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Effect of siRNAs targeting T790M mutation and wild type EGFR in non-small cell lung cancer cell line resistant to gefitinib or erlotinib

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ABSTRACT

The epidermal growth factor receptor (EGFR) is a validated therapeutic target in non-small cell lung cancer (NSCLC). However, some mutations confer resistance to current available agents, especially the T790M mutation. In the current study, we have examined in a NSCLC cell line H1975 containing both L858R and T790M mutations, the effect of T790M-specific-siRNAs. T790M-specific-siRNAs were able to inhibit T790M and EGFR mRNA, to reduce EGFR protein expression, as well as to reduce the cell growth and induce cell caspase activity in H1975 cells. However, this effect showed less potency compared to the EGFR-specific-siRNAs. EGFR-specific-siRNAs offered strong activity to inhibit cell growth and induce apoptosis in H358, H1650, H292, HCC827 and also H1975 cells, which showed weak response to tyrosine kinase inhibitors (TKIs) or cetuximab. The addition of T790M-specific-siRNAs could rescue the sensitivity of T790M mutant H1975 cells to TKIs. The combination of T790M-specific-siRNAs and cetuximab also additively enhanced cell growth regression and induction of apoptosis in H1975 cells. Among the anti-EGFR agents tested, the strongest biological effect was observed when afatinib combined with T790M-specific-siRNAs. Afatinib also offered extra effect when combined with cetuximab in H1975 cells. In a conclusion, knock-down of T790M transcript by siRNAs further decreases the cell growth of T790M mutant lung cancer cells that are treated with TKIs or cetuximab alone. The combination of a potent, irreversible kinase inhibitor such as afatinib, with T790M-specific-siRNAs should be further investigated as a new strategy in the treatment of lung cancer containing the resistant T790M mutation.

1. Background

Lung cancer is one of the most frequent neoplasm worldwide and about 85–90% of lung cancer is non-small cell lung cancer (NSCLC) [1]. Activating mutations in the kinase domain of epidermal growth factor receptor (EGFR) in NSCLC commonly arise as in-frame deletions in exon 19 and L858R exon 21 substitution, and confer sensitivity to the reversible tyrosine kinase inhibitors (TKIs) [2,3]. Despite initial responses, NSCLCs driven by EGFR activating mutations inevitably develop resistance to these TKIs. An acquired substitution mutation (T790M) emerges in approximately 50% of EGFR-mutated patients with TKI resistance [4]. The threonine to methionine change at the 790 amino acid "gatekeeper" residue in the EGFR kinase domain has been shown to confer resistance by increasing the affinity for ATP, compromising the potency of reversible TKIs [5]. In these T790M-harboring cells, inhibition of EGFR by currently available second-generation EGFR-TKIs is still not sufficient to physiologically prevent the emergence of cells that are dependent on EGFR signaling [6]. For instance, afatinib (BIBW 2992, Boehringer Ingelheim GmbH), an panHER inhibitor of EGFR, HER2 and HER4 kinases, retains some activity in tumors with T790M mutations. However, afatinib showed a limited efficacy in NSCLC with T790M [7]. Therefore, strategies to overcome TKI resistance remain practical needs in order to prolong survival of patients with NSCLC. RNA interference (RNAi) has provided a powerful tool with which to modulate gene expression for the study of gene function. Several reports described effects of EGFR-targeted RNAi to inhibit cell growth [3,4,8–10], however attempts to knock-down the T790M-containing allele (using shRNA constructs) were unsuccessful [4]. In the current study we have first investigated the combined effect of RNAi targeting T790M mRNA with T790M-specific-siRNAs, and inactivation of EGFR signaling using different TKIs or a monoclonal antibody cetuximab in the cell line H1975 with T790M mutation.

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2. Material and methods

2.1. siRNAs, cell lines and reagents

Eight siRNAs out of 21 candidate sequences containing the T790M mutation were selected by applying algorithms from Maurice HO Rational siRNA design (http://ihome.ust.hk/~bokcmho/siRNA/siRNA.html), Dharmacon (http://www.dharmacon.com), Reynolds [11], Ui-Tei [12] and Jagla [13]. Eight siRNAs targeting wild type (wt) EGFR sequences were designed using algorithms from Invitrogen, Eurogentec, or Dharmacon or were modified from the literature (Table 1). All siRNAs were purchased from Eurogentec (Liege, Belgium). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) positive control siRNA (used in preliminary experiments to optimize transfection efficiency, data not shown) was from Invitrogen (Merelbeke, Belgium). The negative control siRNA was a proprietary sequence that does not correspond to any eukaryotic gene (Liege, Belgium). The siRNA duplexes with the final concentration 200 nM (for siRNA pool, 50 nM of each individual siRNA were used) were transiently transfected using lipofectamine™ 2000 as described previously [14]. The cell lines information and preparation for different anti-EGFR agents were described previously [14]. Each experiment was performed at least in triplicate and three times independently.

2.2. RT-qPCR and Western blot analysis

RNA isolation, normalization, real time RT-qPCR for T790M, EGFR and GAPDH and western blot were as described previously [14–16]. The following primary antibodies were used: EGFR (Cell Signaling), phospho-EGFR (Tyr1173, clone 9H2, Upstate), phospho-AKT/PKB (pS473, Invitrogen), phospho-ERK1/2 (pTpY185/187, Invitrogen), phospho-STAT3 (Tyr705, 3E2, Cell Signaling), phospho-STAT5 (pY694, BD Biosciences) and β-actin (Sigma–Aldrich N.V.).

