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BRCA1, LMO4, and CtIP mRNA Expression in Erlotinib-Treated Non–Small-Cell Lung Cancer Patients with *EGFR* Mutations

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Introduction: Lung adenocarcinoma patients harboring *EGFR* activating mutations attain improved progression-free survival (PFS) with treatment with epidermal growth factor receptor tyrosine kinase inhibitors. However, patients ultimately relapse, indicating that other genetic factors could influence outcome in such patients. We hypothesized that PFS could be influenced by the expression of genes in DNA repair pathways.

Methods: We examined the mRNA expression of C terminus-binding protein–interacting protein and *Lin11*, *Isl-1*, and Mec-3 domain only 4 (*LMO4*) in pretreatment tumor samples from 91 erlotinib-treated advanced non–small-cell lung cancer patients with *EGFR* mutations in whom breast cancer gene 1 (*BRCA1*) expression and the concomitant presence of the *EGFR T790M* mutation had previously been assessed. Gene expression was analyzed by polymerase chain reaction, using *β-actin* as endogenous gene. Results were correlated with PFS and overall survival.

Results: In patients with low *LMO4* levels, PFS was 13 months, whereas it was not reached for those with high *LMO4* levels (p = 0.03). In patients with low levels of both *BRCA1* and *LMO4*, PFS was 19 months whereas it was not reached in those with low *BRCA1* and high *LMO4* mRNA levels (p = 0.04). In patients with high *BRCA1* and low *LMO4* levels, PFS was 8 months, whereas it was 18 months in those with high levels of both genes (p = 0.03).

Conclusions: Low *BRCA1* and high *LMO4* levels were associated with longer PFS to erlotinib. Baseline assessment of *BRCA1* and *LMO4* mRNA expression can help predict outcome to erlotinib.

Key Words: Breast cancer gene 1, *Lin11*, *Isl-1*, and *Mec-3* domain only 4, *EGFR*, Non–small-cell lung cancer, Erlotinib.

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With a greater understanding of tumor biology, novel molecular-targeted strategies have been evaluated as a therapeutic approach for treating non–small-cell lung cancer (NSCLC).[1] Recent studies have established the efficacy of the epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib in patients with *EGFR* activating mutations (deletion in exon 19 or L858R in exon 21), who have attained progression-free survival (PFS) of 9 to 11 months.[2–7]

However, despite promising initial responses, all patients ultimately progress because the efficacy of *EGFR* TKIs is limited by either primary or acquired resistance after therapy. The detection of biomarkers of acquired resistance is thus critical for maximizing the benefits of TKI therapy.[8] The concomitant *EGFR T790M* mutation in exon 20, mesenchymal epithelial transition factor amplification, hepatocyte growth factor overexpression, activation of insulin-like growth factor 1 receptor, and other factors have been associated with acquired resistance to *EGFR* TKIs.[9–11]

DNA repair as a therapeutic target has recently received considerable attention owing to the promise of drugs that target tumor-specific DNA-repair enzymes and improve the efficacy of chemotherapy.[12] The breast cancer gene 1 (*BRCA1*) plays a central role in DNA repair and is also involved in mitosis and cell division.[13] High *BRCA1* levels are associated with platinum resistance in vitro.[14] In NSCLC patients receiving induction chemotherapy with cisplatin plus gemcitabine, low *BRCA1* mRNA levels were associated with longer overall survival (hazard ratio [HR], 0.206; p = 0.026),[15] and with a

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radiographic response rate (partial response or stable disease) of 100%, whereas in chemonaive patients with resected NSCLC, high *BRCA1* levels correlated with shorter overall survival (HR, 1.98; $p = 0.02$) and a radiographic response rate of 91.6%.[16] The *BRCA1*-C-terminus (*BRCT*) domain and its capability to bind phosphorylated protein is required for the tumor-suppressor function of *BRCA1*.[17]

