p53 Increases Intra-Cellular Calcium Release by Transcriptional Regulation of Calcium Channel TRPC6 in GaQ3-Treated Cancer Cells

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Abstract

p53 and calcium signaling are inter-dependent and are known to show both synergistic and antagonistic effects on each other in the cellular environment. However, no molecular mechanism or cellular pathway is known which shows direct regulation between these important cellular signaling molecules. Here we have shown that in cancer cells treated with antineoplastic drug GaQ3, p53, there is an increase in intracellular calcium levels by transcriptional regulation of a novel calcium channel gene TRPC6. p53 directly binds to a 22 bp response element in the TRPC6 gene promoter and increase its mRNA and protein expression. Over-expression of TRPC6 results in calcium-dependent apoptotic death and activation of apoptotic genes in a variety of cancer cells. This research work shows that p53 and its transcriptional activity is critical in regulation of calcium signaling and an increase in the intracellular calcium level might be one of the anti-cancer strategies to induce apoptosis in cancer cells.

Introduction

Gallium and its organic derivatives have shown high consistency and efficiency as anti-cancer drugs [1–5]. We have recently established a novel organic derivative of gallium “GaQ3” [tris(8-quinolinolato)gallium(III)] (KP46) as an effective anti-cancer drug in cancer cells with Wt-p53 or Mt-p53 protein [6]. We observed that GaQ3 induces calcium signaling in cancer cells by increasing the intracellular calcium levels. Increase in cellular Ca2+ activates p53 protein and increases p53 cellular levels. GaQ3-induced intracellular calcium release was significantly higher in cancer cells with wild-type p53 than in cancer cells with mutant p53 or with p53 gene deletion [6]. Interestingly, it was observed that the rise in intracellular Ca2+ release was p53-dependent and inhibition of p53 transcriptional activity using pifithrin-α abolished the intracellular Ca2+ release. This observation suggested that p53 might transcriptionally regulate intracellular Ca2+ release and Ca2+-signaling in GaQ3-treated cancer cells. p53 and calcium are known to function in synergy, but no direct relation has been established between p53 activation and p53-dependent regulation of calcium signaling at the cellular, biochemical or molecular level. In certain reports Ca2+-induced signals like Ca2+-activated RAF/MEK/ERK pathways mediated p53-independent apoptosis [7]. It is also predicted that p53 works in close relation with cellular calcium signaling, since intracellular calcium release plays an important role in inducing Bcl-2, ROS and mitochondrial pathway of apoptosis [8]. However, no molecular mechanism or pathway of p53-mediated regulation of intracellular calcium release is known.

In this study we have shown that the cellular calcium signaling and intracellular calcium release are under transcriptional control of p53 protein. p53 transcriptionally regulates a novel calcium channel TRPC6 by directly binding to a 22 base-pair p53-RE present 400 base pairs upstream of the +1 transcriptional start site (TSS) at the TRPC6 promoter. We observed that GaQ3 induces apoptosis via p53-dependent upregulation of TRPC6 gene in cancer cells with Wt p53. Over-expression of TRPC6 results in significant apoptosis in cancer cells. Further TRPC6 expression initiates a calcium-dependent regulation of the expression of genes involved in apoptosis.

Materials and Methods

Cell culture

MCF-7, U2OS, HCT, A-431, PC3 and H1299 cells were obtained from National Centre for Cell Science, Pune (India) and were maintained in DMEM medium. The cells were cultured as monolayers in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics, and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. All the transfections were carried out using effectene transfection reagent.

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Ca²⁺ was measured using the cell permeable Ca²⁺ sensitive fluorescent dye Fluo-3 acetoxy-methyl ester diluted in Krebs-Ringer buffer (KRB) (10 mM D-glucose, 120 mM NaCl, 4.5 mM KCl, 0.7 mM NaH₂PO₄, 1.5 mM NaH₂PO₄, and 0.5 mM MgCl₂ (pH 7.4 at 37°C)) (Sigma) for 20 min. The dishes were washed once with 5 ml KRB to remove the residual dye. The medium was removed from the tissue culture plates and replaced with 4 µM Fluo-3 acetoxy-methyl ester diluted in Krebs-Ringer buffer (KRB) (10 mM D-glucose, 120 mM NaCl, 4.5 mM KCl, 0.7 mM NaH₂PO₄, 1.5 mM NaH₂PO₄, and 0.5 mM MgCl₂ (pH 7.4 at 37°C)) (Sigma) for 20 min. The dishes were washed once with 5 ml KRB to remove the residual dye. The cells were harvested by trypsinization, washed in 5 ml of Ca²⁺ free PBS at 37°C, pelleted by centrifugation, re-suspended in 1 ml of Ca²⁺ free PBS at 37°C, and analyzed immediately for Fluo-3 fluorescence intensity by flow-cytometry.

