Repression of androgen receptor in prostate cancer cells by phenethyl isothiocyanate

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Background: Prostate cancer usually progresses to androgen refractory after an initial anti-androgen treatment. The androgen receptor (AR) is a pivotal factor for the androgen-mediated growth and maintenance of the prostate. Abnormality of the AR, such as overexpression has been postulated to be related to the hormone independent growth of the cancer. Although we previously demonstrated that the AR expression could be modulated by isothiocyanates, which are natural constituents of cruciferous vegetables, the mechanism, however, remained to be clarified. We have since investigated the mechanism of phenethyl isothiocyanate (PEITC) in AR regulation. Methods: A human androgen dependent prostate cancer cell line LNCaP (AD) and its sub-line LNCaP (AI), i.e. androgen independent but overexpressing AR, were exposed to PEITC. The effects of PEITC on cell growth and AR expression/transcription were analyzed with MTT assay, real-time PCR and western blotting. The AR promoter activity was analyzed with the reporter activity after transfection with pAR-luc. The effects on Sp1, the major transcription factor of the AR, were tested with Sp1-luc activity, western blotting and electrophoretic mobility shift assay. Results: PEITC induced a significant growth inhibition, with equal IC50, in both AD and AI cells. The AR present in both cells was repressed as demonstrated with real-time PCR and western blot. PEITC mediates dual effects at transcriptional and post-translational levels to regulate the AR. At transcriptional level the AR level was reduced via inhibition of the transcription factor Sp1, and at post-translational level by accelerating protein degradation. Conclusion: PEITC represses AR transcription and expression, and mediates growth arrest in androgen dependent and independent prostate cancer cells. With the AR modulation and growth attenuation, PEITC and possibly other isothiocyanates, may prevent and inhibit hormone sensitive and refractory prostate cancer.

Abbreviations: AR, androgen receptor; CHX, cycloheximide; CSFBS, charcoal-stripped, heat-inactivated FBS; FBS, fetal bovine serum; PEITC, phenethyl isothiocyanate.

Background

Prostate cancer is the most commonly diagnosed cancer among men in the United States. For patients who are not cured by local treatment and are experiencing metastasis, neither androgen ablation nor chemotherapy abrogates progression of the androgen-independent/hormone refractory disease. Thus, creating a novel strategy for preventing prostate cancer initiation, as well as treating cancer in progression, has become a practical goal to reducing morbidity and mortality.

Abnormalities of the androgen receptor (AR) are decisive factors in the failure of treatment for advanced prostate cancer. The mechanisms postulated for the development of hormone refractory prostate cancer include wild-type AR amplification, excessive recruitment of AR transcriptional co-activators and AR gene mutation (1–3). Although hormone refractory, the growth of the cancer cells need the AR (4,5). One such example is a sub-line developed from androgen-dependent prostate carcinoma LNCaP, which becomes androgen-independent, and strongly overexpress functional AR, and is phosphorylated in an androgen-depleted medium (4). It is therefore reasonable to assume that regulating the overexpressed AR and its function may prove to be an effective approach to prevent and overcome the hormone refractory state (6,7).

Recent epidemiological reports indicated that consumption of cruciferous vegetables is associated with reduced incidence of prostate cancer cases (8–10). The cruciferous vegetables include the Brassica family, and examples are broccoli, cabbages, Brussels sprouts, kale, watercress and cauliflower. The isothiocyanates from these vegetables have been investigated as the responsible dietary factors for chemoprevention. The isothiocyanates occur naturally as thioglucoside conjugates, i.e. glucosinolates, in a wide variety of cruciferous vegetables (11). They are released from glucosinolates by the hydrolytic action of the enzyme myrosinase when the vegetable tissues are crushed or masticated (12). Hydrolysis of the glucosinolate gluconasturtin, found in high amounts in watercress, yields phenethyl isothiocyanate (PEITC). The isothiocyanates are potent cancer chemopreventive agents, as demonstrated with a number of carcinogen-induced cancers in rodents (13,14). The major mode of action is cytoprotection, i.e. by inducing enzymes that prevent cells from the assaults of carcinogens (15,16). More recently, isothiocyanates and their metabolites have been demonstrated to mediate growth regulation in rapidly growing cancer cells, suggesting that the isothiocyanates can inhibit post-initiation progression of carcinogenesis. We have described that the growth regulatory mechanism may include inhibition of cdk activity and/or directly upregulating inhibitor p21WAF1/CIP1 and downregulating cyclins (17–19). The upstream mechanisms to regulate cell cycle progression, as well as the relationship with the AR, remain to be elucidated.
With the lack of TATA and CCAAT box of the AR gene promoter, the Sp1 has been described to predominantly and positively influence AR transcription (20–25). Cross talk between AR and growth factor signaling pathways may occur through activation of Sp1, as indicated by our observation that the epidermal growth factor directly stimulates AR transcription (unpublished data). In the present study, we have demonstrated that PEITC significantly downregulates the transcription of the AR via an inhibition of Sp1, and stimulates AR protein degradation, which in turn modulates the cell growth of androgen-dependent and -independent prostate cancer cells. Our observations thus established a new basis for further exploration of PEITC and the isothiocyanates, for preventing and inhibiting the hormone sensitive and hormone refractory prostate cancer.