Our previous findings support a predominant predictive role of *BRCA1* in patients with *EGFR* mutations through an *H2AX*-independent pathway.[18] The DNA breakage caused by erlotinib is different from that caused by radiotherapy or platinum-based chemotherapy, and *BRCA1* by itself can be a relevant predictive biomarker.[18] Erlotinib can suppress homology-directed repair and increase basal levels of *γ-H2AX* foci, which are indicative of an accumulation of DNA double-strand breaks, where *BRCA1* plays an important role. In experimental models, erlotinib sensitivity was highly influenced by *BRCA1* status.[18]

LIM (named for the initials of the three homeodomain proteins *Lin11*, *Isl-1* and *Mec-3*) domain only 4 (*LMO4*) and C terminus-binding protein–interacting protein (*CtIP*) both interact with the BRCT domain of *BRCA1*.[19] The precise contact residues are likely to differ because tumor-derived mutations in the BRCT domain abolish the interaction of *BRCA1* with *CtIP* but not with *LMO4*.[19,20]

LMO4 is a member of the *LIM*-only (*LMO*) family of transcriptional regulators, consisting of *LMO1–4* and is expressed primarily in epithelial-derived tissues.[21] *LMO1–4* act as molecular adaptors, providing a scaffold for multiprotein complexes of DNA-binding factors and transcriptional regulatory proteins, which play essential roles in cell fate determination, tissue patterning, and organ development.[22] The expression profile of *LMO4* suggests that it is an important regulator of epithelial proliferation, with a role in the pathogenesis of cancer.[21] Overexpression of *LMO4* in a subset of sporadic breast tumors was found to be a mechanism of *BRCA1* down-regulation that may contribute to the pathogenesis of breast cancer.[23]

CtIP is a transcriptional coregulator that binds a number of proteins involved in cell-cycle control and cell development, including *BRCA1* and *LMO4*.[24] CtIP represses transcription when recruited to a promoter by the *Gal4* DNA-binding domain, suggesting that it is a corepressor.[25] Moreover, it has been reported to repress *BRCA1*-mediated transactivation of the *p21* promoter when recruited to a *Gal4*-dependent promoter.[26] Although *LMO4* interacts with two regions of *CtIP*, it does not further repress transcription by *CtIP* on the *Gal4* promoter, in contrast to its effect on *BRCA1* activity.[27]

To shed further light on the potential effect of the gene expression of *BRCA1*, *LMO4* and *CtIP* on outcome to *EGFR* TKIs, we have examined the mRNA expression of *LMO4* and *CtIP* in pretreatment tumor samples of erlotinb-treated NSCLC patients with *EGFR* mutations for whom data on *BRCA1* expression and the *EGFR T790M* mutation were available. Gene expression data were correlated with clinical characteristics and outcome, type of *EGFR* activating mutation (deletions in exon 19 or missense mutations in exon 21), and T790M mutational status.

MATERIALS AND METHODS

Tumor Samples

The Spanish Lung Adenocarcinoma Data Base had prospectively screened 2105 NSCLC patient tumor tissues from 91 institutions for *EGFR* mutations. *EGFR* mutations were detected in 350 patients, 217 of whom were treated with erlotinib.[28] Additional genetic analyses were performed in 91 of these patients from whom sufficient tumor tissue was available. All patients signed a written consent form, and approval was obtained from the Institutional Review Board and the Ethics Committee of each hospital.

All analyses were performed centrally at the Pangaea Biotech Laboratory of Molecular Biology, an ISO 15189-certified laboratory. All specimens were formalin-fixed, paraffin-embedded tumor tissues; these were stained with hematoxilin and eosin and assessed by the pathologist of the Pangaea Biotech Laboratory. The histopathological analysis of the tumors, based on the 2004 World Health Organization classification of lung tumors, identified 70 adenocarcinomas, 14 bronchioloalveolar carcinomas, and seven undifferentiated large-cell carcinomas.

