem, La Jolla CA). The supernatant was collected and the protein content was measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Equal amounts of protein were electrophoresed on 10% SDS-PAGE gels and transferred to Hybond-P (Amersham Biosciences Limited, Buckinghamshire, United Kingdom). The membranes were incubated overnight with primary antibody at 4°C, followed by incubation with anti-mouse secondary antibody horseradish peroxidase conjugate (Amersham Biosciences Limited) and detected by chemiluminescence and autoradiography using Hyperfilm obtained from Kodak (Rochester, NY). Densitometric measurements of the bands were carried out using ChemiImager 4400 (Alpha Innotech, San Leandro, CA). To detect exogenous GALR2, the cell lysates were treated with 1000 units of N-Glycosidase F (New England BioLabs, Beverly, MA) and loaded onto the gel without boiling to avoid protein aggregation. The mouse monoclonal anti-HA tag antibody (CONVANCE, Berkeley, CA) was used as the primary antibody. Expression of $p27^{Kip1}$, $p57^{Kip2}$, cyclin D1, and p53 was detected using the mouse monoclonal antibodies for $p27^{Kip1}$, $p57^{Kip2}$ (Lab Vision, Fremont, CA), cyclin D1 (DakoCytomation Norden A/S, Glostrup, Denmark), and p53 (Calbiochem, La Jolla, CA). UM-SCC-10B cells containing a point mutation were used as a p53 high expression control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using the mouse monoclonal anti-GAPDH monoclonal antibody (Chemicom International, Temecula, CA) as internal control.

Immunocytochemistry

The cells were seeded on coverslips. After 24 h of incubation, the cells were fixed with 4% paraformaldehyde and then stained with mouse monoclonal anti-HA tag antibody and Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). After incubation with Alexa Fluor 546 goat anti-mouse IgG$_1$ (Molecular Probes), the localization of exogenous GALR1 was determined using the Olympus FV-500 Confocal Microscope (Olympus Corporation).

BrdU Incorporation Assay

The cells were seeded on coverslips. After 24 h of serum-starved incubation, 1 µM galanin was added to the SFM and the cells were incubated for an additional 24 h. After galanin treatment, the cells were incubated with 10 µM BrdU (Caltag Laboratories, Burlingame, CA) for 45 min, fixed with 70% ethanol and the DNA was denatured with 2N HCl. The cells were stained with mouse monoclonal anti-BrdU antibody (Caltag Laboratories), followed by incubation with Alexa Fluor 546 goat anti-mouse IgG$_1$ (Molecular Probes). For each coverslip, 3000 cells were counted and the percentage of BrdU-positive cells was determined.

Apoptosis Analysis

Induction of apoptosis was evaluated by active caspase-3 staining, annexin-V staining, and DNA fragmentation analysis. Serum-starved cells were treated with galanin for 12 or 24 h, and active caspase-3 and annexin-V in UM-SCC-1-GALR2 and UM-SCC-1-mock cells were detected using the Active Caspase-3 PE MAb Apoptosis Kit (BD Biosciences) and the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. Flow cytometric analysis was carried out using FACS Vantage SE and the data analyzed using Cell Quest and ModFit LT software packages (Verity Software House Inc., Topsham, ME). Genomic DNA was isolated using DNAeasy Tissue Kit (Qiagen, Inc.), and then loaded onto a 2 % agarose gel. The DNA ladders stained with ethidium bromide were visualized under UV light.

Statistical Analysis

Results were tested for statistical significance using two-way ANOVA or Student's *t*-test.

Results

Exogenous GALR2 expresseion

The extracted protein from UM-SCC-1-GALR2 and UM-SCC-1-mock cells pretreated with PNGase-F was assessed for exogenous GALR2 expression by immunoblotting using the monoclonal antibody for HA-tag. Digestion with PNGase-F, a glycosidase that removes N-linked sugars, and solublization without boiling yielded the appropriate GALR2-HA band in UM-SCC-1-GALR2 but not in UM-SCC-1-mock cells (Fig.1A). To assess the cellular localization of exogenous GALR2, cells grown on coverslips were stained with HA-tag antibody. Immunofluorescence revealed that all the GFP-positive cells show exogenous GALR2 expression localized to the cytoplasmic membrane as expected for a GPCR (Fig. 1B). The expression of GALR2 in the parent UM-SCC-1 cells was undetectable and less than that in normal human tissue (data not shown). Thus, the differences in the results that we obtained with the UM-SCC-1-GALR2 and UM-SCC-1-mock cells following galanin stimulation should reflect mainly the function of GALR2.

UM-SCC-1 cells contain mutant p53

Since previous studies had shown that GALR2 signaling caused apoptosis in neuroblastoma cells with wild-type *p53*, we examined the p53 status of UM-SCC-1 cells to determine if the

effects of galanin and GALR2 required p53-mediated signaling. UM-SCC-1 was previously reported by us to have wild type *p53*2 as assessed by sequencing exons 5–8 (Bradford et al. Head Neck 25:654–661, 2003). However, other experiments indicated that UM-SCC-1 failed to respond to DNA damage with up-regulation of p21. Therefore, we sequenced the *p53* transcript (using the method described in Hauser et al. and Mandic et al.3,4) from UM-SCC-1, which revealed a splice site mutation that results in a 46-bp deletion and frame shift mutation beginning in exon 5 that leads to a nonsense sequence after the splice (Fig. 2A). UM-SCC-74B cells have wild-type *p53* and were used as a control for transcript analysis (11). UM-SCC-10B contain a point mutation at exon 245 and overexpress p53 (12). These cells were used as a size marker for p53 protein expression. No p53 protein is detectable in UM-SCC-1 cells suggesting that the truncated transcript fails to produce a protein or that it is degraded (Fig. 2B).

