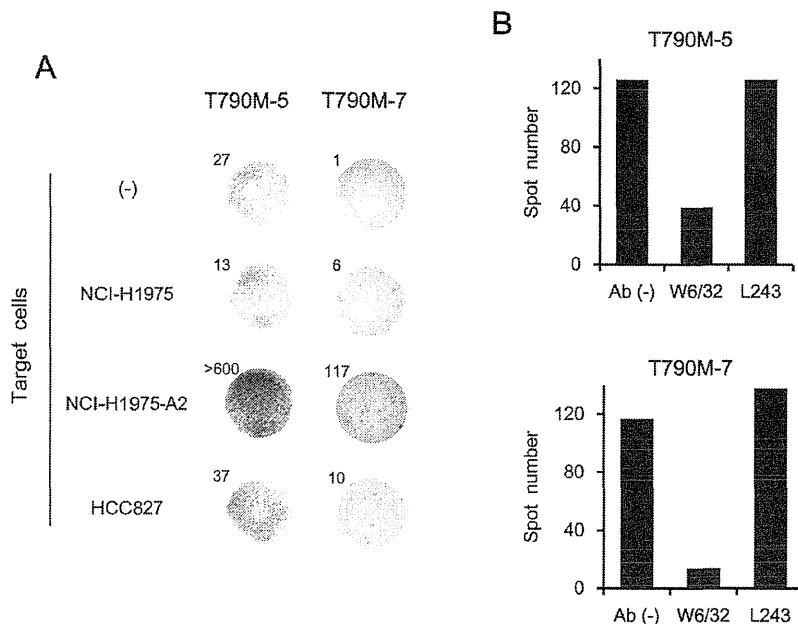

Figure 2. Reactivity of T790M-5- or T790M-7-stimulated T cells against HLA-A2$^+$ NSCLC cells harboring EGFR T790M mutation. (A) CD8$^+$ T cells (2×10^4 cells/well) isolated from the T790M-5-stimulated PBMCs by magnetic beads were examined for their reactivity against different NSCLC cell lines (1×10^4 cells/well), NCI-H1975 (HLA-A2$^-$ T790M$^+$), NCI-H1975-A2 (HLA-A2$^+$ T790M$^+$), or HCC827 (HLA-A2$^-$ T790M$^-$), by IFN-γ ELISPOT assay (left panel). The T790M-7-stimulated PBMCs (2×10^4 cells/well) were similarly examined for their reactivity against the same tumor cell lines by IFN-γ ELISPOT assay (right panel). The assays were carried out in duplicate wells, and representative wells are shown. The numbers of spots are shown for each well. The experiments were repeated with blood from three different donors, and representative results are shown. (B) The T790M-5 and T790M-7-stimulated PBMCs (2×10^4 cells/well) were examined for their reactivity against NCI-H1975-A2 cells (1×10^4 cells/well) by IFN-γ ELISPOT assay in the absence or presence of 10 μg/ml of anti-HLA class I (W6/32) or anti-HLA-DR (L243) mAb. The assays were carried out in duplicate wells, and the means of spot numbers from duplicate wells are shown. The experiments were repeated with blood from three different donors, and a representative result is shown.
doi:10.1371/journal.pone.0078389.g002

an isotype control mAb, anti-HLA-DR (clone L243), confirming that this response was MHC class I-restricted (Figure 2B).

The reactivity of T cells against NSCLC cell lines was also investigated by ^{51}Cr-release assay. Both T790M-5- and T790M-7-stimulated T cells showed higher cytotoxic activity against NCI-H1975-A2 than HLA-A2-negative NCI-H1975 (Figure 3A). Moreover, they demonstrated strong cytotoxic activity against HLA-A2-positive PC9/ZD cells harboring the EGFR T790M mutation (HLA-A2$^+$ T790M$^+$), but not against the original PC9 (HLA-A2$^+$ T790M$^-$) or HCC827 (HLA-A2$^-$ T790M$^-$) without the T790M mutation (Figure 3B). These results suggested that both T790M-5 and T790M-7 epitopes might be expressed on the surface of NSCLC cells harboring the EGFR T790M mutation in an HLA-A2-restricted manner.

Cross-reactivity of T790M-5-stimulated T cells against the wild-type epitope or T790M-8

Since T790M-5 was different from the corresponding wild-type peptide, WT-5, by only a single amino acid sequence, we examined whether T cell lines established after repeated stimulation with T790M-5 could show reactivity against the corresponding wild-type peptide WT-5 (Figure 4). When the T790M-5-stimulated T cell lines were stimulated by a higher dose (1 μg/ml) of peptides for ELISPOT assay, the wild-type peptide WT-5 induced apparent, but weaker antigen-specific T cell responses, compared to the mutant peptide T790M-5. When a lower dose (10 ng/ml) of peptides was used for the assay, the T790M-5-stimulated T cell lines responded only to T790M-5, but not to WT-5. This finding suggested that the T cells established by stimulation with the mutant peptide T790M-5 possessed a substantial, but weaker (around 100 times less) cross-reactivity to the corresponding wild-type peptide. Similarly, the T cell lines established by T790M-5 stimulation possessed an apparent, but weaker (around 100 times less) cross-reactivity to the T790M-8 peptide, which has one additional amino acid extension at the N-terminal of T790M-5 (Figure 4). We did not examine the cross-reactivity of T790M-7-stimulated T cell lines, since the corresponding wild-type peptide, WT-7, could not bind to HLA-A2 (Table 1).

Immunogenicity of the wild type HLA-A2-binding peptides in T cells from HLA-A2$^+$ normal donors

To examine the immunogenicity of the wild type HLA-A2-binding peptides, PBMCs from 6 different HLA-A2$^+$ healthy donors were repeatedly stimulated with the synthetic peptides, WT-5 and WT-7. As shown in Figure 5A, after repeated stimulation, a T cell line secreting IFN-γ in response to WT-5 could be established in 1 of 6 (17%) healthy donors. However, none of the 6 donors showed antigen-specific T cell responses to WT-7 (data not shown), because this peptide could not bind to HLA-A2 (Table 1). When the WT-5-stimulated T cell line was stimulated by a higher dose (1 μg/ml) of WT-5 peptide for ELISPOT assay, it showed a strong antigen-specific response. In contrast, when a lower dose (10 ng/ml) of WT-5 peptide was used for the assay, the T cell line showed a much weaker response

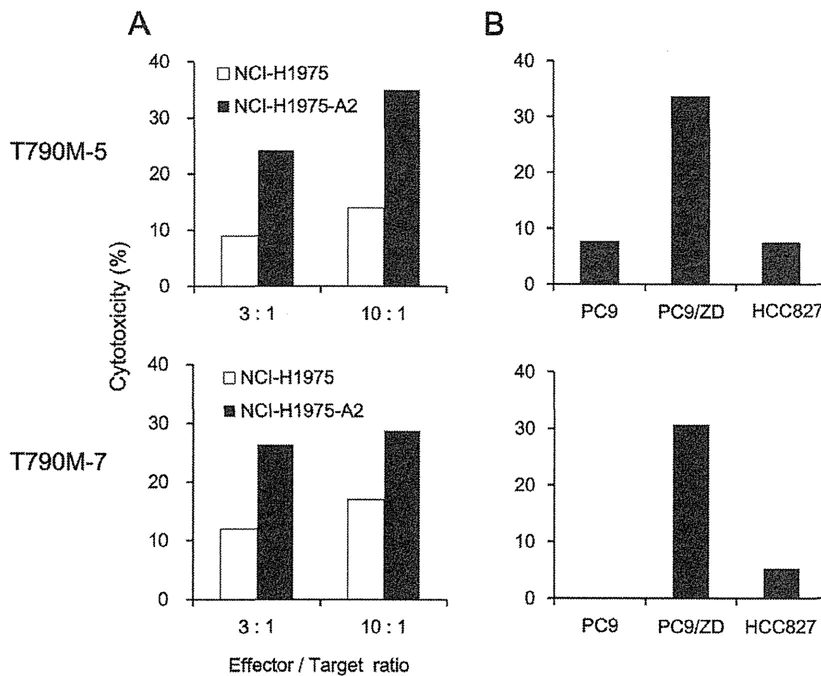

Figure 3. Cytotoxicity of the T790M-5- or T790M-7-stimulated T cells against NSCLC cells harboring EGFR T790M mutation. (A) The T790M-5- or T790M-7-stimulated PBMCs were examined for their cytotoxicity against the NSCLC cell lines (2×10^3 cells/well), NCI-H1975 (HLA-A2$^-$ T790M$^+$) or NCI-H1975-A2 (HLA-A2$^+$ T790M$^+$), at the indicated effector/target ratios. (B) The T790M-5- or T790M-7-stimulated PBMCs (1×10^4 cells/well) were examined for their cytotoxicity against the NSCLC cell lines (2×10^3 cells/well), PC9 (HLA-A2$^+$ T790M$^-$), PC9/ZD (HLA-A2$^+$ T790M$^+$) or HCC827 (HLA-A2$^-$ T790M$^-$), at an effector/target ratio of 5:1. The assays were carried out in duplicate, and the means of duplicate samples were used for calculation. The experiments were repeated with blood from three different donors, and a representative result is shown.
doi:10.1371/journal.pone.0078389.g003

(Figure 5A), suggesting that the avidity of T cell receptor (TCR) of WT-5-stimulated T cells might be low.

