All-trans retinoic acid reduces cancer stem cell-like cell-mediated resistance to gefitinib in NSCLC adenocarcinoma cells

Wenxiu Yao
Sichuan Cancer Hospital and Research Institute
Liyang Wang
Sichuan Cancer Hospital and Research Institute
Huan Huang
Wuming Hospital
Xin Li
Sichuan Cancer Hospital and Research Institute
Pinjia Wang
The Sixth People's Hospital of Chengdu
Kun Mi
Sichuan Cancer Hospital and Research Institute
Jia Cheng
Sichuan Cancer Hospital and Research Institute
Huifen Liu
Sichuan Cancer Hospital and Research Institute
Cuirong Gu
Sichuan Cancer Hospital and Research Institute
Lingxiao Huang
Sichuan Cancer Hospital and Research Institute
Jianming Huang (hjianming@yahoo.com)
Sichuan Cancer Hospital and Research Institute https://orcid.org/0000-0002-6307-4977

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Abstract

Background: The enrichment of cancer stem cell-like cells (CSCs) has been considered to be responsible for tumor progression after an initial response to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) in patients with non-small cell lung adenocarcinoma (NSCLC/ADC). All-trans retinoic acid (ATRA) has been shown to potentiate cancer chemotherapy due to its ability to induce signals for CSC differentiation. We therefore investigated whether ATRA could improve the response of NSCLC/ADC cells to TKI gefitinib.

Methods: Treatment of NSCLC/ADC A549 and H1650 cells with gefitinib enriched the gefitinib surviving cells (GSCs). The expression of ALDH1A1 and CD44 and the IC50 values for gefitinib were determined by flow cytometry (FCM) and crystal violet assay in GSCs and ATRA-treated GSCs, respectively.

Results: GSCs showed higher expression of ALDH1A1 and CD44 and IC50 values for gefitinib than their respective parental cells, suggesting that gefitinib can lead to propagation of CSC-enriched gefitinib-resistant cells. Treatment with ATRA was found to significantly reduce the increased expression of ALDH1A1 and CD44 and the IC50 values for gefitinib in A549GSC and H1650GSC cells.

Conclusion: Our findings suggest that combination treatment with ATRA prevents gefitinib-induced enrichment of ALDH1A1bright/CD44high CSCs and enhances gefitinib-induced growth inhibition of NSCLC/ADC cells. Key words: All-trans retinoic acid, ALDH1A1, CD44, EGFR tyrosine kinase inhibitors, non-small cell lung adenocarcinoma

Background

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for 84% of all lung cancer diagnoses [1]. For advanced NSCLC, median survival remains poor at 7.9 months and only approximately one third of patients survive for one year or more despite conventional combination chemotherapy [2]. About 10% and 30% of NSCLCs in Western and Asian populations, respectively, express an activated mutant epidermal growth factor receptor (EGFRm) and the majority of such patients respond to adenosine triphosphate (ATP)-competitive EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib [3–5].

Based on data from clinical trials that compared EGFR-TKIs to platinum-based chemotherapy in advanced NSCLC patients with EGFR tyrosine kinase domain (TKD) mutations, EGFR-TKI monotherapy such as gefitinib has become the standard front-line treatment for these patients. The response rate of gefitinib to EGFR-sensitive mutations was ~ 77%, while that of wild-type EGFR ($EGFR^{WT}$) patients was 11–25% [4]. Despite initial dramatic efficacy of EGFR-TKIs in NSCLC patients with or without EGFR mutation, emergence of acquired resistance is almost inevitable at a median of 9–13 months [5–8], thereby limiting the benefits of EGFR-TKIs to NSCLC patients' outcomes. Inducible EGFR T790M-mediated gefitinib resistance in NSCLC patients accounts for ~ 50% of resistance, and the remaining 50% of non-

T790M-mediated EGFR-TKIs resistance has been linked, at least in part (~ 20%), to the cancer stem cell-like cell (CSC) [9–11].