Materials and methods

Reagents

The PEITC was purchased from LKT Labs (Minneapolis, MN) with 98% purity. The MG-132 was purchased from EMD Biosciences (San Diego, CA). The paclitaxel and other chemicals were purchased from Sigma (St Louis, MO). The Sp1-luc and mtSp1-luc containing three tandem repeats of consensus Sp1 sites and mutant Sp1 sites driving luciferase gene (26) were kindly provided by Dr Yoshishiro Sowa (University of Medicine, Kamigyo-ku, Kyoto 602, Japan). The AR luciferase reporter pLARS-1 was constructed in our laboratory as described previously (27). AR-Sp1 luciferase reporter pARSp1-3 was constructed by inserting three repeats of the double Sp1 sequences from +429 to +442 or its mutant of 5'-untranslated region (UTR) of the AR gene into a basic luciferase reporter pGL3 and the DNA sequence verified. Effectene transfection reagent and luciferase assay kit were acquired from Qiagen, Valencia, CA. Twenty-four hours after transfection, the cells were exposed for an additional 24 h period to various concentrations of PEITC or mithramycin. The cells were washed, lysed and the lysates used for luciferase activity assay with a Promega luciferase assay system. The luciferase activities were expressed as units per milligram of proteins.

Quantitative real-time PCR analysis

The LNCaP AD and AI cells grown exponentially were treated with different concentrations of PEITC for 24 h. The cells were washed, harvested and total RNA extracted with Qiagen kits (Chatsworth, CA). All RNA was treated with DNase I (Ambion). A total 1 μg RNA was reverse-transcribed per reaction using first-strand complementary DNA synthesis with random primers (Invitrogen, Carlsbad, CA). Quantitative real-time PCR of AR was done using AR specific primers provided by Applied Biosystems (Foster City, CA). All values were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all experiments were done in triplicate.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (30). Briefly, DNA samples of AD and AI cells, purified from 4 μg Sp1-antibody immunoprecipitates, were amplified by PCR using a high fidelity PCR system and the primers hAR5F: 5'-AGC TGC TAA AGA AGA GGA G-3' and hAR3R: 5'-GGA GTT ACC TCT CTT CAA AC-3' that covers DNA sequence containing two adjacent Sp1 sites, located at 429–442 of 5'-UTR of AR gene. PCR products were analyzed by electrophoresis on 2% agarose gels and stained with SYBR Gold nucleic acid gel stain (Molecular Probes).

Electrophoretic mobility shift assay (EMSA)

The assay was performed as described previously (31). Nuclear proteins extracted from AD or AI cells were reacted with 32P-labeled Sp1 oligos, and their mutants corresponding to the 5'-UTR of AR gene containing two adjacent Sp1 sites located at 429–442, in binding buffer containing 1 μg of poly(dI–dC) for 30 min at room temperature. The reaction mixtures were then subjected to electrophoresis in 8% native polyacrylamide gel, and the binding complexes visualized by exposure on X-ray films. Specificity of the Sp1 binding complexes were established using specific and non-specific competitors (31).

