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Full Length Research Paper

Transient receptor potential channels interference has broader implications in safeguarding against genomic instability

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Prostate cancer is a multistep molecular disorder that arises because of a miscellany of proteins. Calcium channels, hotspots in genome and susceptibility to DNA damage, an illegitimate repair of the genome, all these factors work synchronously at various levels to worsen the clinical management of the disease. Fusion transcripts are recently acclaimed candidates for exacerbation of the disease. Erlotinib has been used for the treatment of various molecular pathologies but it has been ineffective in producing desired level of effects. It is therefore used in conjunction with other therapeutic intercessions. Despite the characterization of a number of fusion genes, therapeutic interventions to address the underlying mechanisms are still insufficient. In this particular study, we have applied a combinatorial approach to silence some calcium channels which have been documented earlier to be contributory in disease progression. The siRNA against TRPM2, TRPV6 and TRPC6 were used to evaluate the striking synergy. Simultaneously, the effects were evaluated for ATM activation and a downregulation of the chimeric gene. The expression of channels was blocked effectively as analyzed by RT PCR and Blotting assays. TRPM2 ablation instigated a DNA damage response which was observed by the blotting assay for phosphorylated ATM. Dampening the expression of TRPV6 and TRPC6 by siRNA concomitantly inhibited the genomic rearrangements. We have evaluated the synergistic impact of Erlotinib along with TRP interference on genomic instability. The observations are indicative of the fact that, silencing of calcium channels offer exciting avenues for getting a step closer to personalized medicine.

Key words: ATM, TMPRSS2-ERG, TRPM2, TRPV6, TRPC6, genomic instability, prostate cancer.

INTRODUCTION

Prostate cancer is a multifactorial disease. A wide range of proteins have been characterized and incriminated to be involved in the disease progression. A confluence of observations indicates that, super-families of transient receptor potential (TRPs) channel are instrumental in prostate carcinogenesis. TRPC6 (Yue et al., 2009) is associated with the disease exacerbation and knocking down of TRPC6 (Wang et al., 2010) and TRPV6 (Zhao et al., 2010) was growth inhibitory in prostate cancer cell lines.

The genomic instability arising because of genomic rearrangements results in the subversion of core biological system. TMPRSS2-ERG is a fusion transcript, that is a well documented example of genomic instability outcomes in prostate epithelium (Tomlins et al., 2005; 2006).

Zeng et al. (2010) has recently documented that, TRPM2 is populated in the nucleus in cancerous cells. The exact mechanism that underlies this trafficking of the protein into the nucleus is still unclear yet some

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hypothesis can be made. It might be engaged in the initiation of genomic rearrangements. The ataxia telangiectasia mutated (ATM) gene is known to have a central role in sensing general DNA damage and mediating cell-cycle checkpoint. Dynamic multifaceted functions of ATM act as gatekeepers of genomic stability and preventing tumorigenesis, ATM is impaired in the prostate cancer aggressiveness that results in an unfaithful repair of the DNA (Luedeke et al., 2009; Mani et al., 2009). Epidermal growth factor receptor (EGFR) is indispensable for mediation of both proliferative and survival signals to cells. Moreover, in recent years it became noticeable that, in addition to ligand binding-induced activation of the EGFR, ligand-independent receptor activating processes also exist (Boerner et al., 2003). The most harmful DNA damages after treatment with ionizing radiation are double-strand strands breaks, which are preferentially repaired by non homologous end-joining. They concluded that, majority of genomic rearrangements seen in wild-type cells after an acute 80-Gy X-ray exposure via DNA-PK-dependent end-joining pathway (Rothkamm et al., 2001).

