Biological difference between L858R and exon 19 deletion contributes to recurrence-free survival of resected non-small cell lung cancer

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Abstract

Background:

The differences in biological characteristics among different genotypes of classical *EGFR* mutations have not been clarified. This study aims to clarify the clinical and biological differences between L858R and 19 deletion in NSCLC.

Patients and Methods:

We analyzed a cohort of 170 consecutive cases of surgically resected NSCLC harboring EGFR driver mutations (L858R or 19 deletion) in which curative resection was performed in Aichi Cancer Center Hospital, Nagoya, Japan from January 2006 to February 2013 and in which recurrence subsequently developed. We also subjected 61 surgically resected NSCLC specimens harboring *EGFR* driver mutations (L858R or 19 deletion) to an RNA sequencing analysis.

Results:

In patients with stage I and II disease, the median time to recurrence did not differ to a statistically significant extent between the types of EGFR mutations; however, among those with stage III disease, the median time to recurrence in patients with the L858R genotype tended to be shorter in comparison to those with 19 deletion (log-rank test, P = 0.071). In comparison to 19 deletion tumors, L858R tumors had higher cytological malignancy (e.g., mitotic ability) and showed stronger immunogenicity.

Conclusions:

L858R and 19 deletion tumors are likely to have a slight difference in the time to recurrence. They suggest that even in *EGFR* driver tumors, which are treated as the same disease category, the biological characteristics of the tumors are different, which may leave room for innovations in postoperative treatment and treatment at recurrence.

Introduction

The development of molecular diagnostics has made a great contribution to the diversification of therapeutic strategies for advanced NSCLC. Especially in *EGFR* mutant-NSCLC, which is the most common type of driver mutation, the standard treatment, including molecular targeted agents has matured, resulting in a dramatic improvement of the prognosis [1]. Because of the significant genomic diversity in *EGFR* mutations (even the classical mutations, L858R and 19 deletion, are treated as the same category) [1], it is also an area where further improvement in treatment strategies, such as combination drugs with EGFR-tyrosine kinase inhibitors (TKIs) and indications for immune-checkpoint inhibitor (ICI), is desired.

There are few reports on the biological differences between the two classical mutations, however, the results of a proteomic analysis have been reported. According to this report, 19 deletion tumors showed the involvement of SUMOylation, EMT, ERK/MAP kinase, and Hippo signaling, while various pathways related to cancer cell survival were identified in L858R tumors [2]. There have been several reports on tumor grade using recurrence-free survival (RFS) as an indicator, but the results have been controversial, and moreover, many of them were only observational studies with no biological considerations [3]. In fact, for estimating tumor grade, the analysis of time to recurrence for each postoperative stage in an uncensored model may reduce biases (e.g., the presence of residual tumor and the amount of residual tumor). In addition, the RNA sequences of tumors and the analysis of differences in their expression may reveal differences in background biological characteristics.

This study aims to clarify the clinical outcome of resected *EGFR* mutant NSCLCs with classical *EGFR* mutations (L858R and 19 deletion) and reveal the biological difference between tumors with L858R and 19 deletion using an RNA expression analysis.

Patients And Methods

Patients

We used a cohort of 170 consecutive patients who underwent surgical resection of NSCLC Aichi Cancer Center Hospital, Nagoya, Japan from January 2006 to February 2013. The inclusion criteria of this cohort were patients with primary NSCLC harboring *EGFR* driver mutations (L858R or 19 deletion) who underwent curative resection and who subsequently experienced recurrence. We also used 61 surgically resected NSCLC specimens harboring *EGFR* driver mutations (L858R or 19 deletion) for an RNA sequencing analysis. This retrospective study was approved by the Institutional Review Board of Aichi Cancer Center (No:2018-2-20). The study was conducted under the principles of the Declaration of Helsinki.

Methods

Conventional gene test

EGFR genes were analyzed as previously described [4].