2.3. Cell functional detections

Cell growth was assessed using a colorimetric tetrazolium assay (substrate MTS, CellTiter96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, USA). For the siRNAs + TKI/antibody combinations, siRNA transfection was performed first, and 24 h later the cells were treated with different anti-EGFR agents in the same wells and incubated for another 72 h. Cell viability was detected by fluorimetric detection of resorufin (CellTiter-Blue Cell Viability Assay, Promega, Madison, USA). Caspase-3/7 activity was measured using a synthetic rhodamine labeled caspase-3/7 substrate performed immediately after the detection of viability on the same wells. The effects on apoptosis and nuclear morphology in the cells were assessed by Hoechst 33342 and propidium iodide (PI, Sigma–Aldrich N.V. Bornem, Belgium) double fluorescent chromatin staining. The protocols were as described previously [14].

2.4. Statistical analysis

SPSS19.0 was used for statistical analysis. Values were presented as the mean ± standard deviation (SD). One-way Analysis of Variance (ANOVA) test was performed to analyze significance between groups. The Least Significant Difference (LSD) method

Table 1
EGFR siRNAs used in the study.

Name of siRNA	Exon	Sequence	Location and length (nt)	Designed by	GC content (%)
EGFR siRNA486	2–3	GACCATCCAGGAGGTGGCTGGTTAT	486–510(25)	Invitrogen	56.0
EGFR siRNA604	3	GCAGTCTTATCTAACTATGATGCAA	604–628(25)	Invitrogen	36.0
EGFR siRNA752	4	GCAGTGACTTTCTCAGCAA	752–770(19)	Eurogentec	47.4
EGFR siRNA1247	8–9	GCAAAGTGTGTAACGGAATAGGTAT	1247–1271(25)	Invitrogen	40.0
EGFR siRNA1608	12	GGAGATAAGTGATGGAGAT	1608–1626(19)	Eurogentec	42.1
EGFR siRNA2654	20	GGGAACACAAAGACAATAT	2654–2672(19)	Dharmacon	36.8
EGFR siRNA2708	20–21	TCGCAAAGGGCATGAACTA	2708–2726(19)	Dharmacon	47.4
EGFR siRNA4765	28	AGAATGTGGAATACCTAAGG	4766–4785(20)	*	40.0
Scrambled EGFR siRNA486		GAGGTTAGCTGGGTCCCGATCATGA	NA(25)	☆	56.0
Scrambled EGFR siRNA604		GCATTGTCATTAAACGCAACGTATT	NA(25)	☆	36.0
Scrambled EGFR siRNA752		GGCTAAGCTCGCTATTACA	NA(19)	☆	47.4
Scrambled EGFR siRNA1247		GGTGATTAGGTTATAAACGGACAGA	NA(25)	☆	40.0
T790M siRNA2596	20	TCCACCGTGCAGCTCATCATG	2596–2616(21)	△	57.1
T790M siRNA2597	20	CCACCGTGCAGCTCATCATGC	2597–2617(21)	△	61.9
T790M siRNA2600	20	CCGTGCAGCTCATCATGCAGC	2600–2620(21)	△	61.9
T790M siRNA2601	20	CGTGCAGCTCATCATGCAGCT	2601–2621(21)	△	57.1
T790M siRNA2603	20	TGCAGCTCATCATGCAGCTCA	2603–2623(21)	△	52.4
T790M siRNA2607	20	AGCTCATCATGCAGCTCATGC	2607–2627(21)	△	52.4
T790M siRNA2608	20	GCTCATCATGCAGCTCATGCC	2608–2628(21)	△	57.1
T790M siRNA2612	20	TCATGCAGCTCATGCCCTTCG	2612–2632(21)	△	57.1
Scrambled T790MsiRNA2597		GCTCAGCGACGCCTCATACCT	NA(21)	☆	61.9
Scrambled T790MsiRNA2600		GCGCAGTCGCTAGCTCACTCA	NA(21)	☆	61.9
Scrambled T790MsiRNA2603		GCATTAGGCGTCCCACTCAAT	NA(21)	☆	52.4
Scrambled T790MsiRNA2608		GCCTCTAGTATCCGGACACCT	NA(21)	☆	57.1
Wt for T790MsiRNA2597, EGFRwtsiRNA2597	20	CCACCGTGCAGCTCATCACGC	2597–2617(21)	NA	66.7
Wt for T790MsiRNA2600, EGFRwtsiRNA2600	20	CCGTGCAGCTCATCACGCAGC	2600–2620(21)	NA	66.7
Wt for T790MsiRNA2603, EGFRwtRNA2603	20	TGCAGCTCATCACGCAGCTCA	2603–2623(21)	NA	57.1
Wt for T790MsiRNA2608, EGFRwtRNA2608	20	GCTCATCACGCAGCTCATGCC	2608–2628(21)	NA	61.9

*, Modified from Ref. [4].
△, Different siRNA design algorithms.
☆, By http://www.sirnawizard.com/scrambled.php.
NA, not applicable.

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