Gene Expression Analysis

Microdissection was performed as previously described.[18] Gene expression profiling was performed on RNA isolated from the tumor tissue specimens. RNA extraction, retrotranscription analysis, and real-time polymerase chain reaction were performed as previously described.[18] The primer and probe sets were designed using Primer Express 2.0 Software (Applied Biosystems, Foster City, CA) according to their specifications in Ref Seq in http://www.ncbi.nlm.nih.gov/LocusLink.

Statistical Analyses

Using the median as cutoff, gene expression levels were divided into low and high expression for the purpose of correlation with clinical outcome. Expression levels of *BRCA1*, *LMO4*, and *CtIP* were correlated with the Spearman rho test. The χ^2 test or Fisher's exact test was used to compare qualitative variables. The normality of quantitative variables was analyzed by the Kolmogorov-Smirnov test and compared by Student's *t* test, ANOVA or the Mann–Whitney and Kruskal–Wallis test. Distributions were estimated with the Kaplan–Meier method, and compared with the log-rank test; Binomial distribution was used to calculate 95% confidence intervals (CIs). A multivariate analysis was performed including age, sex, performance status, smoking history, metastatic site, type of *EGFR* mutation (exon 19 deletion or L858R), erlotinib treatment line, T790M mutation status, and median expression levels of *BRCA1*, *LMO4*, and *CtIP*. HRs and 95% CIs were estimated with the use of the Cox proportional-hazards model. All statistical calculations were performed with the SPSS software statistical package, version 17.0 (SPSS Inc., Chicago, IL) and S-PLUS 6.1. Statistical significance was set at two-sided *p* value less than 0.05.

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RESULTS

Patient Characteristics and Gene Expression Levels

The characteristics of the 91 patients are shown in Table 1 and in Tables S1 and S2, (Supplemental Digital Content 1 and 2, http://links.lww.com/JTO/A408, which show patient characteristics according to *LMO4* and *CtIP* expression levels). The majority of the patients (98.9%) were whites, 70.3% women, 67% never-smokers, and 76.9% had adenocarcinoma. Deletions in exon 19 were more frequent (62.6%) than L858R mutation (37.4%). The concomitant *EGFR T790M* mutation was detected in 35 of 91 patients (38.5%). All patients were treated with erlotinib, 49 as first-line and 42 as second-line therapy. The overall response rate was 70.4%, including 14.8% complete responses.

TABLE 1. Patient Characteristics

	All patients N = 91 *n* (%)
Age, yr	
Median (range)	68 (22–85)
Sex	
Male	27 (29.7)
Female	64 (70.3)
Race	
Asian	1 (1.1)
White	90 (98.9)
Smoking history	
Exsmoker	24 (26.4)
Current smoker	6 (6.6)
Never-smoker	61 (67)
ECOG PS	
0	28 (30.8)
1	45 (49.5)
≥2	17 (18.7)
Histology	
Adenocarcinoma	70 (76.9)
Bronchioalveolar carcinoma	14 (15.4)
Large-cell carcinoma	7 (7.7)
Stage	
IIIB	6 (6.6)
IV	85 (93.4)
Treatment line	
First	49 (53.8)
Second	42 (46.2)
T790M mutation	
Detected	35 (38.5)
Not detected	56 (61.5)
EGFR mutation	
Exon 19 deletion	57 (62.6)
L858R	34 (37.4)
Response	
CR	12 (14.8)
PR	45 (55.6)
CR + PR	57 (70.4)
SD	16 (19.8)
PD	8 (9.9)
Response	
CR+PR	57 (70.4)
SD+PD	24 (29.6)

ECOG PS, Eastern Cooperative Oncology Group performance status; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

mRNA expression of *LMO4* and *CtIP* was successfully analyzed in 65 patients (71.4%) and 77 patients (84.6%), respectively. The median mRNA expression was 1.54 (range, 1.51–9.21) for *LMO4* and 1.54 (range, 0.24–2.89) for *CtIP*. Data on *BRCA1* expression were available in 55 patients (60.4%); the median mRNA expression was 7.26 (range, 1.45–20.99). There was a strong correlation between *BRCA1* and *LMO4* expression levels ($\rho = 0.32$; $p = 0.02$) and between *BRCA1* and *CtIP* expression levels ($\rho = 0.31$; $p = 0.001$) but not between *CtIP* and *LMO4* expression levels ($\rho = 0.09$; $p = 0.49$).