The WT-5-stimulated T cell line was then examined for their reactivity against NSCLC cell lines, including NCI-H1975 (HLA-A2$^-$ T790M$^+$), NCI-H1975-A2 (HLA-A2$^+$ T790M$^+$), HCC827 (HLA-A2$^-$ T790M$^-$), PC9 (HLA-A2$^+$ T790M$^-$), PC9/ZD (HLA-A2$^+$ T790M$^+$), 11–18 (HLA-A2$^+$ T790M$^-$), and YM-21 (HLA-A2$^+$ T790M$^-$), by IFN-γ ELISPOT assay. As shown in Figure 5B, the WT-5-specific T cells showed reactivity against none of HLA-A2$^-$ cell lines (HCC827 and NCI-H1975), HLA-A2$^+$ T790M$^+$ cell lines (NCI-H1975-A2 and PC9/ZD), or HLA-A2$^+$ T790M$^-$ cell lines (PC9, 11–18, and YM-21).

Immunogenicity of T790M-5 and T790M-7 epitopes in T cells from HLA-A2$^+$ NSCLC patients

We further investigated the immunogenicity of the T790M-5 and T790M-7 epitopes in 22 HLA-A2$^+$ NSCLC patients harboring the EGFR gene mutations in exon 19 (delE746-A750) or exon 21 (L858R), who had been sensitive ($n = 8$) or resistant ($n = 14$) to the EGFR-TKI treatment at the blood sampling (Table 2). Among the 8 patients who had been sensitive to EGFR-TKIs, 4 patients (50%) showed antigen-specific T cell responses to T790M-5 or T790M-7. In contrast, only 3 of 14 EGFR-TKI-resistant patients (21%) revealed positive T cell responses to these epitopes. Interestingly, the NSCLC patients with antigen-specific T cell responses to these epitopes showed a significantly less frequency of EGFR-T790M mutation than those without them [1 of 7 (14%) vs 9 of 15 (60%); chi-squared test, $p = 0.0449$], indicating the negative correlation between the immune responses to the EGFR-T790M-derived epitopes and presence of EGFR-T790M mutation in NSCLC patients. Of note, the T790M-5 and T790M-7-specific T cell lines established in these NSCLC patients were functional, since they showed substantial cytokine production or cytotoxicity against the NSCLC cell lines that harbored both the EGFR-T790M mutation and HLA-A2 (data not shown).

Discussion

The clinical benefit of EGFR-TKIs has been demonstrated in NSCLC patients with activating EGFR mutations [8–10], but most tumors develop acquired resistance via several different mechanisms, including the secondary T790M mutation that occurs in around 50% of patients with EGFR-TKI resistance [11–15]. However, there have been no effective treatment options for NSCLC patients with the secondary T790M resistance mutation. In the current study, we identified two HLA-A2-restricted cytotoxic T cell (CTL) epitopes containing the mutated methionine residue of the EGFR T790M mutation, T790M-5 and T790M-7. Given their high immunogenicity in human T cells, these epitopes might provide a novel immunotherapeutic approach for NSCLC patients with the T790M mutation. The limitation of the current study is that the T cell epitopes that we identified can be applicable only to NSCLC patients with HLA-A*0201. Although to increase the population coverage, we examined the immunogenicity of T cell epitopes restricted to other prevalent HLA-A types, including HLA-A*2402 and HLA-A*1101, we have not identified immunogenic epitopes restricted to these HLA-A types (data not shown). Nevertheless, HLA-A*0201 is one of the most prevalent HLA-A types in the world (http://

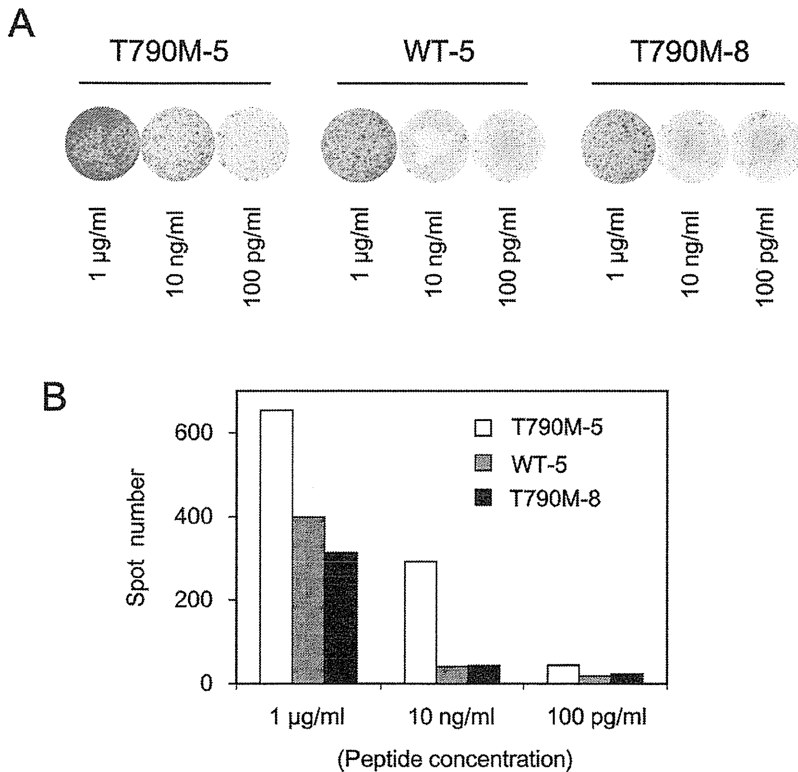

Figure 4. Cross-reactivity of T790M-5-stimulated T cells against the wild-type or T790M-8 peptide. The T790M-5 stimulated PBMCs (2×10^4 cells/well) were examined for reactivity against T2 cells (1×10^4 cells/well) pulsed with T790M-5, WT-5, or T790M-8 at the indicated concentrations by IFN-γ ELISPOT assay. The assays were carried out in duplicate wells. Representative wells (A) and the means of spot numbers from duplicate wells (B) are shown. The experiments were repeated with blood from two different donors, and a representative result is shown.
doi:10.1371/journal.pone.0078389.g004

www.pypop.org/popdata/2008/byfreq-A.php), and many of the peptides binding to HLA-A*0201 with high affinity have been shown to crossreact with other HLA-A2 supertype molecules, including HLA-A*0202, HLA-A*0203, HLA-A*0204, HLA-A*0205, HLA-A*0206, HLA-A*0207, HLA-A*6802, and HLA-A*6901 [42]. It would be of interest to determine whether the T790M-5 and T790M-7 epitopes can crossreact with other HLA-A2 supertype molecules.

For inducing effective anti-tumor immune responses, tumor antigens should be sufficiently immunogenic to stimulate

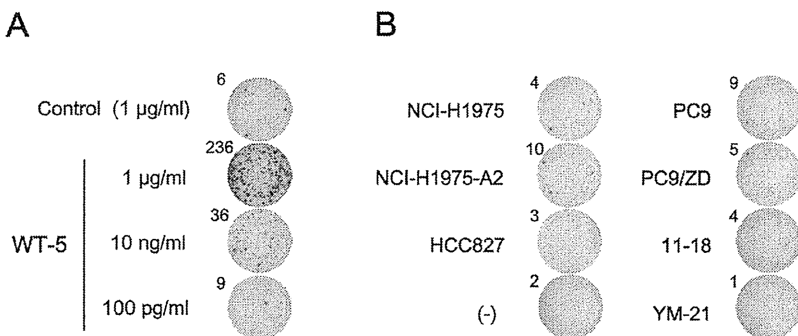

Figure 5. Reactivity of WT-5-stimulated T cells from an HLA-A2$^+$ healthy donor. (A) PBMCs from an HLA-A2$^+$ healthy donor were stimulated 5 times with WT-5 peptide (10 μg/ml) every 3 or 4 days. The WT-5-stimulated PBMCs (2×10^4 cells/well) were examined for reactivity against T2 cells (1×10^4 cells/well) pulsed with WT-5 or control HIV peptide at the indicated concentrations by IFN-γ ELISPOT assay. The assays were carried out in duplicate wells, and representative wells are shown. The numbers of spots are shown for each well. (B) The WT-5-stimulated PBMCs (2×10^4 cells/well) were examined for their reactivity against different NSCLC cell lines (1×10^4 cells/well), including NCI-H1975 (HLA-A2$^-$ T790M$^+$), NCI-H1975-A2 (HLA-A2$^+$ T790M$^+$), HCC827 (HLA-A2$^-$ T790M$^-$), PC9 (HLA-A2$^+$ T790M$^-$), PC9/ZD (HLA-A2$^+$ T790M$^+$), 11–18 (HLA-A2$^+$ T790M$^-$), and YM-21 (HLA-A2$^+$ T790M$^-$), by IFN-γ ELISPOT assay. The assays were carried out in duplicate wells, and representative wells with the spot numbers are shown. The data were from a single donor, since WT-5 was not immunogenic in 5 other healthy donors tested.
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Table 2. Immunogenicity of T790M-5 and T790M-7 peptides in NSCLC patients treated with EGFR-TKIs.