Treatment with EGFR-TKIs, while dramatically resulting in robust cell death, also enriches ALDH1A1^{bright}/CD44^{high} surviving cells that lose the responsiveness to EGFR-TKIs in NSCLC [12–14]. ALDH1A1 and CD44 are involved in the differentiation of normal stem cells, suggesting a link between them and CSC differentiation. It has been found that cancer stemness induced via up-regulation of ALDH1A1 and CD44 expression contributes to the acquisition of gefitinib resistance in EGFR-TKI sensitive NSCLC [15]. Much higher expression of ALDH1A1 and CD44 of EGFR-TKI-resistant NSCLC cells than their respective parental cells confers resistance to EGFR-TKIs, and is correlated with shortened therapy response and duration [13, 16, 17].

The retinoic acid (RA) signaling pathway has been known to be crucial for regulation of differentiation properties of CSC [18–20]. The existence of CSC expressing CD44 and a high ALDH1A1 activity has been demonstrated in NSCLC [21–23]. The ALDH1A-RA signaling is involved in cancer cell proliferation, invasiveness and sensitivity to various chemotherapy drugs [24]. Retinoic acid potentiates the chemotherapeutic effect of cisplatin by inducing differentiation of CSC in several cancers [25], and adding all-trans retinoic acid (ATRA) to chemotherapy based on paclitaxel and cisplatin as first-line treatment could increase response rate (RR) and progression-free survival (PFS) in patients with advanced NSCLC [26]. Therefore, these findings suggest that inducing CSC differentiation is a promising approach to the treatment of CSC-mediated EGFR-TKI resistant NSCLC.

In the current study, we explored if ATRA reduces propagation of CSC-enriched gefitinib-resistant cells and potentiates response of NSCLC/ADC cells to gefitinib.

Methods

Cell culture and reagents

The human NSCLC/ADC H1650 cell line with EGFR$^{delE746-A750}$ mutation and A549 cell line with EGFR^{wt} were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences (CTCCCAS, Shanghai, China). H1650 and A549 cells were chosen because their response to gefitinib have been extensively characterized (primary resistance to gefitinib) [27, 28].

Cell lines were cultured in RPMI 1640 medium (GIBCO) that contained 10% fetal calf serum (FCS), 2 mmol/L L-glutamine and 100 units/mL penicillin and 40 IU/mL gentamycin were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Subconfluent cells (80%) were passaged with a solution containing 0.25% trypsin-0.5 mmol/L Ethylenediaminetetraacetic acid (EDTA). Cell lines were tested for mycoplasma and confirmed to be negative.

Gefitinib (N-[3-Chloro-4-fluorophenyl]-7-methoxy-6-[3-morpholinopropoxy]quinazolin-4- amine, CAS 184475-35-2, MF $C_{22}H_{24}ClFN_4O_3$, MW 446.907 g/mol, HPLC > 98%) and all-trans retinoic acid (ATRA,

[2E,4E,6E,8E]-3,7-dimethyl-9-[2,6,6-trimethylcyclohexen- 1-yl] nona-2,4,6,8-tetraenoic acid, CAS 302-79-4, MF $C_{20}H_{28}O_2$, MW 300.442 g/mol, HPLC > 98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); ALDEFLUOR™ Kit (Cat. No.01700) was purchased from STEMCELL Technologies Inc.; BD Pharmingen™ PE mouse anti-human CD44 monoclonal antibody (Clone 515 Cat No. 550988) and its isotype mouse BALB/c IgG1 were purchased from BD Biosciences (Lake Franklin, NJ, USA).

AmoyDx ARMS EGFR mutation detection kit was purchased from Amoy Diagnostics Co. LTD (Xiamen, China).

Cell viability assay

Cell viability was measured by a colorimetric assay using crystal violet. To a 96-well plate, 5×10^3 cells/well were pre-cultured for 24 h, and then exposed to varying concentrations of gefitinib and ATRA, and 0.1% DMSO was used as a vehicle in triplicate. After 72 h, the supernatant was discarded as much as possible, and 100μL of crystal violet solution (0.5% crystal violet in 30% methanol) was added to each well for 30 min, and then rinsed with tap water and dried at 40 °C. 100μL of 10% SDS solution was added to each well and fully dissolved for 30 min. The absorbance at 595 nm was measured spectrophotometrically using a microplate reader (Infinite M200 Pro TECAN-Reader, Switzerland).