Two exciting documentations prompted us to focus our concentration on a probable connection between EGFR and DNA repair. First, Bandyopadhyay et al. (1998) showed a physical co-existence of EGFR and DNA-PK, after incubation with the EGFR-blocking antibody C225. Second, Lin et al. (2001) demonstrated nuclear localization of EGFR which would interdigitate/tether EGFR to sites of DNA damage. Thus, we analyzed the location of EGFR and its molecular interactions after radiation exposure. Erlotinib, gefitinib are the drugs which are designed for inhibition of RTK's. Heterogeneity of the tumor cell population develops chemoresistance and radioresistance. In addition, uncontrolled cellular growth and constitutive activation of survival pathways severely compromise efficacy of erlotinib monotherapy (Qi et al., 2009). The calcium homeostasis triggers a multiplicity of cellular dynamics together with gene transcription, proliferation and apoptosis. Perturbance of Ca^{2+} signaling may stimulate uncontrolled cell proliferation and suppression of apoptosis providing the basis for cancer development. Interestingly, there is an upregulation of specific calcium channels or pumps associated with certain types of cancer. Consistent with the same documentations, upregulation of TRPV6 channel in prostate cancer cells may symbolize an instrument for maintaining a higher proliferation rate, increasing cell survival and apoptosis resistance. Androgen receptor is engaged in TRPV6 regulation in a ligand-independent way. They found that androgen receptor abrogation by siRNA decreased TRPV6 mRNA and protein levels. Discordantly, ligands DHT, an androgen receptor–selective agonist and antagonist, had no noteworthy consequence on TRPV6 mRNA expression (Lehen'kyi et al., 2007; Schwarz et al., 2006). Taking into consideration, TRPM (melastatin-related trp cation channel) and TRPV sub-families of TRP channels, and review of literature indicates that, TRPM4 was found contributory in cancer. Armisén et al. (2011) stated that, abrogation of TRPM4, resulted in an attenuation of cellular proliferation however reconstituting the cells for TRPM4 resulted in enhanced cellular proliferation. On the contrary, TRPM8, a receptor for cold stimuli and menthol portrays opposite trends. It was considered as an oncogene by Bidaux et al. (2007).

Conflictingly, Yang et al. (2009) appraised it as a tumor suppressor if over-expressed. They documented that PC-3 cell line underwent a decrease in cellular proliferation if this gene was over-expressed. This was further strengthened by a recent study in which Valero et al. (2010) induced an enforced over-expression of TRPM8, that resulted in shuttling of the protein from endoplasmic reticulum to the plasma membrane and retarding cellular proliferation. TRPV6 is strongly expressed in prostate cancer, but not in benign prostate hyperplasia. It is intriguing that, TRPV6 renders higher invasion and metastasizing efficiency to prostate tumors and it is well documented that, drastic calcium influx through TRPC channels, gears up proliferation rate of LNCaP cells. In the present investigation, we adapted a combinatorial targeting of TRPC/TRPM and TRPV along with administration of Erlotinib, to explore probabilistic gene fusions in prostate cancer cell line. Moreover the influence of knock down of the channels and ATM responsiveness was analyzed.

Cell lines and treatments

The prostate cancer cell line, LNCaP, was obtained from the American Type Culture Collection. Human PrECs was maintained in PrEGM media. Transfection of LNCaP and PrECs cells with siRNA was performed with Lipofectamine2000 (Invitrogen). For induction of chromosomal translocation, LNCaP cells or PrECs were grown in charcoal-stripped serum containing media for 48 h followed by mock, DHT (10^{-7} M), g-irradiation (50 Gy) treatment or both. Co-treatment was done because of non-detectable expression of fusion transcripts in single treatments. After treatment, the cells were reincubated for 24 h before being harvested for appropriate assays. Cell viability in cultures before and after irradiation was assessed by trypan blue exclusion. Erlotinib (sc-202154) purchased from santacruz biotech was dissolved in autoclaved water and DMSO (<0.1%) respectively, as stock solutions for *in vitro* studies. LNCaP cell line was treated with varying doses of the drug, however worked well at 5 µM.

Small interfering RNA assay

To examine the effects of ATM on genomic rearrangements, cells were transfected with TRPV6, TRPM2 and TRPC6 specific or scrambled siRNAs. Briefly, cells were seeded in 24 or 6-well plates till they attained a confluency of 60 to 80%. To achieve the finest evaluations transient transfection of cells in 24-well plates was done with 10, 20, 40 µM/well of specific siRNAs (TRPV6, TRPM2 and TRPC6) or scrambled siRNA, using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After incubation at 37°C in 5% CO_2, cells were then harvested for gene expression and protein analysis after 48 h. siRNA for TRPV6,

