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Combined therapeutic potential of nuclear receptors with receptor tyrosine kinase inhibitors in lung cancer

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ABSTRACT

Cancer heterogeneity is a big hurdle in achieving complete cancer treatment, which has led to the emergence of combinational therapy. In this study, we investigated the potential use of nuclear receptor (NR) ligands for combinational therapy with other anti-cancer drugs. We first profiled all 48 NRs and 48 biological anti-cancer targets in four pairs of lung cell lines, where each pair was obtained from the same patient. Two sets of cell lines were normal and the corresponding tumor cell lines while the other two sets consisted of primary versus metastatic tumor cell lines. Analysis of the expression profile revealed 11 NRs and 15 cancer targets from the two pairs of normal versus tumor cell lines, and 9 NRs and 9 cancer targets from the primary versus metastatic tumor cell lines had distinct expression patterns in each category. Finally, the evaluation of nuclear receptor ligand T0901317 for liver X receptor (LXR) demonstrated its combined therapeutic potential with tyrosine kinase inhibitors. The combined treatment of cMET inhibitor PHA665752 or EGFR inhibitor gefitinib with T0901317 showed additive growth inhibition in both H2073 and H1993 cells. Mechanistically, the combined treatment suppressed cell cycle progression by inhibiting cyclinD1 and cyclinB expression. Taken together, this study provides insight into the potential use of NR ligands in combined therapeutics with other biological anti-cancer drugs.

1. Introduction

Clinicopathogical features are used to broadly classify lung cancer into small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC) [1]. However, cancer heterogeneity still exists in tumors within the same histological group, which has important implications in metastasis, clinical diagnostics and therapeutic responses [2]. Attempts have therefore been made to classify lung cancer into distinct molecular subgroups that can be used to guide therapy [3,4]. In the adenocarcinoma of the lung, EGFR gene mutations, KRAS gene mutations and EML4-ALK fusion genes have been identified as driver mutations that drive carcinogenesis and determine response to therapy [5]. The discovery of these gene mutations has led to the development of targeted therapy with specific inhibitor drugs such as gefitinib and erlotinib for EGFR mutations [6,7]. Different types of cancers have been shown to have different driver mutations [8,9] and the food and drug administration (FDA) has approved drugs targeting these genes for use as targeted therapy against cancer [10,11]. Although these therapies are initially effective in cancer treatment, most patients with prolonged exposure to the drug develop relapse of cancer with drug resistance due to tumor heterogeneity [12–14].

Combinational therapy has become a good therapeutic strategy to curb drug resistance caused by tumor heterogeneity [15]. The rationale of combinational therapy is to target several molecules involved in multiple independent and essential pathways in cancer. This strategy widens the therapeutic window and raises the threshold required by any particular tumor clone to acquire resistance. However, the challenge lies in identifying and validating molecular candidates for combinational therapy. Fortunately, the improved tools to detect gene expression and thus screen more drugs have led to significant advances in this field.

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The NR super family consists of 48 members of ligand-controlled transcription factors involved in various physiological processes including development [16], inflammation and immune response [17], drug and nutrient metabolism [18]. Therefore NRs have become valuable drug targets for many diseases including cancer. For example, estrogen and progesterone receptors are currently in use as prognostic and therapeutic markers for breast cancer patients, while androgen receptor is well known for its roles in prostate cancer. In our previous study, we showed that NRs are potential prognostic as well as therapeutic targets for the treatment of lung cancer [1,19].

In this study, we investigated the potential of NR ligands as combinational partners with other anti-cancer drugs in the treatment of lung cancer. The mRNA expression profiles of the NRs and biological anti-cancer targets in pair-matched lung cell lines showed heterogeneity of tumors from the same patient. In addition, using LXR ligand in combination with EGFR and cMET tyrosine kinase inhibitors (TKIs), we show that NR ligands have potential use in combined therapeutics with other anti-cancer drugs. Taken together, this study provides an insight into a strategy for tailored, tumor stage-specific (primary versus metastasis) or patient-specific combinational therapy.

2. Materials and methods

2.1. Quantitative PCR analysis

RNA was isolated from the different cell lines using Qiagen RNeasy Mini kit (Qiagen Sciences, Maryland), according to the manufacturer's instructions. The protocol for cDNA synthesis has been described elsewhere [19]. All the members of the NR family were profiled using the standard curve method as described [19]. The anti-cancer drug target (Table S1 in Supplementary material) gene expression profiling was done using the delta-delta Ct method [20] with 18S as the reference gene.

