表 4. 誤分類バイアスの感度解析の結果(Greenland, 1996, p.1111 より引用). Se, Sp の値ごとのオッズ比 OR_{DX} の推定値.

Cases		Controls				
Se	*Sp*	*Se*:	0.90	0.80	0.90	0.80
		Sp:	0.90	0.90	0.80	0.80
0.90	0.90		2.34*	2.00	19.3	16.5
0.80	0.90		2.83	2.42*	23.3	19.9
0.90	0.80		1.29	1.11	10.7*	9.1
0.80	0.80		1.57	1.34	12.9	11.0*

* Non-differential な誤分類.

となる. $B_0 = M_0 - B_1$ であるため, これにより, (B_0, B_1) を計算することができる. こうして得られる B_0, B_1 は, 感度・特異度についての仮定を置いたもとで得られる推定値(期待値)である. $SeSp = FnFp$ である場合, これらの解は不定となり, $SeSp < FnFp$ の場合, 負値をとる. 後者の場合, 曝露変数の分類が, でたらめ(無作為)な分類よりも悪いということを意味している.

一般的には, 曝露変数の分類は, 無作為な分類よりも正確であるという前提で感度解析を行う. 式(2.4)と同様に, ケース群における, 真に曝露を受けた人数を A_1, 真に曝露を受けていない人数を A_0 とすると,

$$A_1 = (SpA_1^* - FpA_0^*)/(SeSp - FnFp) \tag{2.5}$$

となる. また, ケースの総数 M_1 に対して, $A_0 = M_1 - A_1$ となる. 古典的な誤分類の感度解析は, (Se, Sp) に適当な値を与えたもとで, (2.4)(2.5)式からセル度数の期待値を求め, バイアス調整を行ったオッズ比 OR_{DX} を求める. Greenland(1996)による, いくつかのシナリオのもとでの感度解析の結果を, 表 4 に示した. よく知られているように, Non-differential な誤分類のもとでは, 誤分類によるオッズ比のバイアスは必ず帰無仮説の(保守的な)方向に入る. これについては, 曝露変数が 3 水準以上であったり, 他の変数に誤分類が入ったりする場合には, その限りではない(Dosemeci et al., 1990). また, 表 4 の結果からは, ケース群がコントロール群よりも「より曝露を受けたと誤分類される傾向が大きい」という場合でも, バイアス調整オッズ比が, 無調整オッズ比よりも大きく出るというシナリオも出ている. これは, いわゆるケースコントロール研究での「思い出しバイアス」の設定であり, 思い出しバイアスが必ずしも曝露効果を過大に推定するバイアスを生じさせるわけではないことを示している. また, この事例では, Se よりも Sp のほうが推定値のバイアスに与える影響が大きく出ている. これは, そもそもの曝露を受けた対象者の割合が小さいためである.

この設定でも, 前節と同様に Se, Sp をバイアスパラメータとして, 同じ手順で, モンテカルロ感度解析を実行することができる. ここでは, Non-differential な誤分類の仮定を置き, ケース群・コントロール群の Se, Sp が共通で, 台形分布 Trapezoidal $(0.75, 0.85, 0.95, 1.00)$ に従うという仮定のもとでのモンテカルロ感度解析を行う. ランダムな誤差も組み込んだ OR_{DX} の分布を, 図 1(d) に示す. 誤分類バイアスのみを調整したオッズ比の分布の中央値は 2.45, 95%区間は $(1.87, 14.25)$ であり, ランダムな誤差も含めた調整オッズ比の中央値は 2.56, 95%区間は $(1.49, 14.68)$ であった. バイアス調整オッズ比の 95%区間は, 無調整オッズ比の 95%信頼区間

よりもかなり広く，誤分類バイアスによる不確実性は，この事例に関しては，ランダムな誤差よりもかなり強いと考えられる．

同様の誤分類の感度解析についての研究は，古くから，疾病の誤分類(Greenland, 1996)，交絡因子の誤分類(Savitz and Baron, 1989)，複数の変数についての誤分類(Kristensen, 1992; Chavance et al., 1992)，バリデーション研究のデータを用いた評価方法(Wacholder et al., 1993; Tennenbein, 1970; Green, 1983; Marshall, 1990; Brenner and Gefeller, 1993)など，多くの方法論が開発されている．モンテカルロ感度解析は，これらの方法にも，同様に適用することができる．

2.3 選択バイアス

選択バイアス(selection bias)は，結論を適用したいターゲット集団からの対象者の選択におけるバイアスであり，ケースコントロール研究では，コントロールの選択におけるバイアスの問題が古くから問題とされてきた(Rothman et al., 2008)．例えば，Science 誌での Taubes and Mann(1995)でも，Boston 大学の Charles Poole 教授が，1980 年代の低レベル電磁場の曝露と白血病・脳腫瘍のケースコントロール研究を行った際の Random digit dialing によるコントロールの選択(ランダムに構成した電話番号で，コントロールを選び出す方法)でのバイアスの実例を紹介しており，この研究では，社会経済的に低い階層の人々が，コントロール群に選ばれにくいというバイアスがあったと述べている．貧しい階層のひとは，そもそもこのような研究に参加したがらないという傾向があり，また，日中，自宅に居ることも少なく，当時の留守番電話機能のある電話を持っていないひとが多かったという(Taubes and Mann, 1995)．この選択バイアスにより，この研究では，対象者集団の偏りはもちろん，統計解析の結果でも，貧しい階層に顕著な母乳育児，母親の喫煙などが，リスク要因として上がってきたという．

選択バイアスの感度解析におけるケースコントロール研究のオッズ比の分解方法はよく知られたものがある(Kleinbaum et al., 1984; Rothman et al., 2008)．S_{A1}, S_{B1} を，それぞれ曝露を受けたケース・非ケースが，ケース群・コントロール群へサンプリングされる確率であるとする．このとき，ケースコントロール研究でのそれぞれのサンプリング数の期待値は，A_1/S_{A1}, B_1/S_{B1} となる．同様に，S_{A0}, S_{B0} を，それぞれ曝露を受けていないケース・非ケースのサンプリング確率とすると，それぞれのサンプリング数の期待値は，A_0/S_{A0}, B_0/S_{B0} となる．このとき，選択バイアスを調整したオッズ比は，

$$\frac{(A_1/S_{A1})(B_0/S_{B0})}{(A_0/S_{A0})(B_1/S_{B1})} = \left(\frac{A_1B_0}{A_0B_1}\right)\left(\frac{S_{A1}S_{B0}}{S_{A0}S_{B1}}\right)^{-1}$$

と書ける．すなわち，選択バイアスの調整オッズ比は，単純に無調整オッズ比を，選択バイアスの調整因子 $S^* = S_{A1}S_{B0}/S_{A0}S_{B1}$ で割ったものとして表すことができる．当然ながら，$S^* = 1$ のとき，選択バイアスの影響はなくなり，ケースコントロールオッズ比で，偏りのない推定が可能である．これは，例えば，疾病の有無・曝露の有無と対象者の選択が独立である場合に成り立つ(Greenland, 1996; Greenland and Lash, 2008)．

モンテカルロ感度解析は，S_{A1}, S_{B1}, S_{A0}, S_{B0} をバイアスパラメータとしても実行可能であるが，ここではバイアス調整因子 S^* を直接バイアスパラメータと見なして，感度解析を行う．バイアスモデルは，95%確率区間が $(0.70, 1.50)$ となるような対数正規分布を仮定する．すなわち，$\log(S^*) \sim N(0, 0.21^2)$ とする．ランダムな誤差も組み込んだ OR_{DX} の分布は，図 1(e) となる．選択バイアスを調整したオッズ比の分布の中央値は 1.76，95%区間は $(1.17, 2.65)$，ランダムな誤差を考慮した調整オッズ比の分布の中央値は 1.76，95%区間は $(1.01, 3.10)$ であった．

このバイアスモデルは，単純なケースコントロール研究に限らず拡張可能である．たとえば Density sampling を行ったケースコントロール研究の場合には，S_{A1}, S_{B1}, S_{A0}, S_{B0} が人時間

表 5. Greenland et al. (1994)の事例におけるモンテカルロ感度解析の結果.

バイアスモデル	ランダムな誤差を含めない場合		ランダムな誤差を含めた場合	
	Median	2.5th & 97.5th Percentiles	Median	2.5th & 97.5th Percentiles
1. なし（無調整な解析）	1.77	—	1.77	1.18, 2.64
2. 未測定交絡因子によるバイアス	1.76	1.25, 2.48	1.76	1.05, 2.96
3. 誤分類バイアス	2.45	1.87, 14.25	2.56	1.49, 14.68
4. 選択バイアス	1.76	1.17, 2.65	1.76	1.01, 3.10
5. 複合バイアスモデル（2-4 を同時にモデル化）	4.26	2.24, 27.66	4.32	2.00, 28.30

あたりの率となる．また，Scharfetein et al. (1999)では，線形回帰モデルへの拡張も行われている．同様のバイアスモデルを用いて，Greenland (2003)や Lash and Fink (2003)のように対象者の参加やレスポンス，追跡・脱落に起因するバイアスも調整をすることも可能である．また，メタアナリシスでは，実施された研究の出版における選択プロセスのバイアスは出版バイアス (publication bias)として知られており，多くの感度解析の方法が研究されているが，Bowden et al. (2010) によって，モンテカルロ感度解析を利用した議論も行われている．

2.4 複合バイアスモデリング

ここまでは，それぞれのバイアスを個別に調整する感度解析の方法を説明してきたが，複数のバイアスが同時に懸念される場合，それらを同時に調整した感度解析を行う必要がある．古典的な感度解析の方法では，複数のバイアスを多次元的にモデル化し，調整オッズ比を求めることには，まずは計算上の困難が伴い，解析的な表現が陽に求まらないこともある．仮に，数値的に取り扱うことができたとしても，それぞれのバイアスについて複数のシナリオを考慮する必要があり，そのすべての組み合わせを考慮すると，全体としてのシナリオは膨大なものとなる．自然，解釈も複雑なものとなる．また，バイアス調整の順番が入れ替わると，調整オッズ比の値が変わってしまうという可能性もある (Greenland, 1996; Greenland and Lash, 2008)．この場合にも，モンテカルロ感度解析によるバイアスの同時調整は可能であり，複数のバイアスをモデル化した方法として，複合バイアスモデリング (multiple bias modeling)が提案されている (Greenland, 2005)．次節で解説する通り，これは，ベイズ流の解析としての解釈が可能であり，バイアスパラメータを複数同時にモデル化したベイズモデルにおいて，近似的な事後サンプリングを行うものとなる．バイアス間の関連についても，バイアスパラメータの同時分布における相関のモデル化によって表現することができる．