Please refer to File S1 for full description of material and methods.

Results

GaQ3 induces TRPC6 gene expression in cancer cells with wild-type p53

We had earlier observed that GaQ3 induces high intracellular calcium release selectively in cancer cells with wild-type p53 protein [6]. Silencing of p53 gene using p53 siRNA and inhibition of p53 transcriptional activity using pifithrin-α both abolished the GaQ3-induced rise in the intracellular calcium release [6]. This data suggested that intracellular rise of calcium levels in cancer cells was regulated by p53 and was dependent on p53 transcriptional activity. However the mechanism involved in the regulation of this observed phenomenon is unknown [6]. Since GaQ3 induced significant increase in calcium uptake, using qPCR we analyzed the expression of a large number of known calcium channels in GaQ3-treated MCF-7 cells. We observed significantly high expression of TRPC6 gene in GaQ3-treated cells. Since the expression of TRPC6 calcium channel was high, we asked if TRPC6 might be involved in the increase in cellular calcium mobilization. Where indicated, exposure prior to the loading procedure with Fluo-3 acetoxy-methyl ester (10 µM fluorescent dye Fluo-3 acetoxy-methyl ester. Where indicated, TRPC6 might be involved in the increase in the cellular calcium expression of TRPC6 calcium channel was high, we asked if TRPC6 was involved in the increase in the cellular calcium levels in GaQ3-treated cancer cells. The expression of TRPC6 mRNA and protein was observed in GaQ3-treated MCF-7, H1299 and PC3 cells (Figure 1A). The qPCR analysis shows that TRPC6 mRNA was significantly increased in GaQ3-treated cancer cells [6] ref is now placed. This data suggests that p53 and TRPC6 show temporal relation in their mRNA expression profiling. The direct role of p53 in the expression of TRPC6 in GaQ3-treated MCF-7 cells is observed by silencing p53 gene in GaQ3-treated MCF-7 cells (Figure 1C). The results show that TRPC6 protein expression was significantly high upon GaQ3 treatment (lane 2); however this GaQ3-induced rise in TRPC6 was completely reversed upon p53 silencing (lane 3). This data suggests that p53 has an important role in the regulation of TRPC6 gene. We further analyzed the efficiency of TRPC6 siRNA and TRPC6 cDNA (2 µM) in MCF-7 cells.

p53 transcriptionally activates TRPC6 promoter

TRPC6 (chromosome 11q22.1, reverse strand) and p53 showed temporal relations in their expression profile in GaQ3-treated cancer cells, therefore it was of interest to define the role of p53 in regulation of TRPC6. The TRPC6 promoter region was identified as 650 bp DNA sequence upstream of +1 transcription start site, using matrix matches determined by Mat Inspector (Genomatix). TRPC6 promoter was provided the locus ID GNP_195240 by the Mat Inspector. This region lies between regions 101,374,672–101,375,321 (TSS ref point represented by Mat Inspector is 101,374,772) and is represented by ENST000000527240 and AK027769. Bioinformatics analysis of the TRPC6 promoter using Mat Inspector module of genomatix database showed putative p53 DNA binding site (matrix sim; score > 0.9) (Figure 2A) suggesting that p53 might be a potential TRPC6 regulator. To establish this, we cloned a 0.65 kb putative TRPC6 promoter carrying the p53 response element (p53RE) into a pGL3 basic vector to generate pTRPC6p-luc1. The pTRPC6p-luc1 was transfected in untreated and GaQ3-treated MCF-7, H1299 and PC3 cells. GaQ3 treatment induced 5-fold increase in TRPC6 promoter activity in MCF-7 cells in comparison to the untreated cells (Figure 2B; bar 2 and 3). The increase in TRPC6 promoter activity was p53-dependent since p53 gene silencing using p53 siRNA reversed the GaQ3-induced increase in TRPC6 promoter activity. GaQ3 treatment was unable to induce TRPC6 promoter activity in H1299 and PC3 cells (bar 5, 6, 8 & 9). TRPC6 promoter was activated in GaQ3-treated H1299 cells which were transfected with p53 cDNA (bar 7). This data showed that TRPC6 was regulated by p53 in GaQ3-treated cells. To further confirm the role of p53RE in p53-mediated regulation of TRPC6 promoter, the region between base pairs −74 to −99 (with reference to the TSS seq [101,374,772]) of the TRPC6 promoter carrying the p53RE were cloned into a pGL3 vector to generate the minimal pTRPC6p-luc2. This TRPC6 minimal promoter was induced upon GaQ3-treatment in MCF-7 cells and p53 silencing abolished this increase in the promoter activity (Figure 2C, bar 2–4). In order to establish the role of the newly identified p53RE in p53-mediated regulation of TRPC6 gene, the p53RE sequence was mutated and cloned in pGL3 vector to generate the mutant minimal mmpTRPC6p-luc2. Transfection of mmpTRPC6p-luc2 in GaQ3-treated MCF-7, PC3 and H1299 cells showed no increase in the TRPC6 promoter activity (Figure 2D). This data establishes that p53 transcriptionally regulates TRPC6 in GaQ3-treated cancer cells.