				EGFR-TKI						
Patient	Age	Sex	Activating EGFR mutation	Primary response[a]	Resistance	Treatment period (days)	Timing of sampling (days)[c]	T790M-5[d]	T790M-7[d]	T790M mutation[e]
EGFR-TKI-sensitive patients										
1	59	M	Exon 19	PR	(–)	296[b]	-	89	102	(–)
2	60	M	Exon 19	PR	(–)	153[b]	-	0	0	(–)
3	77	F	Exon 19	PR	(–)	199[b]	-	50	0	(–)
4	65	F	Exon 19	PR	(–)	209[b]	-	0	0	(+)
5	64	F	Exon 19	PR	(–)	209[b]	-	0	0	(+)
6	71	F	Exon 19	PR	(–)	491[b]	-	0	64	(–)
7	67	F	Exon 21	PR	(–)	188[b]	-	68	0	(–)
8	72	F	Exon 21	SD	(–)	429[b]	-	0	0	(–)
EGFR-TKI-resistant patients										
1	60	F	Exon 21	PD	(+)	71	0	0	0	(+)
2	60	M	Exon 21	PD	(+)	51	94	0	0	(–)
3	81	M	Exon 19	SD	(+)	67	0	0	0	(–)
4	78	F	Exon 19	PR	(+)	642	0	0	0	(+)
5	57	F	Exon 19	PR	(+)	802	4	0	0	(+)
6	70	F	Exon 19	PR	(+)	395	152	0	26	(–)
7	64	M	Exon 19	PR	(+)	315	118	0	0	(+)
8	81	F	Exon 19	PR	(+)	457	0	0	0	(+)
9	76	F	Exon 21	PR	(+)	642	0	0	0	(+)
10	79	F	Exon 21	PR	(+)	350	76	0	0	(–)
11	74	M	Exon 21	PR	(+)	678	77	0	0	(–)
12	68	F	Exon 21	PR	(+)	499	26	157	0	(+)
13	59	M	Exon 21	PR	(+)	233	33	367	0	(–)
14	59	M	Exon 21	PR	(+)	230	78	0	0	(+)

Abbreviations: M, male; F, female; PR, partial response; SD, stable disease; PD, progressive disease.
[a]Primary clinical responses to EGFR-TKIs.
[b]Treatment with EGFR-TKIs was ongoing in the EGFR-TKI-sensitive patients at the time of blood sampling.
[c]The period (days) between discontinuance of EGFR-TKI-treatment and blood sampling.
[d]Spot numbers ($/2\times10^4$ cells) by ELISPOT assay.
[e]T790M mutation examined by droplet digital PCR in cell-free DNA from plasma.
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antigen-specific T cells with high avidity. However, the T cell repertoires available for non-mutated "self-antigens" might have relatively low TCR avidity to them, since T cell reactivity against them can occur only when T cell tolerance is incomplete. In contrast, the "neo-antigens" derived from mutated amino acid sequences could be tumor-specific, and T cell repertoires with high TCR avidity to them might be available due to the lack of central and/or peripheral T cell tolerance [23,24]. Indeed, in the current study, 5 of 6 healthy donors (83%) showed T cell responses to the mutated epitope T790M-5, whereas only 1 of 6 (17%) donors responded to the corresponding wild type epitope WT-5, suggesting that the immunogenicity of the wild type epitope may be low. Since WT-5-specific T cells with higher TCR avidity may be deleted by central and/or peripheral tolerance, only those with low TCR avidity could be activated. In fact, the WT-5-stimulated T cells could not react to HLA-A2$^+$ NSCLC cells harboring wild-type EGFR. It may be possible that the TCR avidity of WT-5-stimulated T cells was too low to respond to the copy number of naturally processed WT-5 epitopes arrayed on HLA-A2$^+$ NSCLC cells harboring wild-type EGFR.

In T790M-5 and T790M-7, the threonine residue at the 1st and 3rd positions of wild type epitopes is replaced by the mutated methionine residue, respectively. Although the amino acid residues at the 2nd and C-terminal positions of epitopes have been known to act as the main anchoring sites in HLA-A*0201 [42], those at the 1st or 3rd positions of epitopes have also been reported to substantially affect the binding capability of peptides to HLA-A*0201 [43–47], which might modulate the immunogenicity [48]. Indeed, as shown in Table 1, the cell surface HLA class I stabilization assay demonstrated that T790M-5 and T790M-7 showed higher binding capability to HLA-A*0201 than the corresponding wild-type peptides, WT-5 and WT-7, respectively. In addition, side chains of amino acid residues at the 1st position of some epitopes have been suggested to directly contact with TCRs and affect the interaction between the peptides and TCRs [49]. Therefore, the higher immunogenicity of T790M-5 and T790M-7 epitopes could possibly be explained by these mechanisms.

Recent analyses on T cell reactivity in cancer patients showed that a significant fraction of T cells recognized mutated neo-antigens, but not non-mutated self-antigens, in human cancers [22,24]. In addition, T cell responses against mutated neo-antigens are expected not to show autoimmune toxicity against normal healthy tissues, suggesting that they might be a highly attractive target for immunotherapeutic manipulation. A large series of immunotherapy trials using non-mutated self-antigens demonstrated that clinical response rates were disappointingly low (around 3–5%) [19], but some studies have shown the potential benefit of targeting mutated neo-antigens [25–27]. For example, Sampson et al. demonstrated that peptide vaccines targeting EGFR variant III (EGFRvIII), which is a constitutively activated and immunogenic mutation not expressed in normal tissues but widely expressed in glioblastoma multiforme (GBM), were capable of inducing potent T- and B-cell immunity in GBM patients, and successfully eliminated tumor cells which expressed the targeted antigen, leading to an unexpectedly long survival time without any evidence of symptomatic collateral toxicity [25]. Weden et al. showed that a vaccine composed of synthetic long mutant K-RAS peptides induced long-term immune responses together with a potential clinical benefit in patients with pancreatic cancer after surgical resection [26]. Considering these feasible results of targeting mutated neo-antigens, cancer vaccines using the CTL epitopes derived from the T790M mutation, T790M-5 and T790M-7, might be promising for the treatment of NSCLC patients with this mutation. In particular, since the T790M mutation seems to be critical for acquired resistance to EGFR-TKIs [11–15], the selection of antigen loss variants as immune escape might be avoided during immunotherapy and tumor evolution.

Interestingly, the current study showed that the NSCLC patients with antigen-specific T cell responses to EGFR-T790M-derived epitopes showed a significantly less frequency of EGFR-T790M mutation than those without them, indicating the negative correlation between the immune responses to the EGFR-T790M-derived epitopes and presence of EGFR-T790M mutation in NSCLC patients. This finding could possibly be explained by the hypothesis that immune responses to the mutated neo-antigens derived from T790M might prevent the emergence of tumor cell variants with the T790M resistance mutation in NSCLC patients during EGFR-TKI treatment. Alternatively, it may be possible that the presence/occurrence of EGFR-T790M inhibits immune responses to the EGFR-T790M-derived epitopes. To further clarify the causal relationship between immune responses to the T790M-derived neo-antigens and the presence/occurrence of EGFR-T790M in NSCLC patients, both antigen-specific immune responses and T790M mutation status remain to be monitored at different time points (before, during, and after EGFR-TKI treatment) in the same patients in future study.

In the current study, T cells stimulated with T790M-5 showed cross-reactivity against the corresponding wild-type WT-5 peptide. However, it should be noted that the avidity of TCR to the WT-5 peptide in the T790M-5-stimulated T cells seemed to be around 100-times less than that to the T790M-5 peptide. Indeed, the T790M-5-stimulated T cells reacted to the PC9/ZD cells harboring the EGFR-T790M mutation, but not to the original PC9 cells expressing EGFR without this mutation. Based on this finding, the cross-reactivity of the T790M-5-stimulated T cells might not be high enough to induce autoimmune toxicity against normal tissues expressing wild-type EGFR.

In summary, we identified two novel HLA-A2-restricted, T790M-derived epitopes, which were highly immunogenic for human T cells. Our findings suggested that recognition of T790M-derived neo-antigens can occur in healthy donors and cancer patients, even in the absence of immunotherapy. Although more studies would be required to investigate whether immune responses to the identified epitopes are associated with clinical benefits and can be selectively enhanced by immunotherapies, the current study could provide important information on individualized immunotherapy for NSCLC patients.

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Author Contributions

Conceived and designed the experiments: TS KA. Performed the experiments: TY EM JK GLZ SM YK-K. Analyzed the data: TY EM YY TY KI TS. Contributed reagents/materials/analysis tools: KA SS KN TH. Wrote the paper: TS.

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Proof Only

Prostaglandin receptor EP3 mediates growth inhibitory effect of aspirin through androgen receptor and contributes to castration resistance in prostate cancer cells

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Abstract

Although numerous epidemiological studies show aspirin to reduce risk of prostate cancer, the mechanism of this effect is unclear. Here, we first confirmed that aspirin downregulated androgen receptor (AR) and prostate-specific antigen in prostate cancer cells. We also found Q1 that aspirin upregulated prostaglandin receptor subtype EP3 but not EP2 or EP4. The EP3 antagonist L798106 and EP3 knockdown increased AR expression and cell proliferation, whereas the EP3 agonist sulprostone decreased them, indicating that EP3 affects AR Q2 expression. Additionally, *EP3* (*PTGER3*) transcript levels were significantly decreased in human prostate cancer tissues compared with those in normal human prostate tissues, suggesting that EP3 is important to prostate carcinogenesis. Decreased EP3 expression was also seen in castration-resistant subtype CxR cells compared with parental LNCaP cells. Finally, we found that aspirin and EP3 modulators affected prostate cancer cell growth. Taken together, aspirin suppressed LNCaP cell proliferation via EP3 signaling activation; EP3 downregulation contributed to prostate carcinogenesis and to progression from androgen-dependent prostate cancer to castration-resistant prostate cancer by regulating AR expression. In conclusion, cyclooxygenases and EP3 may represent attractive therapeutic molecular targets in androgen-dependent prostate cancer.