事例として，2.1 節から 2.3 節で考慮したバイアスを独立にモデル化した複合バイアスモデルにおけるモンテカルロ感度解析を考える．バイアスパラメータは独立であるという仮定になるため，前節までのアルゴリズムの単純な拡張として，未調整のオッズ比に対して，個々のバイアス調整の方法を順に適用していき，それぞれのバイアスの不確実性を加算していくだけでよい (Greenland and Lash, 2008)．バイアスの不確実性のみを考慮した調整オッズ比の分布では，中央値は 4.26，95%区間は (2.24, 27.66) となり，複数のバイアスを同時に考慮している分，不確実性は大きくなる．ランダムな誤差も考慮すると，中央値は 4.32，95%区間は (2.00, 28.30) とな

る．後者の OR_{DX} の分布は，図 1(f) となる．

2 節での感度解析の結果を，表 5 にまとめた．いずれのバイアスモデルのもとでも，95%区間の下限は，帰無仮説を跨いでいない．特に，すべてのバイアスを複合バイアスモデルで組み込んだ場合でも，この結果は一貫して成り立っている．すなわち，今回の解析で考慮したシナリオのもとでは(概ね Greenland and Lash(2008)によるものと同等)，結論は概ね不変であり，これらの仮定のもとでのバイアスに対するロバストネスが確認できたと言うことはできるだろう．感度解析の結果の解釈・報告の方法についての詳細は，Lash et al.(2009) が参考になるだろう．

3. 確率的感度解析の理論

ここまで Greenland et al.(1994)の事例をもとにして説明を行ってきた確率的感度解析は，もともとは，金融や経済における投機リスク評価，原子炉の安全性評価などリスク評価の分野で用いられてきた方法である(Committee on Risk Assessment of Hazardous Air Pollutants, Commission on Life Sciences, National Research Council, 1994; Vose, 2008)．疫学研究では，近年になって，電磁場への曝露と小児白血病の関連評価への適用(Phillips, 2003; Greenland, 2003)やシリカ曝露と肺がんの関連評価への適用(Steenland and Greenland, 2004)をはじめ，Chu et al.(2006)，Eddy et al.(1992)，Fox et al.(2005)，Greenland(2001, 2005)，Hoffman and Hammonds(1994)，Lash and Fink(2003) などによる研究が行われてきた．

モンテカルロ感度解析の数学的定式化として，まず一連の観測データ (D) を用いて興味あるパラメータ $\theta = \theta(\alpha)$ を推定することを考える．従来の解析では，系統的バイアスはないという仮定を置き D のみによって θ が識別できるものとして，統計モデル $L(D|\alpha)$ を用いて θ を推定する．これに対してバイアスモデルでは，バイアスパラメータを η としてモデルに導入し，データの分布はバイアスモデルを組み込んだ $L(D|\alpha,\eta)$ によってモデル化されるとする．

バイアスモデルを用いたバイアス調整をベイズ流に扱うことを考えると，(α,η) の事前分布 $p(\alpha,\eta)$ を与えて $\theta = \theta(\alpha)$ 周辺事後分布を求めることになる(Gelman et al., 2013; Greenland, 2005)．

$$p(\alpha|D) \propto \int L(D|\alpha,\eta)p(\alpha,\eta)d\eta$$

その際は，事前分布 $p(\alpha,\eta)$ を説明するのに，バイアスパラメータ η 自身が既知の共変量と未知のハイパーパラメータ β の関数としてモデル化されることになることもある(Parmigiani, 2002)．このようにして導かれる周辺事後分布における推測では，複雑な階層モデルなどでは特に，マルコフ連鎖モンテカルロ法の実装や収束判断などの計算上の問題が伴うが，モンテカルロ感度解析法では，比較的簡便なモンテカルロサンプリングを組み合わせるだけで実行することができ，それらの問題点を回避することができる．また，後に述べるとおり，複数のバイアスを複合的にモデル化する場合には，階層ベイズモデルでの事後サンプリングのアルゴリズムは，それに比例して複雑なものとなるが，モンテカルロ感度解析は，単純に複数のバイアスに対してのモンテカルロシミュレーションを組み合わせるだけで実装できるという利点がある．

ここまでに述べてきたモンテカルロ感度解析法は，前述の事前分布 $p(\alpha,\eta)$ をバイアスパラメータの周辺事前分布 $p(\eta)$ のみの特定で代用したものに相当する．一般に，α は η の情報なしには部分的にも特定されず，α についての推定は η の値に完全に依存する(逆に η も α の情報なしには部分的にも特定されない)．モンテカルロ感度解析では，周辺事前分布 $p(\eta)$ からの η の各サンプリング値に対して，η を固定して推定した興味あるパラメータの推定値を最尤推定量であるとみなす．この場合，α には一様事前分布(無情報事前分布)を仮定していることになり，$p(\eta|D) \fallingdotseq p(\eta)$ と近似できる．したがって，

$$p(\alpha|D,\eta) \propto \frac{L(D|\alpha,\eta)p(\alpha,\eta)}{p(\eta|D)} \propto L(D|\alpha,\eta)$$

が成り立ち，

$$p(\theta|D,\eta) \propto \int_{\theta(\alpha)=\theta} L(D|\alpha,\eta)d\alpha$$

となる．

大標本の状況では，事後分布 $p(\theta|D,\eta)$ は正規近似 $[\sim N(\hat{\theta}_\eta, \hat{s}^2_\eta)]$ が可能であり，$p(\theta|D,\eta)$ を得るための積分が不要となる．モンテカルロ感度解析では以下の3つの手順を踏むことで以下の事後分布 $p(\theta|D)$ からの擬似的なサンプリングとみなすことができる．

$$p(\theta|D) = \int p(\theta|D,\eta)p(\eta|D)d\eta$$

1. $p(\eta)$ から η の値をランダムサンプリングする
2. 各 η から $\hat{\theta}_\eta$ と標準誤差 $\hat{s}_\eta$ を算出する
3. $\hat{\theta}_\eta$ を $N(\hat{\theta}_\eta, \hat{s}^2_\eta)$ から再サンプリングするか $\hat{\theta}_\eta$ の分布に $N(0, \hat{s}^2_\eta)$ を加える

このように，モンテカルロ感度解析は，ベイズ流の枠組みで解釈することができる．一方で，さまざまな観点からの考察はされているが，頻度論的な方法としての解釈はできない(Greenland, 2001)．また，α についての事前分布を導入した方法への拡張は容易である．

複合バイアスモデリングにおけるモンテカルロ感度解析は，特定のバイアスに対して，上記のサンプリングとバイアス調整を実施し，その結果を他のバイアスモデルでの無調整パラメータとして，個別に同様のサンプリングを繰り返すことで，複数のバイアスの同時調整が可能となる．

ここで紹介した解析手法の多くは，Orsini et al.(2008)による STATA モジュール EPISENS (http://nicolaorsini.altervista.org/stata/tutorial/e/episens.htm)で実行することができる．また，Lash et al.(2009)は，疫学における確率的感度解析の教科書であり，背景の理論や計算ツールについてのより詳細な解説がまとめられている．

4. おわりに

観察研究でのバイアスの存在およびその影響については，生物統計の専門家であるとなしとに関わらず，広く重要であると認識されている．その一方で定量的な評価はシナリオ依存的な感度解析になることが多く，ほとんどの場合は実施されずに考察部分で限界としての議論がなされるに留まることが多い．バイアスモデルの定式化及び事前分布を仮定してモンテカルロサンプリングを行うことで複数のバイアスを同時考慮することは，このような定性的議論を打開し，観察研究の結果に含まれる不確実性を定量的に評価することにつながる．選択バイアスに関しては，公的な統計データなどを外部データとの比較で事前分布に関する情報を得ることが可能であろう．未測定の交絡に関しては，すべての対象者で測定されていない共変量を未測定の交絡因子とみなし，測定されている一部対象者の情報を事前情報とした欠測補完の手法として利用するという利用方法もある．または，架空の交絡因子を想定し，結果を覆さない交絡因子はどの程度の影響(未測定の交絡因子のオッズ比)をもつものかを逆算することで，そのような交絡因子が存在する可能性を議論することも可能である．測定誤差については，このようなバイアスモデルを用いることで，デザイン段階で一部対象者に対して精度の高い測定を併せて実施しておくことで，それを妥当性データとして利用することもできる．既存の観察研究データおよび計画中の観察研究に対してぜひ適用を検討いただきたい．

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参 考 文 献

Axelson, O. and Steenland, K. (1988). Indirect methods of assessing the effect of tobacco use in occupational studies, *American Journal of Industrial Medicine*, **13**, 105–118.

Barron, B. A. (1977). The effects of misclassification on the estimation of relative risk, *Biometrics*, **33**, 414–418.

Bowden, J., Jackson, D. and Thompson, S. D. (2010). Modelling multiple sources of dissemination bias in meta-analysis, *Statistics in Medicine*, **29**, 945–955.

Brenner, H. and Gefeller, O. (1993). Use of positive predictive value to correct for disease misclassification in epidemiologic studies, *American Journal of Epidemiology*, **138**, 1007–1115.

Bross, I. D. J. (1966). Spurious effects from an extraneous variable, *Journal of Chronic Diseases*, **19**, 637–647.

Bross, I. D. J. (1967). Pertinency of an extraneous variable, *Journal of Chronic Diseases*, **20**, 487–495.

Carroll, R. J., Spiegelman, C. H., Lan, K. K. G., Bailey, K. T. and Abbott, R. D. (1984). On errors-in-variables for binary regression models, *Biometrika*, **71**, 19–25.

Carroll, R. J., Ruppert, D. and Stefanski, L. A. (1995). *Measurement Error in Nonlinear Models*, Chapman and Hall/CRC, London.

Chavance, M., Dellatolas, G. and Lellouch, J. (1992). Correlated nondifferential misclassifications of disease and exposure, *International Journal of Epidemiology*, **21**, 537–546.

Chu, H. Z., Wang, S. R. and Greenland, S. (2006). Sensitivity analysis of misclassification: A graphical and a Bayesian approach, *Annals of Epidemiology*, **16**, 834–841.

Committee on Risk Assessment of Hazardous Air Pollutants, Commission on Life Sciences, National Research Council (1994). *Science and Judgment in Risk Assessment*, National Academy Press, Washington, D.C.

Copeland, K. T., Checkoway, H., Holbrook, R. H. and McMichael, A. J. (1977). Bias due to misclassification in the estimate of relative risk, *American Journal of Epidemiology*, **105**, 488–495.

Cornfield, J., Haenszel, W., Hammond, E. C., Lillienfeld, A. M., Shimkin, M. B. and Wynder, E. L. (1959). Smoking and lung cancer: Recent evidence and discussion of some questions, *Journal of the National Cancer Institute*, **22**, 173–203.

Dosemeci, M., Wacholder, S. and Lubin, J. H. (1990). Does nondifferential misclassification of exposure always bias a true effect toward the null values?, *American Journal of Epidemiology*, **132**, 746–749.

Eddy, D. M., Hasselblad, V. and Shachter, R. D. (1992). *Meta-analysis by the Confidence Profile Method: The Statistical Synthesis of Evidence*, Academic Press, Boston.

Flanders, W. D. and Khoury, M. J. (1990). Indirect assessment of confounding: Graphic description and limits on effect of adjusting for covariates, *Epidemiology*, **1**, 239–246.