2.2. MTT assay

H1993 (1000 cells/well) and H2073 (2000 cells/well) cell lines were seeded into 96 well plates in RPMI media containing 5% charcoal stripped serum. The following day, the cells were treated with pioglitazone (PPARγ ligand), T0901317 (LXR ligand), gefitinib (EGFR inhibitor), or PHA665752 (cMET inhibitor). Six days after treatment, MTT assay was performed as previously described [1].

2.3. Western blot

For protein expression analysis, cells were treated with the various compounds for 48 h. A standard protocol was used to assay the expression of various proteins. Primary antibodies used are: PPARγ (#2435, Cell Signaling), LXRα (PP-K8607-00, Perseus Proteomics), LXRβ (PP-K8917-00, Perseus Proteomics), EGFR (#2232, Cell Signaling), cMET (#3127, Cell Signaling), b-actin (ab6276, Abcam), Lamin A/C (sc-7292, Santa Cruz), CyclinD1 (#2926, Cell Signaling), CyclinA (sc-239, Santa Cruz) and CyclinB (#4135, Cell Signaling).

2.4. Statistical analysis

All results are expressed as mean ± SEM and they were analyzed using GraphPad Prism 5.03 (GraphPad Software Inc.). QPCR data was analyzed using Student's *t*-test with Welch's correction being used for data that showed significant differences in the variance. MTT assay data was analyzed using one-way ANOVA with Tukey's post hoc test. Results with *P*-value <0.05 were considered to be statistically significant.

3. Results

3.1. Nuclear receptor expression

To explore the expression of the NR super family in the pair-matched normal versus tumor cell lines and primary versus metastatic tumor cell lines, quantitative real-time PCR for the 48 NRs was performed as described previously. The pair-matched cell lines used in this study were obtained from the same patient and their characteristics are shown in Table 1. One set of paired cell lines includes two pairs of normal versus tumor cell lines and the other set includes two pairs of primary versus metastatic tumor cell lines. Analysis of the expression profile revealed that subsets of NRs showed distinct patterns of expression in the pair-matched panel of cell lines, normal versus tumor and primary versus metastatic cell lines (Fig. 1 and see Fig. S1 in the Supplementary material). We found that 37 NRs (normal versus tumor cell lines) and 39 NRs (primary versus metastatic cell lines) did not exhibit any common difference between the two groups of pair-matched cell lines in each set (Fig. S1 in the Supplementary material). By contrast, 11 NRs (normal versus tumor cell lines) and 9 NRs (primary versus metastatic cell lines) showed distinct expression patterns in each set of pair-matched cell lines (Fig. 1). Germ cell nuclear factor (GCNF), mineralocorticoid receptor (MR), nur-related factor 1 (NURR1), reverse-erb (REV-ERB)β, retinoic acid-related orphan receptor (ROR)β, retinoic X receptor (RXR)β and thyroid hormone receptor (TR)α showed higher expression in the tumor cell lines compared to the normal counterpart (Fig. 1A first panel), while PPARδ, TRβ and vitamin D receptor (VDR) showed higher expression in the normal cell lines compared to the corresponding tumors cell lines (Fig. 1A second panel). On the other hand, chicken ovalbumin upstream promoter-transcription factor (Coup-TF)β, Coup-TFγ, GCNF, hepatocyte nuclear factor 4 (HNF4)γ, PPARδ, retinoic acid receptor (RAR)α, RXRα, TRβ and RXRβ expression levels were higher in the metastatic tumor cell lines compared to the pair-matched primary tumor cell lines (Fig. 1B). This result suggests that subsets of NRs are differentially expressed upon tumorigenesis or tumor progression.

3.2. Expression profile of molecular targets for anti-cancer drugs

Molecular targeted therapy has become a popular strategy to treat cancer in the clinic since it is considered to be more selective thus increasing therapeutic efficacy while keeping toxicity at the minimum [21]. Thus, we wondered if the expression profile of molecular targets for FDA-approved anti-cancer drugs can be utilized to develop a strategy for patient- or tumor stage-tailored anti-cancer therapy. We first selected 48 biological targets from

Table 1
Characteristics of paired lung cell lines.