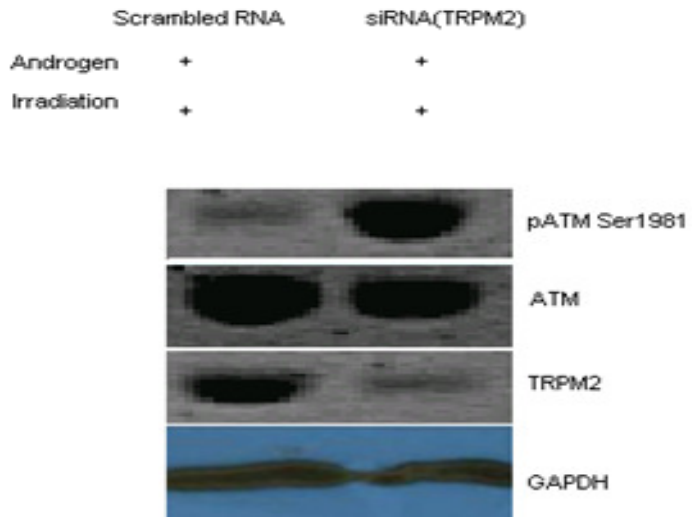


Figure 1. Treatment of androgen treated and irradiated LNCaP cells with scrambled RNA and siRNA (TRPM2).

TRPM2 and TRPC6 were purchased form Santacruz Biotechnology.

Western blot analysis

Cell collection was done by adding trypsin and subsequent centrifugation for 5 min at 13,000 rpm. Pellets obtained after discarding supernatant were treated with 1XPBS. 100 µl of lysis buffer was added incubated in ice for 20 min and subjected to centrifugation for 30 min at 13,000 rpm. Supernatant obtained was shifted carefully to new tube and stored at -20°C. Protein samples were mixed with 5X reducing dye in microtubes and placed in a boiling water bath for 2 min, then placed on ice and again centrifuged. Samples were then carefully loaded in the wells and run at 80 V until the samples passed the stacking gel, with a slight increase in voltage up to 100 V, samples seperated completely. After SDS-PAGE and electrophoretic transfer, the membrane was blocked in 5% skim milk in 1XTBS with 0.05% Tween-20 for 1 h at RT or at 4°C overnight. The antibody for ATM, pATM and TRPM2 were purchased from Santa Cruz biotechnology.

RNA extraction and RT PCR based amplifications

RNA extraction was done from RNeasy Mini kit (Qiagen) from LNCaP cells. There was a collection of cultured cells by preliminary centrifugation for 5 min at 5000 g, 5 min at 5000 g washing in phosphate-buffered saline (PBS). RNAse-free water (Sigma–Aldrich) was used to dissolve all RNA samples. The purity and concentration were evaluated by A260 nm measurement. 2 µg of each sample in a total volume of 50 µl was subjected to reverse transcription using the cDNA Archive Kit. Incubation was given to the mixtures for 10 min at 25°C and for 2 h at 37°C. cDNA products of 2 µl were used as template for polymerase chain reaction (PCR) quantification in all samples. The primers for TMPRSS2-ERG and conditions for PCR amplification were reported earlier by Hessels et al. (2007). The effect of genes and their siRNA were detected by using specific primers of TRPV6 and TRPC6 genes on ABI 7500 real time PCR, using SYBR Green mix (Fermentas) according to manufacture's instructions. Temporarily, 2X SYBR Green RT-PCR Master Mix, template RNA, primers, and RNase-free water were thawed. After mixing the individual solutions, all regents were placed on ice. In the end, template cDNA of 0.5 µl were added to the individual PCR vessels. The relative gene expression analysis was done by using SDS 3.1 software, provided by ABI. Each real time PCR assay was performed in triplicate. Level of significance and standard error was determined by SPSS software.

RESULTS

Western blot analysis showed that, TRPM2 protein expression was decreased in siRNA TRPM2 treated cells. GAPDH is used as an internal control. There is a remarkable activation of ATM in siRNA treated cells, as evaluated by western blot analysis using ATM and ser 1981 ATM antibodies. RT PCR analysis showed that siRNA treatment efficiently inhibited the expression of TRPV6 and TRPC6. However, scrambled RNA did not inhibit the expression. Down regulation in TMPRSS2-ERG expression is also evident from RT PCR based analysis. GAPDH was used as an internal control.

Explanation

Figure 1 shows the protein expressions of TRPM2 and its influence on ATM activation. The Figure 1 displays that there is a blockade of TRPM2 after pretreatment with siRNA. Sequence specific inhibition was obvious in the TRPM2 RNA interference mediated LNCaP cell line. No results of blockade can be seen in the cells treated with scrambled RNA. This is indicative of the fact that, TRPM2 is actively involved in desensitizing the ATM to DNA damage. ATM once rendered inactive can not contribute in genome repair rather another kinase enzyme (DNA-PK) repairs genome in an unfaithful manner.