Fox, M. P., Lash, T. L. and Greenland, S. (2005). A method to automate probabilistic sensitivity analyses of misclassified binary variables, *International Journal of Epidemiology*, **34**, 1370–1376.

Gail, M. H., Wacholder, S. and Lubin, J. H. (1988). Indirect corrections for confounding under multiplicative and additive risk models, *American Journal of Industrial Medicine*, **13**, 119–130.

Gelman, A., Carlin, J. B., Stern, H. S., Funson, D. B., Vehtari, A. and Rubin, D. B. (2013). *Bayesian data analysis*, 3rd ed., Chapman and Hall/CRC, London.

Green, M. S. (1983). Use of predictive value to adjust relative risk estimates biased by misclassification of outcome status, *American Journal of Epidemiology*, **117**, 98–105.

Greenland, S. (1982). The effect of misclassification in matched-pair case-control studies, *American Journal of Epidemiology*, **116**, 402–406.

Greenland, S. (1987). Quantitative methods in the review of epidemiologic literature, *Epidemiologic Review*, **9**, 1–30.

Greenland, S. (1990). Randomization, statistics, and causal inference, *Epidemiology*, **1**, 421–429.

Greenland, S. (1996). Basic methods for sensitivity analysis of biases, *International Journal of Epidemiology*, **25**, 1107–1116.

Greenland, S. (2001). Sensitivity analysis, Monte Carlo risk analysis, and Bayesian uncertainty assessment, *Risk Analysis*, **21**, 579–583.

Greenland, S. (2003). The impact of prior distributions for uncontrolled confounding and response bias: A case study of the relation of wire codes and magnetic fields to childhood leukemia, *Journal of the American Statistical Association*, **98**, 47–54.

Greenland, S. (2005). Multiple-bias modeling for analysis of observational data (with discussion), *Journal of the Royal Statistical Society, Series A*, **168**, 267–306.

Greenland, S. and Kleinbaum, D. G. (1983). Correcting for misclassification in two-way tables and matched-pair studies, *International Journal of Epidemiology*, **12**, 93–97.

Greenland, S. and Lash, T. L. (2008). Bias analysis, *Modern Epidemiology* (eds. K. J. Rothman, S. Greenland and T. L. Lash), 345–380, Lippincott-Raven, Philadelphia.

Greenland, S., Salvan, A., Wegman, D. H., Hallock, M. F. and Smith, T. J. (1994). A case-control study of cancer mortality at a transformer-assembly facility, *International Archives of Occupational and Environmental Health*, **66**, 49–54.

Hoffman, F. O. and Hammonds, J. S. (1994). Propagation of uncertainty in risk assessments: The need to distinguish between uncertainty due to lack of knowledge and uncertainty due to variability, *Risk Analysis*, **14**, 707–712.

Kleinbaum, D. G., Kupper, L. L. and Morgenstern, H. (1984). *Epidemiologic Research: Principles and Quantitative Methods*, Wiley, Van Nostrand.

Kristensen, P. (1992). Bias from nondifferential but dependent misclassification of exposure and outcome, *Epidemiology*, **3**, 210–215.

Lash, T. L. and Fink, A. K. (2003). Semiautomated sensitivity analysis to assess systematic errors in observational data, *Epidemiology*, **14**, 459–466.

Lash, T. L. and Silliman, R. A. (2000). A sensitivity analysis to separate bias due to confounding from bias due to predicting misclassification by a variable that does both, *Epidemiology*, **11**, 544–549.

Lash, T. L., Fox, M. P. and Fink, A. K. (2009). *Applying Quantitative Bias Analysis to Epidemiologic Data*, Springer, New York.

Marshall, R. J. (1990). Validation study methods for estimating exposure proportions and odds ratios with misclassified data, *Journal of Clinical Epidemiology*, **43**, 941–947.

Orsini, N., Bellocco, R., Bottai, M., Wolk, A. and Greenland, S. (2008). A tool for deterministic and probabilistic sensitivity analysis of epidemiologic studies, *The Stata Journal*, **8**, 29–48.

Parmigiani, G. (2002). *Modeling in Medical Decision Making — A Bayesian Approach*, Wiley, New York.

Phillips, C. V. (2003). Quantifying and reporting uncertainty from systematic errors, *Epidemiology*, **14**, 459–466.

Rosenbaum, P. R. (1995). *Observational Studies*, Springer-Verlag, New York.

Rothman, K. J., Greenland, S. and Lash, T. L. (2008). *Modern Epidemiology*, 3rd ed., Lippincott

Williams & Wilkins, Philadelphia.

Savitz, D. A. and Baron, A. E. (1989). Estimating and correcting for confounder misclassification, *American Journal of Epidemiology*, **129**, 1062–1071.

Scharfstein, D. O., Rotnitzky, A. and Robins, J. M. (1999). Adjusting for nonignorable drop-out using semiparametric nonresponse models, *Journal of the American Statistical Association*, **94**, 1096–1120.

Schlesselman, J. J. (1978). Assessing effects of confounding variables, *American Journal of Epidemiology*, **108**, 3–8.

Simon, R. (1980). Re: 'Assessing effect of confounding variables', *American Journal of Epidemiology*, **111**, 127–128.

Steenland, K. and Greenland, S. (2004). Monte Carlo sensitivity analysis and Bayesian analysis of smoking as an unmeasured confounder in a study of silica and lung cancer, *American Journal of Epidemiology*, **160**, 384–392.

Taubes, G. and Mann, C. C. (1995). Epidemiology faces its limits, *Science*, **269**, 164–169.

Tennenbein, A. (1970). A double sampling scheme for estimating from binomial data with misclassification, *Journal of the American Statistical Association*, **65**, 1350–1361.

Vose, D. (2008). *Risk Analysis*, John Wiley and Sons, New York.

Wacholder, S., Armstrong, B. and Hartge, P. (1993). Validation studies using an alloyed gold standard, *American Journal of Epidemiology*, **137**, 1251–1258.

Yanagawa, T. (1984). Case-control studies: Assessing the effect of a confounding factor, *Biometrika*, **71**, 191–194.

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Sensitivity Analysis for Biases in Observational Studies

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Most researchers recognize that conventional statistical analysis of observational data require assumptions like no selection bias, no information bias, no unmeasured, missing at random... etc. It is almost impossible to assess that these assumptions are met with study data. If these assumptions are unmet, the results from conventional analysis have uncertainty and are biased. In this article, we reviewed bias analysis, focusing on probabilistic sensitivity analysis.

Key words: Observational study, epidemiology, uncertainty, bias, probabilistic sensitivity analysis.

Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced non-squamous non-small-cell lung cancer harbouring *EGFR* mutations (JO25567): an open-label, randomised, multicentre, phase 2 study

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Summary

Background With use of EGFR tyrosine-kinase inhibitor monotherapy for patients with activating *EGFR* mutation-positive non-small-cell lung cancer (NSCLC), median progression-free survival has been extended to about 12 months. Nevertheless, new strategies are needed to further extend progression-free survival and overall survival with acceptable toxicity and tolerability for this population. We aimed to compare the efficacy and safety of the combination of erlotinib and bevacizumab compared with erlotinib alone in patients with non-squamous NSCLC with activating *EGFR* mutation-positive disease.

Methods In this open-label, randomised, multicentre, phase 2 study, patients from 30 centres across Japan with stage IIIB/IV or recurrent non-squamous NSCLC with activating *EGFR* mutations, Eastern Cooperative Oncology Group performance status 0 or 1, and no previous chemotherapy for advanced disease received erlotinib 150 mg/day plus bevacizumab 15 mg/kg every 3 weeks or erlotinib 150 mg/day monotherapy as a first-line therapy until disease progression or unacceptable toxicity. The primary endpoint was progression-free survival, as determined by an independent review committee. Randomisation was done with a dynamic allocation method, and the analysis used a modified intention-to-treat approach, including all patients who received at least one dose of study treatment and had tumour assessment at least once after randomisation. This study is registered with the Japan Pharmaceutical Information Center, number JapicCTI-111390.

Findings Between Feb 21, 2011, and March 5, 2012, 154 patients were enrolled. 77 were randomly assigned to receive erlotinib and bevacizumab and 77 to erlotinib alone, of whom 75 patients in the erlotinib plus bevacizumab group and 77 in the erlotinib alone group were included in the efficacy analyses. Median progression-free survival was 16·0 months (95% CI 13·9–18·1) with erlotinib plus bevacizumab and 9·7 months (5·7–11·1) with erlotinib alone (hazard ratio 0·54, 95% CI 0·36–0·79; log-rank test p=0·0015). The most common grade 3 or worse adverse events were rash (19 [25%] patients in the erlotinib plus bevacizumab group *vs* 15 [19%] patients in the erlotinib alone group), hypertension (45 [60%] *vs* eight [10%]), and proteinuria (six [8%] *vs* none). Serious adverse events occurred at a similar frequency in both groups (18 [24%] patients in the erlotinib plus bevacizumab group and 19 [25%] patients in the erlotinib alone group).

Interpretation Erlotinib plus bevacizumab combination could be a new first-line regimen in *EGFR* mutation-positive NSCLC. Further investigation of the regimen is warranted.

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Introduction

Lung cancer is a leading cause of death worldwide; it is the primary cause of cancer deaths in men and the secondary cause in women.[1] Most patients with lung cancer have non-small-cell lung cancer (NSCLC) and a clinically significant proportion of patients have activating mutations of *EGFR*.[2] In this subgroup of patients, EGFR tyrosine-kinase inhibitors have consistently led to better outcomes than has standard chemotherapy.[3–6] Erlotinib and gefitinib have been shown to prolong progression-free survival compared with chemotherapy in several phase 3 trials.[7–10] Unfortunately, most patients with NSCLC with activating *EGFR* mutations who are given EGFR tyrosine-kinase inhibitors eventually develop resistance and relapse within about 1 year of initiation of treatment.[5,7–11] To improve outcomes, the foundation treatment of EGFR tyrosine-kinase inhibitors should be built on through investigation of biologically synergistic combinations.

The anti-angiogenic monoclonal antibody bevacizumab targets the VEGF signalling pathway and has been shown to provide additional efficacy when used in combination with first-line platinum-based chemotherapy in several trials in non-squamous NSCLC.[12–14] The combination of erlotinib and bevacizumab has the potential to prolong progression-free survival in unselected populations of patients with NSCLC.[15,16] In a subgroup analysis of *EGFR*

mutation-positive participants in the phase 3 BeTa study of second-line treatment of NSCLC (12 patients treated with erlotinib and bevacizumab and 18 with erlotinib alone), median progression-free survival with erlotinib plus bevacizumab in patients with *EGFR* mutation-positive disease was substantially higher than with erlotinib alone (17·1 months *vs* 9·7 months).[16,17] However, this analysis was post-hoc and *EGFR* mutation status was not a prespecified stratification factor in this trial. Because of this limitation, we undertook this phase 2 trial to examine the combination of erlotinib and bevacizumab in patients with *EGFR* mutation-positive NSCLC.