Key Words

- androgen receptor
- aspirin
- castration-resistant prostate cancer
- cyclooxygenase
- EP3
- prostate cancer

Endocrine-Related Cancer
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Introduction

Prostate cancer is the most commonly diagnosed non-skin cancer among men in developed countries (Jemal *et al.* 2008). In its early stages, prostate cancer cell growth is androgen dependent, and androgen deprivation therapies cause prostate tumor regression. Unfortunately, the majority of prostate cancers eventually transit to castration-resistant prostate cancer (CRPC). Androgen receptor (AR) plays a vital role in cell growth and survival of both androgen-dependent prostate cancer and CRPC. Especially, in CRPC, AR is thought to be inappropriately activated under the condition of castration levels of androgens (Kung & Evans 2009); AR inhibition represses tumor growth in both androgen-dependent prostate cancer and CRPC (Chen *et al.* 2003, Scher & Sawyers 2005).

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Inflammation by various insults is thought to be a major cause and promoter of various cancers including prostate cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases (COXs) and suppress prostaglandin (PG) synthesis, thus fighting inflammation (Majima *et al.* 2003, Wang & DuBois 2010). NSAIDs have been reported to reduce risk of developing some solid tumors, including breast, colon, lung, and prostate cancer (Harris 2009), as well as exert antitumor effects. The COX2 inhibitor celecoxib decreased patients' rising prostate-specific antigen (PSA) rates after radical therapies (Pruthi *et al.* 2004, Smith *et al.* 2006). NSAIDs may suppress prostate cancer incidence and tumor development by suppressing *AR* transcription and promoting apoptosis in prostate cancer cells (Lim *et al.* 1999, 2003, Pan *et al.* 2003). However, the precise mechanism of NSAIDs' preventative and therapeutic effects is not well understood.

COXs have two distinct isoforms: COX1 and COX2. COX1 is expressed constitutively in several tissues, whereas COX2 is induced by cytokines, mitogens, and tumor promoters (Katori & Majima 2000, Gupta & DuBois 2001, Subbaramaiah & Dannenberg 2003), resulting in enhanced synthesis of PGs in inflamed and neoplastic tissues (Sharon *et al.* 1978, Bennett 1986, Rigas *et al.* 1993). The expression of COX2 is higher in prostate cancer tissues than in benign prostate tissues (Gupta *et al.* 2000, Yoshimura *et al.* 2000) and increases as differentiation attenuates (Shappell *et al.* 2001). PGE_2 produced by COXs is reportedly crucial to angiogenesis and oncogenesis in prostate cancer (Jain *et al.* 2008). Consistent with the finding of COX2 overexpression in prostate cancer, PGE_2 content is also almost tenfold higher in malignant prostatic tissues than in benign prostatic tissues (Chaudry *et al.* 1994). PGE_2 acts through four G-protein-coupled receptors: EP1, EP2, EP3, and EP4. EP2 and EP4 bind to stimulative G proteins and increase intracellular cAMP whereas EP3 couples to an inhibitory G protein and decreases cAMP. EP1 increases intracellular calcium (Cha & DuBois 2007). However, EP1 was not detectable in human prostate cancer cell lines (Wang & Klein 2005). On the other hand, EP3 expression was decreased compared with normal mucosa in colon cancer of mice, rats, and Q3 humans (Shoji *et al.* 2004). Furthermore, Ines *et al.* revealed that mouse EP3 reduced tumor cell proliferation and tumorigenesis *in vivo* (Macias-Perez *et al.* 2008) and contributed to growth inhibition or cellular senescence (Fulton *et al.* 2006). Taken together, these results suggest that EP3 signaling could be the basis of a novel anticancer therapy. However, the contribution of EP3 in prostate cancer is not well understood.

To establish the rationale of aspirin use in chemoprevention of prostate cancer, we investigated the mechanism of aspirin's effect on AR expression in prostate cancer and in preventing prostate transformation, using human prostate cancer cells and tissues. Furthermore, we tried to show the therapeutic effect of aspirin, as well as its downstream target on prostate cancer, including CRPC, for which effective therapeutics are limited.

Materials and methods

Cell culture

Human prostate cancer DU145 (MEM), LNCaP (RPMI-1640), and 22Rv1 (RPMI-1640) cells were purchased from the American Type Cell Collection (Manassas, VA, USA) and were cultured under media in parenthesis purchased from Invitrogen supplemented with 10% fetal bovine serum. LNCaP cells that propagated between 10 and 40 times were used. Castration-resistant derivatives of LNCaP cells (LNCaP-CxR cells, referred to as CxR cells) were established and maintained as described previously (Shiota *et al.* 2009*b*). All cell lines were maintained in a 5% CO_2 atmosphere at 37 °C.

Antibodies and drugs

Antibody against AR (sc-815) was purchased from Santa Cruz Biotechnology. Anti-EP2, anti-EP3, and anti-EP4 antibodies, sulprostone and PGE_2, were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti β-actin and anti-PSA antibodies were purchased from Sigma and Epitomics, Inc. (Burlingame, CA, USA) respectively; L798106 was purchased from Sigma.

RNA isolation and RT

Total RNA was prepared from cultured cells using an RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1.0 μg total RNA using a Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol.

Quantitative real-time PCR

Synthesized cDNA was diluted to 1:2 ratio; 2.0 μl of the diluted sample was used. TissueScan Prostate Cancer Tissue qPCR array III (HPRT303) was purchased from OriGene (Rockville, MD, USA). This product contains first-strand cDNAs prepared from 48 human prostate tissues,

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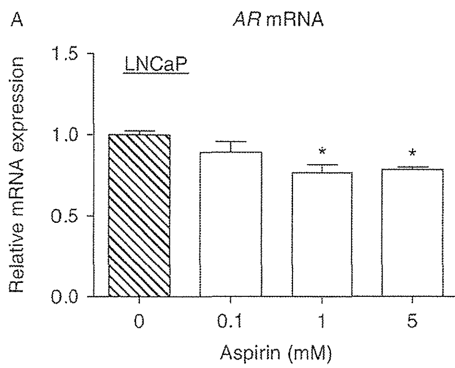
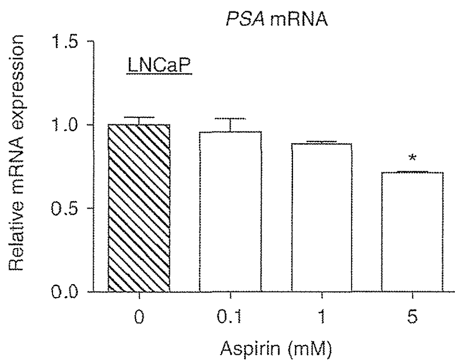
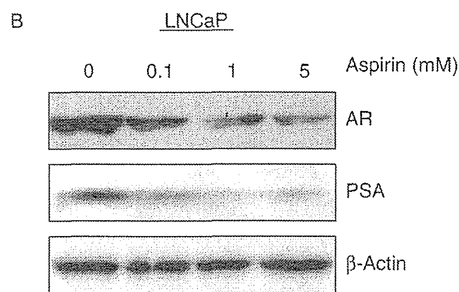
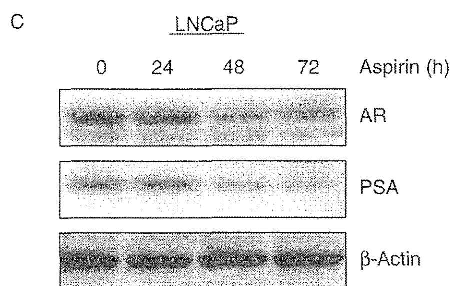
including both malignant and healthy controls. These 48 cDNAs had been normalized against β-actin by RT-PCR Q4 and arrayed onto PCR plates. Quantitative real-time PCR with TaqMan Gene Expression Assay (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) was performed using an ABI 7900HT System; *GAPDH* values were used for normalization. Results are representative of at least three independent experiments.









Western blot analysis

Western blot analysis was performed as described previously (Kashiwagi *et al.* 2010). To prepare whole cell lysates, cells were sonicated for 20 s with buffer-Y. Whole cell lysates (30 μg) were separated by SDS–PAGE and transferred onto PVDF membranes. Western blot analysis was performed using appropriately diluted antibodies. The membrane was developed using a chemiluminescence protocol (GE Healthcare, Waukesha, WI, USA). Images were obtained using an image analyzer (LAS-3000 Mini; Fujifilm, Tokyo, Japan).