Cell Line	Clinical Features	Genetic features	Treatment
HBEC30KT	Normal		
HCC4017	Tumor		
HBEC34KT	Normal		
HCC4018	Tumor		
H2073	Primary	EGFR high	VP16/CDDP
H1993	Metastasis	cMET high	
H2085	Primary		
H2086	Metastasis	p53 mutation	

Abbreviations: VP16, etoposide; CDDP, cisplatin.

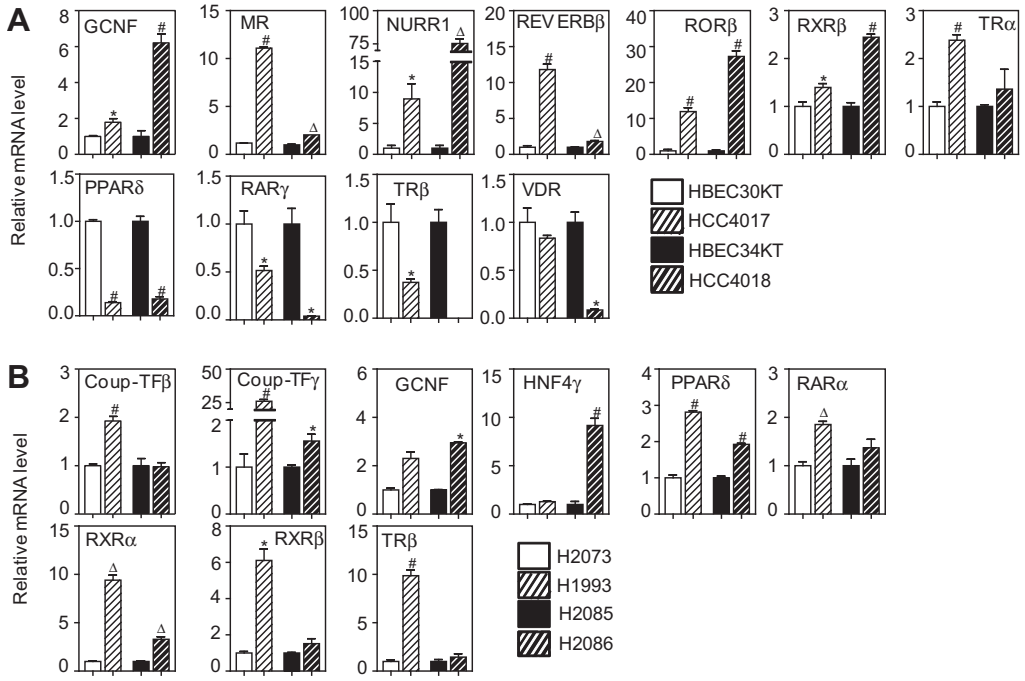

Fig. 1. Expression profile of the NR superfamily in the lung panel. Quantitative PCR was performed for the 48 NRs in the four pairs of lung cell lines (A) NRs with different expression between normal and the corresponding tumor cell lines. The upper panel shows receptors whose expression is higher in the tumor cell lines than in the normal cell lines, while the lower panel shows higher expression of the NRs in the normal cells compared to the tumor counterpart. (B) NRs with different expression between primary and the corresponding metastatic tumor cell lines. Both panels show receptors whose expression is higher in the metastatic cell lines than in the primary tumor cell lines. Values are mean ± SEM. $^{*}P < 0.05$; $^{\triangle}P < 0.01$; $^{\#}P < 0.001$ HCC4017 vs. HBEC30KT; HCC4018 vs. HBEC34KT; H1993 vs. H2073 and H2086 vs. H2085; Student's *t*-test with Welch's correction for data with significantly different variances.