Among patients with low *LMO4* expression, the frequency of the concomitant T790M mutation (57.6%) was higher than among those with high *LMO4* expression (25%; $p = 0.01$). No other differences were observed in clinical characteristics according to the expression levels of any of the three genes.

Gene Expression Levels and Clinical Outcome

We have previously reported that low levels of *BRCA1* mRNA correlated with a prolonged PFS of 27 months, whereas a shorter disease-free survival of 11 months was associated with high levels of *BRCA1* ($p < 0.03$).[18] In the present study, PFS was 13 months (95% CI, 6.7–19.3) in patients with low *LMO4* levels and was not reached for those with high *LMO4* levels ($p = 0.006$; Fig. 1). In the multivariate analysis, the presence of high *BRCA1* levels (HR, 5.13; $p < 0.02$) and low *LMO4* levels (HR, 6.02; $p < 0.01$) emerged as markers of shorter PFS (Table 2).

There was a similar, but nonsignificant, trend toward shorter overall survival in patients with low levels of *LMO4* ($p = 0.17$) (see Fig. S1, Supplemental Digital Content 3, http://links.lww.com/JTO/A408, which shows overall survival according to *LMO4* levels).

No significant differences in median PFS or overall survival were observed according to *CtIP* expression levels (see Fig. S2, Supplemental Digital Content 4, http://links.lww.com/JTO/A408, which shows PFS according to *CtIP* levels).

Among the 28 patients with low *BRCA1* expression, median PFS was 19 months (95% CI, 12.7–25.3) in those with low *LMO4* levels and not reached in those with high *LMO4* levels ($p = 0.04$; Fig. 2A). Among the 27 patients with high *BRCA1* expression, median PFS was 8 months (95% CI, 2.9–13) in those with low *LMO4* levels, and 18 months in those with high *LMO4* levels ($p = 0.03$; Fig. 2B). Similar, but nonsignificant, results were observed for overall survival (see Figs. S3 and S4, Supplemental Digital Content 5 and 6, http://links.lww.com/JTO/A408, which show overall survival in patients with low [Fig. S3] and high [Fig. S4] *BRCA1* expression according to *LMO4* levels).

No significant correlation was found between mRNA expression levels of any of the three genes and response (data not shown).

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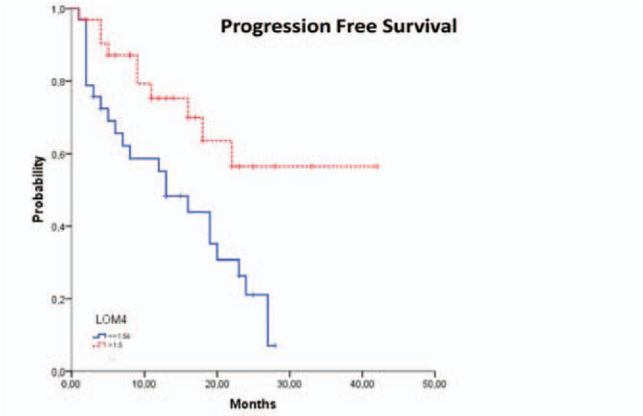

LMO4 levels	N	PFS (mos)	95% CI	*p*
≤1.54	33	13	6.7-19.3	0.006
>1.54	32	NR	-	

FIGURE 1. Progression-free survival to erlotinib in 65 non–small cell lung cancer patients according to *LMO4* expression levels. CI, confidence interval; PFS, progression free survival; *LMO4*, *Lin11*, Isl-1 and *Mec-3* domain only 4.