WT p53 directly binds to its response element at the TRPC6 promoter

The binding of p53 at the 22 base pair region at the TRPC6 promoter was analyzed under in-vitro conditions using EMSA (Figure 3A). The data showed binding of p53 at the TRPC6-p53-RE sequence as a clear shift and super-shift of the complex was
observed (lane 2). The binding between p53 and its response element was lost upon heat denaturing p53 protein (lane 3). The binding between p53 and its response element was very specific since mutation of the 22 base pair p53 RE sequence abolished the binding between p53 and its RE (lane 5–8). The binding between p53 and its response element at the p21 5' promoter element serves as positive control (lane 9–12). To further define the role of TRPC6-p53RE in p53-mediated TRPC6 induction under in-vivo conditions, we performed chromatin immunoprecipitation (ChIP) assays in GaQ3-treated MCF-7 cells. Consistent with luciferase results, we detected one specific PCR product derived from TRPC6-p53RE (Figure 3B, upper panel). Input serves as the control, in absence of GaQ3 treatment p53 showed no binding to the p53RE at the TRPC6 promoter. Upon treatment of MCF-7 cells with GaQ3, p53 shows binding to its RE at the TRPC6 promoter, PCR with scrambled primers serves as the negative control. On the other hand no binding between p53 and p53RE at the TRPC6 promoter is observed in H1299 and PC3 cells (Figure 3B, lower panel). These results established that TRPC6-p53RE was responsible for p53-mediated induction of TRPC6 promoter activity and that p53 transcriptionally induces TRPC6 through promoter binding in GaQ3-treated cells. Since GaQ3 induces an exponential increase in the intracellular calcium release in cancer cells with Wt p53 and p53 is now shown to transcriptionally regulate the calcium channel TRPC6 in the GaQ3-treated cells, the role of TRPC6 in the p53-mediated calcium release is observed. Flow-cytometric analysis of the intracellular calcium release shows that GaQ3 treatment induces an exponential increase in the release of intracellular calcium at the 8th hr of its incubation (Figure 3D, red line), upon silencing TRPC6 gene using TRPC6 siRNA we observed that the 8 hr exponential increase in the calcium levels was reversed and the increase in calcium levels became linear as previously observed in p53 null (H1299) and p53 mutant (PC3) cells. This data shows that the GaQ3-induced and p53-mediated rise in the intracellular calcium release is due to the p53-dependent transcriptional up-regulation of TRPC6 protein in the treated cancer cells.
Figure 2. p53 transcriptionally regulates TRPC6 promoter. A) A putative p53 binding site is observed in the TRPC6 promoter using Genomatix, MatInspector module. TRPC6-p53RE lies between −2422 to −2400 bp on the 0.6 kb TRPC6 promoter. The DNA seq of the TRPC6 promoter

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GXP-193240 (TRPC6/human TRPC6 Homo Sapiens)
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B) Full Promoter (0.65 Kb)

- PC + GaQ3
- PC
- H1299 + GaQ3 + p53 cDNA
- H1299 + GaQ3
- H1299
- MCF-7 + GaQ3 + p53 siRNA
- MCF-7 + GaQ3
- MCF-7
- Empty Vector

C) Minimal Promoter (25 bp)