the FDA-approved anti-cancer drug list in which the corresponding anti-cancer drugs have been developed for and utilized in the cancer clinics (Table S1 in the Supplementary material) [10,11,22]. Using the same QPCR approach as the NR profiling, we completed the mRNA expression profile of the 48 molecular targets in the same pair-matched lung cancer cell panel as described in Table 1. Note that some NRs were excluded from the analysis of the molecular target profile since they are overlapped in the NR profile data. The NRs are androgen receptor (AR), estrogen receptor (ER)α, ERβ, RXRα, RXRβ, and RXRγ. From the analysis of the profile, we found that 27 genes (normal vs. pair-matched tumor line) and 33 genes (primary vs. pair-matched metastatic tumor line) exhibit no difference between the pairs or the pair-specific difference for the expression (Fig. S2 in the Supplementary material). By contrast, subsets of genes showed distinct expression pattern between the pairs; 15 molecular targets in the first panel of normal vs. pair-matched tumor lines and 9 molecular targets in the second panel of primary vs. metastatic cell lines. Eight of the 14 genes from the first panel showed tumor specific increased expression compared to the pair-matched normal cell lines. Included in this subgroup were aminolevulinate dehydratase (ALAD), colony stimulating factor 1 receptor (CSF1R), dihydrofolate reductase (DHFR), DNA methyltransferase 1 (DNMT1), fms-related tyrosine kinase 1 (FLT1), lymphocyte-specific protein tyrosine kinase (LCK), ribonucleotide reductase M2 (RRM2), DNA topoisomerase 2-alpha (TOP2A), and thymidylate synthetase (TYMS), whereas 6 of the 14 genes are EGFR, EPH receptor A2 (EPHA2), FYN oncogene related to SRC, FGR, YES (FYN), histone deacetylase 6 (HDAC6), v-src avian sarcoma viral oncongene homolog (SRC), and v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 (YES1) that showed decreased expression in the tumor vs. normal cell lines (Fig. 2A). The 9 genes from the second panel include 6 genes – chromodomain helicase DNA binding protein 1 (CHD1), DHFR, DNMT1, glycinamide ribonucleotide formyltransferase (GARFT), proteasome subunit beta type 5 (PSMB5), and YES1- with increased expression in the metastatic pair, and 3 genes – c-KIT, CSFR1, and fms-related tyrosine kinase 4 (FLT4)-with decreased expression in the metastatic tumor line compared to the matched primary tumor line (Fig. 2B).

3.3. Functional evaluation of nuclear receptors and anti-cancer targets in the primary and pair-matched metastatic tumor line

We next investigated whether the expression profiles of NRs and anti-cancer drug targets could be utilized to treat lung cancer. As a proof-of-principle approach, we first chose LXR and PPARγ as NR targets and EGFR and cMET as anti-cancer drug targets since these NRs are well-known for their biological functions with pharmacological ligands available [23–26]. For functional evaluation of the NRs and anti-cancer drug targets, we used H2073 (primary tumor cell line) and H1993 (pair-matched metastatic tumor cell line) cells which had been derived from the same patient. As discussed previously, the NRs showed distinct expression patterns of mRNA and protein between the primary and the secondary lung cancer cells. The mRNA expression of PPARγ, LXRα and LXRβ was dramatically increased in H1993 compared to H2073 and the corresponding protein expression was confirmed in the same cell panel (Fig. 3A and B). For the anti-cancer drug targets, the H2073 cells showed higher EGFR expression, whereas H1993 cells showed higher cMET expression for both mRNA and protein (Fig. 3A and B). In growth response assays, treatment of both cell lines with LXR ligand T0901317 revealed that there was growth inhibition in the metastatic H1993 cells but not in the primary H2073 tumor cells. By contrast and interestingly, treatment with a PPARγ ligand pioglitazone exhibited minor growth inhibitory response in H1993 cells that showed very high PPARγ expression, suggesting that other factors might be involved in determining growth response to PPARγ activation in H2073 and H1993. Note that fluorobexarotene was co-treated with pioglitazone or T0901317 since its

