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14. ABSTRACT Majority of human prostate cancers show STAT3 activation, which promotes more aggressive castration-resistant phenotype. In addition, STAT3 is activated in diverse immune cell associated with prostate tumors. Therefore, STAT3 is a highly desirable target for prostate cancer therapy. Within the first year, we made significant progress developing CpG-siRNA for STAT3 targeting in human prostate cancer cells and in the tumor microenvironment. Our studies using xenotransplanted models of TLR9+ CRPCs validated CpG-STAT3 siRNA as therapeutic approach to induce tumor cell death. In addition, we identified another molecular target, NF-κB/RELA, a transcription factor activated downstream from TLR9, which contributes to STAT3 activation. We have also undertaken immunohistochemical studies to compare TLR9 expression and STAT3 activation in both tumor and lymph node specimen from. Our analyses indicated that increased TLR9 levels correlate with higher Gleason grade of prostate cancer specimens. TLR9 expression pattern indicates therapeutic opportunity for targeting not only primary but also disseminated prostate cancer cells in lymph nodes or bones. Finally, all tested prostate cancer patients' samples indicated increased infiltration of CD68+ macrophages with increased levels of TLR9 levels and STAT3 phosphorylation. These results underscore the feasibility of using CpG-siRNA strategy to simultaneously target prostate cancer cells and tumor-associated immune cells.					
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CpG-STAT3 siRNA for Castration-Resistant Prostate Cancer Therapy

Progress Report: 28/09/2012 – 26/09/2013

Introduction

Majority of human prostate cancers show STAT3 activation, which strongly correlates with tumor progression towards hormone-refractory/castration-resistant phenotype (CRPC) and poor patients' survival¹⁻³. In addition, STAT3 is activated in diverse immune cell associated with prostate tumors promoting immune tolerance, angiogenesis and metastasis^{4,5}. Because STAT3 operates in both cancer cells and non-malignant tumor-associated cells, it represents a highly desirable target for cancer therapy⁶. Inhibiting STAT3 has potential to eliminate the aggressive castration-resistant prostate cancer (CRPC) cells, which are not amenable to existing hormonal treatments or standard chemotherapeutics¹ but undergo apoptosis when STAT3 signaling is inhibited⁷⁻⁹. Pharmacological inhibition of proteins lacking enzymatic activity, including STAT3, is difficult and requires alternative approaches, such as silencing genes using siRNA. We previously generated a strategy for cell-specific delivery of siRNA using CpG oligonucleotides (ODNs) for targeting cells positive for Toll-like receptor 9 (TLR9)¹⁰. CpG-siRNAs are internalized specifically by TLR9-positive human/mouse immune cells and malignant cells, such as blood cancer cells or CRPCs^{10,11}. We also demonstrated that *in vivo* administration of CpG-STAT3 siRNA breaks tumor immune tolerance, thereby inducing antitumor immune responses in syngeneic tumor models in mice¹⁰. In addition, intratumoral injections of CpG-STAT3 siRNA were shown to induce direct tumor cell death in TLR9-positive human leukemia xenotransplants¹¹. These results suggested the feasibility of using human-specific CpG-STAT3 siRNA to simultaneously target both castration-resistant prostate cancer cells and tumor-associated immune cells, such as myeloid-derived suppressor cells (MDSCs). We hypothesized that targeting oncogenic and tolerogenic signaling using cell-specific CpG(A)-siRNA strategy will suppress tumor progression and generate potent antitumor immunity, with minimal adverse effects.

Body

The main goals of this study are: optimization of the CpG(A)-siRNA strategy for targeting survival signaling in metastatic prostate cancer cells and for breaking immunosuppressive effects of the prostate cancer microenvironment.

Task 1. To optimize CpG(A)-siRNA strategy for direct inhibition of human CRPC cell survival and proliferation (months 1-12). Within this aim, **we successfully validated STAT3 and NF- κ B/RELA transcription factors as optimal targets for CpG-siRNA strategy in xenotransplanted CRPC models *in vivo*.** We have also generated new stable cell lines PC3- and DU145-mCherry/Luciferase for *in vivo* imaging studies on disseminated human prostate cancers in NSG mice.