- PC + GaQ3
- PC
- H1299 + GaQ3
- H1299
- MCF-7 + GaQ3 + p53 siRNA
- MCF-7 + GaQ3
- MCF-7
- Empty Vector

D) Mutant minimal promoter (25 bp)

- PC + GaQ3
- PC
- H1299 + GaQ3
- H1299
- MCF-7 + GaQ3
- MCF-7
- Empty Vector
along with the location of p53 RE (shown in red) is diagrammatically represented. B) pTRPC6p-luc1 (TRPC6 0.6 kb promoter luciferase construct) is transfected in GaQ3-treated MCF-7 cells and effect of p53 on luciferase activity is measured. GaQ3-induced p53 gene activation induces 8-fold increase in pTRPC6p-luc1 luciferase activity (bar 3). p53 gene silencing using p53 siRNA reverses this effect and TRPC6 promoter activation is abolished (bar 4). Transfection of pTRPC6p-luc1 in H1299 and PC3 cells in absence or presence of GaQ3 has no effect on the activity of TRPC6 promoter. Upon exogenous addition of Wt p53 cDNA in GaQ3-treated H1299 cells the TRPC6 promoter shows 9-fold increase in the luciferase activity (bar 7). This data show that p53 regulates TRPC6 promoter transcriptionally. C) pTRPC6p-luc2, the TRPC6-p53RE (~422 to ~400) cloned in luciferase vector is transfected in GaQ3-treated MCF-7 cells. Results show that p53 induces a 6-fold increase in the TRPC6-p53RE luciferase activity (bar 3). p53 gene silencing using p53 siRNA abolishes the increase in luciferase activity (bar 4). The increase in TRPC6 promoter activity is absent in H1299 and PC3 cells. The data show that p53 regulates TRPC6 promoter via TRPC6-p53RE. D) The mmpTRPC6p-luc2 construct with mutated sequence of TRPC6-p53RE is transfected in GaQ3-treated MCF-7 cells. No increase in the luciferase activity is observed, showing the specificity of TRPC6-p53RE. For figure 2b–2d, *(red) represents significant difference in the results all values p<0.05, n = 7, Anova, error bars represent S.D).

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Figure 3. p53 directly binds to the TRPC6 promoter. A) EMSA is conducted to study the binding between p53 and its response element at the TRPC6 promoter under in-vitro conditions. A shift and a super-shift of the p53, p53AB and the p53-RE are observed in lane 2. The binding between p53 and its RE is lost after a mutant p53 RE sequence is used for EMSA, showing high specificity of this interaction (lane 6). Binding between p53 and its known p215RE is used as a control (lane 9–12). (B) Chromatin immunoprecipitation is conducted in GaQ3-treated MCF-7 cells to confirm in-vivo binding of p53 to TRPC6 promoter. p53 shows positive TRPC6-p53RE binding exclusively in GaQ3-treated cells. Input serves as positive control and scrambled primers for PCR were used as negative control (n = 5). The binding between TRPC6 and p53 RE is not observed in H1299 and PC3 cells in presence of GaQ3 (n = 5). D) Time course analysis of intracellular calcium release is observed in the GaQ3-treated MCF-7 cells (red line) and GaQ3-treated MCF-7 cells where TRPC6 gene is silenced using TRPC6 siRNA (black line). Results show that GaQ3 induces the sharp increase in the intracellular calcium release in p53 (+/−) MCF-7 cells by 6th hr of its incubation. The silencing of the TRPC6 abolishes this 6th hr increase in the intracellular calcium release *(red) represents significant difference in the results between red and black line at the 8th hr time point, p<0.029, n = 5, Anova, error bars represent S.D).

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**Discussion**

In this research work we have shown that the intracellular calcium release is under the transcription control of the p53 tumor suppressor, via p53-dependent transcriptional regulation of TRPC6 gene. Genome wide analysis of p53 binding sites in the promoter regions of calcium channels showed presence of p53 RE in TRPC6 gene. We have shown that GaQ3 induces expression of TRPC6 only in cancer cells with wild-type p53 and p53 gene silencing reverses the GaQ3-induced increase in TRPC6 expression. This data also establishes that TRPC6 is a direct transcriptional target of p53 and p53 binds to its response element in the TRPC6 promoter. We have found the p53 binding site in the 0.6 Kb TRPC6 gene promoter which lies 400 base pairs upstream of the +1 transcription start site. The sudden rise in intracellular calcium release and calcium-mediated cellular apoptosis in GaQ3-treated cancer cells is due to p53-dependent transcriptional activation of TRPC6 gene. In this research work we have shown a direct connection between the p53 transcriptional ability and calcium signalling.