Knockdown analysis using siRNAs

Knockdown analysis using siRNAs was performed as described previously (Kashiwagi *et al.* 2010). The following double-stranded RNA 25 bp oligonucleotides were commercially generated (Invitrogen): *EP3* (*PTGER3*) siRNA: siEP3 #2, 5′-CGAACAGCUAUUAAGAAGAAGUUGC-3′ (sense) and 5′-GCAACUUCUUCUUAAUAGCUGUUCG-3′ (antisense); siEP3 #3, 5′-UUAACAGCAGGUAAACCCAAGGAUC-3′ (sense) and 5′-GAUCCUUGGGUUUACCUGCUGUUAA-3′ (antisense). LNCaP cells were transfected with various amounts of the siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cytotoxicity analysis

Cytotoxicity analysis was performed as described previously (Kashiwagi *et al.* 2010). LNCaP and CxR cells

Q8 **Figure 1**
Aspirin reduces *AR* and *PSA* expression in LNCaP and CxR cells. (A) LNCaP cells were cultured with the indicated concentrations of aspirin for 24 h. The mRNA levels of *AR* and *PSA* were analyzed using quantitative real-time PCR. All values represent means of at least three independent experiments. Boxes, mean; bars, ±s.d. *$P<0.05$ (compared to untreated LNCaP cells). (B) LNCaP cells were cultured with indicated concentrations of aspirin for 48 h; cell lysates (30 μg) were analyzed for AR and PSA using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (C) LNCaP cells were cultured with 1 mM aspirin for the indicated times (in hours); cell lysates (30 μg) were analyzed for AR and PSA using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control.

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(2×10^3) were seeded into 96-well plates. The following day, various concentrations of aspirin were applied in medium. After 48 h, the surviving cells were stained with the Alamar Blue assay (TREK Diagnostic Systems, Cleveland, OH, USA) for 180 min at 37 °C. Absorbance of the wells was measured using a plate reader (ARVO MX; Perkin Elmer, Inc., Waltham, MA, USA).

Cell proliferation assay

Cell proliferation assay was performed as described previously (Shiota *et al.* 2008, 2009*a*). Briefly, LNCaP cells (2.5×10^4) were seeded into 12-well plates and transfected with the indicated siRNA or added indicated drugs under androgen-deprived conditions. After 96 h, the cells were harvested with trypsin and counted using a cell counter (Beckman Coulter, Fullerton, CA, USA). The results were normalized by cells transfected with control siRNA or treated with vehicle at 96 h and are representative of at least three independent experiments.

Statistical analysis

The Mann–Whitney *U* test was used for statistical analysis. Significance was set at $P\leq0.05$.

Results

Aspirin reduces AR and PSA expression in prostate cancer cells

Q5

To confirm aspirin's effects on AR expression, we examined mRNA and protein levels of AR in LNCaP cells after aspirin treatment. Both *AR* and *PSA* mRNA, and both AR and PSA protein levels, were decreased by aspirin treatment in a dose-dependent manner (Fig. 1A and B respectively). Aspirin (1 mM) also decreased AR and PSA protein in a time-dependent manner (Fig. 1C).

PGE_2 does not induce AR expression in prostate cancer cells

As aspirin – a COX inhibitor – decreases PGE_2 levels, we supposed that aspirin might regulate AR expression through PGE_2. However, when we examined AR expression after exposure to PGE_2, we found that PGE_2 did not affect AR mRNA or protein levels in LNCaP cells (Fig. 2A and B).

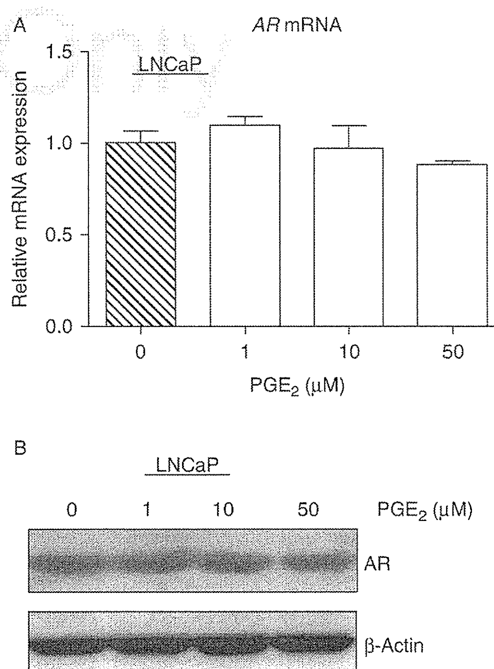

Figure 2
PGE_2 does not induce *AR* expression in LNCaP and CxR cells. (A) LNCaP cells were cultured with indicated concentrations of PGE_2 in serum-free medium for 24 h. The mRNA level of *AR* was analyzed using quantitative real-time PCR. All values represent means of at least three independent experiments. Boxes, mean; bars, ±s.d. $*P<0.05$ (compared to untreated LNCaP cells in serum-free medium). (B) LNCaP cells were cultured with indicated concentrations of PGE_2 in serum-free medium for 48 h; cell lysates (30 μg) were analyzed for AR using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control.

Q9

Aspirin induces EP3 expression in prostate cancer cells

Based on the results mentioned earlier, we hypothesized that COX-independent pathway regulates AR expression in prostate cancer cells and speculated that aspirin may affect expression of PG receptors. We then examined effects of aspirin on PG receptor subtypes, EP2, EP3, and EP4. Surprisingly, aspirin reduced EP2 expression and induced EP3 expression but not EP4 in dose- and time-dependent manners in LNCaP cells (Fig. 3A and B). We also examined aspirin's effect on expressions of AR and PG receptors using another androgen-sensitive prostate cancer cell line 22Rv1 cells. Expectedly, aspirin induced EP3 expression concurrently with AR suppression, similar to LNCaP cells; however, EP2 expression was only marginally reduced by aspirin treatment in 22Rv1 cells (Fig. 3C). Consistently, AR transactivity represented by *PSA* transcription in 22Rv1 cells was also decreased by aspirin treatment in a dose-dependent manner (data not shown).

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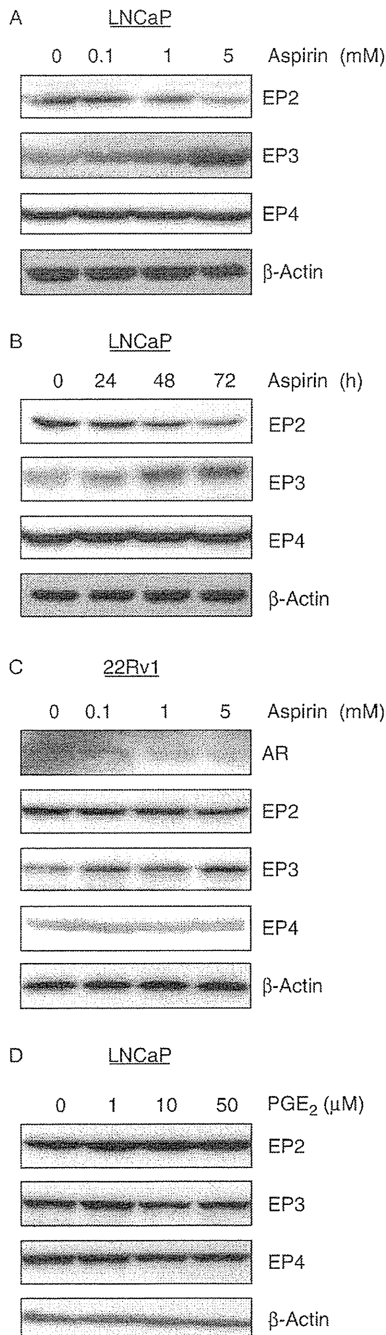
Furthermore, EP3 expression was similarly induced also in AR-null DU145 cells. Expression of PG receptors in LNCaP cells was not influenced by exposure to PGE_2 (Fig. 3D), indicating that PGE_2 did not affect PG receptor expression as well as AR did. These findings indicate that aspirin affects AR expression through PG receptor expression, but not through PGE_2. Because aspirin treatment commonly increased EP3 expression in two prostate cancer cell lines, LNCaP and 22Rv1, we thereafter focused on EP3 as a regulator of AR expression, but not EP2 and EP4.



EP3 regulates AR expression in prostate cancer cells

We initially investigated the effects of EP3 on AR expression using pharmacological manipulation. The EP3 agonist sulprostone suppressed *AR* mRNA and AR and PSA protein expressions in LNCaP cells (Fig. 4A and B). Inversely, the EP3 antagonist L798106 increased *AR* mRNA (Fig. 4C) and AR and PSA protein expressions (Fig. 4D). We then employed a knockdown method using EP3-specific siRNAs, which successfully suppressed EP3 expression and upregulated AR expression (Fig. 4E). Thus, it was confirmed that EP3 negatively regulates AR expression, using both pharmacological and knockdown methods.

EP3 expression decreases in clinical samples of prostate cancer and in castration-resistant cells

Because EP3 expression was negatively correlated with AR expression, which is known to promote prostate cancer development and progression, we hypothesized that EP3 is decreased in prostate cancer samples. Therefore, we examined EP3 expression in normal prostate tissues and prostate cancer tissues using a prostate cancer tissue array containing cDNAs derived from 9 normal prostate tissues and 39 prostate cancer tissues and real-time quantitative PCR. EP3 expression was remarkably downregulated in prostate cancer compared with normal prostate tissues (Fig. 5A), suggesting that EP3 downregulation may contribute to cancerous changes in prostate tissues.

Next, because AR expression is closely implicated in prostate cancer progression to CRPC, we examined

Figure 3
Aspirin induces EP3 expression in LNCaP and 22Rv1 cells. (A) LNCaP cells were cultured with indicated concentrations of aspirin for 48 h; cell lysates (30 μg) were analyzed for EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (B) LNCaP cells were cultured with 1 mM aspirin for indicated times; cell lysates (30 μg) were analyzed for EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (C) 22Rv1 cells were cultured with indicated concentrations of aspirin for 48 h; cell lysates (30 μg) were analyzed for AR, EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (D) LNCaP cells were cultured with indicated concentration of PGE_2 in serum-free medium for 48 h; cell lysates (30 μg) were analyzed for EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control.