Results

Androgen-dependent and independent growth of AD and AI cells

We established an androgen-independent (AI) prostate cancer cell line from androgen-dependent (AD) LNCaP cells, and characterized the differences between the AD and AI lines. The major differences of AI cells from their parental AD cells are androgen-independent growth and resistance to chemotherapeutic agents, due to at least in part the over-expression of AR (5,27,28,32). Figure 1 shows the growth characteristics of AD, AI and PC-3 cells, in the presence or absence of androgen, as measured by MTT. An identical growth rate between AD and AI cells was observed when both cell lines were incubated in the culture medium containing androgens (10% FBS) (Figure 1, left panel). However, the AD cell culture became static when the cells were supplied with an androgen-depleted medium (10% CSFBS), confirming that androgens are required for the growth of the AD cells (Figure 1 right panel). In contrast, AI cells grew well in the charcoal stripped medium just as that with regular FBS medium, demonstrating that the cells became androgen-independent (Figure 1, left and right panels). The growth pattern of AI cells is comparable with the androgen-independent PC-3 cells (Figure 1).

Effects of PEITC on AD and AI cell growth

The effects of PEITC on the cell growth of AD and AI were examined by MTT assay. As shown in Figure 2 panel A, a significant growth inhibition was achieved in both cell lines after exposure to PEITC for 7 days, exhibiting an identical IC50 of ~0.6 μM. The two cell lines were also exposed to paclitaxel, which showed a stronger growth inhibitory
activity than PEITC. The AD and AI cells displayed different sensitivity toward paclitaxel (4,5), with IC$_{50}$ < 0.01 nM for AD cells and 0.6 nM for AI cells, representing ~60-fold difference (Figure 2 panel B). The results are in line with previous observations that the AI cells are more resistant to chemotherapeutic agents, such as paclitaxel, retinoic acid and doxorubicin, than it’s parental AD cells (4,28).

**PEITC represses AR transcription via Sp1 inhibition**

The effects of PEITC on the expression of AR were examined by a real-time PCR and western blotting. Figure 3 panel A shows that exposure of AD and AI cells to PEITC for 24 h resulted in a concentration-dependent decrease of the mRNA of AR. The maximal transcriptional repression occurred at 10 μM, with ~60% inhibition in both AD and AI cells. Concurrently, the protein levels of AR were also decreased, to a similar extent after exposure to PEITC (Figure 3, panel B).

To further examine, whether the decrease in mRNA and protein levels of the AR was the result of repression of the AR promoter activity, AD cells were transfected with pLARS-1 (pAR-luc) that covers the human AR promoter from −741 to +907 (27). The exposure of the transfected AD
and AI cells to PEITC for 24 h, resulted in a significant decrease of the reporter activities (Figure 3C), with AI cell line appearing to be more sensitive to PEITC than AD (Figure 3C, right panel). The decreased pLARS-1 activity matches the results obtained from real-time PCR (Figure 3A), indicating that repression of AR transcription may be the result of inhibiting the AR promoter.

Since Sp1 predominantly drives AR transcription, we speculated that the repression of AR transcription by PEITC could be mediated by an inhibition of Sp1. To examine this possibility, we tested the effects of PEITC on the activities of Sp1-luc, containing three repeats of Sp1 consensus sites, or its mutant mSp1-luc. Figure 4A shows that exposure of AD cells to a specific Sp1 inhibitor, mithramycin, caused a strong inhibition of the Sp1-luc activity, whereas trichostatin A (TSA) mediated a strong induction, as that described by others (4,26). Similar to mithramycin, PEITC mediated a significant inhibition of Sp1-luc activity in both AD and AI cells, in a concentration-dependent manner (Figure 4B). Mithramycin, TSA or PEITC showed no effect when the mutant mSp1-luc was used (data not shown).

To analyze whether the inhibition of Sp1 by PEITC was a reflection of reduced Sp1 protein level, western blotting was performed. As revealed in Figure 4C, exposure for 24 h to 5.0 or 10 μM of PEITC caused, respectively, >50 and 70% reduction of Sp1 protein in both cell lines. The data thus suggest that the decrease of Sp1 activity may associate with an inhibition of Sp1 expression.