Methods

Study design and patients

JO25567 was a randomised, open-label, multicentre, phase 2 study in patients with stage IIIB/IV (according to the 7th edition of the General Rule for Clinical and Pathological Record of Lung Cancer[18]) or recurrent NSCLC with activating *EGFR* mutations. Patients were enrolled from 30 centres across Japan.

See Online for appendix

Eligible patients had histologically or cytologically (excluding sputum cytology) confirmed stage IIIB/IV or postoperative recurrent non-squamous NSCLC with activating *EGFR* mutation (either exon 19 deletion or Leu858Arg mutation). Tumour samples were screened for *EGFR* mutation by PCR-based hypersensitive *EGFR* mutation testing in local laboratories, according to standard testing practices. Other criteria included age 20 years or older when giving informed consent; Eastern Cooperative Oncology Group performance status 0 or 1; adequate haematological, hepatic, and renal function; and life expectancy 3 months or more at the time of registration. No previous chemotherapy for advanced disease was allowed, but postoperative adjuvant or neoadjuvant therapy of 6 months or more previously was allowed. Previous radiotherapy was also allowed, but only for non-lung lesions. Patients had to have one or more measurable lesion based on Response Evaluation Criteria in Solid Tumors (RECIST 1.1).

Major exclusion criteria included confirmation of Thr790Met mutation, presence of brain metastases, history or presence of haemoptysis or bloody sputum, any coagulation disorder, tumour invading or abutting major blood vessels, coexistence or history of interstitial lung disease, and previous receipt of EGFR inhibitors or VEGF receptor inhibitors.

This study was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The study protocol was reviewed and approved by the institutional review boards of the participating institutions (appendix p 10), and written informed consent was obtained from all patients.

Randomisation and masking

Patients were randomly assigned (1:1) to receive either erlotinib plus bevacizumab or erlotinib alone with a dynamic allocation method. Central randomisation was done by a clinical research organisation (EPS Corporation, Tokyo, Japan). Patients were stratified according to sex (men *vs* women), disease stage (stage IIIB *vs* stage IV *vs* postoperative relapse), smoking history (never smokers or former light smokers *vs* others), and type of *EGFR* mutation (exon 19 deletion *vs* Leu858Arg mutation). All patients and investigators were unmasked to treatment allocation.

Procedures

Patients assigned to the erlotinib plus bevacizumab group received bevacizumab 15 mg/kg by intravenous infusion on day 1 of a 21-day cycle and erlotinib orally once daily at 150 mg/day, starting from day 1 of cycle 1. Patients in the erlotinib alone group received erlotinib orally once a day at 150 mg/day. Patients remained on treatment until disease progression or unacceptable toxicity. Changes to dose of erlotinib or bevacizumab because of adverse events were allowed, as per the protocol. The dose of bevacizumab was not to be reduced except when dose adjustment was needed because of change in bodyweight. Dose reduction of erlotinib was allowed for up to two doses (100 mg/day and 50 mg/day) in a stepwise decrease. After two steps of dose reduction, erlotinib was discontinued. Patients who required suspension of erlotinib for more than 3 weeks consecutively, or of bevacizumab for more than 6 weeks from the date of previous administration, were discontinued from study treatment. In the erlotinib plus bevacizumab group, if either drug was discontinued, the other could be

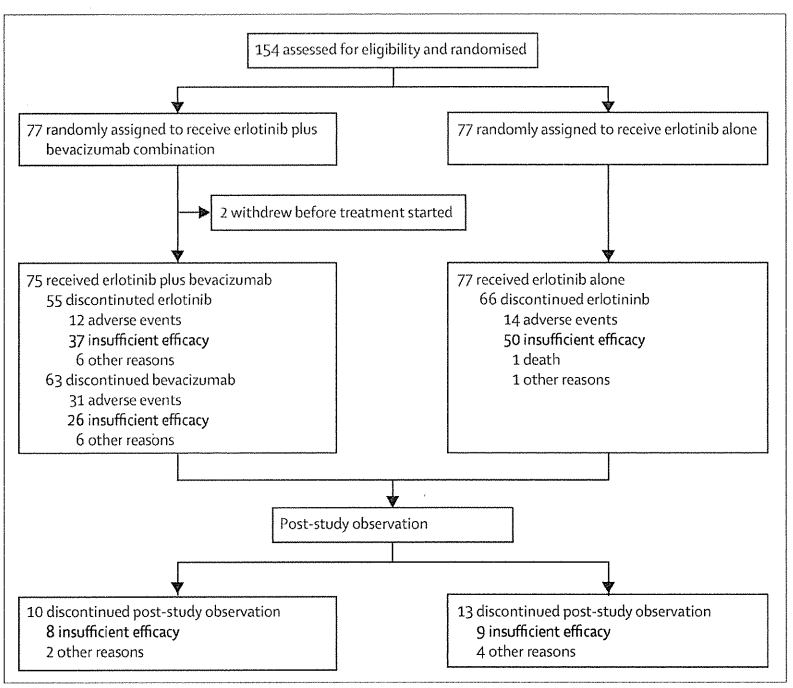

Figure 1: **Trial profile**

continued. Tumour lesions were assessed radiologically at baseline, week 4, week 7, every 6 weeks from week 7 to 18 months, and every 12 weeks thereafter until disease progression according to RECIST 1.1.

Patient-reported outcomes were assessed with the Functional Assessment of Cancer Therapy for patients with Lung cancer (FACT-L) scale until disease progression. An independent review committee of clinicians and radiologists masked to treatment assignment reviewed all tumour images and determined tumour response and progression status. Laboratory studies including blood and urine tests were done at days 1, 8, and 15 in cycles 1 and 2, and day 1 in cycle 3 and thereafter. Adverse events were monitored throughout the study period and were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTC-AE) version 4.03.

Outcomes

The primary endpoint was progression-free survival, as determined by an independent review committee. Secondary endpoints were overall survival, tumour response (the proportion of patients with an objective response and disease control, and duration of response) according to RECIST 1.1, quality of life, symptom improvement measured by the FACT-L scale, and safety profile.

	Erlotinib plus bevacizumab group (n=75)	Erlotinib alone group (n=77)
Age (years)		
Median	67·0 (59–73)	67·0 (60–73)
<75	63 (84%)	62 (81%)
≥75	12 (16%)	15 (19%)
Sex		
Male	30 (40%)	26 (34%)
Female	45 (60%)	51 (66%)
Smoking status		
Never smoker	42 (56%)	45 (58%)
Former light smoker	9 (12%)	6 (8%)
Other	24 (32%)	26 (34%)
ECOG performance status		
0	43 (57%)	41 (53%)
1	32 (43%)	36 (47%)
Histopathological classification		
Adenocarcinoma	74 (99%)	76 (99%)
Large-cell carcinoma	0	1 (1%)
Adenosquamous carcinoma	1 (1%)	0
Clinical stage at screening		
IIIB	1 (1%)	0
IV	60 (80%)	62 (81%)
Postoperative recurrence	14 (19%)	15 (19%)
***EGFR* mutation type**		
Exon 19 deletion	40 (53%)	40 (52%)
Exon 21 Leu858Arg mutation	35 (47%)	37 (48%)

Data are n (%) or median (IQR). ECOG=Eastern Cooperative Oncology Group.

Table 1: **Baseline demographics and clinical characteristics**

Statistical analysis

A median progression-free survival of 13 months was estimated for the erlotinib alone group, and 89 events were deemed necessary to detect a hazard ratio (HR) of 0·7 in favour of erlotinib plus bevacizumab, with a one-sided significance level of 0·2 and a power of 0·8. The target sample size was set at 150 patients (75 patients in both groups), allowing for dropouts. Median progression-free survival was estimated by the Kaplan-Meier method and compared between groups with an unstratified log-rank test. Greenwood's formula was used to calculate 95% CIs. HRs were calculated by unstratified Cox proportional hazard methodology.

In the safety analysis, adverse events were converted to Medical Dictionary for Regulatory Activities (version 14.0) preferred terms, and tabulated by grade. Changes in laboratory test data with time were summarised in tables and graphs.

All patients who received at least one dose of the study treatment were included in the safety analysis population. The modified intention-to-treat population for the efficacy analysis included all patients who received at least one dose of study treatment and had tumour assessment at least once after randomisation. Statistical analyses were done with SAS version 9.2.

The study is registered with the Japan Pharmaceutical Information Center, number JapicCTI-111390.

Role of the funding source

The study was designed and funded by Chugai Pharmaceutical Co Ltd and monitored by a clinical research organisation (Niphix Corp, Tokyo, Japan) who

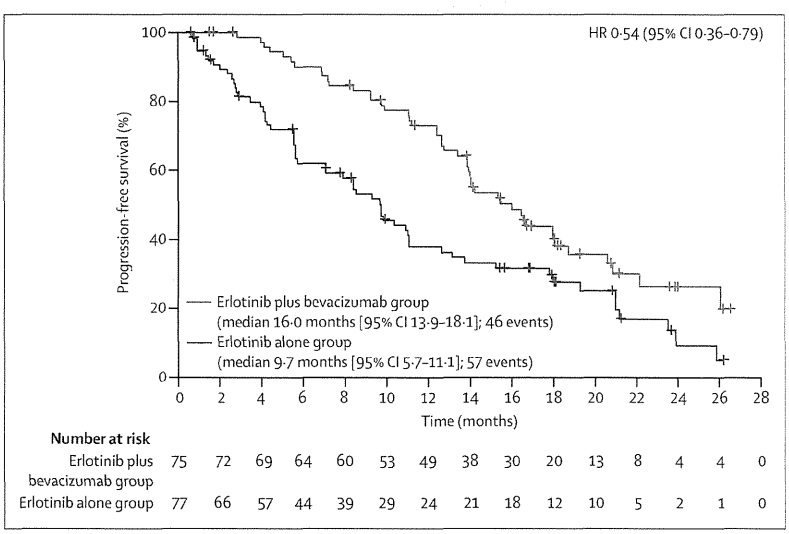

Figure 2: **Progression-free survival, as determined by independent review committee, in the modified intention-to-treat population**
HR=hazard ratio.