EGFR mutation testing

Genomic DNA from A549 and H1650 cells was manually extracted using a TIANamp Genomic DNA Kit (DP304, TIANGEN, China.) according to the manufacturer's protocol. DNA was isolated by elution with 50 μl of Tris/Acetate/EDTA (TAE). EGFR mutations were detected with the AmoyDx Human EGFR Gene 29 Mutations Detection kit with fluorescence polymerase chain reaction (PCR) (Amoy Diagnostics, Xiamen, China) and assays were performed on CFX96 Touch (Bio-Rad, USA) real-time fluorescence quantitative PCR instrument according to the manufacturer's instructions. Positive results were defined as [Ct (sample)-Ct (control)] \ Ct (cut-off).

Preparation of GSC and ATRA treatment

H1650 and A549 cells were passaged with 15 μmol/L of gefitinib twice weekly for three consecutive weeks, and the resultant gefitinib surviving cells (A549GSC cells and H1650GSC cells) were pre-incubated with 5 μmol/L of ATRA for 1–5 days. These cells were respectively harvested to test the expression of ALDH1A1 and CD44 by flow cytometer (FCM). The GSCs with enhanced expression of ALDH1A1 and CD44 are defined as CSC-enriched gefitinib-resistant cells.

Flow cytometry for ALDH1A1 and CD44 expression

Expression of ALDH1A1 and CD44 by A549 and H1650 cells were determined using ALDEFLUOR™ kit (FITC) and CD44 mAb (PE), respectively according to the manufacturer's protocols. Briefly, A549 and H1650 cells (1×10^6) were harvested and stained with ALDH (DEAB as the negative control) and PE anti-human CD44 mAb (mouse IgG1 as the isotype control) staining. The stained cells were resuspended in 1 ml of Assay Buffer and subjected respectively to flow cytometrical analysis on FACSCanto II Flow Cytometer (Becton–Dickinson).

Results

Growth inhibition of H1650 and A549 cells by gefitinib and ATRA

As shown in Table 1 and Fig. 1A-D, we showed that there was no significant difference between H1650 cells and A549 cells for the response to gefitinib (IC50 5.26 vs. 8.42 μmol/L), however the IC50 values of gefitinib for H1650GSC and A549GSC cells significantly increased by 5.15-fold (from 5.26 to 27.11 μmol/L) and 4.39-fold (from 8.42 to 36.97 μmol/L), respectively as compared to their untreated cells (both $P < 0.01$). We found that pre-incubation with ATRA significantly enhanced gefitinib-induced growth inhibition and decreased the IC50 values for gefitinib by up to 2.27-fold (from 27.11 to 11.94 μmol/L) ($P < 0.01$), and 2.04-fold (from 36.97 to 18.17 μmol/L) ($P < 0.01$) for H1650GSC and A549 GSC cells, respectively. Interestingly, we found that ATRA significantly inhibited the growth of H1650GSC and A549GSC cells but did not obviously impact the growth of H1650 and A549 cells. These results suggest that both H1650GSC and A549GSC cells have a higher resistance to gefitinib, and ATRA could re-sensitize H1650GSC and A549GSC cells to gefitinib.

CSC-enriched cell population mediates gefitinib resistance

As shown in Fig. 2, H1650GSC and A549GSC cells showed increased expression of ALDH1A1 and CD44, compared to the untreated cells ($P < 0.05$). The expression of ALDH1A1 and CD44 in A549GSC cells increased from 2.8–4.8% and from 55.2–73.9%, respectively (Fig. 2A); the expression of ALDH1A1 and CD44 in H1650GSC cells increased from 3.1–11.2% and from 40.3–70.2%, respectively (Fig. 2B). However, no EGFR T790M mutation in A549GSC and H1650GSC cells was detected using quantitative fluorescence PCR as described in methods (Figure S1). These results indicate that resistance of A549GSC and H1650GSC cells to gefitinib could be associated with $ALDH1A1^{bright}/CD44^{high}$ but not associated with EGFR T790M mutation.