RNA sequence

For RNA sequencing (n = 61), an RNA sequence library was prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) in accordance with the manufacturer's protocols. The enriched libraries were sequenced as 150-bp paired-end reads using NovaSeq (Illumina, San Diego, CA, USA) at Veritas Genetics (Danvers, MA, USA). RNA-seq data were analyzed using the CLC genomics workbench 20.1 software program (Qiagen). Control sequencing data were obtained from the ENA database (https://www.ebi.ac.uk/ena/browser/home). A gene set enrichment analysis was performed at RaNA-seq (https://ranaseq.eu/).

Statistics

To evaluate the risk factors associated with the time to recurrence and the prognosis, a Cox proportional hazards regression model with a step-down procedure was used. The time to recurrence and survival curves were determined using the Kaplan–Meier method. A log-rank test was performed to evaluate differences between survival curves. All statistical analyses were performed using the JMP 12 software program (SAS Institute, Cary, NC, USA).

Results

The clinicopathological findings of patients with *EGFR*-mutated NSCLC who received curative surgery and who developed recurrent disease are listed in Table 1. In brief, all patients (male, n = 63 [37.1%]; female, n = 107[62.1%] median age, 66 [range, 39–85] years) underwent curative surgery. The number of dominant adenocarcinomas was 169 (99.4%). Approximately two-thirds of patients (112, 70.6%) were never smokers. Eighty-nine cases (52.3%) harbored L858R and the remining 81 cases (47.7%) had the 19 deletion.

Table 1
Clinicopathological characteristics of 170 patients who developed recurrence after the resection of EGFR-mutant NSCLC.

	Value	p.L858R	p.exon19 deletion
	170	89 (52.3)	81 (47.7)
Age (years)			
Median	66	66	65
Range	39–85	46–84	39–85
Sex, n (%)			
Female	107 (62.9)	51	56
Male	63 (37.1)	38	25
Smoking status, n (%)			
Current/former	58 (29.4)	30	28
Never	112 (70.6)	59	53
p-Stage, n (%)			
IA1	4	3	1
IA2	11	8	3
IA3	9	5	4
IB	42	21	21
IIA	4	1	3
IIB	39	18	21
IIIA	64	30	34
IIIB	7	3	4
Histology, n (%)			
Adenocarcinoma			
Papillary predominant	85	47	38
Acinar predominant	60	29	31
Solid predominant	19	10	9
Lepidic predominant	2	1	1
Invasive mucinous	2	1	1
Cribriform	1	0	1
Clear cell carcinoma	1	1	0
Site of recurrence			

* Multiple metastases was defined as three or more metastases.

p-Stage, pathological stage; L, Leucine; R, Arginine.

	Value	p.L858R	p.exon19 deletion
Adrenal	2	2	0
Bone	24	14	10
Brain	30	16	14
Liver	1	1	0
Lung	56	25	31
Lymph node	33	17	16
Pancreas	1	0	1
Pleural effusion	11	7	4
Skin	2	2	0
*Multiple	10	5	5
* Multiple metastases was defined as three or more metastases.			
p-Stage, pathological stage; L, Leucine; R, Arginine.			

The clinicopathological findings of the *EGFR*-mutated cases for which the RNA sequence analysis was performed are listed in Table 2. In brief, all 6l patients (male, n = 27 [44.2%]; female, n = 34 [55.8%], median age, 70 [range, 45–86] years) underwent curative surgery. The number of dominant adenocarcinomas was 59 (96.7%). Thirty-four cases (55.8%) harbored L858R, while the remaining 27 cases (43.2%) had 19 deletion.