Understanding the relation between calcium signalling and p53 is important in cancer perspective since both these factors regulate cell-growth, differentiation, ageing, proliferation at the physiological, cellular and molecular level [12,13]. In past a Ca^{2+}-permeable TRPC channel has been shown to participate in a diverse array of cellular functions by regulating intracellular Ca^{2+} signaling [14]. TRPC6-mediated Ca^{2+} signaling activates cell-proliferation gene like calmodulin-dependent protein kinase and mitogen-activated protein kinase [15]. The role of TRPC6 in cancer growth and development is not clear; however multiple mechanisms are involved in TRPC6 channel activation and regulation in cancer cells. The physiological and cellular factors involved in cancer disease progression are also known to regulate the cellular TRPC6 expression [16-20]. The cellular factors like membrane receptor activation via TFN-α and cellular Ca^{2+} store depletion involved in cancer progression have been known to induce TRPC6 expression [16-20]. Recently role of an important signaling molecule ROS in TRPC6 activation is observed [18]. Since ROS is involved in both cancer progression and its regulation, thus it is important to identify the role of TRPC6 protein in the cancer disease development or regulation. Recent studies have shown that several pro-apoptotic factors, including members of the Bcl-2 family proteins and reactive oxygen species (ROS) regulate the Ca^{2+} sensitivity of both the Ca^{2+} release channels in the ER and mitochondria [21]. Further no data related to the relation between the tumor suppressor p53 and regulation of calcium signalling transcriptional regulation of TRPC6 gene in cancer cells is available. It is important to identify the relation between p53 and its control of the intracellular calcium release and the role played by TRPC6 gene in GaQ3-treated cancer cells. The p53 and TRPC6 induced since changes in the cytosolic concentrations of Ca^{2+} can induce signaling pathways that regulate a broad range of cellular events, including those important in tumorigenesis [21].

Further a new relation between the intracellular Ca^{2+} release and p53 was observed where Ca^{2+} release stabilized the binding of p53 to its transcriptional co-activator p300 and also stabilized the binding of p53-p300 transcriptional complex to the p53-DNA binding site on p53-minimal promoter [22]. In this research work we have elucidated the presence of a cellular cross-talk between p53 and calcium signalling, where calcium signalling activates p53 transcriptionally [6] and the active p53 increase the intracellular calcium release by transcriptionally regulating the promoter of TRPC6 gene. Thus calcium-dependent apoptosis might be
mediated by p53 and calcium signalling might play a crucial role in p53-mediated apoptosis. Recently TRPC6 gene has shown crucial role in pathological cardiac hypertrophy and remodelling in response to stress [23,24]. Deletion of TRPC6 prevents stress-induced exaggerated cardiac remodelling and overexpression of TRPC6 develop spontaneous cardiac hypertrophy in the mice model, suggesting towards a possible link between TRPC6 expression and cell-death and division, since cardiac remodelling involves both these processes. TRPC6 expression is also known to induce podocyte cytoskeletal remodelling [25], human keratinocyte differentiation [26], and hippocampal neuron differentiation [27]. An important role of TRPC6 has also been discovered in wound healing where a genome-wide screen identified TRPC6 important for myofibroblast transformation and TRPC6 gene-deleted mice showed impaired dermal and cardiac wound healing after injury. The previous reports suggest towards a link between TRPC6 expression and regulation of cell-division and cell-death related processes, in this report we are suggesting a strong role of TRPC6 as a p53-regulated pro-apoptotic protein in the cancer model. In conclusion, here we have elucidated a novel role of TRPC6 calcium channel to function as a p53 downstream effector protein which induces apoptosis by regulating cellular calcium levels in cancer cells. This pathway appears to be a strategy adopted by p53 protein to utilize calcium signalling as an effective pro-apoptotic anti-cancer means by transcriptionally regulating TRPC6 calcium channel, thus using TRPC6 as a possible mechanism to function as a tumor suppressor.

Supporting Information

File S1 Supplementary material and methods. (DOC)

Author Contributions

Conceived and designed the experiments: RG. Performed the experiments: EM, RG, BK. Analyzed the data: RG, UP. Contributed reagents/materials/analysis tools: RG, EM, RG, BK. Wrote the paper: RG, EM.

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