GALR2-mediated galanin signaling inhibits cell proliferation and induces changes in cell morphology and adhesion

To determine the role of GALR2 in cell proliferation, cells starved for 24 hours were treated with various concentrations of galanin, and counted with a Coulter counter. Fig. 3A shows proliferation of UM-SCC-1-GALR2 and UM-SCC-1-mock cells after galanin treatment in dose-dependent and time-dependent assays. As the concentration of galanin increased, the proliferation UM-SCC-1-GALR2 cells decreased significantly when compared to the mock transfected control cells. Proliferation was reduced by 58% compared to the control UM-SCC-1-mock cells when treated with 1 µM of galanin ($P<0.01$). Furthermore, UM-SCC-1-GALR2 cells treated with a fixed concentration of galanin (1 µM) and counted at 24, 48 and 72 hours after galanin treatment, also exhibited a progressive decrease in cell number with time. Compared with galanin treated UM-SCC-1-mock cells, galanin treated UM-SCC-1-GALR2 cells showed almost no increase in cell number from control levels at 24 hours after galanin treatment. The inhibitory effect on galanin treated UM-SCC-1-GALR2 cells increased in a time dependent manner, and was significantly different from UM-SCC-1-GALR2 cells without galanin treatment and UM-SCC-1-mock cells (Fig. 3A) ($P<0.01$). Obvious morphology changes also were seen in UM-SCC-1-GALR2 but not UM-SCC-1-mock cells after treatment with various concentrations of galanin. Within 24 hours after addition of 1 µM of galanin, UM-SCC-1-GALR2 cells started to round up and exhibited decreased adhesion to the culture dishes. By 36 hours after treatment, 60–80% of cells had detached from the culture dishes and were floating in the medium (Fig. 3B). These results indicate that galanin and GALR2 affects cell adhesion in UM-SCC-1 cells.

GALR2 inhibits BrdU incorporation, induces p27^{Kip1}, p57^{Kip2} up-regulation and Cyclin D1 down-regulation

To clarify the mechanism of GALR1-induced inhibition of cell proliferation, we examined BrdU incorporation as an index of cell cycle arrest and inhibition of DNA synthesis. As compared with UM-SCC-1-mock cells, UM-SCC-1-GALR2 cells exhibited significantly lower BrdU incorporation after 1 µM of galanin treatment ($P<0.01$). However, this decrease of BrdU incorporation did not occur in UM-SCC-1-mock regardless of galanin treatment (Fig. 4A). These data indicate that GALR2-mediated inhibition of proliferation is associated with suppression of DNA biosynthesis. Deregulation of the cell cycle is a common feature of human cancers. Tumor-associated alterations in this process frequently affect cyclin-

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dependent kinases (Cdks) and their regulators such as p27^{Kip1}, p57^{Kip2} and cyclin D1. To better understand the role of GALR2 in arrest of tumor cell proliferation, starved cells were treated with 1 µM of galanin for 24 hours and the expression of these regulatory proteins was measured by immunoblotting using specific antibodies. As shown in Fig. 4B, p27^{Kip1} expression in UM-SCC-1-GALR2 was increased by galanin treatment, but this increase was not seen in UM-SCC-1-mock cells. Similarly, p27^{Kip1}, p57^{Kip2} expression in UM-SCC-1-GALR2 was obviously increased by galanin, but not in UM-SCC-1-mock cells. Densitometric measurements indicate that galanin significantly enhanced p27^{Kip1} expression at 1.8 fold and p57^{Kip2} at 1.7 fold ($P<0.05$). In contrast, cyclin D1 expression decreased in UM-SCC-1-GALR2, but not in UM-SCC-1-mock. Densitometric measurements indicate that galanin significantly suppressed cyclin D1 expression by 0.28 fold ($P<0.01$). These data suggest that galanin and GALR2 induced arrest of proliferation is associated with regulation of p27^{Kip1}, p57^{Kip2} and cyclin D1.

GALR2 activation leads to apoptosis

To investigate whether UM-SCC-1-GALR2 cells die through apoptosis, galanin treated cells were assessed for annexin-V expression by flow cytometry. Annexin-V positive cells increased with galanin concentration in UM-SCC-1-GALR2 but not in UM-SCC-1-mock cells (Fig. 5A) ($P<0.01$). Furthermore, galanin and GALR2 induced DNA ladder formation, a characteristic of apoptosis. Cells were treated with 100 nM galanin for 24 hours, then genomic DNA was extracted and loaded onto a 2 % agarose gel. As shown in Fig. 5B, galanin treated UM-SCC-1-GALR2 cells exhibited obvious DNA ladder formation. These results provide the evidence that galanin and GALR2 induce apoptosis in UM-SCC-1 cells.