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expressions of PG receptors in CPRC using castration-resistant LNCaP derivative CxR cells (29, 30). EP3 expression was downregulated concurrently with upregulation of AR in CxR cells compared with LNCaP cells (Fig. 5B); however, EP2 and EP4 expressions were upregulated, suggesting that EP3 downregulation is also involved in prostate cancer progression to CRPC. Accordingly, neither EP3 agonist sulprostone nor EP3 antagonist L798106 affected AR expression at both mRNA and protein levels (Fig. 5C and D), indicating an abolished EP3 signaling in CRPC.

EP3 signaling modulates cell growth of prostate cancer

Based on the result mentioned earlier, we hypothesized that proliferation of prostate cancer under castration

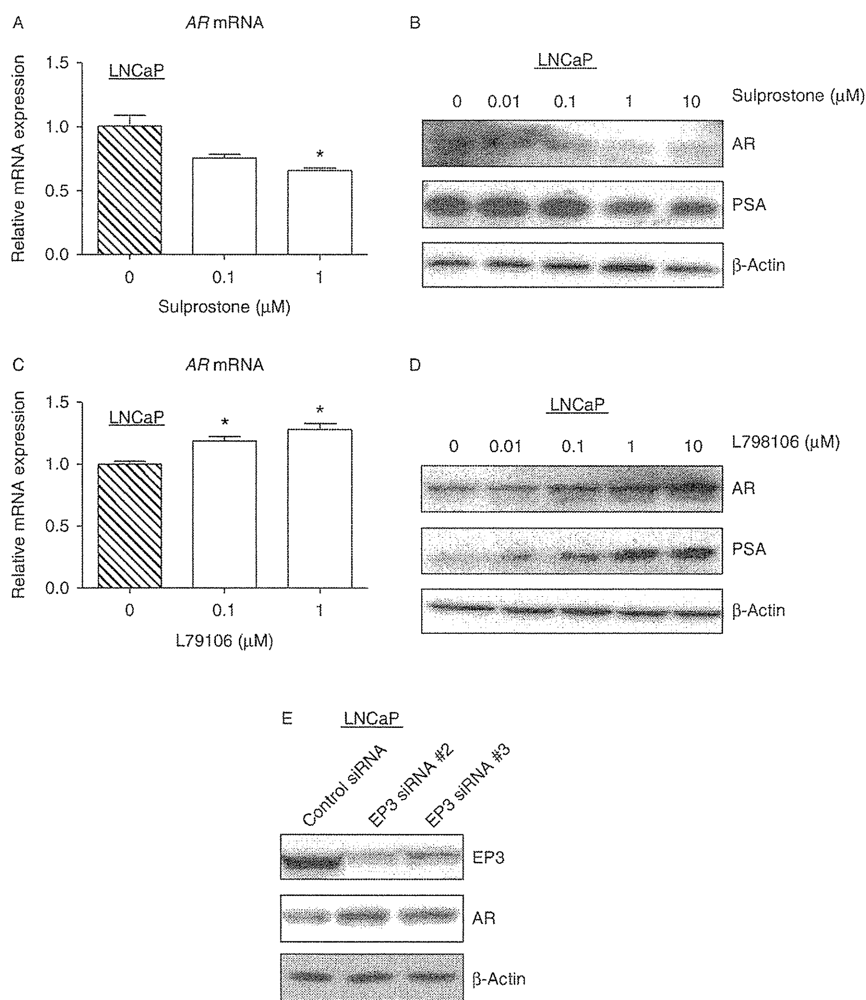

Figure 4

The EP3 agonist sulprostone decreases AR expression and the EP3 antagonist L798106 increases AR expression in LNCaP cells. (A) LNCaP cells were cultured with indicated concentrations of sulprostone for 24 h. The mRNA level of *AR* was analyzed using quantitative real-time PCR. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±S.D. *$P<0.05$ (compared to untreated LNCaP cells). (B) LNCaP cells were cultured with indicated concentrations of sulprostone for 48 h; cell lysates (30 μg) were analyzed for AR and PSA using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (C) LNCaP cells were cultured with indicated concentrations of L798106 for 24 h. The mRNA level of *AR* was analyzed using quantitative real-time PCR. All values represent means of at least three independent experiments. Boxes, mean; bars, ±S.D. *$P<0.05$ (compared to untreated LNCaP cells). (D) LNCaP cells were cultured with indicated concentrations of L798106 for 48 h; cell lysates (30 μg) were analyzed for AR and PSA using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (E) LNCaP cells were transfected with 40 nM of control siRNA, *EP3* siRNA #2, or *EP3* siRNA #3. At 72 h after transfection, whole cell extracts (30 μg) were analyzed for EP3 and AR using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control.

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conditions is affected by EP3 signaling. We therefore examined LNCaP cell proliferation under castration conditions after modulating EP3 signaling by pharmacological and knockdown methods. Surprisingly, the EP3 agonist sulprostone suppressed LNCaP cell growth under an androgen deprivation medium in a dose-dependent manner (Fig. 6A), while the EP3 antagonist L798106 increased (Fig. 6B). In addition, EP3 knockdown accelerated LNCaP cell growth in an androgen deprivation medium after 96 h (Fig. 6C). Thus, these data indicate that EP3 signaling is a critical regulator of prostate cancer growth under androgen-deprived conditions.

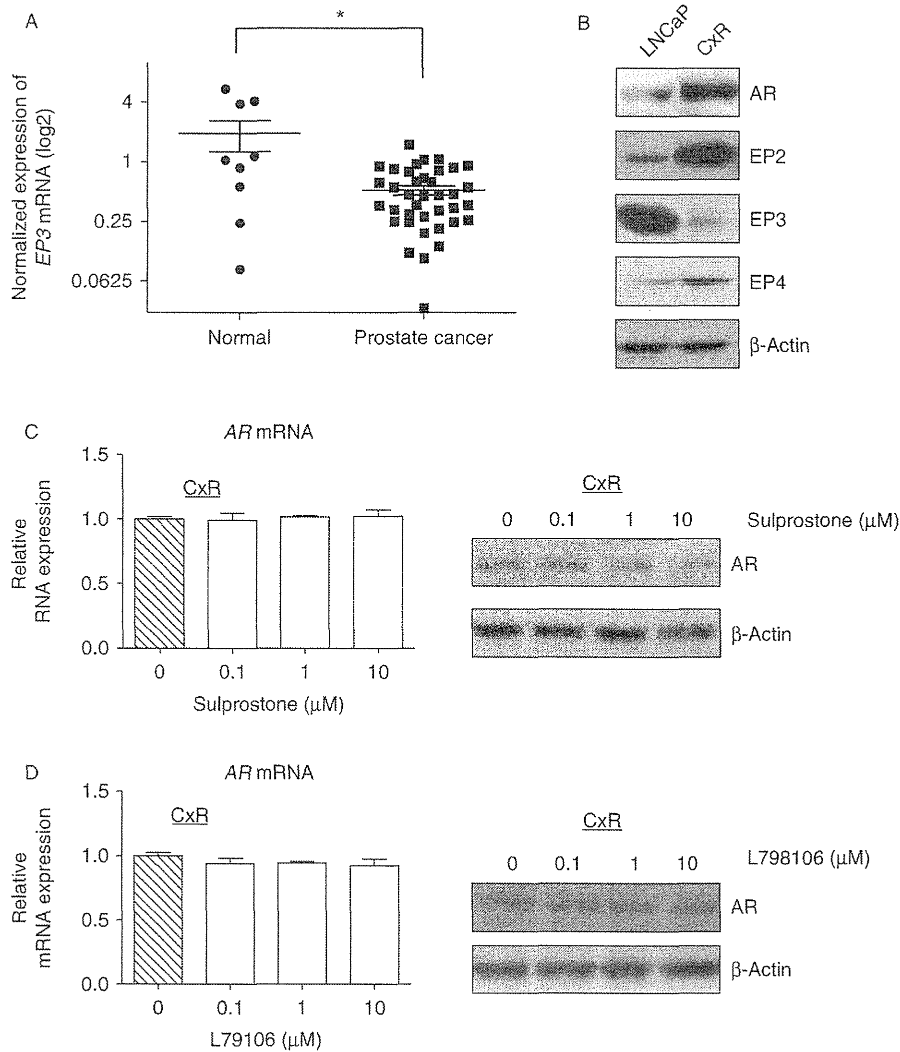

Figure 5

EP3 expression in prostate cancer samples and CxR cells. (A) Expression of *EP3* mRNA in normal prostate and prostate cancer was determined. The log_2 of normalized (to β-actin) expression values relative to the mean expression of the normal is presented in normal prostate vs prostate cancer. The line in the middle represents the median; bars, ±s.d. *$P<0.05$ (compared to normal prostate). (B) Whole cell extracts (30 μg) from LNCaP and CxR cells were analyzed for AR, EP2, EP3, and EP4 using SDS–PAGE and western blot analysis with specific antibodies. The anti-β-actin antibody was used as a loading control. Dividing lines were used. (C) CxR cells were cultured with indicated concentrations of sulprostone for 24 h. The mRNA level of *AR* was analyzed using quantitative real-time PCR. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. Cell lysates (30 μg) were analyzed for AR using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control. (D) CxR cells were cultured with indicated concentrations of L798106 for 24 h. The mRNA level of *AR* was analyzed using quantitative real-time PCR. All values represent means of at least three independent experiments. Boxes, mean; bars, ±s.d. Cell lysates (30 μg) were analyzed for AR using SDS–PAGE and western blotting with specific antibodies. β-Actin was used as a loading control.