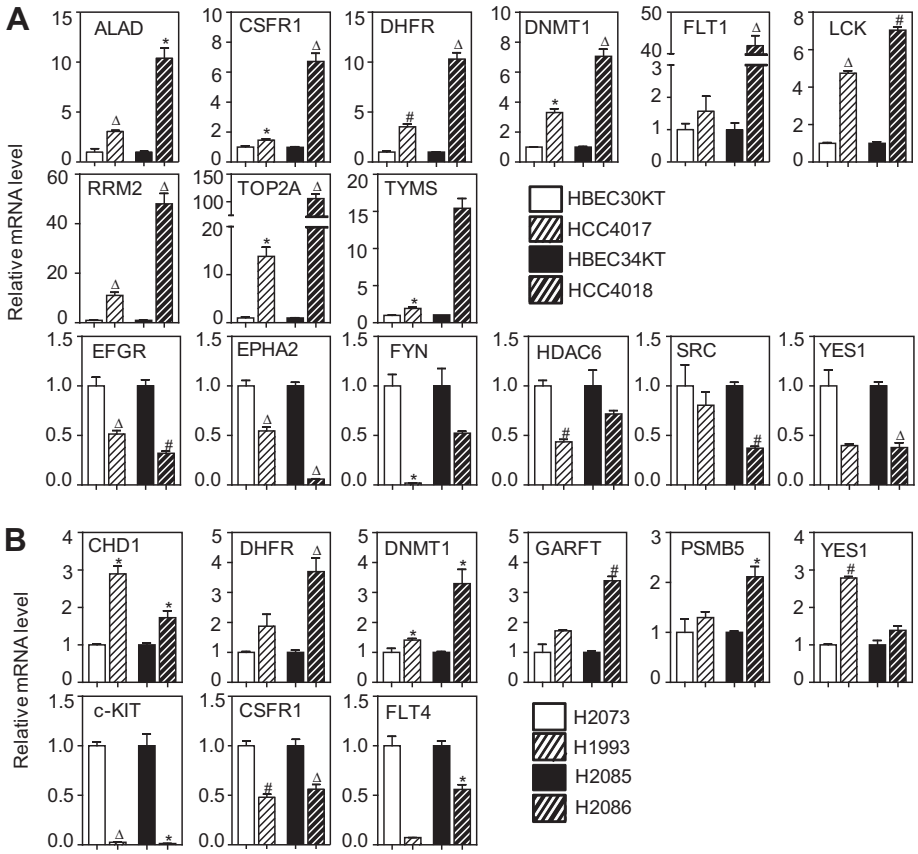

Fig. 2. Expression profile of the drug target genes in the lung panel. Quantitative PCR was performed for the 48 drug target genes in the four pairs of lung cell lines. Figures show drug target genes with different expression between the normal and the corresponding tumor cell lines (A) or between the primary tumor and the pair-matched metastatic tumor cell lines (B). (A) The upper two panels show genes with higher expression in the tumor cell lines compared to the normal counterpart while the third panel shows genes with lower expression in the tumor cell lines compared to the normal cell lines. (B) The first panel shows genes with higher expression in the metastatic cell lines while the second panel shows genes with higher expression in the primary cell lines. Values are mean ± SEM. $^{*}P < 0.05$; $^{\triangle}P < 0.01$; $^{\#}P < 0.001$ HCC4017 vs. HBEC30KT; HCC4018 vs. HBEC34KT; H1993 vs. H2073 and H2086 vs. H2085; Student's t-test with Welch's correction for data with significantly different variances.

cognate receptor, RXR, forms heterodimers with PPARγ and LXR. Treatment of receptor tyrosine kinase inhibitors, gefitinib for EGFR or PHA665752 for cMET, showed growth inhibitory response in a target gene expression-dependent manner, suggesting the therapeutic potential of the drug target expression profile as a patient-tailored cancer therapeutic strategy (Fig. 3C).

3.4. Effect of combined treatment of T0901317 and gefitinib/PHA665752

Recently, combined therapeutics has been developed in the clinic as a strategy to overcome potential side effects or drug resistance due to drug toxicity as well as cancer heterogeneity. Thus, in this study, we also explored the combinational therapeutic potential of nuclear receptor LXR ligand and molecular targeted drugs, gefitinib and PHA665752, in the primary H2073 and the secondary metastatic H1993 tumor cell lines. In H2073 cells that have high EGFR expression, low expression of both LXRα and β, and no expression of cMET, the combined treatment of T0901317 with PHA665752 or gefitinib showed more than additive growth inhibitory response when compared to either drug alone. In a similar manner, PHA66572 or gefitinib treatment with T0901317 also showed additive growth inhibitory effect in H1993 cells (Fig. 4A). In the molecular analysis of cell cycle factors, cyclinD1 expression was down regulated by combined treatment of T0901317 and gefitinib or PHA66572 in H2073, while the combined treatment of gefitinib and T0901317 suppressed cyclinB expression in H1993 (Fig. 4B).

4. Discussion

In this study, we show that tumor cell lines derived from the same patient have different gene expressions which cause different response to single-drug regimens underscoring the impact of tumor heterogeneity on cancer therapy. Since no tumor is exactly the same, patient-specific genotype-based predictions would be ideal for designing cancer therapy. However, this would not be economically viable and the process would be time consuming. An alternative is to build representative sample sets that can be used for genetic and therapeutic testing and which can be used to build prediction models for the general population [21]. The panel of pair-matched cell lines used in this study provides a sample set of cell components that are important in understanding tumor progression. The inclusion of normal versus tumor set or primary versus metastatic tumor gives representative cells for studying tumor stage- or patient-specific therapy. Using this panel of cells (1) we analyzed the mRNA expression of the 48 members of the NR superfamily and 48 biological anti-cancer drug targets during the different stages of tumor progression; and (2) we investigated the potential of NR ligands as combinational partners with biological anti-cancer drugs in the treatment of cancer.