To assess the effect of targeting STAT3 with intratumoral injections of CpG-STAT3 siRNA, we used CRPC tumor models xenotransplanted into immunodeficient NSG mice. The repeated daily *i.t.* injections of 5 mg/kg CpG-STAT3 siRNA resulted in inhibition of DU145 tumor growth with 4 days after initial treatment (Fig. 1A). These effects correlated with both significant STAT3 gene silencing and increase in the number of apoptotic tumor cells as measured by qPCR and flow cytometry, respectively. The intravenously delivered CpG-STAT3 siRNA had only limited effect likely due to serum half-life of the conjugate. To overcome this obstacle, we have recently generated new chemically modified CpG-siRNA(21mer) with increased serum stability as originally planned. The *in vivo* testing for antitumor effects of this conjugate is ongoing.

Previous reports from other groups suggested that in addition to STAT3, NF- κ B signaling provides important survival signal for prostate cancer cells^{12,13}. We confirmed these observations in tested CRPC cell lines. Both TLR9-positive PC3 and DU145 but not TLR9-negative LNCaP-S17 cells showed high levels of NF- κ B/RELA activity, which is likely reflecting TLR9-induced NF- κ B signaling (Kortylewski, unpublished data). To assess whether targeting NF- κ B/RELA/p65 will lead to antitumor effect as observed for STAT3 blocking, we generated a new CpG-RELA siRNA conjugate. As shown in Fig. 1B, targeting NF- κ B/RELA/p65 signaling using intratumoral injections of CpG-siRNA completely suppressed *in vivo* growth of

PC3 xenotransplants. We also assessed the effect of the simultaneous targeting of both STAT3 and NF- κ B/RELA using CpG-siRNA. However, this approach did not generate synergistic antitumor effects (Kortylewski, unpublished data). This is either an effect of limited capacity of intracellular RNAi processing machinery or a result of the functional overlap of both survival pathways in cancer cells. Studies in immunocompetent mice planned in the Specific Aim 3 should more precisely assess therapeutic value of targeting STAT3 and/or NF- κ B/RELA, taking into consideration systemic immune activation, which is critical for the overall antitumor efficacy¹¹.