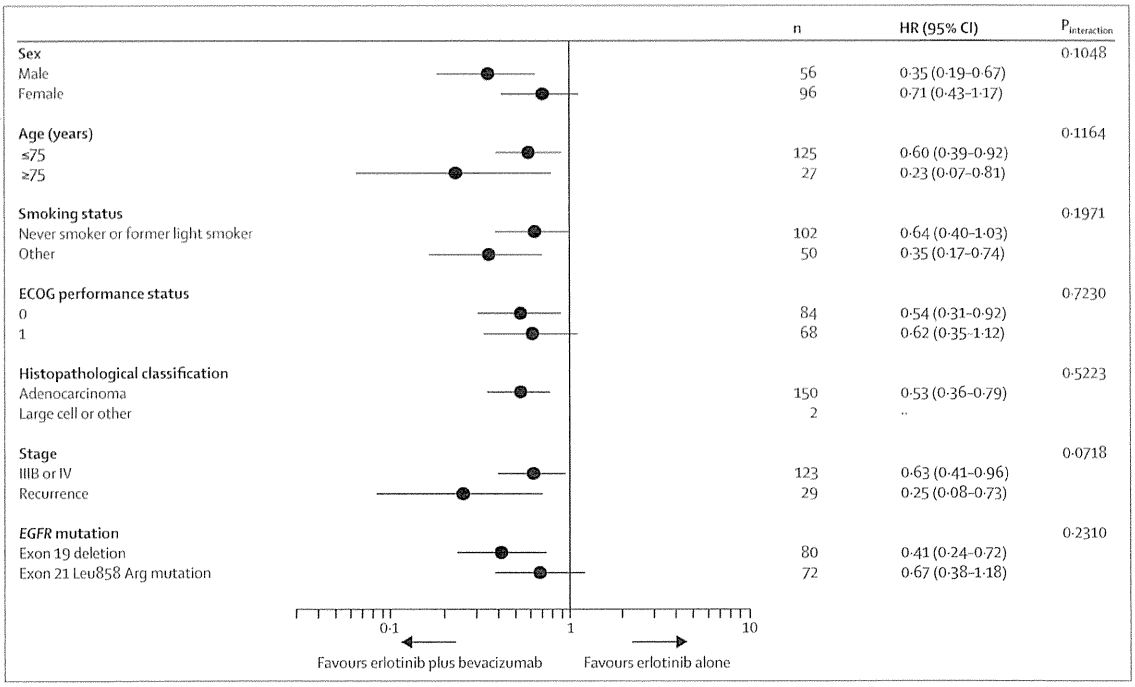

Figure 3: Forest plot of hazard ratios for progression-free survival by baseline characteristics
HR=hazard ratio.

	Erlotinib plus bevacizumab group (n=75)	Erlotinib alone group (n=77)
Complete response	3 (4%)	1 (1%)
Partial response	49 (65%)	48 (62%)
Stable disease	22 (29%)	19 (25%)
Progressive disease	0	6 (8%)
Non-evaluable	1 (1%)	3 (4%)

RECIST=Response Evaluation Criteria in Solid Tumors.

Table 2: **Best RECIST response, as determined by independent review committee**

obtained all data and did all initial data analyses; further analysis and interpretation was done by the funder, with input from the authors and investigators. The initial draft of the report was reviewed and commented on by all authors and by employees of Chugai Pharmaceutical Co Ltd. NobuY had full access to all data, and had final responsibility for the decision to submit the results for publication.

Results

Between Feb 21, 2011, and March 5, 2012, 154 patients were enrolled, of whom 77 were randomly assigned to receive erlotinib plus bevacizumab and 77 to erlotinib alone. Two patients withdrew before treatment started and were excluded (one had multiple thrombosis and the other had increased pleural effusion). Thus, data from 152 patients (75 patients in the erlotinib plus bevacizumab group and 77 in the erlotinib alone group) were included in the analysis population (figure 1). The cutoff date for the primary analysis was June 30, 2013, when 103 progression events had occurred; median follow-up was 20·4 months (IQR 17·4–24·1).

The baseline characteristics of patients were well balanced between the groups (table 1). Median age was 67 years (IQR 60–73), and 27 (18%) patients were aged 75 years or older. *EGFR* mutation subtypes were balanced between the two groups.

Progression-free survival was significantly prolonged with erlotinib plus bevacizumab compared with erlotinib alone (log-rank test p=0·0015; figure 2). When subgroup analyses were done by baseline clinical characteristics, most patient subgroups seemed to have greater benefit from erlotinib plus bevacizumab compared with erlotinib alone. No significant difference was noted between any of the subgroups ($p_{interaction}$>0·05 for all subgroups; figure 3).

Analysis of progression-free survival by mutation subtype showed that in patients whose tumours had an exon 19 deletion (40 [53%] of 75 patients in the erlotinib plus bevacizumab group and 40 [52%] of 77 patients in the erlotinib alone group), median progression-free survival was significantly longer with erlotinib plus bevacizumab than with erlotinib alone (18·0 months [95% CI 14·1–20·6] *vs* 10·3 months [95% CI 8·0–13·1]; HR 0·41 [95% CI 0·24–0·72]; p=0·0011; appendix p 1). In patients whose tumours harboured the Leu858Arg mutation (35 [47%] patients in the erlotinib plus bevacizumab group; 37 [48%] patients in the erlotinib alone group), median

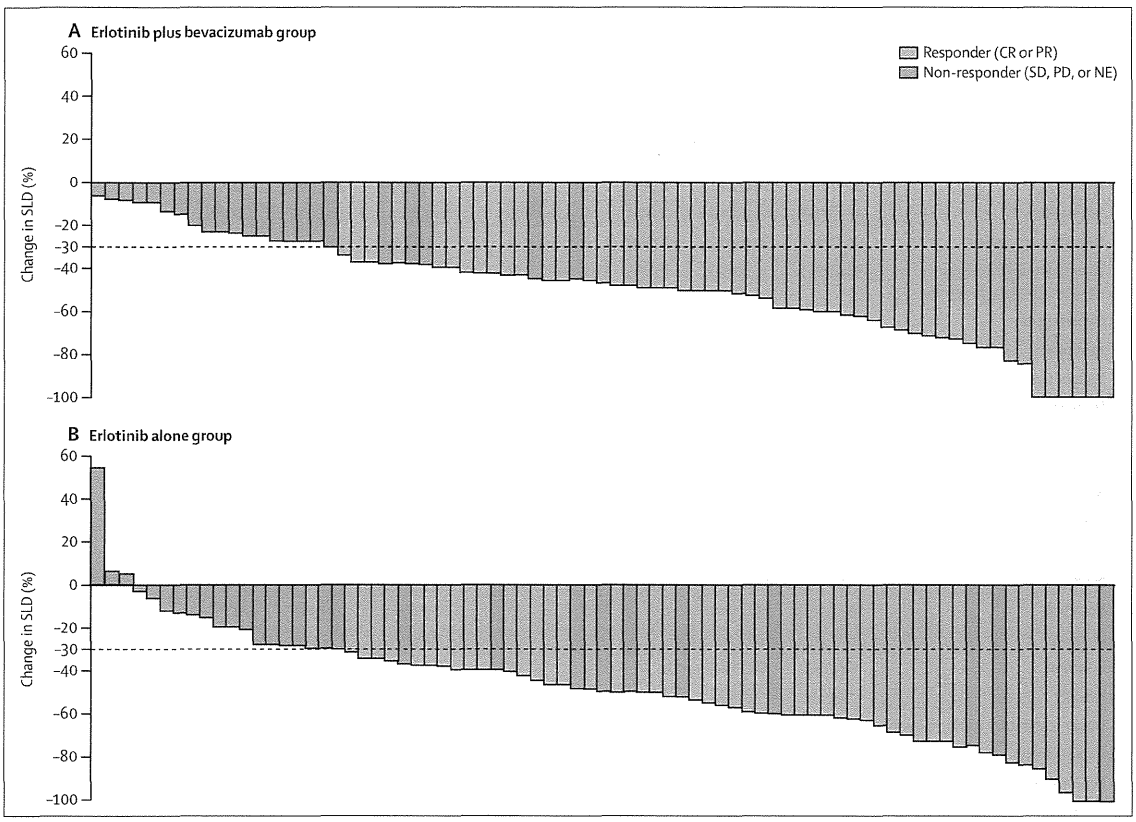

Figure 4: **Waterfall plot of best percentage change from baseline in the sum of longest tumour diameters**
Responders were confirmed by Response Evaluation Criteria in Solid Tumors. CR=complete response. PR=partial response. SD=stable disease. PD=progressive disease. NE=non-evaluable. SLD=sum of longest diameters.

progression-free survival was numerically longer with erlotinib plus bevacizumab than with erlotinib alone, but the difference was not significant (13·9 months [95% CI 11·2–20·9] *vs* 7·1 months [95% CI 4·3–15·2], respectively; HR 0·67 [95% CI 0·38–1·18]; p=0·1653; appendix p 2).

52 (69% [95% CI 58–80]) patients in the erlotinib plus bevacizumab group had an objective response, as did 49 (64% [52–74]) patients in the erlotinib alone group (p=0·4951), although median duration of response was not significantly longer with erlotinib plus bevacizumab than with erlotinib alone (13·3 months [95% CI 11·6–16·5] *vs* 9·3 months [6·9–13·8]; p=0·1118). A greater proportion of patients achieved disease control with erlotinib plus bevacizumab (74 [99%] *vs* 68 [88%]; p=0·0177). Best responses to treatment are shown in table 2.

Figure 4 shows change in tumour size from baseline in the two groups. All patients in the erlotinib plus bevacizumab achieved tumour reduction, but three patients in the erlotinib alone group did not. Of patients who had a 30% or greater reduction in tumour size during treatment, six (8%) patients in the erlotinib plus bevacizumab group and 12 (16%) patients in the erlotinib alone group did not meet the criteria for complete or partial response according to RECIST.

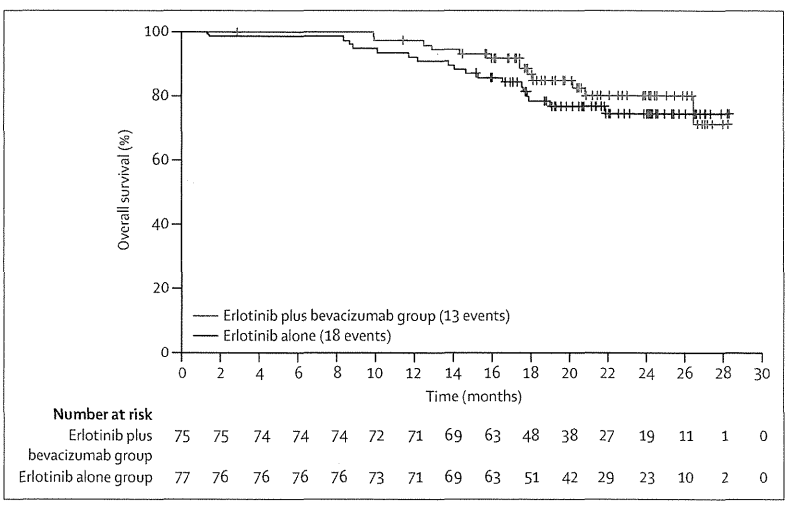

Figure 5: **Overall survival, as determined by independent review committee, in the modified intention-to-treat population**

Overall survival data are immature at present and so we cannot present any statistical analyses. At data cutoff, only 13 events (17%) had occurred in the erlotinib plus