ATRA reduces increased ALDH1A1 and CD44 expression of GSC cells

As shown in Figs. 3 and 4, following incubation with ATRA for a different time, the expression of ALDH1A1 and CD44 of A549GSC and H1650GSC cells showed a significant decrease in a time-dependent manner. Approximate 12-fold and 2.6-fold decrease in expression of ALDH1A1 and CD44 (from 4.8–0.4% and from 73.9–28.5%) (Fig. 3A and B) and 7-fold and 2.5-fold (from 11.2–1.6% and 70.2–28.4%) (Fig. 4A and B) were observed in A549GSC and H1650GSC cells, respectively, compared to the control (both $P < 0.01$), suggesting that ATRA reduces propagation of A549GSC and H1650GSC cells showing ALDH1A1^{bright}/CD44^{high}.

Discussion

CSC cell-mediated drug resistance of cancers is a major cause leading to the failure in cancer therapies. Resistance to molecular targeted drugs of NSCLC is a common characteristic of CSC cells. Among EGFR non-T790M mutation NSCLC cases, approximately 20% of NSCLC patients with CSC features come into being a more resistant phenotype during12 months after an initial response to the EGFR-TKIs [4]. Since potential relapse of NSCLC may occur due to the enrichment of CSCs following TKI initial therapy, the generation of more effective therapeutic interventions based on CSC cell-mediated resistance of NSCLC to EGFR-TKIs is an urgent requirement [29].

There is increasing evidence to show that ALDH1A1^{bright}/CD44^{high} CSC-mediated EGFR-TKI resistance may be a major obstacle for EGFR-TKIs maintenance therapy of NSCLC. Loss of responsiveness to EGFR-TKIs in NSCLC with non-T790M EGFR mutation can be explained in terms of EGFR-TKI-resistant ALDH1A1^{bright}/CD44^{high} CSC that evolutionally possesses drug resistance and is often referred to as a tumor-initiating cell and associated with EGFR-TKIs non-responder [5–10]. Recent studies showed that ALDH1A1^{bright} CSCs promote EGFR-TKI resistance in NSCLC [13, 17]. With respect to CD44, it has been reported that modulation of CD44 is detrimental to CSCs self-renewal and differentiation and NSCLC cells expressing CD44 are enriched for stem-like properties, suggesting that ALDH1A1^{bright}/CD44^{high} CSC linked to tumor progression and EGFR-TKI resistance is associated with a significantly poor prognosis factor in NSCLC [30]. Several clinical studies revealed significantly increased proportions of ALDH1A1^{bright} NSCLC cells displaying resistance to EGFR-TKIs and chemotherapy drugs [26, 31, 32].

Differentiating CSCs may provide such an approach to modulating or converting the phenotypes of CSCs for sustained treatment response of NSCLC to EGFR-TKIs, although the mechanisms underlying CSCs contribution to resistance of NSCLC to TKIs remain unclear [33].

The ALDH-retinoic acid pathway plays an important role in differentiation of CSCs. It has been shown that treatment of lung adenocarcinoma A549 cell with ATRA led to the downregulation of ALDH1A1[34]. RA can reduce the ALDH activity and CD44 expression, thus affecting cell proliferation, cancer invasiveness and sensitivity to various chemotherapy drugs [24, 35, 36]. ALDH1A1 has been shown to convert/oxidize retinaldehyde into retinoic acid (RA) in several tissues, and to be one of the target proteins of ATRA [15]. Treatment with ATRA increased the C/EBP homologous protein (GADD153) and GADD153-CCAAT-enhancing binding protein-β (C/EBP-β) interaction resulting in a decreased cellular

availability of C/EBP-β for binding to the Raldh1 CCAAT box and high ATRA levels inhibit Raldh1 gene expression by sequestering C/EBP-β through its interaction to GADD153 [37, 38]. CD44 expression was highly responsive to ATRA as it was down regulated following treatment. ATRA treatment also resulted in decreased migration and invasion of cancer cells and promoted tumor regression by inducing differentiation [35].