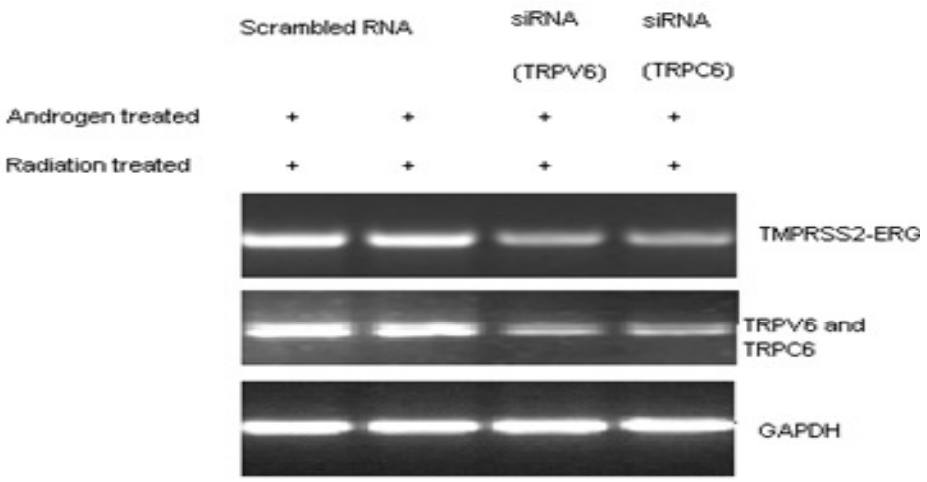


Figure 2. Treatment of androgen and radiation treated LNCaP cells with siRNA (TRPV6) and TRPC6 respectively.

Figure 1 explains that, TRPM2 abolishes the activation of ATM. As the autophosphorylation at serine 1981 is absent in the LNCaP cell line treated with scrambled RNA. This is underlining the aspect that TRPM2 abrogation, stimulates the activation of ATM and there is no illegitimate genome repair.

Another fact that cannot be ruled out is that, TRPM2 activities severely compromise the activation of ATM and incapable of being converted into its autophosphorylated form. Consistent with the same approach in Figure 2, we ablated TRPV6 and TRPC6 and the effect on the genomic instability was studied. In the cells treated with scrambled RNA, there was no down regulation of the fusion transcripts. This genomic rearrangement was induced by pretreatment of cell line with androgen and radiation. Both the treatments induced DNA breaks and subsequent generation of the chimeric transcripts. It seems obvious that DNA repair protein activities are hampered as a result of which, there is no surveillance in terms of genome repair. GAPDH is used as an internal control. Cells treated with TRPV6 specific RNA interference gave remarkable results in terms of attenuation of the genomic instability and genomic rearrangements. This was observed by the fact that, expression of the TMPRSS2-ERG was reduced because of the treatment of the cells with TRPV6 specific siRNA. It seems to be down regulated presumably because of the silencing of the TRPV6 because cells competent for TRPV6, do not show any decrease in the expression profile of the fusion transcripts.

On a similar note, TRPC6 treated cells were also positive for an inhibition of the fusion transcripts. Cells were priorly treated with androgen and radiation and scrambled RNA. The non specific RNA was inefficient in retarding the expression pattern of the chimeric transcripts. However, treatment of the cells with the TRPC6 specific RNA resulted in expression inhibition of the fusion genes. This means that genomic rearrangements are actively mediated by TRPC6 and TRPV6 and both the genes are negatively regulating the activation of ATM. Figure 3 shows remarkable shut down of fusion transcript (TMPRSS2-ERG) expression after pretreatment with siRNA (TRPV6) in lane 2 and with siRNA (TRPC6) in lane 4. Lane 5 depicts expression profile of TMPRSS2-ERG after pretreatment with androgen treatment and irradiation. Moreover, lane 3 shows a co-treatment with siRNA (TRPV6+ TRPC6). Lanes 7 and 8 are treated with scrambled RNA. Statistical analysis shows relative effect of RNA interference. Asterisks indicate significant change. Figures 4a, b and c, shows LNCaP cell line treated with Erlotinib (E) alone and in combination either with siRNA TRPV6(V) or siRNA TRPC6(C) and activation of ATM was evaluated after various time frames.