	Erlotinib plus bevacizumab group (n=75)					Erlotinib alone group (n=77)				
	All	Grade 1–2	Grade 3	Grade 4	Grade 5	All	Grade 1–2	Grade 3	Grade 4	Grade 5
Rash	74 (99%)	55 (73%)	19 (25%)	0	0	76 (99%)	61 (79%)	15 (19%)	0	0
Diarrhoea	61 (81%)	60 (80%)	1 (1%)	0	0	60 (78%)	59 (77%)	1 (1%)	0	0
Paronychia	57 (76%)	55 (73%)	2 (3%)	0	0	50 (65%)	47 (61%)	3 (4%)	0	0
Dry skin	56 (75%)	54 (72%)	2 (3%)	0	0	45 (58%)	45 (58%)	0	0	0
Stomatitis	47 (63%)	46 (61%)	1 (1%)	0	0	46 (60%)	44 (57%)	2 (3%)	0	0
Haemorrhagic event	54 (72%)	52 (69%)	2 (3%)	0	0	22 (29%)	22 (29%)	0	0	0
Liver function disorder or abnormal hepatic function	33 (44%)	27 (36%)	5 (7%)	1 (1%)	0	39 (51%)	25 (32%)	7 (9%)	7 (9%)	0
Hypertension	57 (76%)	12 (16%)	45 (60%)	0	0	10 (13%)	2 (3%)	8 (10%)	0	0
Pruritus	34 (45%)	33 (44%)	1 (1%)	0	0	32 (42%)	32 (42%)	0	0	0
Weight decreased	33 (44%)	33 (44%)	0	0	0	19 (25%)	19 (25%)	0	0	0
Decreased appetite	26 (35%)	25 (33%)	1 (1%)	0	0	26 (34%)	25 (32%)	1 (1%)	0	0
Proteinuria	39 (52%)	33 (44%)	6 (8%)	0	0	3 (4%)	3 (4%)	0	0	0
Dysgeusia	20 (27%)	20 (27%)	0	0	0	17 (22%)	17 (22%)	0	0	0
Nasopharyngitis	20 (27%)	20 (27%)	0	0	0	15 (19%)	15 (19%)	0	0	0
Constipation	17 (23%)	17 (23%)	0	0	0	15 (19%)	14 (18%)	1 (1%)	0	0
Alopecia	13 (17%)	13 (17%)	0	0	0	14 (18%)	14 (18%)	0	0	0
Nausea	12 (16%)	12 (16%)	0	0	0	15 (19%)	15 (19%)	0	0	0
Vomiting	14 (19%)	14 (19%)	0	0	0	7 (9%)	7 (9%)	0	0	0
Malaise	10 (13%)	10 (13%)	0	0	0	10 (13%)	10 (13%)	0	0	0
Insomnia	8 (11%)	8 (11%)	0	0	0	8 (10%)	8 (10%)	0	0	0
Pyrexia	7 (9%)	7 (9%)	0	0	0	9 (12%)	9 (12%)	0	0	0
Upper respiratory tract infection	9 (12%)	9 (12%)	0	0	0	7 (9%)	7 (9%)	0	0	0
Conjunctivitis	8 (11%)	8 (11%)	0	0	0	7 (9%)	7 (9%)	0	0	0
Peripheral oedema	8 (11%)	8 (11%)	0	0	0	6 (8%)	6 (8%)	0	0	0
Fatigue	10 (13%)	9 (12%)	1 (1%)	0	0	3 (4%)	3 (4%)	0	0	0
Nail disorder	9 (12%)	9 (12%)	0	0	0	4 (5%)	4 (5%)	0	0	0
Dry eye	8 (11%)	8 (11%)	0	0	0	3 (4%)	3 (4%)	0	0	0
Dysphonia	8 (11%)	8 (11%)	0	0	0	1 (1%)	1 (1%)	0	0	0

Data are n (%).

Table 3: **Adverse events reported by 10% or more patients for grades 1 and 2 and all adverse events for grades 3–5 (safety population)**

bevacizumab group and 18 events (23%) in the erlotinib alone group (figure 5).

68 (91%) patients in the erlotinib plus bevacizumab group and 41 (53%) patients in the erlotinib group had grade 3 or 4 adverse events. The most common adverse events of any grade in the erlotinib plus bevacizumab group were rash, diarrhoea, hypertension, and paronychia, and in the erlotininb alone group were rash, diarrhoea, and paronychia (table 3). The most common grade 3 or worse adverse events in the erlotinib plus bevacizumab group were hypertension, rash, proteinuria, and liver function disorder or abnormal hepatic function, and in the erlotinib group were rash, liver function disorder or abnormal hepatic function, and hypertension (table 3). Substantially higher (>40%) incidences of hypertension, haemorrhagic events, and proteinuria were noted in the erlotinib plus bevacizumab group compared with the erlotinib alone group (table 3). Serious adverse events were reported by 18 (24%) patients in the erlotinib plus bevacizumab group and 19 (25%) patients in the erlotinib group.

12 (16%) patients in the erlotinib plus bevacizumab group and 14 (18%) patients in the erlotinib group discontinued erlotinib because of adverse events. 31 (41%) patients discontinued bevacizumab because of adverse events (figure 1). Ten patients discontinued both erlotinib and bevacizumab because of adverse events in the erlotinib plus bevacizumab group. Of these patients, seven discontinued erlotinib and bevacizumab simultaneously because of adverse events (liver function disorder or abnormal hepatic function in two patients, and infection, pancreatic cancer, rash, interstitial lung disease, and cerebral infarction in one patient each). In the remaining three patients, bevacizumab was initially discontinued, and patients continued on erlotinib monotherapy, although this was also subsequently discontinued. The dose of erlotinib was reduced to 100 mg for 34 (45%) of 75 patients in the erlotinib plus bevacizumab group and 33 (43%) of 77 patients in the erlotinib alone group; and to 50 mg for 17 (23%) of patients in the erlotinib plus bevacizumab group and eight (10%) patients in the erlotinib alone group.

The major adverse events leading to discontinuation of erlotinib in both groups were liver function disorder or abnormal hepatic function (two [3%] patients in the erlotinib plus bevacizumab group, eight [10%] in the erlotinib alone group), interstitial lung disease (two [3%], three [4%]), and rash (two [3%], none). Major adverse events leading to discontinuation of bevacizumab were proteinuria (11 [15%] patients), haemorrhagic events (nine [12%]), and hypertension (two [3%]). Most haemorrhagic events were low-grade epistaxis or haemorrhoidal bleeding. All of the 11 patients who discontinued bevacizumab because of proteinuria had grade 3 or lower events, and five of these patients recovered during the study period. All of the nine patients who discontinued because of haemorrhagic events had grade 3 or lower events; eight patients improved or recovered during the study period.

The median duration of erlotinib treatment was 431 days (range 21–837) in the erlotinib plus bevacizumab group and 254 days (18–829) in the erlotinib group, whereas median duration of bevacizumab was 325 days (1–815). The median duration of bevacizumab in patients who discontinued treatment because of proteinuria was 329 days (113–639) and because of haemorrhagic events was 128 days (23–357).

The relative dose intensity of erlotinib (calculated as [totally administered dose / total treatment duration] / 150×100) was similar in both groups (95·3% [range 34·7–100·0] in the erlotinib plus bevacizumab group and 98·7% [33·3–100·0] in the erlotinib alone group), whereas that of bevacizumab (calculated as totally administered dose/planned dose×100) was 93·9% (72·4–99·7).

Haemoptysis was reported in six (8%) patients in the erlotinib plus bevacizumab group (five [7%] patients had grade 1 events and one [1%] had a grade 2 event); one patient (1%) had a grade 1 event in the erlotinib alone group. Interstitial lung disease was reported for five (3%) of all patients. One patient in the erlotinib alone group had grade 3 interstitial lung disease, but all other cases were grade 1 or 2, and all patients recovered. During the study period, one patient in the erlotinib group died by drowning, and a potential association with the study drug was confirmed.

No significant difference was noted between the two groups in terms of quality of life, including total FACT-L score, trial outcome index score, and all other subscores, since the standard deviations at each time point overlapped (appendix pp 3–9).

Discussion

In this study, the addition of bevacizumab to erlotinib significantly prolonged progression-free survival in patients with NSCLC with activating *EGFR* mutation-positive disease compared with erlotinib alone. To our knowledge, this is the first randomised study to show a clinically significant treatment effect of combining an EGFR tyrosine-kinase inhibitor with another biological drug in patients with activating *EGFR* mutation-positive NSCLC (panel). We noted clear separation of the Kaplan-Meier survival curves from the start of treatment, despite the use of erlotinib in both groups.

Multivariate analysis according to baseline patient characteristics showed a consistent treatment benefit, with longer progression-free survival noted with erlotinib plus bevacizumab across most subgroups of patients. Previous studies have reported that erlotinib tends to be more effective in tumours with *EGFR* exon 19 deletions versus those with Leu858Arg mutations,[7,8,21] which is consistent with our results.

No new safety signals were identified and the incidence of adverse events (any grade) and serious adverse events was similar between the two groups. There were more grade 3 or worse adverse events in the erlotinib plus bevacizumab group. Discontinuation of bevacizumab because of adverse events was more common than that reported in previous studies.[13,14] One possible reason for this discrepancy could be the longer duration of treatment than in previous studies: the median treatment duration of bevacizumab was 325 days (16 cycles), which is substantially longer than that in previous studies. Furthermore, proteinuria was one of the major adverse events that led to discontinuation of bevacizumab, and the time to onset of bevacizumab discontinuation because of proteinuria tended to be in the later treatment phase (median 329 days [range 113–639]). Nevertheless, despite the high incidence of bevacizumab discontinuation because of adverse events, most of these events (mainly proteinuria and haemorrhagic events) were deemed non-serious and reversible.

The incidence of grade 3 or greater hypertension and proteinuria were higher than those in previous studies, again possibly related to the prolonged duration of treatment. Another potential factor that could explain the difference in the incidence of hypertension is in the

Panel: Research in context

Systematic review

We searched PubMed for articles published in English until Feb 1, 2014 (with no restrictions for the starting date), using the search terms "bevacizumab", "erlotinib", "NSCLC", and "EGFR". We identified two studies that had assessed the efficacy of erlotinib plus bevacizumab in the first-line setting.[19,20] However, no previous study had assessed the efficacy of the combination of erlotinib and bevacizumab as first-line therapy for patients with activating *EGFR* mutation-positive NSCLC.

Interpretation

To our knowledge, this study is the first to show that the combination of erlotinib and bevacizumab can significantly prolong progression-free survival compared with erlotinib alone in patients with non-squamous *EGFR* mutation-positive NSCLC. Some degree of increased toxicity, particularly hypertension, proteinuria, and haemorrhagic events, was noted with the addition of bevacizumab. Our findings suggest that the combination of erlotinib and bevacizumab could be a new first-line regimen in *EGFR* mutation-positive NSCLC. Two clinical trials, BELIEF (NCT01562028) and ACCRU RC1126 (NCT01532089) are ongoing and the results are awaited to confirm the efficacy and safety shown in our study.

definition of grading used; we used CTC-AE version 4.03, whereas previous studies[14,16] used CTC-AE version 3. Akhtar and colleagues[22] showed that the change in CTC-AE version from 3 to 4 could lead to a significant shift in the severity of adverse events in clinical trials. Furthermore, despite the somewhat higher incidence of hypertension observed in this study, only two (3%) of 75 patients discontinued bevacizumab administration because of hypertension.