Retinoids prevent the development of several tumors and enhance the efficacy of cytotoxic drugs such as cisplatin and docetaxel [39, 40]. Retinoids bind to specific nuclear receptors, which function as transcriptional regulators controlling the expression of numerous genes. The retinoid X receptors (RXRs) and retinoic acid receptors (RARs) are selectively expressed in $ALDH^{bright}$ CSCs, indicating RA signaling mainly occurs via $ALDH^{bright}$ CSCs of lung cancer, which provides a mechanism to selectively target CSCs [41]. RA signaling is modulated by two classes of nuclear retinoid receptors, RARs and RXRs. Both RXRs and RARs interact with multiple co-activator and co-repressor proteins to promote increased cell stemness or cell differentiation. Retinoic acid showed feedback inhibition of the ALDH1 gene through RARα and C/EBP-β [42]. Specifically, RA signaling regulates ALDH via the binding of ATRA to RXR and RAR that transcriptionally control ALDH gene expression [38].

Loss of retinoid receptors expression happens frequently in the development of carcino- genesis and induction of resistance to apoptosis. The known effect of ATRA on differentiation of cells is mediated through RARβ. RARβ belongs to the nuclear receptor (NR) superfamily of transcription factors. Upregulation of RARβ within the drugresistant cancer cells, which exhibits loss of RARβ expression, has been shown to increase the susceptibility of cells to apoptosis induced by chemotherapeutic agents. Activation of RARs or RXRs contributes to induction of RARβ, growth inhibition and apoptosis by retinoids. It evidenced that the therapeutic anti-CSC and proapoptotic effects of ATRA are dependent on receptor class-selective retinoids and the expression of RARβ plays a role in mediating retinoid response in NSCLC cells. [38, 42]. The loss of RARβ might contribute to enhanced cancer stemness and the apoptosis resistance of CSCs to gefitinib in NSCLC cells. ATRA can induce the apoptosis of NSCLC CSCs through activation of RARβ and its ability to down-regulate the CSCs markers in lung cancer cells [42, 43]. The expression of RARβ as well as RXRβ was reported to be downregulated in NSCLC, which enabled the cancer cell to evade apoptosis [45, 46].The RARβ is also known as tumor suppressor and the major target gene of retinoid action, and an enhanced level of RARβ protein exhibited its growth inhibitory action of lung cancer cells [47, 48]. RARβ can mediate retinoid action in lung cancer cells by promoting apoptosis. However, a fundamental question that remains unanswered is how ATRA and RARβ trigger apoptosis in lung cancer cells. Studies showed the overexpression of RARβ was accompanied by an increase in c-Myc and Bax but not TP53 protein expression and associated with an increase in the Bax/Bcl2 ratio, and that ATRA enhanced G1 growth arrest, up-regulated p21and p27 and downregulated cyclin D1. These data suggest that the expression of RARβ is positively associated with ATRA-induced apoptosis and growth inhibition in lung cancer cells [38, 42–45]. It has been shown that RA inhibits EGFR expression at the transcriptional level by targeting the EGFR promoter leading to inhibition of lung cancer cell growth and arrests EGFR-TKI resistant NSCLC cells in the G0/G1 phase of the cell cycle by altering the expression of

GATA-binding factor 6 (GATA6) and inhibits the activation of two important pathways involved in lung cancer progression namely EGFR and Wnt signaling to overcome TKI resistance [48]. Combinatorial treatment of retinoids with EGFR-TKIs drugs in EGFR-TKIs resistance lung cancer cells promotes the activation of GATA6 and then inhibits the activation of EGFR/Wnt signaling pathways and favors the association of RXR, RARβ, and cellular retinoic acid binding protein-2 (CRABP2). This complex inhibits the proliferation and promotes the differentiation of lung tumor cells via inhibiting activating protein-2 (AP-2), which result in re-sensitization of EGFR-TKIs resistant lung cancer cells [49].