TABLE 2. Multivariate Analysis

	HR	95% CI	*p*
BRCA1			
≤4.92	[1]		
4.92–10.7	5.32	1.45–19.52	0.03
>10.7	5.13	1.25–20.99	0.02
CtIP			
≤1.21	[1]		
1.21–2.1	0.69	0.24–2.02	0.50
>2.1	1.10	0.42–2.89	0.84
LMO4			
≤1.29	6.02	1.51–23.94	0.01
1.29–1.86	2.81	0.85–9.21	0.09
>1.86	[1]		

HR, hazard ratio; *BRCA1*, breast cancer gene 1; *LMO4*, *Lin11*, *Isl-1* and *Mec-3* domain only 4; *CtIP*, C terminus-binding protein–interacting protein.

Both *BRCA1* and *LMO4* expression emerged as significant factors for PFS in the multivariate analysis (HR for high *BRCA1* levels, 5.23; 95% CI, 1.57–17.45; $p = 0.007$; HR for low *LMO4* levels, 4.31; 95% CI, 1.69–10.7; $p = 0.002$).

DISCUSSION

Notwithstanding the success of erlotinib and gefitinib in cases of NSCLC with activating *EGFR* mutations, patients eventually progress despite such treatment. Erlotinib can cause double-strand breaks that are repaired mainly by homologous recombination, where *BRCA1* plays an important role, and in experimental models, erlotinib sensitivity is highly influenced by *BRCA1* status.[18] We have previously reported that elevated *BRCA1* mRNA levels predict poor prognosis in resected NSCLC[16] and shorter PFS to erlotinib in metastatic *EGFR*-mutant NSCLC patients.[18] In the present study, based on the expression of genes involved in DNA-repair pathways, we have defined a favorable subgroup of *EGFR*-mutant NSCLC patients with impressively longer PFS to erlotinib.

LMO4, initially described as a human breast tumor autoantigen, is a repressor of *BRCA1*-mediated transcriptional activity, with a potential role as a negative regulator of *BRCA1* function in sporadic breast cancers.[27] Deregulation of *LMO4* occurs in several tumors, and high *LMO4* nuclear expression is an independent prognostic factor in breast cancer.[27] *LMO4* interacts with the cofactor *CtIP* and *BRCA*, and inhibits the transcriptional activity of *BRCA1* in both yeast and mammalian cells.[27] Immunohistochemistry studies have shown that *LMO4* is highly expressed in epithelial tissues, specifically in cells lining the airways of the developing and adult lung.[29] In a large cohort of patients with primary operable squamous cell carcinoma of the anterior tongue, treated with surgery and adjuvant radiotherapy, low *LMO4* expression (by immunohistochemistry) was observed in 34%. However, this was not significantly associated with disease-free survival or overall survival.[22]

Of the 91 erlotinib-treated *EGFR*-mutant NSCLC patients included in the present study, those with low *BRCA1* expression had significantly longer PFS, as did those with high *LMO4* expression, whereas low *LMO4* levels were associated with shorter PFS, which is consistent with findings in breast cancer and squamous cell carcinoma of the anterior tongue.[22,27] Moreover, the combination of low *BRCA1* and high *LMO4* mRNA levels identified a favorable subgroup of patients in whom PFS was not reached. In addition, although overall patients with high *BRCA1* levels had shorter PFS, the 17 patients with high levels of both *BRCA1* and *LMO4* had a significantly longer PFS of 18 months and a trend toward better overall survival.