Activation of Caspase-3 in GALR2-Mediated Apoptosis

Caspases are a family of cysteine-proteases, which upon activation will cleave cellular protein targets, leading to the biochemical and morphological alterations typical of apoptosis (13). To examine the contribution of caspase-3 to galanin stimulated GALR2-mediated apoptosis, we counted active caspase-3 positive cells by flow cytometry in galanin-treated UM-SCC-1-mock cells and UM-SCC-1-GALR 2 cells. Active-caspase-3 positive cells were significantly increased in a galanin concentration dependent manner in UM-SCC-1-GALR2 (Fig. 6A) ($P<0.01$). Furthermore, preincubation of cells with 50 µM DEVD prior to 0.1 µM galanin treatment could prevent the apoptotic effect of galanin and GALR2 (Fig. 6B). Thus, these results pointed to the activation of caspase-3 by galanin as a critical step in the process of apoptotic cell death occurring in UM-SCC-1-GALR2 cells.

Discussion

In this study, we demonstrated that re-introduction of GALR2 into HNSCC cells makes the tumor cells susceptible to galanin-induced growth inhibition and apoptosis. These results suggest that the galanin-GALR2 pathway has significant growth regulatory activity and that loss of this pathway could be an important factor in tumor development and progression. Furthermore, since GALR2 can induce apoptosis in *p53* mutant HNSCC cells, the loss or inactivation of the galanin-GALR2 signaling cascade provides tumor cells with another mechanism of avoiding cell death. Our results also suggest the possibility that reactivation of the galanin receptor(s) pathway could be a therapeutic strategy in HNSCC. In fact, our recent data demonstrate that the galanin receptors are frequently silenced by hypermethylation in HNSCC (14), and that galanin and GALR1 can inhibit cell proliferation (6).

It was reported earlier that GALR2 can initiate a complex signaling cascade by coupling to G_q, G_i and G_{12} proteins (5), but that in small cell lung cancer, galanin activated MAP kinase

and had mitogenic effects (7). Recently, it was reported that in neuroblastoma cells, exogenous expression of GALR2 inhibited cell proliferation by inducing apoptosis (4). It is known that some kinds of GPCRs, including the *N*-formyl peptide receptor (FPR), and the V2 vasopressin, angiotensin II, and CXCR2 receptors are capable of initiating apoptosis upon stimulation (15). Although the mechanism by which the GALR2 pathway induces antiproliferative effects is not completely understood, a few studies about other GPCRs have been reported. Angiotensin II-induced apoptosis is mediated via its receptors through the induction of transforming growth factor-β production, which is followed by increased transcription of cell death genes such as Fas, FasL, Bax and caspase-3 (16). The FPR receptor also induces apoptosis by the activation of several signaling proteins, such as p38, MEK and caspases (17). Recently, To fighi *et al.* demonstrated exogenous GALR2-transfected pheochromocytoma cells induced apoptosis, by decrease in the phosphorylated Bad level followed by suppression of the PI3K/Akt pathway (18). In our preliminary data, galanin induced activation of the extracellular signal regulated kinase (Erk) pathway activation in UM-SCC-1-GALR2 cells (data not shown), and Erk pathway and PI3K/Akt pathway could cross-talk in cancer cells (19), but their roles for GALR2 induced apoptosis in HNSCC cells are unclear.

In our study, GALR2 up-regulated $p27^{Kip1}$, $p57^{Kip2}$ while down-regulating cyclin D1. $p27^{Kip1}$, $p57^{Kip2}$ and $p21^{Cip1}$ belong to the Cip/Kip family of inhibitors of cyclin-dependent kinases. Their roles are not fully understood but many studies have been reported. One recent finding has shown that $p21^{Cip1}$ is implicated in the antiapoptotic response to chemotherapy (20). As a negative regulator of apoptosis, $p21^{Cip1}$ interacts with several caspases, such as caspase-3, caspase-8 and caspase-10 (20). We could not detect $p21^{Cip1}$ expression in either UM-SCC-1-GALR2 or UM-SCC-1-mock cells (data not shown), but its absence is consistent with the fact that UM-SCC-1 cells have mutated *p53*. Meanwhile, it has been reported that $p27^{Kip1}$ positively regulates the apoptotic response (21, 22). Actually, galanin and GALR2 mediated $p27^{Kip1}$ up-regulation and caspase-3 dependent apoptosis in our experimental models, but the role of $p27^{Kip1}$ for GALR2-mediated apoptosis is unclear. Clinically, *p27^{Kip1}* and *p57^{Kip2}* are known to be tumor suppressor genes, and their expression levels are related to prognosis in many different tumors, including non-small cell lung carcinoma, gastric carcinoma, and HNSCC (23–26). Overexpression of cyclin D1 occurs at a high frequency in a variety of carcinomas, including those arising in the breast, colorectum, and head and neck, and is associated with poor prognosis (27–29). The fact that galanin and GALR2 can regulate these genes suggests GALR2 is a tumor suppressor in head and neck cancer cells.