The mRNA expression profiling revealed that the level of NR gene expression and anti-cancer target gene expression is dynamic during tumorigenesis or tumor progression. Some of these genes showed distinct expression patterns in the two pairs of cell lines in a given set, which could indicate that these genes have specific roles in tumorigenesis. Other genes lacked specific patterns of

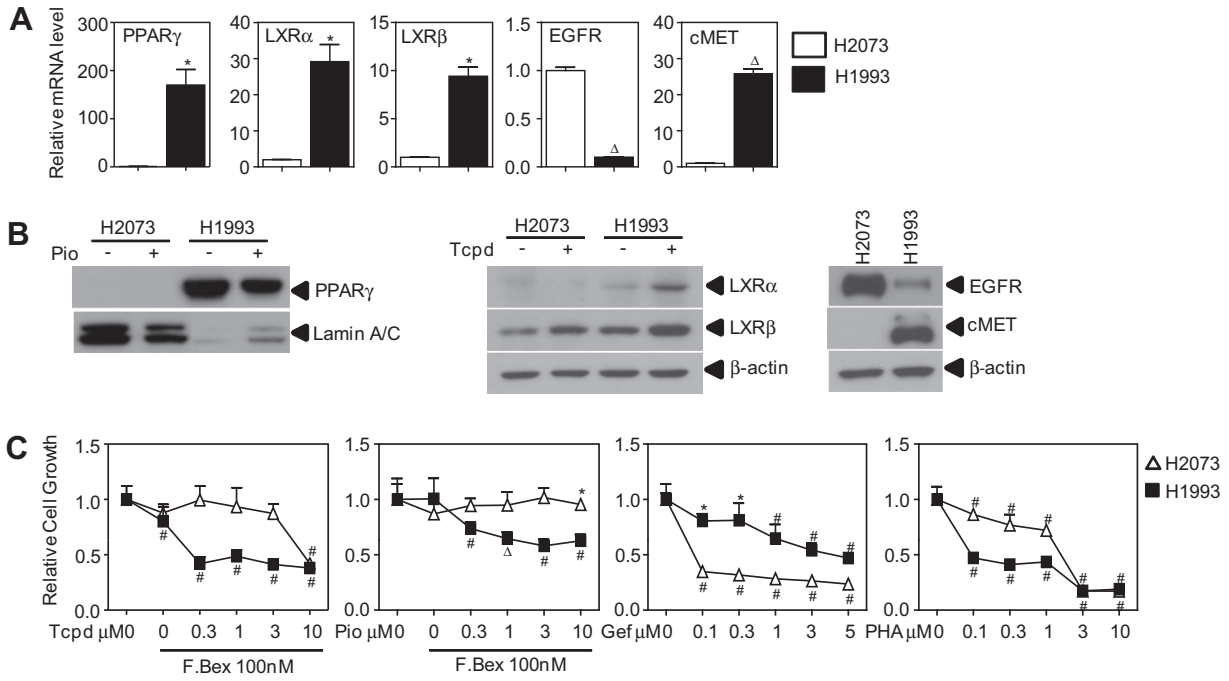

Fig. 3. Therapeutic evaluation of nuclear receptors and drug targets in H2073 and H1993 cells. The expression of genes of interest was confirmed in the cell line panel. The mRNA expression levels of PPARγ, LXR, EGFR, and cMET were measured using QPCR assay in the primary H2073 and the pair-matched metastatic H1993 tumor cells (A). The corresponding protein expressions were assayed using western blot analysis in the same panel of cells (B). Both cell lines were treated or non-treated with 3 μM of pioglitazone or T0901317. (C) Growth response of H2073 and H1993 cells to the treatment with cognate receptor ligands. MTT assay was performed after treatment with pioglitazone (Pio), T0901317 (Tcpd), gefitinib (Gef) and PHA665752 (PHA), in a dose-dependent manner. Fluorobexarotene (F. bex, 100 nM), a RXR ligand, was co-treated with pioglitazone and T0901317. Values are mean ± SEM. (A) $^{*}P < 0.05$ and $^{\triangle}P < 0.01$ H1993 vs. H2073; Student's *t*-test. (C) $^{*}P < 0.05$; $^{\triangle}P < 0.01$; $^{\#}P < 0.001$ vs. control; one-way ANOVA with Tukey's post hoc test.