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Next, we examined aspirin's effect on the viability of prostate cancer cells. Expectedly, aspirin treatment suppressed LNCaP cell viability. However, surprisingly, aspirin had less effect on the viability of CxR cells (Fig. 6D). This discrepancy between LNCaP and CxR cells may result from downregulated EP3 expression in CxR cells (Fig. 5B), suggesting that aspirin does not exert therapeutic effects after progression to CRPC.

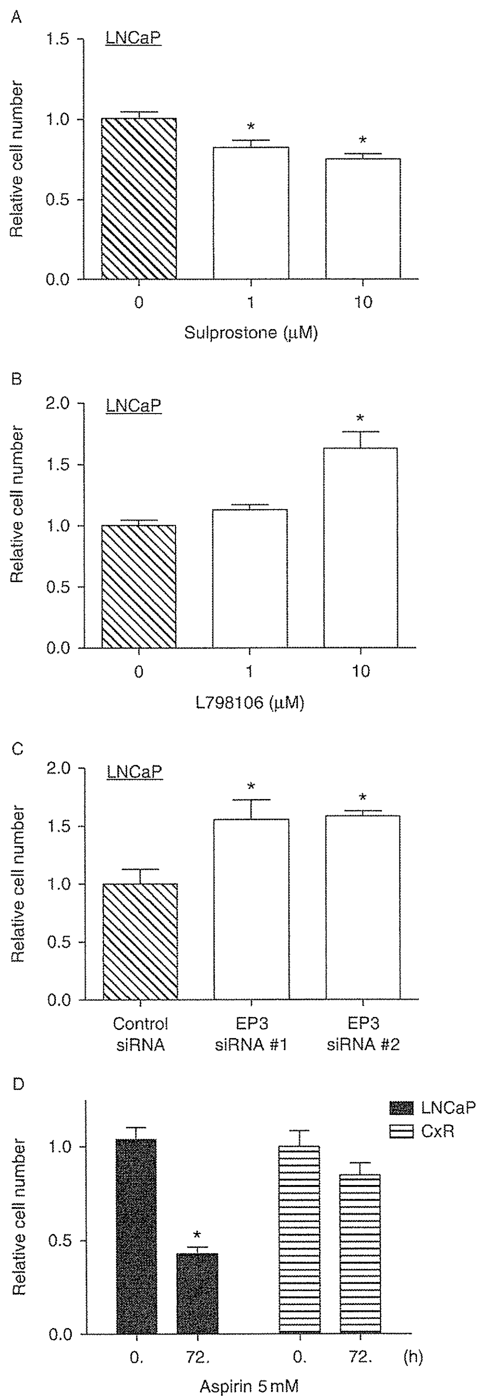

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Figure 6
Intracellular signaling via EP3 receptor is a key to cell growth in androgen-deprived condition. (A) LNCaP cells were cultured with sulprostone in a charcoal-stripped medium. After 72 h, the cell numbers were counted. The relative number of untreated LNCaP cells was set as 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ±S.D. *$P<0.05$ (compared to untreated LNCaP cells). (B) LNCaP cells were cultured with L798106 in a charcoal-stripped medium. After 72 h, the cells numbers were counted. The relative number of untreated LNCaP cells was set as 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ±S.D. *$P<0.05$ (compared with untreated LNCaP cells). (C) LNCaP cells were transfected with 40 μM of control siRNA, *EP3* siRNA #2, or *EP3* siRNA #3 and cultured in a charcoal-stripped medium. After 96 h, the cell numbers were counted. The relative number of LNCaP cells transfected with control siRNA was set as 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ±S.D. *$P<0.05$ (compared to LNCaP cells transfected with control siRNA). (D) LNCaP and CxR cells (2×10^3) were seeded into 96-well plates. The following day, 5 mM aspirin were applied in the medium. After 48 h, the surviving cells were stained with the Alamar Blue assay. Cell survival in the absence of drugs corresponded to 100%. All values represent means of at least three independent experiments.

Discussion

NSAIDs are thought to exert anticancer activities through both COX-dependent and -independent pathways. COX2 has been shown to affect cancer development by promoting cell division, inhibiting apoptosis, stimulating neoangiogenesis, and altering cell adhesion (Grösch *et al.* 2006). Inversely, inhibition of COX2 activity is supposed to block these activities and exert tumor-preventative effects. Thus, through COX-dependent pathway, NSAIDs can show anticancer effects. Conversely, a selective COX2 inhibitor, celecoxib, was shown to target COX-independent pathway such as Ca^{2+} ATPase, protein-dependent kinase 1, and cyclin-dependent kinases, resulting in inhibitions of antiapoptosis cell cycle progression, angiogenesis, and metastasis (Hwang *et al.* 2002, Grösch *et al.* 2006). Furthermore, NSAIDs inhibited cell growth of COX2-null colorectal cancer (Chan *et al.* 1998), indicating a COX2-independent anticancer effect of NSAIDs. Similarly, in prostate cancer cells, Patel *et al.* (2005) demonstrated an anticancer activity by a COX2 inhibitor through both COX-dependent and -independent pathway. Previous demonstrations that NSAIDs block LNCaP cell proliferation despite low levels of COX2 protein in LNCaP cells (Hsu *et al.* 2000, Tanji *et al.* 2000,

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Fujita *et al.* 2002) also imply COX-independent anticancer effects of NSAIDs. Taken together, these results show that NSAIDs have anticancer effects through a COX-independent pathway.

In this report, we confirmed that aspirin suppressed AR expression at a transcript level (Lim *et al.* 1999, Pan *et al.* 2003). We had speculated that aspirin regulates AR expression through PGE_2, as NSAIDs suppress PGE_2 synthesis by inhibiting COX. However, actually, PGE_2 did not affect AR mRNA or protein levels, suggesting that aspirin regulates AR expression in a COX-independent pathway. A previous report showed that a classical COX inhibitor, indomethacin, affected PG receptor expression (Chang *et al.* 2004). Consistently, we showed that aspirin upregulates EP3 expression, leading to an anticancer effect. Cumulatively, and in line with previous reports that NSAIDs' anticancer effect comes through a COX-independent pathway, this study showed that aspirin regulates AR expression in a COX-independent manner by modulating EP3 expression.

We have shown, for the first time, that EP3 signaling regulates AR expression, although its mechanism is still unknown. EP3 is a G-protein-coupled receptor, and a negative regulator of cAMP, which has been shown to positively regulate *AR* transcription (Mizokami *et al.* 1994). Therefore, EP3 may negatively regulate AR expression by modulating cAMP concentration. Alternatively, NF-κB might mediate AR suppression through EP3 because EP3 signaling is known to inactivate the NF-κB pathway (Wang *et al.* 2010), which, in turn, positively regulates AR expression (Zhang *et al.* 2009). Taken together, these findings suggest that EP3 signaling negatively regulates AR expression through downstream pathways in prostate cancer cells.

In cancers other than prostate cancer, EP3 is recognized as a negative key mediator of cancer progression (Shoji *et al.* 2004, Fulton *et al.* 2006, Macias-Perez *et al.* 2008). Consistent with these previous findings, this study as well as recent another study showed that EP3 was suppressed in prostate cancer samples compared with normal prostate samples (Huang *et al.* 2013) and in castration-resistant cells compared with androgen-dependent cells. Inversely, when EP3 signaling was suppressed, LNCaP cells kept proliferating in androgen-deprived conditions, similar to castration-resistant cells. Taken together, these results imply that EP3 downregulation contributes to cancer development and progression to castration resistance through AR overexpression (Fig. 7). We have also shown that a EP3 agonist downregulated AR expression and suppressed prostate cancer cell growth,

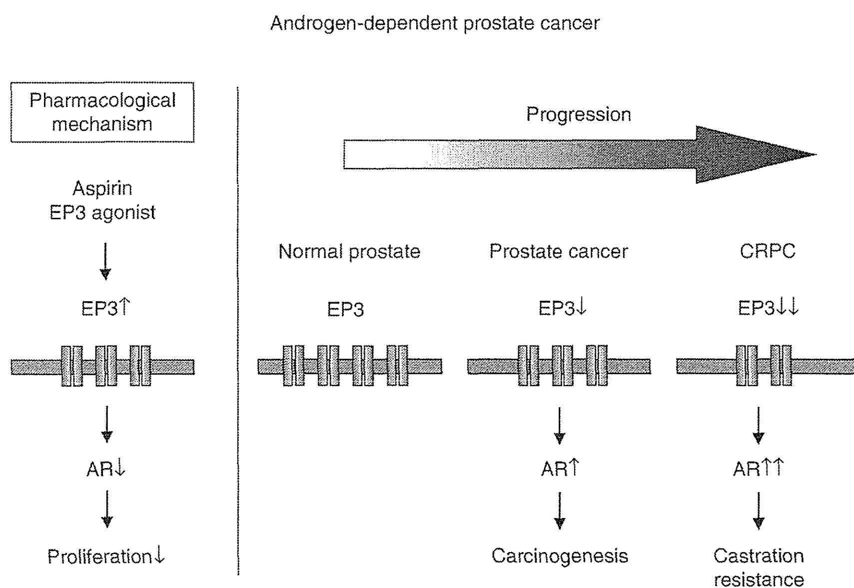

Figure 7
Schematic representation of the relationship between EP3 and AR expression. (Left) NSAIDs or EP3 agonist activates EP3 signaling and suppresses AR expression, thus contributing to suppression of cancer cell proliferation. (Right) Progression from normal to prostate cancer or to castration-resistant prostate cancer; EP3 expression gradually decreases, while AR expression is upregulated. As a result, cancer proliferation is potentiated.