It is significant that GALR2 can mediate this potent anti-proliferative effect in the absence of p53 function. Most studies have focused on the involvement of p53 in regulating apoptosis, because the therapeutic action of many anti-neoplastic agents depends on p53-induced apoptosis. However, *p53* is frequently mutated in HNSCC, and thus it would be valuable to find a molecule that can kill tumor cells in a p53-independent manner. Although the p53-independent mechanism activated by GALR2 has not yet been clearly defined, it is known that some kinds of receptors and signaling proteins, such as adenovirus E1A, E2F1 and p73, induce apoptosis independent of p53 (30–33). Conflicting results have been reported regarding GPCR- induced apoptosis and p53 status. Wang *et al*. reported a potential role for GPCR signaling in the modulation of p53-mediated apoptosis and indicated that β-arrestin 2 may serve as a signaling linker between the GPCR and p53 pathways via sequestration of MDM2 (17). On the other hand, the somatostatin analogue AN-238 and its GPCR receptor induce apoptosis though a p53-independent pathway (34). The orexin receptor was also reported to induce p53-transcription-independent apoptosis though p38 activation (35). Until now, it had not been determined whether GALR2 needs p53 function to induce apoptosis, because GALR2-mediated apoptosis was demonstrated in the

neuroblastoma cell line SH-SY5Y (4). In general, *p53* is wild-type in human neuroblastoma cell lines (36). SH-SY5Y cells are known to have wild-type *p53* and were used as a typical model to study the mechanism of p53-induction of apoptosis (37). In the present study, GALR2 induced-anti-proliferative and apoptotic effects in UM-SCC-1, a cell line that expresses only a truncated p53 message that lacks the C-terminal sequence encoding the nuclear localization signal and lacks detectable p53 protein. Our results demonstrated that GALR2 can induce apoptosis and cell cycle arrest though a p53-independent but caspase-3 dependent pathway. These results suggest that GALR2 might act through a novel apoptotic pathway, that is not yet well-defined.

In conclusion, we have shown that galanin and GALR2 induce apoptosis and cell cycle arrest independent of p53. These results suggest that GALR2 probably controls normal cell proliferation and survival in the epithelium and that its loss may facilitate tumor development and progression. Furthermore, this pathway is a possible target in head and neck cancer.

Translational Relevance

In this study, we investigated the role of GALR2 in HNSCC, a tumor type that frequently contains mutated *p53*. HA-tagged GALR2 was stably expressed in a human oral carcinoma cell line UM-SCC-1 that contains mutant *p53* and does not express GALR1 (Misawa, et al. Clin Can Res, In Press). Galanin treatment of GALR2 transfected UM-SCC-1 cells caused distinct morphological changes and a marked decrease in cell number. Galanin and GALR2 inhibited BrdU incorporation and induced p27^{Kip1} and p57^{Kip2} up-regulation and cyclin D1 down-regulation. These effects were similar to GALR1 signaling,[1] however, GALR2 also induced caspase-3-dependent apoptosis, which was not observed with GALR1. The results demonstrate that exogenous GALR2 expression can inhibit cell proliferation and induce apoptosis in HNSCC cells with mutant *p53*, and thus GALR2 may be a feasible target for HNSCC therapy.