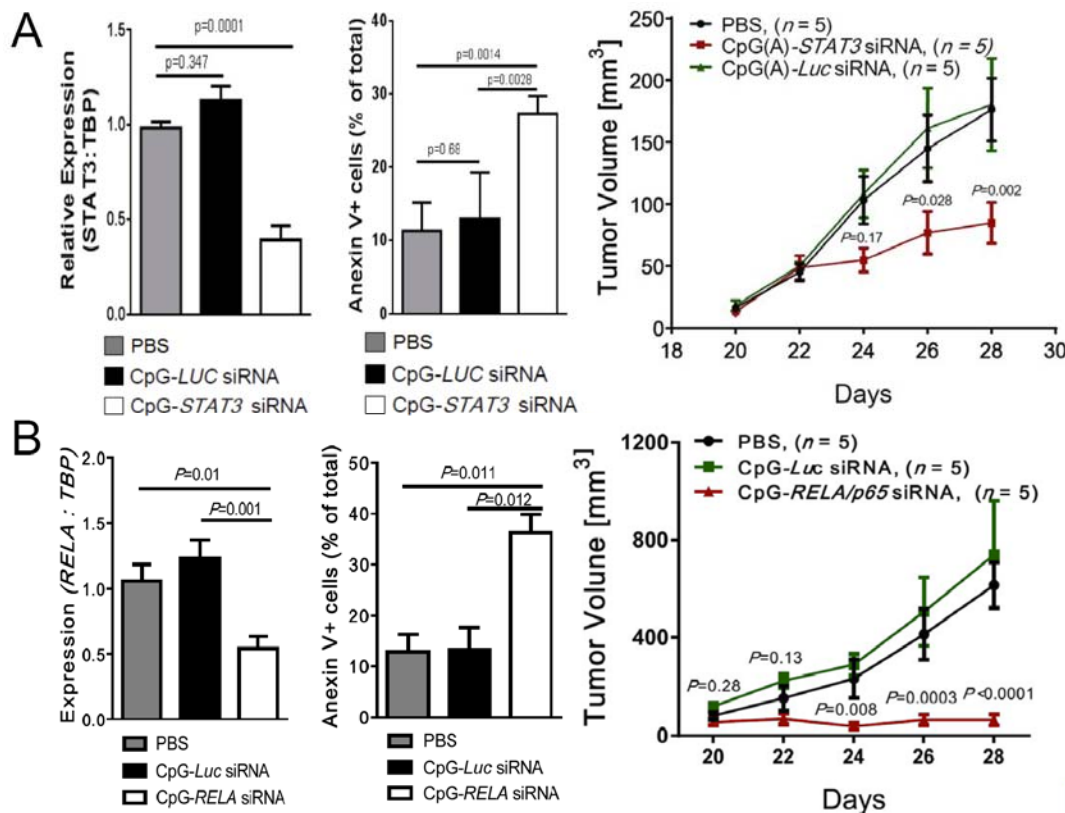


Figure 1. Both STAT3 and NF- κ B/RELA are suitable therapeutic targets for CpG(A)-siRNAs in CRPC tumors *in vivo*. (A, B) Intratumoral injections of CpG(A)-siRNAs targeting STAT3 (A) or NF- κ B/RELA (B) genes inhibit growth of xenotransplanted prostate tumors. NSG mice were injected with 5×10^6 DU145 (A) or PC3 (B) CRPC cells. After tumors were established, mice were treated daily with *i.t.* injections of CpG(A)- siRNAs (5 mg/kg) as indicated. Left panels - target gene silencing in samples from 5 individual tumors was verified using qPCR and normalized to *TBP* expression. Middle panels - the percentage of apoptotic cancer cells was assessed in tumor cell suspensions using

staining with Annexin V and flow cytometry. Right panels - tumor growth kinetics after treatment with indicated oligonucleotides. Shown are means $\pm$ SEM ($n = 5$) for all experiments described above; P values from 2-way ANOVA plus Bonferroni post-tests for CpG-STAT3 (or *RELA*) siRNA vs CpG-Luc siRNA were indicated in both graphs.

Task 2. To assess the feasibility of using CpG(A)-STAT3 siRNA to break tumor immune tolerance in metastatic CRPC (months 6-24). Our initial immunohistochemical studies within this aim, confirmed that TLR9 expression and STAT3 activation is common in tumor and lymph node specimen from CRPC patients.

To verify that TLR9 is expressed in advanced prostate cancer as recently reported^{14,15}, we analyzed tumor specimens from a cohort of 21 prostate cancer patients kindly provided by Dr. Sumanta Pal (Molecular Oncology, City of Hope). The immunohistochemical analysis and evaluation by collaborating pathologist, Dr. Massimo D'Apuzzo (Pathology, City of Hope), confirmed **a potential positive correlation between the level of TLR9 expression in tumor cells and the Gleason grade** (Fig. 2A-C). In addition, prostate cancer cells forming lymph node and bone metastases retained expression of TLR9 (Fig. 2DE). We conclude that TLR9 expression pattern indicates therapeutic opportunity for targeting not only primary but also disseminated prostate cancer cells.

Next, we analyzed lymph node biopsy specimens from a cohort of 20 late-stage metastatic prostate cancer patients comparing them to control lymph node tissues from tumor-free subjects. In comparison to controls, **all tested prostate cancer patients' samples indicated increased infiltration of CD68⁺**

macrophages with upregulation of TLR9 levels and STAT3 phosphorylation in similar areas (Fig. 3). These results support the possibility of blocking STAT3 activation in human TLR9-positive tumor-associated macrophages using CpG(A)-STAT3 siRNA. To corroborate these data, we used flow cytometry to analyze blood samples from early- and late-stage prostate cancer patients. The initial results indicate that, in addition to previously reported plasmacytoid DCs, TLR9⁺/STAT3P⁺ myeloid-derived suppressor cells accumulate in blood samples from late stage prostate cancer patients (Kortylewski, unpublished data). Studies in the next funding period will assess the feasibility of using CpG-STAT3 siRNA to target human prostate cancer-associated MDSC to overcome tumor immune evasion.