The interaction of the Sp1 site with Sp1 protein was examined by in vivo ChIP assay shown in Figure 5A. A clear PCR product covering the Sp1 site (429–442) was obtained when Sp1 monoclonal antibody was used to

Fig. 3. Repression of AR transcription in LNCaP AD and AI cells by PEITC. (A) AD and AI cells at exponential growth phase were exposed to the indicated concentrations of PEITC for 24 h. Total RNA was extracted, and the mRNA of AR was determined by a real-time PCR as described in Materials and methods. All experiments were done in triplicate, and the data represent mean ± SD. (B) Effects of PEITC on protein levels of the AR in AD and AI cells: AD and AI cells at exponential growth phase were exposed to the indicated concentrations of PEITC for 24 h and the levels of AR were measured by western blotting (β-actin was used as internal loading control) as described in Materials and methods. (C) Effects of PEITC on the activity of AR-luciferase reporter. AD and AI cells were transfected with 1 μg/6-well dish of pLARS-1 in serum-free medium using effectene. Twenty-four hours after the transfection, the cells were exposed to the indicated concentrations of PEITC in a serum containing medium for additional 24 h. The cells were then washed and lysed for determination of luciferase activity using Promega assay kit. Data represent mean ± SD from two independent experiments.
immunoprecipitate the chromatin cross-linked proteins from AD or AI cells.

To further examine whether a decreased Sp1 activity was involved in the PEITC-induced AR downregulation, the interaction of the Sp1 with Sp1 element in AR promoter was studied by EMSA using 32P-labeled double strands of ARSp1-3 oligonucleotide, corresponding to the sequence located 429–442 in the 5'-UTR of AR gene, as a probe. As shown in Figure 5B, a significant decrease in the formation of Sp1-nuclear binding complex was observed, when the nuclear extracts from the mithramycin-treated AD cells were used to react with 32P-labeled Sp1 oligonucleotide probe. Similar to mithramycin, PEITC induced a moderate decrease of the Sp1-nuclear complex formation, in a concentration-dependent manner, suggesting that the decreased Sp1 binding to the AR promoter could account for, at least in part, the decrease of AR transcription. This interpretation was supported by the data shown in Figure 5C, that PEITC inhibited the AD cell reporter activity of pARSp1-3, a Sp1 specific AR promoter driven luciferase reporter.

**PEITC accelerates degradations of AR and Sp1 protein**

To further establish the relationship between Sp1 and AR expression, a dynamic effect of PEITC on the protein levels of AR and Sp1 was studied. After exposure of AD cells to 10 μM PEITC, the level of AR protein showed a time-related alteration. A >80% reduction of AR protein was observed after 1 h, with maximal reduction reached by 7 h. Then the AR level was recovered to some extent at 24 h until full restoration by 48 h (Figure 6A). In comparison, the magnitude of Sp1 reduction, mediated by the same PEITC treatment, was less extensive and slower, with the maximum reduction observed at 24 h, and the Sp1 level was fully recovered by 48 h. The data suggest that the PEITC-mediated AR reduction does not result solely from a decrease of Sp1 level.

To examine whether the suppression of the AR and Sp1 expression by PEITC requires protein synthesis, or is a result of proteasome-dependent protein degradation, AD cells were exposed for 24 h to 10 μM PEITC, protein synthesis inhibitor cycloheximide (CHX, 25 μM), or proteasome inhibitor MG-132 (2.5 μM) alone, or to the combination of PEITC plus CHX or MG-132. As described in Figure 6B, the PEITC inhibitory effect for Sp1 was completely abrogated by the presence of PEITC plus either CHX or MG-132. The data suggest that downregulation of Sp1 by PEITC may require protein synthesis for a proteasome activity, thereby enhancing Sp1 protein degradation. The results showed further that the level of Sp1 was unchanged in the presence of either CHX or MG-132 without PEITC, indicating that the steady-state levels of Sp1 protein could not be altered by either a mechanism involving protein synthesis inhibition, or by inhibiting protein-degradation alone.