Although we noted no significant difference in the proportion of patients achieving an objective response between the erlotinib plus bevacizumab group and erlotinib alone groups, all patients in the erlotinib plus bevacizumab group had a reduction in tumour size. Of those patients who had a greater than 30% reduction in the sum of longest diameter of their target lesions from baseline, more patients in the erlotinib alone group failed to meet the criteria for complete or partial response. These findings suggest that the addition of bevacizumab to erlotinib might help to maintain the tumour-suppressing effect after reduction in tumour size, which might explain the difference in progression-free survival between the two groups.

One possible mechanism to explain this effect could be improved drug delivery. Bevacizumab changes tumour vessel physiology, resulting in increased intratumoral uptake of drugs.[23,24] The results of a preclinical study suggested that patients on lower doses of EGFR tyrosine-kinase inhibitors tend to develop treatment resistance earlier than those who receive higher doses.[25,26] Therefore, achieving a higher intratumoral concentration of erlotinib could delay the appearance of resistant cells. Another possible mechanism that could explain these findings is the effective blocking of angiogenesis signalling via the VEGF receptor and EGFR signalling pathways, which is thought to promote tumour growth.[27,28] In addition to synergistic inhibition of tumour growth signalling, VEGF signal inhibition is still effective for tumours harbouring EGFR tyrosine-kinase inhibitor resistance mutations. In preclinical studies, blocking the VEGF receptor signalling pathway overcame resistance for EGFR signalling blockage by Thr790Met EGFR mutation in vivo.[29,30]

Another treatment strategy that has been recently investigated is the combination of an EGFR tyrosine-kinase inhibitor with chemotherapy. Wu and colleagues[31] reported that platinum doublet chemotherapy with intercalated erlotinib increased progression-free survival compared with platinum doublet chemotherapy alone. In a subset analysis of the *EGFR* mutation-positive population in this study, progression-free survival was 16·8 months. In our study, median progression-free survival with erlotinib and bevacizumab was 16·0 months. The first-line use of erlotinib and bevacizumab could allow chemotherapy to be reserved for subsequent lines of treatment, which might further improve survival outcomes in these patients.

Our study has several limitations. First, the analysis of *EGFR* mutations was not done at a central laboratory and various methods were used, including the peptide nucleic acid, locked nucleic acid PCR clamp method, the PCR invader method, and the cycleave method. However, on the basis of previous evidence, these methods are generally judged to provide consistent results.[32] Second, because some patients are still receiving the first-line treatment and overall survival data are still immature, assessment of subsequent treatment effects after progression is not possible. Data relating to post-study treatment will be reported in due course with updated overall survival results. Third, we did not use the EQ-5D questionnaire developed by the EuroQol group for quality-of-life assessment. Therefore, we could not formally estimate quality-adjusted life-years for a cost-effectiveness analysis. The health economics related to the combined use of erlotinib and bevacizumab remains unclear and should be discussed in future studies. Additionally, follow-up for overall survival is still ongoing and these results are needed before the clinical value of this combination can be determined.

In summary, our study provides, to the best of our knowledge, the first evidence that the addition of bevacizumab to erlotinib confers a significant improvement in progression-free survival when used as first-line treatment for patients with non-squamous NSCLC with activating *EGFR* mutation-positive disease. Some degree of increased toxicity, particularly hypertension, proteinuria, and haemorrhagic events, seems to be associated with the addition of bevacizumab. Our findings suggest that the combination of erlotinib and bevacizumab could be a new first-line regimen in *EGFR* mutation-positive NSCLC, and that further investigation of the regimen is warranted. Two clinical trials, BELIEF (NCT01562028) and ACCRU RC1126 (NCT01532089), are ongoing and the results are awaited to confirm the efficacy and safety shown in our study.

Contributors

NobuY was the principal investigator. TS, TK, MN, KG, NoboY, IO, TY, KT, RH, MF, and NobuY contributed to the study design and data analysis and data interpretation. TS, TK, MN, KG, SA, YH, NoboY, TH, MM, KN, SN, IO, and NobuY contributed to patient recruitment and data collection. NobuY, TS, KT, and RH prepared the initial draft of the report input from other authors. All authors approved the final version of the report.

Declaration of interests

TS received research grants and honoraria from Chugai Pharmaceutical. TK received research grants and honoraria from Chugai Pharmaceutical; honoraria from Eli Lilly, Ono Pharmaceutical, Novartis Pharma, Taiho Pharmaceutical, and AstraZeneca; and research grants from Nippon Boehringer Ingelheim, Kyowa Hakko Kirin, Pfizer, and Shionogi. MN received research grants and honoraria from Chugai Pharmaceutical, Pfizer, Novartis Pharma, Taiho Pharmaceutical, Nippon Boehringer Ingelheim, and AstraZeneca; research grants from MSD and Bristol-Myers Squibb. KG received research grants and honoraria from Chugai Pharmaceutical, Taiho Pharmaceutical and Nippon Boehringer Ingelheim; honoraria from AstraZeneca, Sanofi, Novartis Pharma, Pfizer, Yakult Honsha, Ono Pharmaceutical and Eli Lilly. SA received honoraria from Chugai Pharmaceutical, Eli Lilly, Taiho Pharmaceutical, Sawai Pharmaceutical, and Novartis Pharma. YH received research grants and honoraria from Chugai Pharmaceutical, Ono Pharmaceutical, and Taiho Pharmaceutical; honoraria from AstraZeneca, Eli Lilly, Novartis Pharma, and Takeda Pharmaceutical; research grants form Yakult Honsha, MSD, Kyowa Hakko Kirin, and Daiichi Sankyo. NoboY received research grants form Chugai Pharmaceutical, Pfizer, Takeda Bio, Astellas Pharma, Taiho

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References

1 WHO. 10 facts about cancer. http://www.who.int/features/factfiles/cancer/en/ (accessed June 26, 2014).

2 Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350:** 2129–39.

3 National Comprehensive Cancer Network. NCCN Drugs & Biologics Compendium (NCCN Compendium). http://ww.nccn.org/professionals/drug_compendium/content/contents.asp (accessed June 26, 2014).

4 National Institute for Health and Care Excellence: Lung cancer (non small cell, EGFR-TK mutation positive)—erlotinib (1st line) (TA258). http://guidance.nice.org.uk/TA258 (accessed June 26, 2014).

5 Sequist LV, Yang JC, Yamamoto N, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. *J Clin Oncol* 2013; **31:** 3327–34.

6 Wu YL, Zhou C, Hu CP, et al. Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial. *Lancet Oncol* 2014; **15:** 213–22.

7 Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation–positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): A multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2011; **12:** 735–42.

8 Rosell R, Carcereny E, Gervais R, et al. Spanish Lung Cancer Group in collaboration with Groupe Français de Pneumo-Cancérologie and Associazione Italiana Oncologia Toracica: Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): A multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 2012; **13:** 239–46.

9 Maemondo M, Inoue A, Kobayashi K, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010; **362:** 2380–38.

10 Mitsudomi T, Morita S, Yatabe Y, et al. Gefitinib versus cisplatin plus docetaxel in patients with non–small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010; **11:** 121–28.

11 Fukuoka M, Wu YL, Thongprasert S, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non–small-cell lung cancer in Asia (IPASS). *J Clin Oncol* 2011; **29:** 2866–74.

12 Sandler A, Gray R, Perry MC, et al. Paclitaxel–carboplatin alone or with bevacizumab for non–small-cell lung cancer. *N Engl J Med* 2006; **355:** 2542–50.

13 Reck M, von Pawel J, Zatloukal P, et al. Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAiL. *J Clin Oncol* 2009; **27:** 1227–34.

14 Niho S, Kunitoh H, Nokihara H, et al, for the JO19907 Study Group. Randomized phase II study of first-line carboplatin–paclitaxel with or without bevacizumab in Japanese patients with advanced non-squamous non-small-cell lung cancer. *Lung Cancer* 2012; **76:** 362–67.

15 Herbst RS, O'Neill VJ, Fehrenbacher L, et al. Phase II study of efficacy and safety of bevacizumab in combination with chemotherapy or erlotinib compared with chemotherapy alone for treatment of recurrent or refractory non small-cell lung cancer. *J Clin Oncol* 2007; **25:** 4743–50.

16 Herbst RS, Ansari R, Bustin F, et al. Efficacy of bevacizumab plus erlotinib versus erlotinib alone in advanced non-small-cell lung cancer after failure of standard first-line chemotherapy (BeTa): a double-blind, placebo-controlled, phase 3 trial. *Lancet* 2011; **377:** 1846–54.

17 Herbst R, Stern H, Amler L. Biomarker evaluation in the phase III, placebo-controlled, randomized BeTa Trial of bevacizumab and erlotinib for patients with advanced non-small cell lung cancer (NSCLC) after failure of standard 1st-line chemotherapy: correlation with treatment outcomes *J Thorac Oncol* 2009; **4:** S323.

18 The Japan Lung Cancer Society. General Rule for Clinical and Pathological Record of Lung Cancer, 7th edn. Tokyo: Kanehara Press, 2010.

19 Zappa F, Droege C, Betticher D, et al. Bevacizumab and erlotinib (BE) first-line therapy in advanced non-squamous non-small-cell lung cancer (NSCLC) (stage IIIB/IV) followed by platinum-based chemotherapy (CT) at disease progression: a multicenter phase II trial (SAKK 19/05). *Lung Cancer* 2012; **78:** 239–44.

20 Dingemans AM, de Langen AJ, van den Boogaart V, et al. First-line erlotinib and bevacizumab in patients with locally advanced and/or metastatic non-small-cell lung cancer: a phase II study including molecular imaging. *Ann Oncol* 2011; **22:** 559–66.

21 Rosell R, Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009; **361:** 958–67.

22 Akhtar NH, Singh B, Ocean AJ, et al. Effect of CTCAE v4 grading of hypertension on reported toxicity in advanced cancer patient receiving vascular endothelial growth factor (VEGF)-targeting agents. *J Clin Oncol* 2013; **31:** e15600.

23 Wildiers H, Guetens G, DeBoeck G, et al. Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11. *Br J Cancer* 2003; **88:** 1979–86.

24 Dickson PV, Hamner JB, Sims TL, et al. Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. *Clin Cancer Res* 2007; **13:** 3942–50.

25 Furugaki K, Iwai T, Moriya Y, Harada N, Fujimoto-Ouchi K. Loss of an EGFR-amplified chromosome 7 as a novel mechanism of acquired resistance to EGFR-TKIs in EGFR-mutated NSCLC cells. *Lung Cancer* 2014; **83:** 44–50.

26 Hayakawa H, Ichihara E, Ohashi K, et al. Lower gefitinib dose led to earlier resistance acquisition before emergence of T790M mutation in epidermal growth factor receptor-mutated lung cancer model. *Cancer Sci* 2013; **104:** 1440–46.

27 Herbst RS, Johnson DH, Mininberg E, et al. Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. *J Clin Oncol* 2005; **23:** 2544–55.

28 Larsen AK, Ouaret D, El Ouadrani K, Petitprez A. Targeting EGFR and VEGF(R) pathway cross-talk in tumor survival and angiogenesis. *Pharmacol Ther* 2011; **131:** 80–90.