Table 2
Clinicopathological characteristics of 61 patients with resected EGFR-mutant NSCLC who underwent RNA-seq

	Value	p.L858R	p.exon19deletion
	61	34 (55.8)	27 (44.2)
Age (years)			
Median	70	70	67
Range	45–86	45–86	48–82
Sex, n (%)			
Female	34 (55.8)	17	17
Male	27 (44.2)	17	10
Smoking status, n (%)			
Current/former	30	19	11
Never	30	15	15
Unknown	1	0	1
p-Stage, n (%)			
IA1	3	3	0
IA2	15	12	3
IA3	10	7	3
IB	8	5	3
IIA	4	1	3
IIB	4	3	1
IIIA	4	3	1
IIIB	1	0	1
IV	2	0	2
Histology, n (%)			
Adenocarcinoma	59	34	25
Papillary predominant	29	20	9
Acinar predominant	14	9	5
Solid predominant	2	1	1
Lepidic predominant	4	4	0
Adenosquamous cell carcinoma	2	0	2

p-Stage, pathological stage; L, Leucine; R, Arginine.

Among patients with pathological stage I and II disease, the median time to recurrence of patients with the two types of EGFR mutations did not differ to a statistically significant extent (Fig. 1A and 1B); however, among those with stage III

disease, the median time to recurrence in patients with the p.L858R genotype tended to be shorter in comparison to those with 19 deletion (log-rank test, P = 0.071) (Fig. 1C).

The results of clustering according to the *EGFR* genotypes are shown in Fig. 2A.

A volcano-plot of the gene expression according to *EGFR* genotypes is shown in Fig. 2B. The difference in the gene expression between tumors with L858R and 19 deletion is shown in Fig. 3). The gene expression of AREG was higher in L858R tumors than in 19 deletion tumors. On the other hand, the expression of NPTX1, PRR4, and ADCY1 in L858R tumors were lower than those in 19 deletion tumors. The expression of HLA-C, HLA-DRA, and HLA-DPB1, which may reflect the immune environment around the tumor, were higher in 19 deletion tumors (Table 3).

Table 3
The gene expression according to *EGFR* genotype

ID	Symbol	Exp Mean	Exp p.L858R	Exp p.19del	log2FC	lfcSE	Stat	p-value	padj
ENSG00000206452.10	HLA-C	116.138	81.009	161.025	0.982	0.866	9.072	1.20E-19	3.70E-15
ENSG00000206478.5	IER3	7.664	4.228	12.055	1.32	2.938	8.192	2.60E-16	4.10E-12
ENSG00000285515.1	VARS2	0.551	0.682	0.383	-0.282	2.947	-7.864	3.70E-15	4.00E-11
ENSG00000228987.9	HLA-DRA	67.021	24.333	121.565	2.274	0.787	5.462	4.70E-08	3.80E-04
ENSG00000137860.11	SLC28A2	0.858	1.229	0.384	-0.687	0.55	-5.376	7.60E-08	4.90E-04
ENSG00000171246.5	NPTX1	3.779	0.88	7.484	2.174	0.598	4.913	8.90E-07	0.005
ENSG00000111215.12	PRR4	890.285	7.748	2017.972	7.85	0.831	4.856	1.20E-06	0.005
ENSG00000109321.10	AREG	66.671	99.674	24.501	-1.981	0.428	-4.507	6.60E-06	0.026
ENSG00000133055.8	MYBPH	2.553	4.006	0.698	-1.56	0.594	-4.408	1.00E-05	0.037
ENSG00000237710.10	HLA-DPB1	50.49	49.897	51.248	0.038	1.41	4.346	1.40E-05	0.041
ENSG00000164742.15	ADCY1	1.125	0.752	1.601	0.57	0.268	4.329	1.50E-05	0.041
ENSG00000127831.10	VIL1	2.953	4.616	0.828	-1.62	0.915	-4.322	1.50E-05	0.041

Exp, expression; FC, scaled fold change; Lfc, log2 scaled fold change calculated by Deseq2; SE, the standard error; Stat, statistic; padj, adjusted p value.

GSEA was carried out to explore the biological differentiation between L858R and 19 deletion tumors. The pathways and gene ontology were analyzed in our study. Pathway enrichment demonstrated that L858R tumors were mainly involved in the cell cycle and cell cycle checkpoint pathways (Fig. 4A). Gene ontology enrichment demonstrated that L858R tumors was involved in cell cycle activity and synapse assembly (Fig. 4B).