In Figure 3 we have used an individual and combinatorial blockade. We come to the conclusion that, cotreatment of the cell line with siRNA for TRPV6 and TRPV6 worked with striking synergy. The results displayed in Figure 3 are the RT PCR based amplicons run on agarose gel using suitable ladder. There is a substantial inhibition of the chimeric transcripts after treatment with TRPV6 and TRPC6 individually, yet more pronounced inhibitory effect was observed after synergistic RNA interference. This draws attention towards the application and efficacy of the combinatorial drug design. Henceforth, expression profile of multiple fusion transcripts after treatment with inhibitors of TRPV6 and TRPC6, was evaluated. It is quite impressive that RNA interference of TRP´s has wide inhibitory effects. As multiple transcripts generated because of the aberrant genomic rearrangement patterns are inhibited in this particular experiment. It is interesting to note that inhibition/interference of TRPV6 and TRPC6, retards induction of wide range of rearranged genome patterns. It

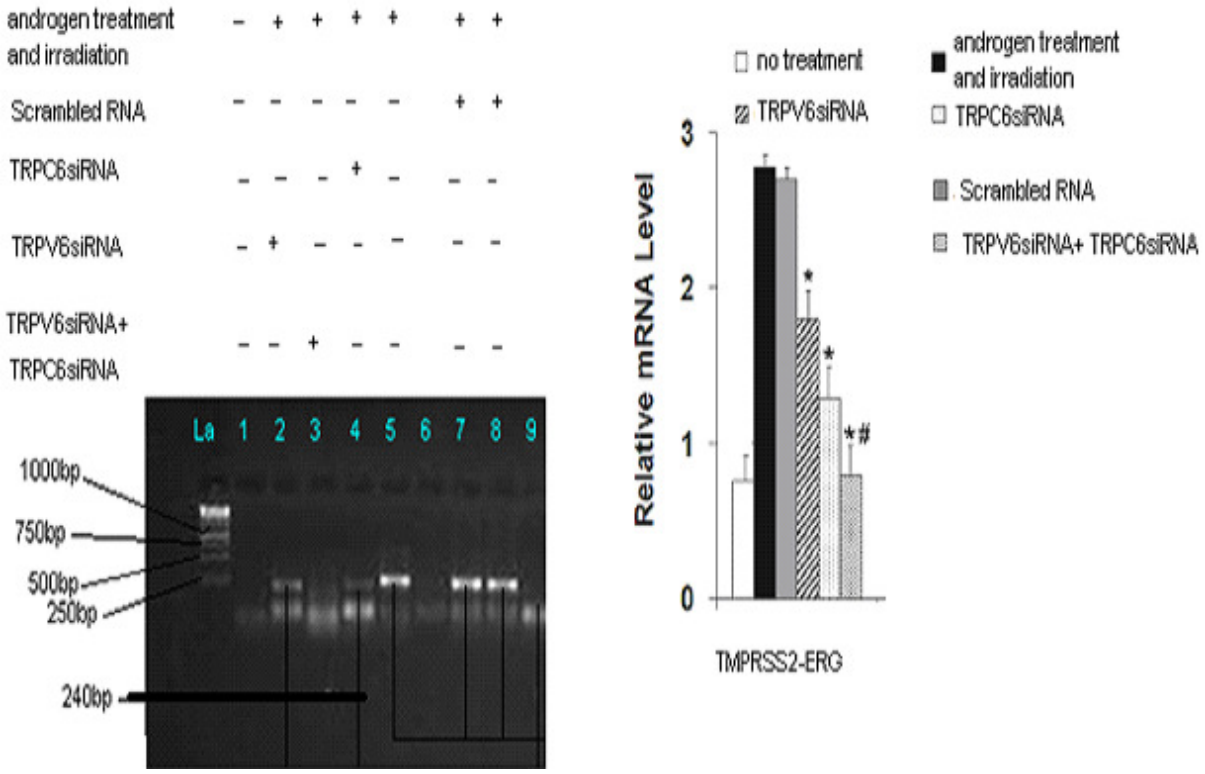


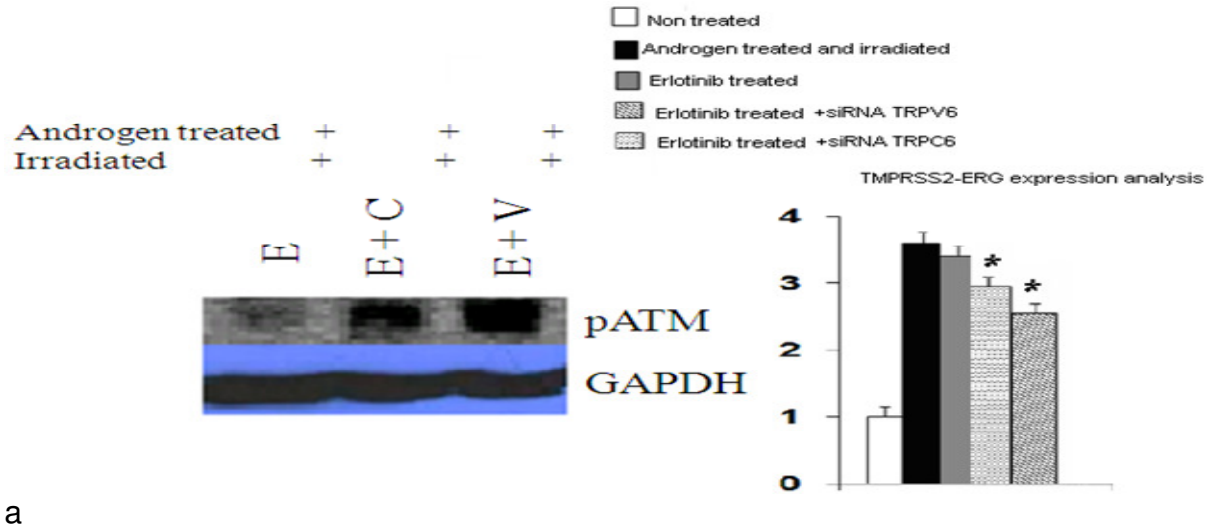
Figure 3. shows expression profile of RT PCR based amplicons of the fusion transcripts after treatment with siRNA's. Statistical analysis displays significant reductions in the fusion transcripts expression. Asterisks indicate.

is also noted in Figure 4 that, Erlotinib treatment acts as a mild stimulus for the activation of ATM. Nonetheless, in combination with siRNA for TRPC or TRPV, the influence is potentiated outstandingly. It is also attention-grabbing that, time span is influential in the activation of ATM, ATM was activated robustly after 72 h. Maximum activation of ATM was achieved after pretreatment with Erlotinib and siRNA TRPV6. It was also observed that, ATM activity in Erlotinib treated cells was similar at 24 and 48 h but there was a sudden increase in the activity at 72 h.

DISCUSSION