Figure 6C shows that in contrast to Sp1, the combination of PEITC with CHX or MG-132 revealed a synergistic effect to downregulate the AR protein level, as compared with PEITC, CHX or MG-132 alone (Figure 6B). A control experiment performed in parallel showed that PEITC prevented degradation of cyclin inhibitor p27, which required new protein synthesis (Figure 6B). Our observation, that AR level is sensitive to protein synthesis inhibitor CHX, is consistent with a previous report (33). However, contrary to our observation, the proteasome inhibitor MG-132 was described to accumulate AR protein in LNCaP cells after a 2 h exposure (34). Considering that the difference of exposure time could be a factor, a time course study of MG-132 on the level of AR protein was initiated. As described in Figure 6C, the expression of the AR protein was found to be a function of exposure time to MG-132. The AR protein gradually accumulated after exposure to MG-132 for up to 7 h, which was followed by a decrease at 16 h, with the lowest level reached by 24 h. As a control experiment, the level of p27WAF1/CIP1 protein under the same experimental conditions was shown accumulates in direct proportion to the MG-132 exposure time, with the highest level reached at 16–24 h (Figure 6C). This indicates that post-translational regulation...
of AR and p21WAF1/CIP1 is mediated by different mechanisms. To examine whether MG-132 can prevent a PEITC-mediated decrease of AR protein at its optimal time points, AD cells were treated with 10 μM PEITC, in the presence or absence of MG-132 for 3 h, and the AR protein level determined. As depicted in Figure 6D, the PEITC-mediated AR decrease was blocked by MG-132. Thus, our data indicated that the degradation of the AR protein by PEITC could be reduced or accelerated by the presence of proteasome.

Discussion

We have demonstrated in this article, for the first time to our knowledge, that PEITC may have dual effects to downregulate the AR, at transcriptional level via an inhibition of Sp1 expression, and at post-translational level by mediating protein degradation. These PEITC activities were demonstrated in both androgen dependent and independent prostate cancer cells. Several lines of evidence have been presented in this regard. On the transcriptional level, the exposure to PEITC decreased the AR mRNA level and the AR promoter activity. In parallel, the Sp1 consensus-driven luciferase reporter was inhibited, the protein level of Sp1 reduced and the interaction of Sp1 with the AR promoter decreased. On the post-translational level, the data revealed that the degradation of the AR and Sp1 proteins by PEITC could be blocked by a proteasome inhibitor, indicating that PEITC mediates a proteasome-dependent degradation. The down-regulation of AR and Sp1 by PEITC is consistent with our recent observations in rats. Oral feeding of rats with PEITC was shown to inhibit the testosterone-stimulated prostatic cell growth via a decrease of Sp1 expression, and the Sp1 binding complex formation which modulates the AR and cell cycle progression (35). Recently, the effect of other chemopreventive agents, such as green tea extracts and indole-3-carbinol, were also reported to downregulate the AR (36,37).

The AR belongs to the family of genes that do not contain the classical regulatory sequences TATA and CCAAT boxes for its transcription. Instead, there are multiple Sp1 located near the transcriptional initiation site (38–43), that are known to predominantly drive AR transcription (20,21,44). We recently identified two adjacent Sp1 sites located at 429–442 of 5'-UTR of the AR gene and named as ARSp1-3. To examine the transcriptional activity of ARSp1-3, we constructed a pARSp1-3 luciferase reporter by inserting three repeats of the two adjacent Sp1 sites, or its mutant into a basic vector pGL3-basic and observed 7- and 11-fold increase of the reporter activities in AD and AI cells, respectively, as
compared with its mutant (45). The pARSp1-3 luciferase activity in both AD and AI cells was also found to correlate with the Sp1-nuclear binding complex formation (45). In this study, we further confirmed by in vivo ChIP (Figures 5A) the binding of Sp1 to the two adjacent Sp1 sites. In addition, the formation of ARSp1-3 nuclear binding complex could be blocked moderately by PEITC in a concentration-dependent manner (Figures 5B), which paralleled the inhibition of pARSp1-3 luciferase reporter activity. The data thus demonstrated that inhibition of Sp1 activity by PEITC led to a repression of AR transcription. As a Sp1 inhibitor, the mechanism by which PEITC inhibits Sp1 expression is different from a classical Sp1 inhibitor, mithramycin. Mithramycin interferes the binding of Sp1 protein to its CG rich regulatory sites, via its specific association with the CG rich DNA sequence (46).