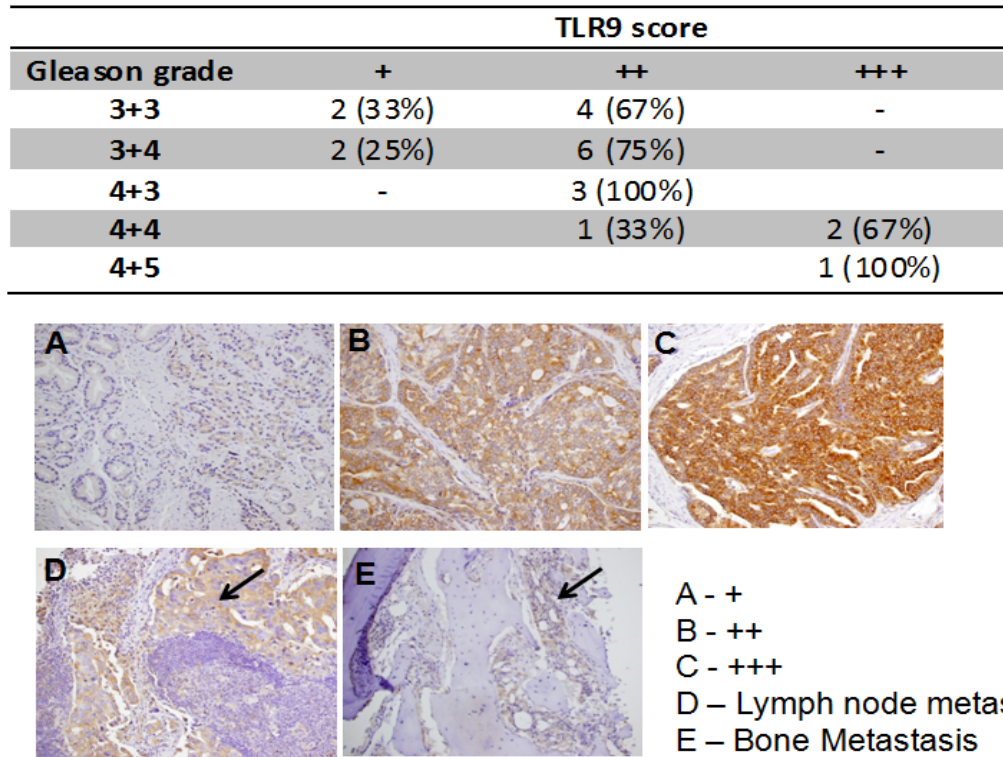


Figure 2. TLR9 expression is increased in less differentiated prostate cancers with higher Gleason grade. 4 μm formalin-fixed/paraffin-embedded (FFPE) sections from 21 primary (A, B, C) prostate cancers plus two metastatic tumors (D, E) were stained using monoclonal antibodies to human TLR9 (Imgenex) and detected using immunohistochemistry. Stained tissue sections were evaluated by pathologist and graded. Shown in brackets are percentages of differently TLR9 scored sections for each Gleason grade.