Prostate cancer is a multidimensional molecular disease that arises because of deviant activities of broad spectrum of mediators. TRP channels have attracted the attention of researchers. Recently, Amantini et al. (2009) documented a tight association of TRPV with ATM. They registered that activation of TRPV1 with capsaicin, resulted in the activation of ATM along with induction of apoptosis. A closer look at the credentials of ATM indicates that, it has bipartite activities. Either it is involved in the induction of apoptosis or it is engaged in DNA repair in a faithful manner. Despite the growing evidence that these channels and genomic rearrangements have wide contributions in disease aggressiveness, no study to date addresses the tight association of the abrogation of channels and genomic rearrangements. Presuming the same assumption we designed our experiments that encompassed all these activities.

siRNA-TRPM2 was used to knock down TRPM2 expression by RT PCR. Figure indicates successful blockade of TRPM2 protein by siRNA-TRPM2. TRPM2 protein, as observed in Western blot analysis, was significantly reduced in LNCaP cells treated with siRNA-TRPM2 in comparison with treatments of scrambled siRNA. LNCaP cells treated with scrambled siRNA presented low levels of ATM phosphorylation, which according to Shiotani et al. (2006) is dependent on ADP-ribosylation of DNA. On the contrary, substantial fraction of ATM activation was observed in TRPM2 compromised cells suggesting that, an increase in ADP-ribosylation occurred. We abolished TRPV6 in order to evaluate its effect on the genomic rearrangements. It was observed that, siRNA (TRPV6) stimulated the activation of ATM and suppression of TMPRSS2-ERG. In accordance with the same concept, we treated cells with siRNA (TRPC6). The results were very much concordant in interpretations. There was a substantial downregulation of the fusion transcript expression which underscores the fact that, faithful genomic repair instigate in TRPC6 knocked down cells. It is interesting to note that, ATM activation is