In this study, a short-term gefitinib treatment was used to enrich A549GSCs and H1650GSCs. FCM assay showed that A549GSCs and H1650GSCs have a significant increase in proportions of ALDH1A1^{bright}/CD44^{high} cells (Fig. 2), and we further confirmed that these ALDH1A1^{bright}/CD44^{high} GSCs exhibit increased IC50 values for gefitinib compared to that of their respective parental cells (Table 1 and Fig. 1A and B), and are involved in CSCs but not in EGFR T790M-mediated gefitinib resistance (Supplementary Fig. 1), suggesting that ALDH1A1^{bright}/CD44^{high} CSCs in NSCLC/ADC contribute to resistance to gefitinib[17]. Interestingly, treatment with ATRA significantly reduced ALDH1A1 and CD44 expression of A549GSCs and H1650GSCs, and their IC50 values for gefitinib, thus returning to sensitization to gefitinib (Table 1 and Fig. 1A and B). These results showed that in contrast to the known tendency of EGFR-TKIs, such as gefitinib, to target the non-stem-like ALDH1A1-negative cell population, ATRA can modulate the ALDH1A1^{bright}/CD44^{high} cell population in NSCLC/ADC. Therefore, the synergistic antitumor effect of ATRA in combination with gefitinib might be a promising therapeutic strategy to prevent or re-sensitize CSC-mediated gefitinib resistant NSCLC/ADC [49, 50].

Conclusions

Our findings indicate that combinatorial treatment of ATRA with gefitinib could reduce CSCs-mediated resistance by down-regulating expression of ALDH1A1 and CD44 and potentiate the anti-tumor effect of gefitinib in NSCLC/ADC.

List Of Abbreviations

EGFR-TKD Epidermal growth factor receptor- tyrosine kinase domain
TKIs Tyrosine kinase inhibitors
ATRA All-trans retinoic acid
NSCLC/ADC Non-small cell lung adenocarcinoma
ALDH1A1 Aldehyde dehydrogenase 1 family member A1
CD44 Cluster of differentiation 44
FCM Flow cytometry
IC50 Half maximal inhibitory concentration
NSCLC Non-small cell lung cancer
EGFRm mutant epidermal growth factor receptor
ATP Adenosine triphosphate

EGFR-TKIs EGFR tyrosine kinase inhibitors
ADC Adenocarcinoma
EGFRWT wild-type EGFR
SCLC Small cell lung cancer
MET Cellular-mesenchymal to epithelial transition factor
ERBB2 Erythroblastic leukemia viral oncogene homolog 2
PIK3CA Phosphatidylino-sitol 3-kinases, catalytic, alpha polypeptide
EMT epithelial to mesenchymal transition
CSC Cancer stem cell-like cell
RAs Retinoic acids
RR Response rate
PFS Progression-free survival
APL Acute promyelocytic leukemia
CTCCCAS The committee on type culture collection of Chinese academy of sciences
FBS Fetal bovine serum
EDTA Ethylenediaminetetraacetic acid
TAE Tris/Acetate/EDTA
PCR Polymerase chain reaction
GSC Gefitinib surviving cell
TME Tumor microenvironment
GADD153 C/EBP homologous protein, CHOP
EBPb CCAAT-enhancing binding protein-B
RARs Retinoic acid receptors
GATA6 GATA-binding factor 6
CRABP2 cellular retinoic acidbinding protein-2
AP-2 activating protein-2

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WY, JH and LW conceived and designed this study; HH, HL and CG performed the experiments; KM and JC contributed to the quality control of data and algorithms; LH and XL analyze the data/results; HH and XL wrote the manuscript; JH improved and revised the manuscript. All authors read and approved the final manuscript.

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Table

Table 1 not included with this version.