Acknowledgments

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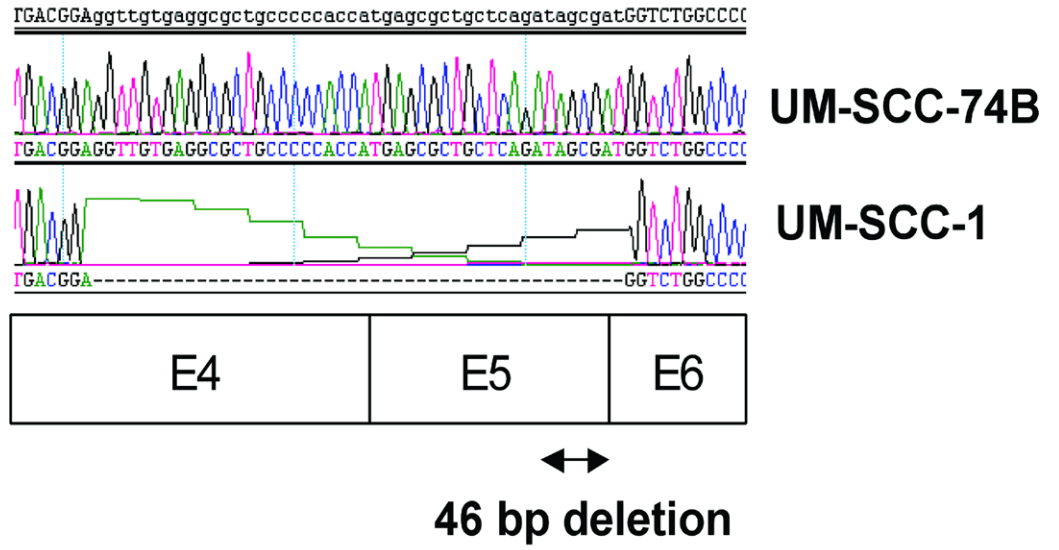
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A
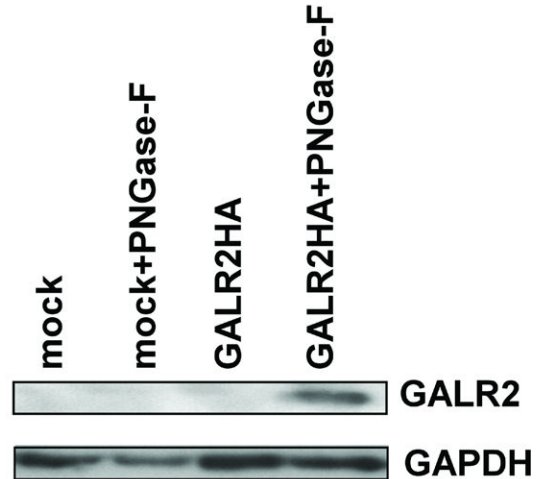
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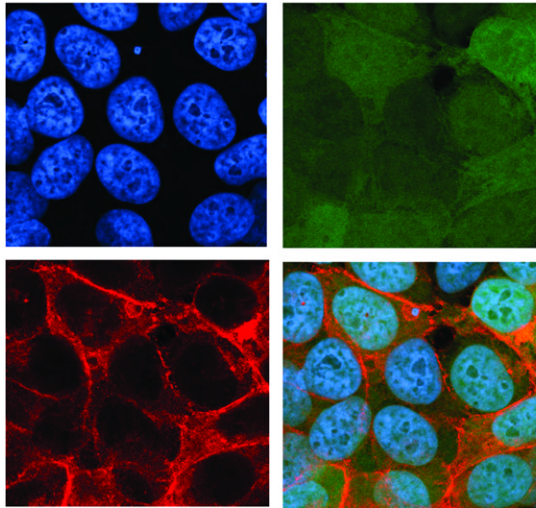
Fig. 1.
Exogenous GALR2 expression in transfected UM-SCC-1 cells. *A,* Immunoblotting shows
exogenous GALR2 expression in pCMVGALR2IresGFP-transfected cells detected using
antibody to HA-tag. Lanes labeled mock and GALR2HA contain protein lysates from mock-
transfected and GALR2HA-transfected UM-SCC-1 cells (UM-SCC-1-GALR2). Lanes
labeled mock+F and GALR2HA+F contain protein from cell lysates that were digested with
N-glycosidase F. *B,* Exogenous GALR2 localizes to the cell membrane in UM-SCC-1-
GALR2 cells. UM-SCC-1-GALR2 cells were stained with mouse monoclonal anti-HA-tag
antibody and Hoechst 33342. Photographs show cells stained with Hoechst 33342 (*upper*

left), GFP (*upper right*), HA-tag (*lower left*), and the merged image (*lower right*) (magnification × 400).
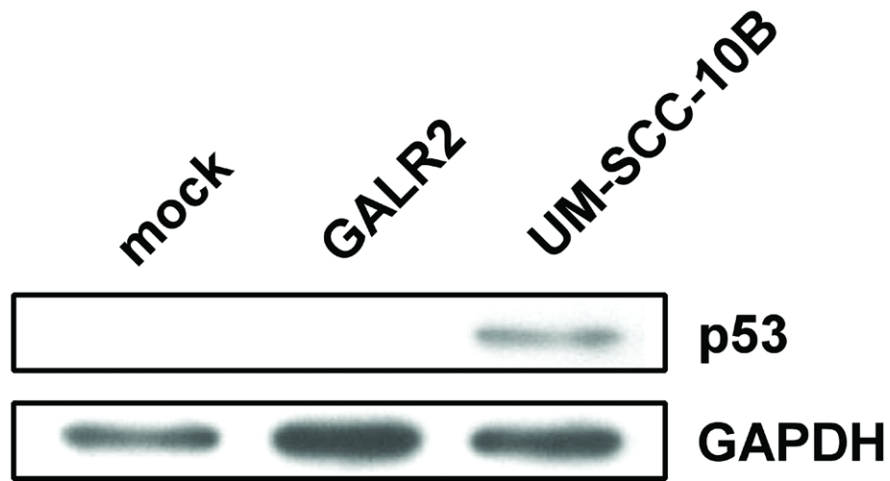
A