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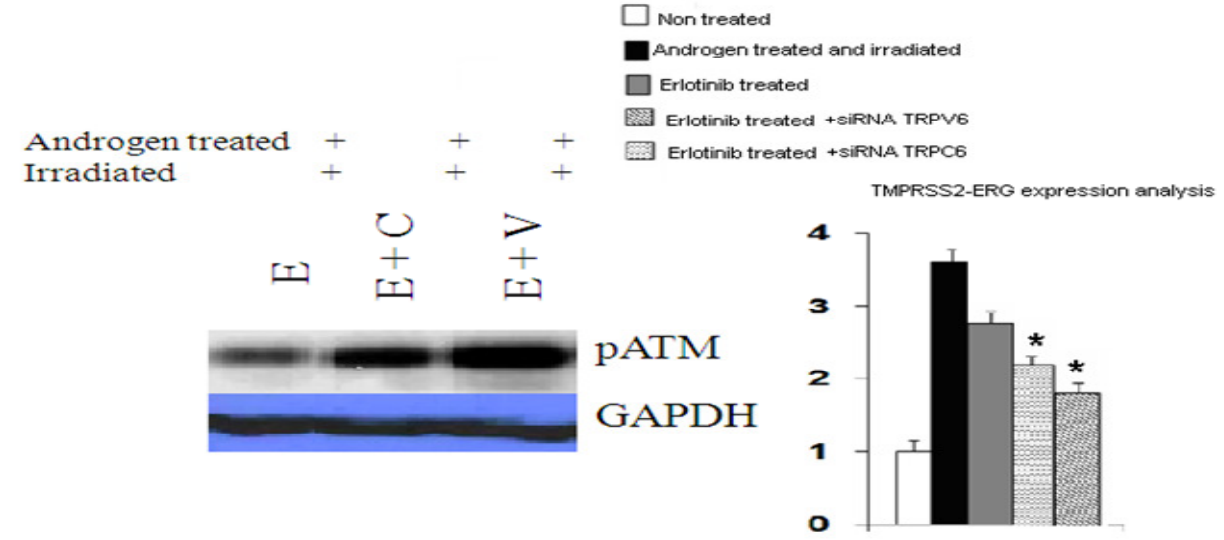

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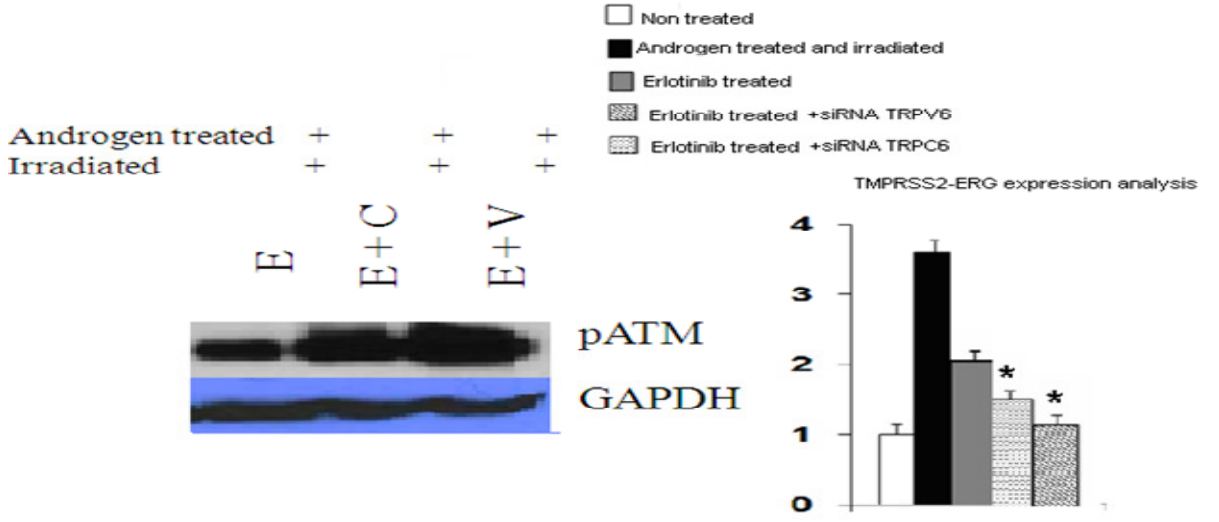

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Figure 4. Treatment of LNCaP cell line with Erlotinib alone and in combination with siRNA. (a) After 24 h (b) after 48 h (c) after 72 h. Erlotinib is more effective as displayed by statistical analysis. Asterisks indicate significant change.

impaired in the TRPM2 competent LNCaP cell line. The presence of TRPM2 does not allow the autophosphorylation of ATM at serine 1981. However, treatment of cells with siRNA of TRPM2, recapitulates the activity of ATM and there is a successive retardation of genomic instability. TRPV6 and TRPC6 exclusively and synergistically hold tremendous potential to inhibit the illegitimate genome repair.