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indicating that activation of EP3 signaling may offer a novel therapy against androgen-dependent prostate cancer, especially when combined with androgen deprivation therapy. This is supported by previous reports that EP3 agonists suppressed cell growth in colorectal cancer (Shoji *et al.* 2004) and hepatocellular carcinoma (Cusimano *et al.* 2009) and vasculogenesis in inflammatory breast cancer (Robertson *et al.* 2010). COX2 selective inhibitors were expected to act as a 'super aspirin' that would not exert the adverse effects typical of classical NSAIDs (DeWitt 1999, Majima *et al.* 2003). However, in some organs such as kidneys, COX2 is expressed constitutively (Okumura *et al.* 2002) and is necessary for the kidney to mature after birth (Norwood *et al.* 2000), suggesting that even 'super aspirin' can cause adverse effects. Thus, selective activation of EP3 receptor signaling may be a more promising treatment than COX2 inhibition.

In conclusion, aspirin represses AR expression by Q11 upregulating EP3 expression in prostate cancer cells and exerts preventative and anticancer effects in prostate cancer by downregulating AR expression. Modulated EP3 signaling affected both AR expression and prostate cancer growth under androgen deprivation conditions. Taken together, this study showed a rationale for use of NSAID aspirin in preventing prostate cancer. We also identified a potential treatment for androgen-dependent prostate cancer in combining EP3-targeting therapy with androgen deprivation therapy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Shiota M, Yokomizo A, Naito S 2011 Increased androgen receptor transcription: a cause of castration-resistant prostate cancer and a possible therapeutic target. Journal of molecular endocrinology 47 25-41.

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Interaction Between Docetaxel Resistance and Castration Resistance in Prostate Cancer: Implications of Twist1, YB-1, and Androgen Receptor

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BACKGROUND. Taxanes, including docetaxel, are currently the only cytotoxic chemotherapeutic agents proven to confer survival benefit in patients with castration-resistant prostate cancer (CRPC). However, the merits of taxanes remain modest, and efforts are needed to improve their therapeutic efficacy.
METHODS. We evaluated the sensitivity of prostate cancer cells to various agents using cytotoxicity assays. Gene and protein expression levels were evaluated by quantitative real-time polymerase chain reaction and Western blotting analysis, respectively.
RESULTS. Hydrogen peroxide-resistant and castration-resistant cells that overexpressed Twist1 and Y-box binding protein-1 (YB-1) were cross-resistant to cytotoxic agents, including docetaxel. Twist1 regulated YB-1 expression in prostate cancer cells, supported by the induction of Twist1 and YB-1 by transforming-growth factor-β, which is critical for taxane resistance. Twist1 and/or YB-1 were activated in docetaxel-resistant prostate cancer cells, and YB-1 was activated by docetaxel treatment. Conversely, Twist1 and YB-1 knockdown sensitized prostate cancer cells to cytotoxic agents, including docetaxel. In addition, androgen receptor (AR) knockdown increased cellular sensitivity to docetaxel, though AR expression in docetaxel-resistant LNCaP cells was paradoxically lower than in parental cells. Intriguingly, androgen deprivation treatment was more effective in docetaxel-resistant LNCaP cells compared with parental cells.
CONCLUSIONS. Twist1/YB-1 and AR signaling promote docetaxel resistance in CRPC cells. However, docetaxel-resistant cells were collaterally sensitive to androgen deprivation because of down-regulation of AR expression, suggesting that the therapeutic effect of initial taxane treatment in hormone-naïve prostate cancer may be superior to that of salvage taxane treatment in CRPC. *Prostate 73:1336–1345, 2013.* © 2013 Wiley Periodicals, Inc.

KEY WORDS: **androgen receptor; CRPC; docetaxel; Twist1; YB-1**

INTRODUCTION

Androgen-deprivation therapy (ADT) is currently the gold standard treatment for recurrent or advanced prostate cancer. Although about 90% of prostate cancers are dependent on androgens for tumor growth and respond well to ADT, most androgen-dependent prostate cancers eventually overcome low circulating levels of androgens and recur in a castration-resistant manner during ADT. Taxanes, including docetaxel and cabazitaxel, are currently the only chemotherapeutic

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agents that have been shown to confer survival benefit in castration-resistant prostate cancer (CRPC) patients. However, their efficiencies are modest, and they only prolong survival by a few months as a result of intrinsic and acquired resistance to taxanes [1,2]. Novel strategies are therefore required to treat recurrent and advanced prostate cancers.

Numerous studies have revealed the involvement of various molecular factors, including P-glycoprotein (P-gp), βIII-tubulin and clusterin, in taxane resistance [3]. We therefore targeted key molecules regulating multiple factors involved in taxane resistance, and focused on transcription factors that simultaneously regulate the expression of several genes. Previous studies have identified Twist1 and Y-box binding protein-1 (YB-1) transcription factors as nodal transcription factors regulating taxane-resistant factors in prostate cancer [4,5]. The basic helix-loop-helix transcription factor Twist1 has been proposed as an oncogenic transcription factor, especially responsible for metastasis, and has been shown to be up-regulated in prostate cancer [4]. YB-1 is known to function in the nucleus as a transcription factor that binds the Y-box sequence (5′-ATTGG-3′) and activates or represses the expression of its target genes [6]. YB-1 was up-regulated during prostate tumor progression and after androgen ablation in a mouse xenograft model [7]. In addition, YB-1 is involved in cellular resistance to various stresses in response to ultraviolet irradiation, paclitaxel, cisplatin, and actinomycin D, and becomes phosphorylated and activated by paclitaxel and ionizing radiation [8]. We previously demonstrated that Twist1 was involved in tumor growth, as well as cisplatin and taxane resistance, through YB-1 expression [5]. Conversely, Twist1 was shown to be regulated translationally by YB-1 [9], indicating a close functional link between Twist1 and YB-1. Furthermore, we reported that both Twist1 and YB-1 were up-regulated and associated with increased levels of androgen receptor (AR) expression in CRPC cells, as well as in human prostate cancer tissues treated with ADT [10–12]. These findings indicate a close link between castration resistance and taxane resistance through Twist1, YB-1, and AR. In addition, a relationship between castration resistance and radiation resistance has been reported [13]. In this study we therefore investigated the interaction between castration resistance and taxane resistance through Twist1/YB-1 and AR signaling.

MATERIALS AND METHODS

Cell Culture

Human prostate cancer PC-3 (Eagle's minimal essential medium, MEM), DU145 (MEM) and LNCaP (RPMI-1640) cells were cultured in the noted media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS). LNCaP cells that had been propagated about 10–40 times were used. Castration-resistant and hydrogen peroxide-resistant derivatives of LNCaP cells, LNCaP-CxR, and LNCaP-HPR50 cells (referred to as CxR and HPR50 cells, respectively), were established and maintained as described previously [10]. Docetaxel-resistant derivatives of LNCaP and DU145 cells (LNCaP/DTX and DU145/DTX cells) were established as described previously [8]. Similarly, docetaxel-resistant derivatives of PC-3 cells (PC-3/DTX cells) were established by long-term culture in appropriate media containing gradually increasing concentrations of docetaxel, and maintained in medium containing 1 ng/ml docetaxel. The cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies

Antibodies against AR (sc-815) and Twist1 (sc-81417) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-YB-1, anti-phosphorylated YB-1 (p-YB-1), and anti-β-actin antibodies were obtained from Epitomics (Burlingame, CA), Cell Signaling Technology (Cambridge, MA), and Sigma (St. Louis, MO), respectively.

Plasmid Construction

The Twist1-Myc-Flag plasmid expressing C-terminally Myc-Flag-tagged Twist1 protein was obtained from OriGene (Rockville, MD).

Knockdown Analysis Using Small Interfering RNAs (siRNAs)

The following 25-base-pair double-stranded siRNA oligonucleotides were generated commercially (Invitrogen): 5′-CUUCCUCGCUGUUGCUCAGGCUGUC-3′ (sense) and 5′-GACAGCCUGAGCAACAGCG-AGGAAG-3′ (antisense) for Twist1 siRNA #1; 5′-UUGAGGGUCUGAAUCUUGCUCAGCU-3′ (sense) and 5′-AGCUGAGCAAGAUUCAGACCCUCAA-3′ (antisense) for Twist1 siRNA #2; 5′-UGGAUAGCGU-CUAUAAUGGUUACGG-3′ (sense) and 5′-CCGU AACCAUUAUAGACGCUAUCCA-3′ (antisense) for YB-1; 5′-UAGAGAGCAAGGCUGCAAAGGAGUC-3′ (sense) and 5′-GACUCCUUUGCAGCCUUGCUCU-CUA-3′ (antisense) for AR siRNA. Prostate cancer cells were transfected with the siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western Blotting Analysis

Whole-cell extracts were prepared as described previously [10–12,14]. Briefly, the concentrations of the