TGACGGAggttgtgaggcgctgcccccaccatgagcgctgctcagatagcgatGGTCTGGCCCC

UM-SCC-74B

TGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGGCCCC

UM-SCC-1

TGACGGA--GGTCTGGCCCC

E4	E5	E6

↔

46 bp deletion

B

mock GALR2 UM-SCC-10B

p53

GAPDH

Fig. 2.
p53 mutation analysis. *A,* Direct cDNA sequencing of the RT-PCR products of control wild-type *p53* expressed by UM-SCC-74B cells and mutant *p53* expressed in UM-SCC-1 cells *B,* p53 protein expressions in UM-SCC-1-mock, UM-SCC-1-GALR2, and UM-SCC-10B cells as positive control.
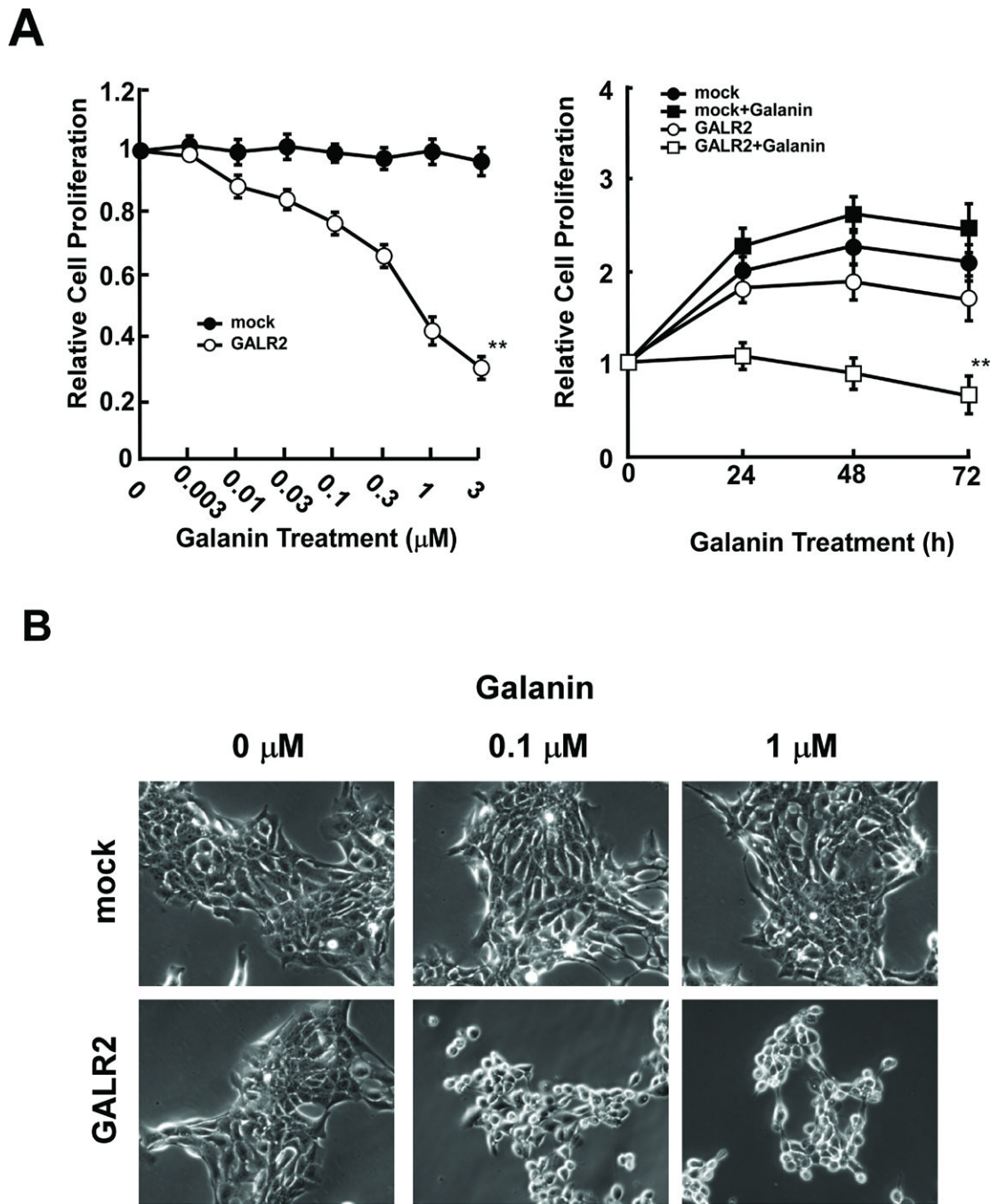
Fig. 3.