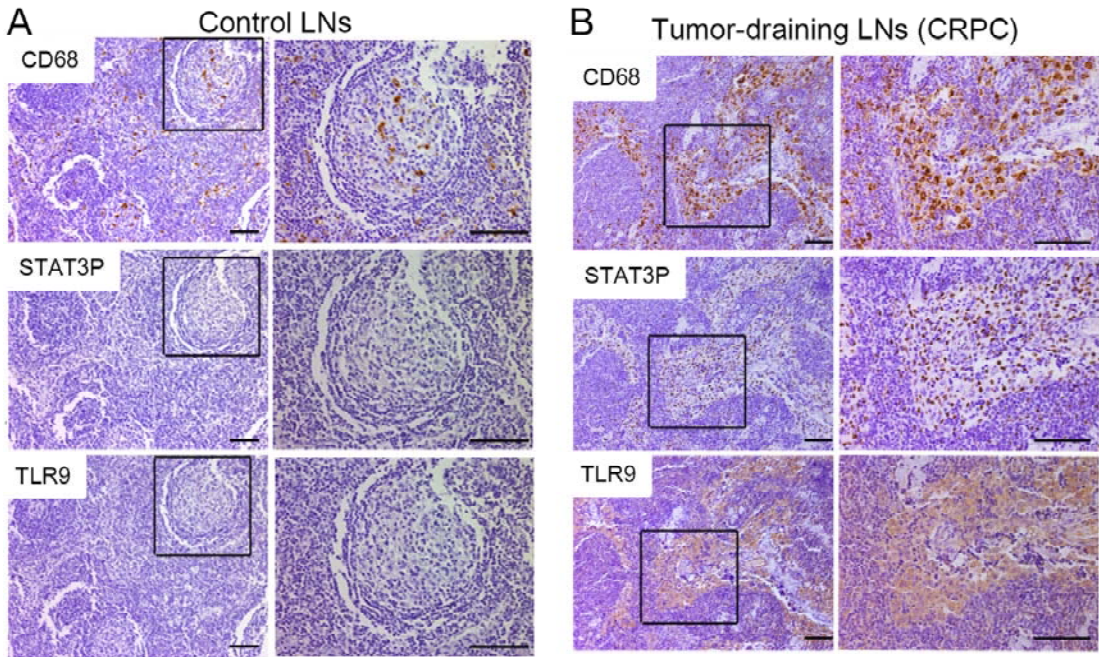


Figure 3. Increased macrophage infiltration, TLR9 expression and STAT3 activation in lymph nodes from prostate cancer patients. 4 μm FFPE sections from 12 tumor-free control lymph nodes (US Biomax) (A) or from 20 prostate cancer patients (B) were stained using antibodies against human macrophage marker CD68 (AbD Serotec), tyrosine-phosphorylated STAT3 (Cell Signaling) and TLR9 (Imgenex). Stained tissue sections were evaluated by pathologist.

Key Research Accomplishments

- STAT3 and NF- κ B/RELA were validated as therapeutic targets for CpG(A)-siRNA conjugates in xenotransplanted TLR9-positive CRPC models
- Upregulated expression of TLR9 correlated with higher Gleason grade and lower differentiation of prostate cancers, including metastases
- Accumulation of macrophages, upregulation of TLR9 and activation of STAT3 are common in lymph nodes from prostate cancer patients

Reportable Outcomes

1. D. Moreira, S. Pal, C. Gao, Q. Zhang, D.M.S. Hossain, C.M. Kowolik, A. Vultur, M. D'Apuzzo, Kortylewski M: Toll-like receptor 9/NF- κ B Signaling Promotes *In Vivo* Growth and Invasion of Castration-Resistant Prostate Cancers (manuscript in preparation).
2. D.M.S. Hossain, S. Pal, D. Moreira, M. D'Apuzzo, Kortylewski M: CpG-STAT3 siRNA for targeting granulocytic myeloid-derived suppressor cells in prostate and renal cancer patients (manuscript in preparation).
3. M. Kortylewski: CpG-STAT3 siRNA for Castration-Resistant Prostate Cancer Therapy (presentation at Prostate Cancer Foundation Meeting, City of Hope, July 2013).
4. M. Kortylewski: CpG-siRNA for Therapy of Castration-Resistant Prostate Cancers (presentation at Genitourinary Research Retreat, City of Hope, September 2013).
5. Generation of stable prostate cancer cell lines:
 - TLR9-positive and TLR9-negative variants of human prostate cancer cells (LNCaP-S17/TLR9, PC3/shTLR9, DU145/shTLR9),
 - PC3-and DU145-mCherry/Luciferase cells for *in vivo* imaging studies.

Conclusion

During the previous year of funding, we accomplished two most critical goals of our project. First, we have validated previously selected and identified new molecular targets for CpG-siRNA strategy. These targets will be further tested for their capacity to overcome immune suppression in the prostate cancer microenvironment. Second, we have successfully verified that previous reports of TLR9 expression in tumor-associated immune cells as well as in human tumor cells. These results, together with common STAT3 activation in the tumor microenvironment, provide us a strong rationale for further development of CpG(A)-siRNA approach for two-pronged CRPC therapy. These initial results underscore the possibility that our studies will produce a technology platform for broad application in cancer immunotherapy.

So What:

There are no direct pharmacological inhibitors of oncogenic and immunosuppressive transcription factors such as STAT3 transcription factor. CpG(A)-siRNA oligonucleotides provide a unique and cell-specific method for targeting both prostate cancer cells and the tumor microenvironment. This two-pronged strategy is paradigm-shifting and highly likely to inspire other combinatorial cancer immunotherapies.

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