29 Naumov GN, Nilsson MB, Cascone T, et al. Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. *Clin Cancer Res* 2009; **15:** 3484–94.

30 Ichihara E, Ohashi K, Takigawa N, et al. Effects of vandetanib on lung adenocarcinoma cells harboring epidermal growth factor receptor T790M mutation in vivo. *Cancer Res* 2009; **69:** 5091–98.

31 Wu YL, Lee JS, Thongprasert S, et al. Intercalated combination of chemotherapy and erlotinib for patients with advanced stage non–small-cell lung cancer (FASTACT-2): a randomised, double-blind trial. *Lancet Oncol* 2013; **14:** 777–86.

32 Goto K, Satouchi M, Ishii G, et al. An evaluation study of EGFR mutation tests utilized for non–small-cell lung cancer in the diagnostic setting. *Ann Oncol* 2012; **23:** 291–94.

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Development of On-Chip Multi-Imaging Flow Cytometry for Identification of Imaging Biomarkers of Clustered Circulating Tumor Cells

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Abstract

An on-chip multi-imaging flow cytometry system has been developed to obtain morphometric parameters of cell clusters such as cell number, perimeter, total cross-sectional area, number of nuclei and size of clusters as "imaging biomarkers", with simultaneous acquisition and analysis of both bright-field (BF) and fluorescent (FL) images at 200 frames per second (fps); by using this system, we examined the effectiveness of using imaging biomarkers for the identification of clustered circulating tumor cells (CTCs). Sample blood of rats in which a prostate cancer cell line (MAT-LyLu) had been pre-implanted was applied to a microchannel on a disposable microchip after staining the nuclei using fluorescent dye for their visualization, and the acquired images were measured and compared with those of healthy rats. In terms of the results, clustered cells having (1) cell area larger than 200 μm^2 and (2) nucleus area larger than 90 μm^2 were specifically observed in cancer cell-implanted blood, but were not observed in healthy rats. In addition, (3) clusters having more than 3 nuclei were specific for cancer-implanted blood and (4) a ratio between the actual perimeter and the perimeter calculated from the obtained area, which reflects a shape distorted from ideal roundness, of less than 0.90 was specific for all clusters having more than 3 nuclei and was also specific for cancer-implanted blood. The collected clusters larger than 300 μm^2 were examined by quantitative gene copy number assay, and were identified as being CTCs. These results indicate the usefulness of the imaging biomarkers for characterizing clusters, and all of the four examined imaging biomarkers—cluster area, nuclei area, nuclei number, and ratio of perimeter—can identify clustered CTCs in blood with the same level of preciseness using multi-imaging cytometry.

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Introduction

Finding irregular cells in blood is fundamental to achieving non-invasive health checks, such as cancer and immune diagnostics. For example, circulating tumor cells (CTCs) are expected to form additional seeds for subsequent growth of tumors [1–3], and quantitative detection of CTCs in the blood [4–8] has the potential to achieve minimally invasive cancer diagnosis in comparison with conventional biopsies. One major approach to finding irregular cells is the targeting of specific molecules, molecular biomarkers, on the cell surface [1,3,6,9,10]; however, its application has sometimes had the difficulty of false-negative detection because of the variety of molecular expression properties of targeted cells.

To overcome these difficulties, we developed another system for the recognition of target cells [11–13]. In this system, cell samples were applied to a microchannel fabricated on a small microchip, cellular images were taken with a high-speed CCD camera, and target cells were identified depending on their morphological characteristics, such as cellular area and perimeter. These morphological parameters, referred to as "imaging biomarkers" hereafter, are other indexes to identify specific target cells. For example, a large cellular size was indicated for some tumor cells [14–17], and a larger nucleus than in healthy cells is known as one common property of the morphometric phenotype of cancer cells [18–24]; therefore, finding target cells using imaging biomarkers, especially using both cell size and nucleus conformation, is useful for the identification of tumor cells. In this study, a real-time cell sorting system to achieve simultaneous processing of imaging biomarkers for both optical image (i.e., total cell configuration) and fluorescent image (i.e., nucleus configuration) was developed, and it was applied to identify irregular cells, especially clustered cells, in a blood sample. According to previous reports on CTC detection, the possibility of the CTCs forming clusters was suggested [7]; however, clear evidence had not been identified and there have been no quantitative studies on the identification of clustered cells in the blood. Here, a quantitative approach for cluster detection was suggested using imaging biomarkers as detection indexes.

Materials and Methods

Fabrication of microchip

The microchip was fabricated by the following procedure. A mask blank, which was a glass substrate coated with both chromium for light interception and positive photo-resist (AZP1350) for the fabrication of patterns (CBL4006Du-AZP, Clean Surface Technology Co., Kanagawa, Japan), was set to a laser lithography system (DDB-3TH, Neoark, Co., Tokyo, Japan) and a laser (405 nm wavelength) was irradiated onto the mask blank in the same pattern as the microchannel used in this study. After the irradiation, the mask blank was immersed in a developer of the resist (NMD-3, Tokyo Ohka Kogyo Co., Kanagawa, Japan) to remove the resist on which the laser was irradiated; then, a chromium layer was bared at this position. Next, the mask blank was immersed in chromium etching solution (MPM-E350, DNP Fine Chemicals, Co., Kanagawa, Japan), after which the bared chromium layer was removed and a transparent pattern of the microchannel was formed on the substrate. Finally, the whole resist on the mask blank was removed by light irradiation onto the whole of the substrate and immersion of the substrate in the developer; then, a photo mask of the microchannel was fabricated.

On the other hand, a light-curing resin (SU-8 3025, Nippon Kayaku Co., Tokyo, Japan) was spin-coated using a spin coater (1H-DX2, Mikasa, Co., Tokyo, Japan) of 25 μm thickness on a clean Si substrate. The resin-coated substrate was pre-baked at 95°C for 15 min, set in a mask aligner with the fabricated photo mask (MA-20, Mikasa), and the light (365 nm wavelength) was irradiated through the mask to harden the resin with the pattern of the microchannel. The substrate was heated at 65°C for 1 min and 95°C for 5 min sequentially to promote hardening of the resin, and excess resin was removed by immersing the substrate in SU-8 developer (Nippon Kayaku). A mold of the microchannel was then fabricated using the resin on the Si substrate.

To fabricate the chip, poly(dimethylsiloxane) (PDMS; SYLGARD 184 silicon elastomer, Dow Corning Co., Midland, MI, USA) was dropped onto the fabricated mold in sol state, and heated at 90°C for 1 h to harden the PDMS. The PDMS on which the pattern of the microchannel was transferred was peeled off from the mold and stuck with cleaned cover glass. Finally, plastic columns for the application of solvents including sample blood were pasted on the PDMS with epoxy resin; then, the microchip to be used in this study was fabricated.

Preparation of sample blood

This study was carried out in strict accordance with the Act on Welfare and Management of Animals of the Ministry of the Environment, Japan. The protocol was approved by the animal experiment committee of the Kanagawa Cancer Center (permit number 21-02). MAT-LyLu is a rat prostate cancer cell line established from the original Dunning R3327 tumor maintained by *in vivo* passage of a prostate cancer that spontaneously occurred in a Copenhagen rat [25]. This cell line was a generous gift from the original founders through Hisao Ekimoto, Ph.D., at the Oncology Section, Laboratory of Biology, Nippon Kayaku Co., Ltd., and was maintained in our laboratory.

To obtain blood containing cancer cells, the MAT-LyLu was adjusted to 5×10^6 cells in 200 μL of cell culture medium (RPMI 1640, Life Technologies Co., Grand Island, NY, USA), and implanted into the dorsal subcutaneous tissue of a Copenhagen rat (male, 6 weeks old). At 2 weeks after implantation, blood of the rat was collected from the subclavian vein using a collection tube containing heparin. The blood was hemolyzed using commercial reagent (BD Pharm Lyse, without fixative, BD Biosciences, San Jose, CA, USA) for 10 min, washed along with 200× g centrifugation for 5 min and re-suspended two times in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, suspended in PBS containing 100 ng/mL Hoechst 33258, and then incubated for 10 min to stain cellular nuclei. The sample was washed again along with centrifugation 3 times, suspended in 5% glucose solution, and applied to the sample inlet on the chip.

Flow cytometry

The prepared sample blood was applied to the sample inlet on a fabricated microchip with a sample volume of 50 μL in an assay. The same buffer with the sample cell suspension (i.e., 5% glucose) was also used as a sheath buffer, and was applied to the sheath buffer inlet. Air pressure was applied onto both sample and sheath buffer inlets simultaneously using a syringe pump to introduce these liquids into the microchannels. Before starting the experiments, flow velocity was calibrated by taking images of calibration beads using a CCD camera (Ditect Co., Tokyo, Japan) as the shift of bead position in the microchannel within a few frames of the images, and typically, 1 kPa pressure achieved flow velocity of about 3 mm/sec at the position after the meeting of sample and sheath flows. Multi-imaging observations of sample blood were then performed through the multi-view unit with 3 mm/sec flow velocity and 200 fps acquisition rate.

Comparative genomic hybridization analysis

Rat genome comparative genomic hybridization (CGH) microarray 244A (Agilent Technologies, Santa Clara, CA, USA) was used to perform array CGH on genomic DNA obtained from the MAT-LyLu cell line according to the manufacturer's instructions. A DNA sample obtained from liver tissue of a healthy Copenhagen rat was used as a reference. Genomic DNAs were extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined with PicoGreen dsDNA Quantitation Reagent (Life Technologies). Agilent Genomic Workbench (Agilent Technologies) was used to analyze chromosomal patterns using an ADM-2 algorithm setting a threshold of 5.0.

Copy number assay

The gene copy numbers for *csrp2* and *zdhhc17* were determined using TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The gene-specific primers and TaqMan probes were used in the experiments had the following sequences. Rat *csrp2* primers were: sense, 5′-GGACTAAATGGATTGATGCCACTCT-3′; antisense, 5′-GTCCCTGCTTCAAAGAACTGTCT-3′; probe, 5′-FAM-AAGAGCAAGAAAGGAAACCC-MGB-NFQ-3′. Rat *zdhhc17* primers were: sense, 5′-GCCCTACTGCATGCATGATACA-3′; antisense, 5′-GGGCTGTTTTGCACATGAAATTCAA-3′; probe, 5′-FAM-CTGGACAGCATCTGCTAGTATAC-MGB-NFQ-3′. Rat *rpp40* primers were: sense, 5′-GTATGACACTGGCATGGAAGTCT-3′; antisense, 5′-CTTGCAGGTCCTCTGTGGAT-3′; probe, 5′-FAM-CCTGGCAATCAAAGTTAGGCTTAG-MGB-NFQ-3′. Genomic DNAs obtained from collected samples using the cell sorting system were extracted using a QIAamp DNA Micro kit (Qiagen) according to the manufacturer's instructions. The DNA concentrations were determined with PicoGreen dsDNA Quantitation Reagent. Rat *rpp40* was used as an internal control. Genomic DNAs obtained from MAT-LyLu cell line and healthy rat liver were used as positive and negative controls, respectively.