Galanin-induced growth inhibition and cytotoxicity in UMSCC-1-GALR2 cells. *A,* Relative cell proliferation as a function of galanin concentration. The cells were serum-starved for 24 h and then cultured with various concentrations of galanin for 24 h (*left*) or with 1 µM galanin for the indicated periods of time (*right*). Relative cell number is the ratio of cell number after treatment to that before treatment. Data were analyzed by two-way ANOVA (**$P<0.01$). *B,* Cell morphology changes induced by galanin in UM-SCC-1-mock and UM-SCC-1-GALR2 cells. The photographs show cells that were cultured with various concentrations of galanin for 24 h (magnification × 200).
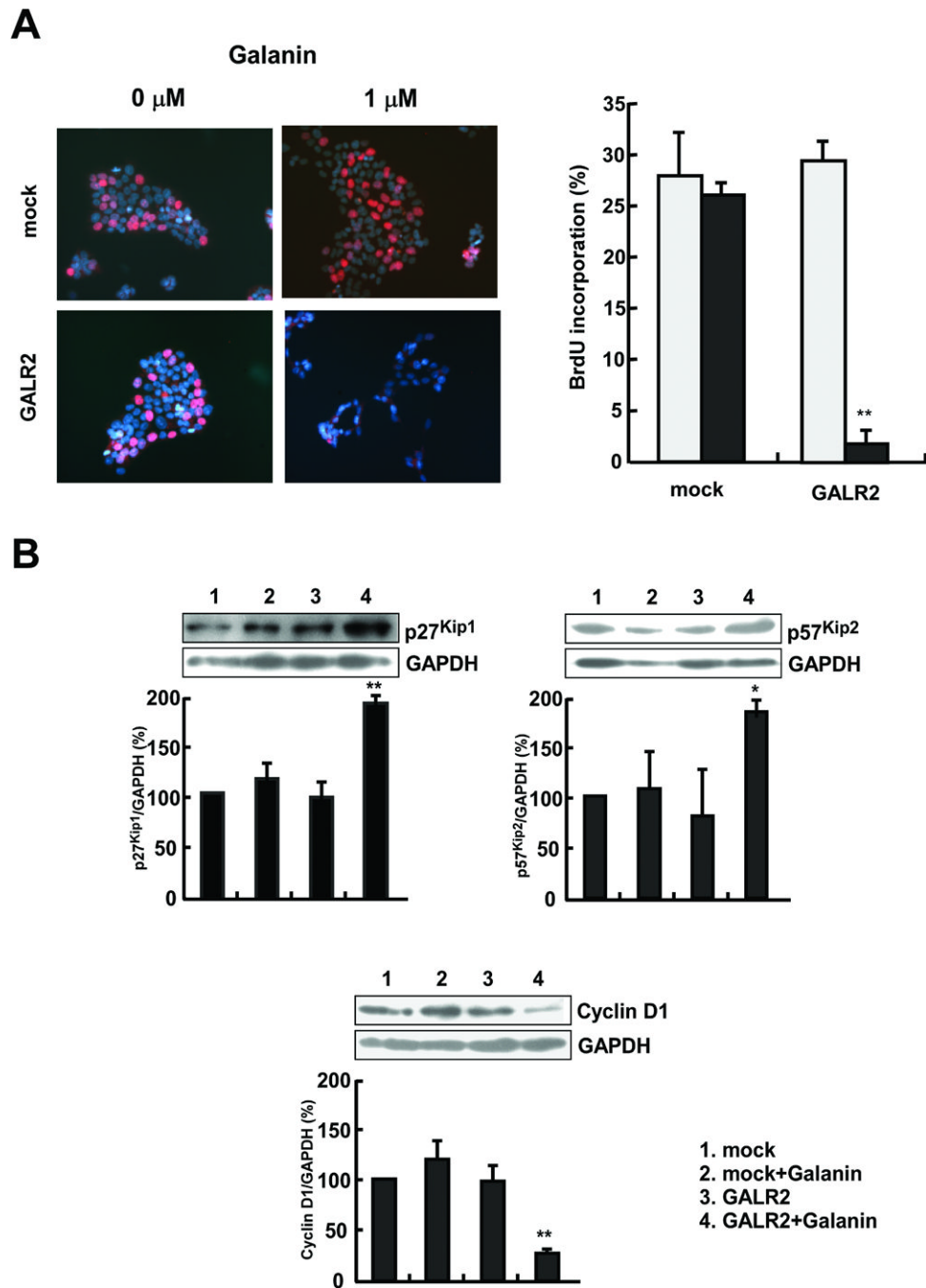
Fig. 4.