Although a correlation was found between *BRCA1*, *LMO4*, and *CtIP* mRNA levels, no differences in median PFS or overall survival were observed according to *CtIP* expression levels. Intriguingly, the concomitant *EGFR* T790M mutation has been identified in approximately 30% of patients before treatment.[11,18,30] In the present study, median PFS for patients with *EGFR* T790M mutation was 12 months, and 23 months for those without the T790M mutation ($p = 0.01$). Overall survival was 27 months for the subgroup with positive T790M, and 33 months for the subgroup negative for T790M ($p = 0.12$). Overall response rate for the T790M positive subgroup was 55.6%, and 73.3% for the negative subgroup ($p = 0.17$). The frequency of T790M mutations was 57.6% in patients with low *LMO4* expression, although in the multivariate analysis, the presence of the T790M mutation (HR, 2.25; $p = 0.29$) was not a marker of shorter PFS. A positive correlation between *BRCA1* and *LMO4* levels was found ($\rho = 0.32$, $p = 0.02$). Intriguingly, *LMO4* levels were lower in the subgroup of patients with the presence of T790M in comparison with the subgroup negative for T790M ($p = 0.03$). No differences were observed in *BRCA1* mRNA levels according to T790M status ($p = 0.47$). The relationship between the T790M mutation and *LMO4* expression warrants further study.

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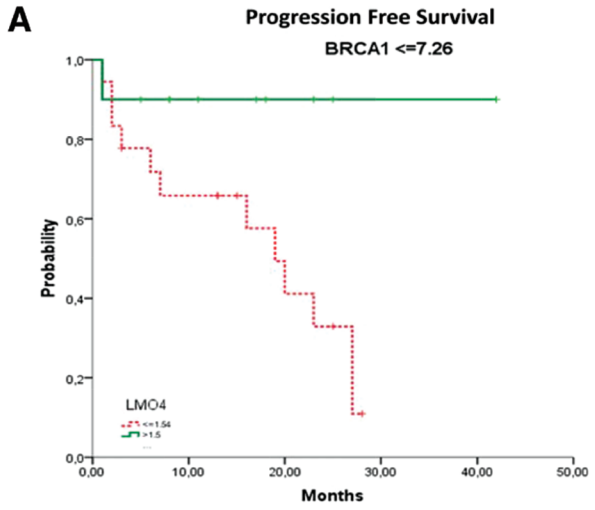
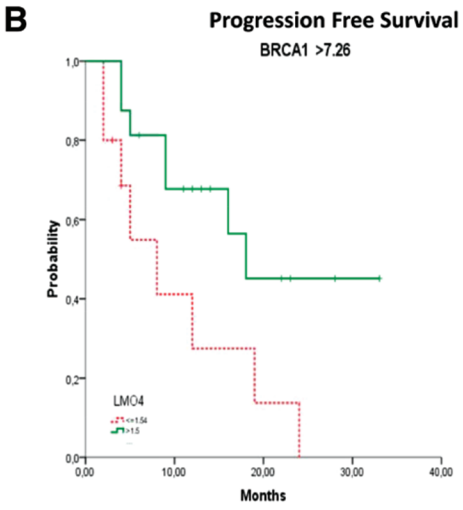


LMO4 levels	N	PFS (mos)	95% CI	*p*
≤1.54	18	19	12.7-25.3	0.04
>1.54	10	NR	-	



LMO4 levels	N	PFS (mos)	95% CI	*p*
≤1.54	10	8	2.9-13	0.03
>1.54	17	18	-	

FIGURE 2. Progression-free survival according to *LMO4* expression levels in (*A*) 28 non–small cell lung cancer patients with low BRCA1 expression (<=7.26) and in (*B*) 27 NSCLC patients with high *BRCA1* expression (>7.26). *BRCA1*, breast cancer gene 1; CI, confidence interval; PFS, progression free survival; *LMO4*, *Lin11*, *Isl-1* and *Mec-3* domain only 4.

Although the sample size in this study was relatively small, which may possibly explain the lack of significant differences in response rate and overall survival according to *BRCA1* and *LMO4* expression levels, the finding that low *BRCA1* and high *LMO4* levels were associated with longer PFS indicates that a potential two-gene model based on *BRCA1* and *LMO4* expression merits further investigation to confirm its role in predicting the efficacy of EGFR TKIs in lung adenocarcinomas with *EGFR* mutations.

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