We have demonstrated that the dynamic characteristics of Sp1 and the AR are different. While the steady-state Sp1 level was stable and unchanged by inhibitors for protein synthesis or for proteasome alone, the effects of PEITC to reducing the Sp1 level could be blocked by CHX or MG-132 (Figure 6B). This finding indicated that the PEITC-mediated Sp1 degradation involves new protein synthesis and is proteasome dependent. We have described that the reduction of the AR after 24 h exposure to PEITC was synergistically enhanced by CHX or MG-132, supporting the conclusion that the AR is a fast turnover protein. On the other hand, exposure to a protein synthesis or proteasome inhibitor resulted in a reduction of the steady-state AR level. In fact, the activity of the proteasome inhibitor MG-132 on the AR stability was intriguing, i.e. short time exposure to MG-132 resulted in accumulation of the AR protein while longer exposure caused reduction (Figure 6C). This has indicated that mechanism independent of proteasome may also be involved in the regulation of AR stability. A similar observation of a conflicting effect of MG-132 on AR stability was reported previously (47).

The detailed processes of proteasome-dependent AR degradation by PEITC, however, need to be further analyzed. One possible mechanism could involve the chaperone complex, which binds to the AR and is essential for AR stability and maturation. The proteasome is known to associate with the chaperone complex including the heat-shock protein 90 (Hsp90) (48–51). Chen et al. (47) recently reported that while the histone deacetylase inhibitor LAQ824 repressed AR transcription, LAQ824 also stimulated AR degradation via inactivation of the Hsp90 molecular chaperone. The inactivation by LAQ824 was due to an enhanced acetylation of Hsp90, thereby inhibiting its ATP binding activity. The study also showed that LAQ824 reduced the stability of Akt, which stimulates AR phosphorylation and increases AR stability. Since we recently reported that PEITC is an inhibitor of histone deacetylases (52), PEITC could induce similar acetylation of Hsp90 molecular chaperone.

![Fig. 6. Dynamic effects of PEITC on degradation of Sp1 and AR proteins. (A) Time course effect of PEITC on Sp1 and AR proteins: AD cells were exposed to 10 μM PEITC for different time length and western blot performed to measure the levels of Sp1 and AR proteins. (B) Effect of inhibitors of protein synthesis or proteasome on Sp1 and AR degradation by PEITC: AD cells were exposed to PEITC (10 μM), CHX (25 μM) or MG-132 (2.5 μM) alone, or to PEITC plus CHX or MG-132 for 24 h, and the protein levels of Sp1 and AR determined by western blotting. (C) Time course effect of proteasome inhibitor on AR degradation; AD cells were exposed to MG-132 (2.5 μM) for indicated time period, and the protein levels of AR and p21WAF1 measured by western blotting. (D) AD cells were exposed to PEITC (10 μM) for 3 h, in the presence or absence of MG-132 (2.5 μM), and the level of AR protein determined by western blotting.](https://academic.oup.com/carcin/article-abstract/27/10/2124/2392446)
and inactivate its binding to AR, promoting AR degradation. Since our data described that the AR protein level was diminished earlier than Sp1 by PEITC, the AR could be degraded by proteasome ahead of Sp1. As a transcription factor, Sp1 is mainly located in the nucleus rather than cytoplasm, and it is unlikely that Sp1 plays a direct role in proteasome-dependent AR degradation. One possible interpretation could be that the AR reduction is mediated by Sp1-independent proteasome degradation, and compounded by a downregulated Sp1 effect on AR transcription. In considering PEITC as an inhibitor of histone deacetylases, experiments need to be performed for the effects of PEITC on acetylation of AR and Sp1 proteins, which could affect their stability (53). In addition, the effect of PEITC on AR transcription, in the presence or absence of the protein synthesis inhibitor CHX, at different time points, may further help to clarify the role of Sp1.

With the information that AR overexpression plays a predominant role in the progression to advanced prostate cancer, novel therapies aiming at AR repression have drawn much attention (6,7,54–56). Since PEITC has been demonstrated to induce growth arrest and apoptosis in prostate cancer cells, our current observation has provided the basis for further exploring PEITC in preventing and inhibiting the androgen-independent progression of prostate cancer. The experimental results also support the speculation that the isothiocyanates present in cruciferous vegetables, by their action to modulate AR and the excessive growth of the prostate, are important dietary factors for prostate cancer chemoprevention.

**Authors’ contributions**

L.G.W. was instrumental in study design, performing experiments and drafting manuscript. X.M.L. performed experiments of cell transfection, culture analyses and EMSA. J.W.C. participated in study design and manuscript preparation.

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