Galanin and GALR2 are associated with arrest of cell proliferation. *A,* BrdU incorporation after galanin treatment. The BrdU-positive cells (*left*) were detected as described in "*Materials and Methods*" (magnification × 200). The BrdU incorporation rate is the ratio of BrdU-positive cell number to the total cell number. Closed bars indicate galanin treatment and open bars indicate no treatment (*right*) (**$P<0.01$). *B,* Galanin and GALR2 regulate cell cycle control genes. Serum-starved cells were treated with 100 nM galanin for 24 h, and the expressions of p27^{Kip1} (*upper left*), p57^{Kip2} (*upper right*), and cyclin D1 (*lower*) were assessed by immunoblotting. The band intensities were determined by densitometry and normalized to GAPDH (**$P<0.01$).
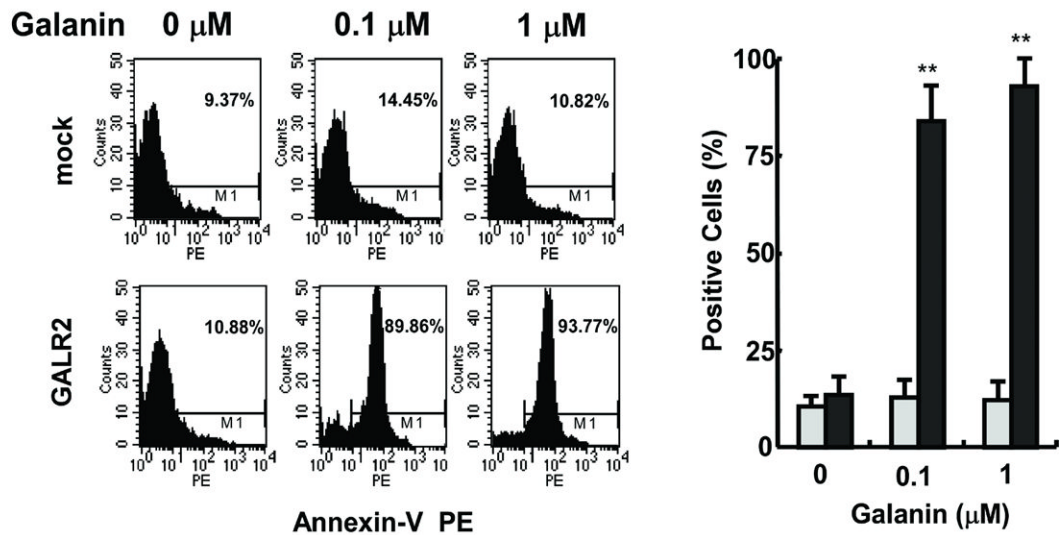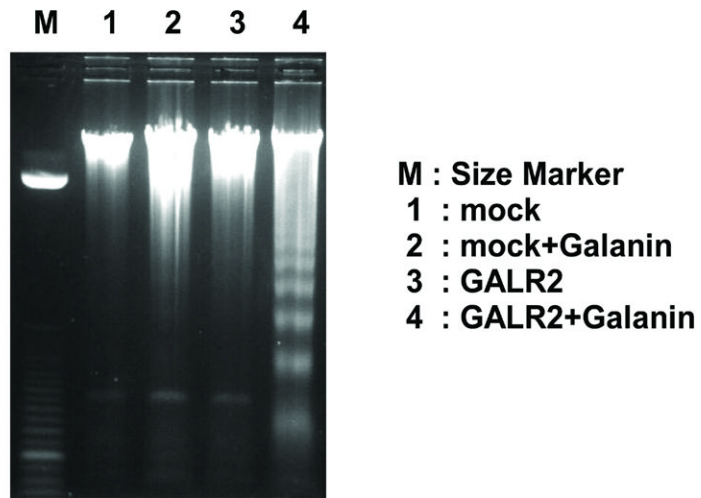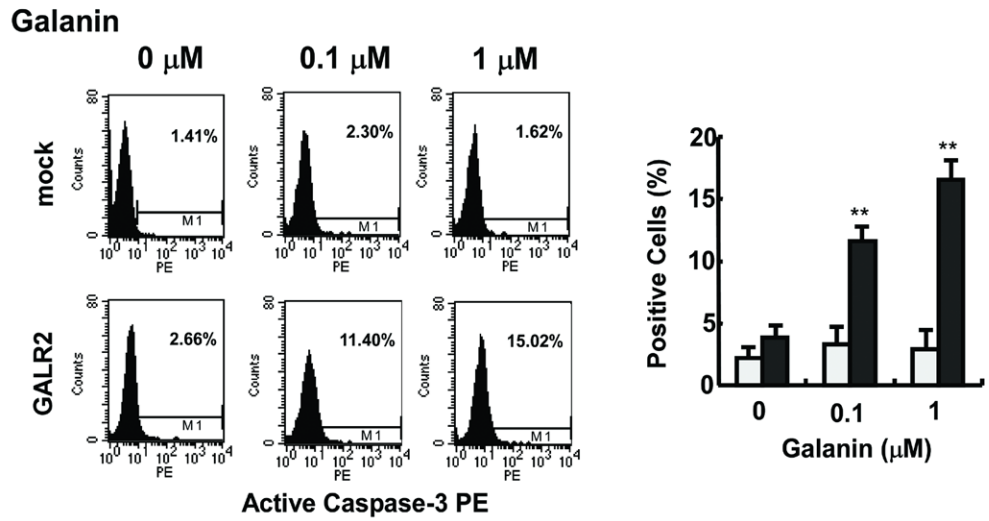
Fig. 5.
Induction of apoptosis in galanin-treated UM-SCC-1-GALR2 cells. *A,* Annexin-V staining. The annexin-V-positive cells were stained and counted by flow cytometry (*left*). Percent positive is the ratio of annexin-V-positive cells to total cell number (*right*) (**P<0.01). *B,* DNA fragmentation analysis. Genomic DNA was isolated and electrophoresed on a 2 % agarose gel. The DNA ladders were visualized under UV light with ethidium bromide. Lane M: Size marker. Lane 1: DNA isolated from UM-SCC-1-mock cells without galanin treatment. Lane 2: UM-SCC-1-mock cells with galanin treatment. Lane 3: UM-SCC-1-GALR2 cells without galanin treatment. Lane 4: UM-SCC-1-GALR2 cells with galanin treatment.
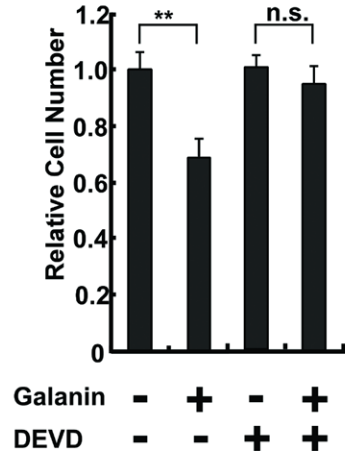
Fig. 6.
GALR2 induced apoptosis through caspase-3 activation. *A,* Active caspase-3 staining. The active-caspase-3-positive cells were stained and counted by flow cytometry (*left*). Percent positive is the ratio of active-caspase-3-positive cells to total cell number (*right*) (**$P<0.01$).
B, Effects of galanin and caspase-3 inhibition on GALR2-induced cell death. Serum-starved UM-SCC-1-GALR2 cells were pretreated with 50 µM DEVD before galanin treatment. Relative cell number is the ratio of the cell number after treatment to that before treatment (**$P<0.01$).