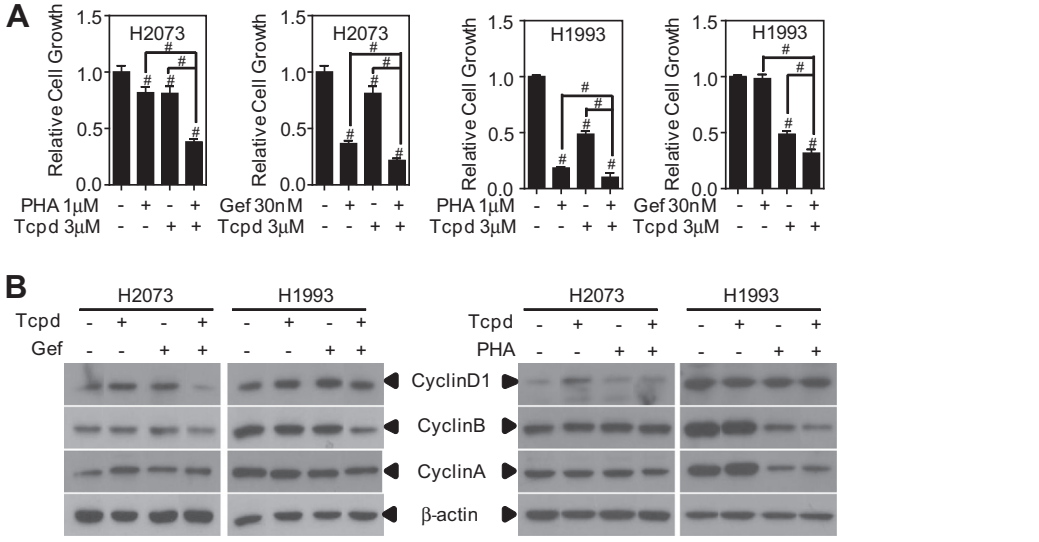

Fig. 4. Therapeutic evaluation of combined treatment of LXR ligand and receptor tyrosine kinase inhibitors (A) MTT assay was performed after treatment of H2073 and H1993 cells with T0901317 (3 μM) alone, or with gefitinib (30 nM) or PHA665752 (1 μM). (B) The expressions of cell cyclins were assayed using immunoblot analysis. Cells were treated with T0901317 (3 μM), gefitinib (30 nM for H2073; 3 μM for H1993) and PHA665752 (1 μM for H2073; 0.1 μM for H1993) for 48 h before isolating protein. Values are mean ± SEM. $^{\#}P < 0.001$comparisons between the respective treatments; one-way ANOVA with Tukey's post hoc test.

expression between the two pairs of cells in each set, indicating the presence of intra- and inter-patient heterogeneity. The Food and Drug Administration (FDA) has approved more than 260 anti-cancer drugs to treat various types of cancers. Among these, we found 48 biological targets that have been utilized for molecular targeted therapy in the cancer clinic. In this study we completed the mRNA expression of the 48 biological anti-cancer targets in our panel of pair-matched cell lines. From the expression profile, we demonstrate that, although targeted therapy was initially tailored to address driver mutations in specific cancer types, the same drugs can be used to treat other cancers which show the appropriate gene expression of the target receptor. However, cancer patients mostly relapse with drug resistance to single-targeted regimens. In this regard, a last approach in this work was to evaluate the

combinational therapeutics of NR ligands with the biological targeted drugs. To this end, the primary H2073 tumor cell and its pair-matched metastatic H1993 tumor cell line were employed in growth inhibition assays. We showed that these two cell lines exhibited sensitivity to growth inhibition by PPARγ, LXR, EGFR and cMET ligands in a receptor expression-dependent manner. In addition, the combination of LXR ligand, T0901317, with the TKIs showed additive growth inhibition when compared to either drug alone, suggesting that NR ligands can be used in combined therapeutics with other anti-cancer drugs. Collectively, our studies improve the idea of cancer type- or patient-tailored therapeutic strategy by expanding the use of single biological targeted drugs that were originally developed for treating the corresponding tumor types in other tumor types, and further multiple combinations of the targeted drugs to overcome potential drug resistance. Our work provides a new strategy to develop oncotherapeutics in cancer clinics.

Conflict of interest

We have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.018.

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