Another important finding is that, the silencing of the channels inhibits the expression of a wide range of fusion transcripts. There are tremendous efforts which are being made to induce apoptosis in castration resistant prostate cancer cells. Keeping in view the critical roles of ATM, we have to design target specific interventions. Another thing that can be ruled out is the differential activities of ATM. This study highlights the involvement of ATM in attenuation of genomic rearrangements. Blockade of the channels resulted in the activation of ATM and the DNA damage repair, instead of apoptosis. Study unfolds a different pathway of addressing the genomic insult by abrogation of TRP channels. Yet, there are some outstanding questions which are to be answered. The intermediate players in TRP channels interference mediated activation of ATM have not been identified. We have gone through the DNA repair mode after targeting of calcium channels; however, the predisposition of apoptosis is still controversial.

It is interesting to note that TRPV forms a complex with EGFR, that results in an exacerbated signaling (Cheng et al., 2010). Moreover, TRPV activation results in activation of EGFR, that stimulates downstream signaling (Yang et al., 2010). Erlotinib (OSI-774 or Tarceva), an EGFR tyrosine kinase inhibitor, has strong antineoplastic and chemopreventive efficacies in a variety of cancer types. Erlotinib is involved in the activation of p53. It modulates expression of cell cycle regulatory proteins p21 and p27 and apoptosis-regulatory protein Bim, in a p53-dependent manner. EGCG enhances the proapoptotic potential of erlotinib by inhibiting the expression of p21 and p27 (Amin et al., 2009). This is indicative of the fact that, monotherapeutic approach is inefficient in yielding desired results. Here, we have explored that Erlotinib activates an upstream kinase of DNA damage pathway, ATM because of its antineoplastic activities. Erlotinib is an EGFR inhibitor, that hampers activation of the native receptor. Moreover, Tanaka et al. (2008) did not observe any suppression of ATM after treatment of on-small cell lung cancer cells by gefitinib (EGFR inhibitor). Consistent with the interpretations in this regard are the reports that C225, a humanized antibody (Dittmann et al., 2005 a, b) that binds to the EGFR and blocks relevant downstream transduction cascade, that causes a redistribution of DNA-PK from the nucleus to the cytosol and blocks the transport of EGFR to the nucleus (Friedmann et al., 2006). It is a matter of great significance that exposure to genotoxic stress results in ligand-independent phosphorylation and activation of the EGFR (Boerner et al., 2003). Our results indicate that, EGFR inhibitor administration along with TRP channel interference mechanism remarkably retarded genomic rearrangements. EGFR is documented to be involved in crosstalk with DNA-PK. DNA PK is the key player, that contributes in genomic rearrangements. DNA-PK overrides the activities of ATM in prostate cancer and chromosomal rearrangements are enhanced in ATM compromised cells. However, recapitulation of ATM dynamics results in faithful repair of the genome (Ammad et al., 2010a). Furthermore, we have previously documented that ATM activities are severely impaired by various key mediators in prostate cancer. Negative regulators of TGF signaling are engaged in hampering ATM autophosphorylation in LNCaP cell line. Yet, silencing of negative regulators of TGF signaling, resulted in reinvigoration of quiescent ATM. (Ammad et al., 2010b). The current study indicates that, TRP channels are undoubtedly the negative regulators of ATM activation. However, silencing of TRP channels resulted in an effective repair of the genome and a marked decrease in genomic rearrangements. It is worth mentioning that, dual inhibition of the channels brought more prominent attenuation of genomic rearrangements. On a similar note, Erlotinib administration alone is inefficient in triggering robust ATM autophosphorylation. But results are quite different if there is a combinatorial administration. Erlotinib in combination with TRPV/TRPC inhibitors, outstandingly increase autophosphorylation activity of ATM after 72 h. It is therefore effective if Erlotinib is administered in combination, as it significantly upregulates ATM dynamics and simultaneously retarding genomic rearrangements.

This is the first documented study that unfolds an interaction between calcium channels and ATM activation. In prostate cancer, these channels are remarkably upregulated and inhibit the activation of ATM after DNA damage. The exact mechanisms underlying ATM inhibition are presently unclear but further studies will add substantial information into the existing pool of negative regulators of DNA damage response. Moreover, phytonutrients based studies on suppressing or promoting calcium channel activities still lack. Hopefully, future studies will converge upon exploring effect of phytonutrients on a broad range of TRP channels and their dynamics.

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