Discussion

Among early-stage resected *EGFR* mutant NSCLCs, the time to recurrence in patients with L858R tumors was shorter than that in those with 19 deletion tumors. In comparison to 19 deletion tumors, L858R tumors had higher cytological malignancy (e.g., mitotic ability) and showed stronger immunogenicity, which may be due to the short recurrence-free time in early-stage NSCLCs.

Among patients with completely resected NSCLC, patients with 19 deletion tumors had better RFS than those with L858R tumors [3]. Disease-free survival in patients with pN1-pN2 19 deletion tumors was better than that in those with pN1-pN2 L858R tumors {Isaka, 2016 #5}. Another study reported that 19 deletion tumors were associated with better disease-free survival in comparison to L858R tumors among stage III lung cancer patients who received lung resection {Zhang, 2017 #4}. In terms of advanced stage, EGFR-TKIs are reported to be more beneficial for patients with 19 deletion tumors than those with L858R tumors {Li, 2020 #6}. These previously reported data are compatible with our results and a gene expression analysis of our data may explain the background of these phenomena.

The GSEA analysis demonstrated that L858R tumors have high AMRG expression levels, and it is possible that the growth signal is activated in a ligand-dependent manner, which may explain why EGFR-TKIs are less effective for L858R tumors {Li, 2020 #6}.

Other genes that are more highly expressed in L858R are as follows. Gene Ontology (GO) annotations related to MYBPH (Myosin Binding Protein H) include structural constituent of muscle. Among *BRAF* V600E-positive papillary thyroid carcinoma cases with brain metastasis, MYBPH was confirmed by a microarray analysis as one of the proteins that were elevated [5]. Gene ontology (GO) annotations related to SLC8A2 (Solute Carrier Family 8 Member A2) include calmodulin binding and calcium:sodium antiporter activity. In the tumor environment of advanced colorectal cancer, SCL28A2 has been identified as one of the proteins that increase Th17 cells and cause immunosuppression. SCL28A2 has been identified as one of these proteins [6]. VIL1 (Villin1) represents a dominant part of the brush border cytoskeleton, which functions in the capping, severing, and bundling of actin filaments. In advanced colorectal cancer, lymph node metastasis and elevated VIL1 have been reported by based on the results of an mRNA expression analysis by RT-PCR [7].

In the environment around the 19 deletion tumor, the expression of MHC class 1and 2 is increased, and the acquired immune activity of both Class 1 and 2 may be high, which may reflect the high immunogenicity. In other words, this immunogenicity may be due to the efficacy in suppressing recurrence. It was also reported that more myeloid dendritic cells (DCs) were present in patients with EGFR 19 deletion than in those with L858R-mutation, which suggests that the TIME of EGFR L858R NSCLC was more immunosuppressive [8].

Other genes that are more highly expressed in 19 deletion tumors are as follows. ADCY (Adenylate Cyclase 2) is insensitive to Ca(2+)/calmodulin, and is stimulated by the G protein beta and gamma subunit complex. It has been reported that ADCY was decreased in patients with distant metastasis of rectal cancer in comparison to those without distant metastasis [9]. PRR4 (Proline Rich 4) may have a protective function in the eye. It has been reported that suppression of circulating RNA PRH1-PRR4 in NSCLC cell lines increased cell malignancy and promoted apoptosis [10]. NPTX1 (Neuronal Pentraxin 1) is a member of the neuronal pentraxin gene family. It has been reported that NPTX1 is decreased in a hepatocellular carcinoma cell line and that blocking AKT1 increases NPTX1 and suppresses cell growth [11]. IER3 (Immediate Early Response 3) functions in the protection of cells from Fas-induced or tumor necrosis factor type alpha-induced apoptosis. It has been reported that knockout of IER3 in a tongue cancer cell line reduced the level of VEGF-C and decreased the invasive capacity [12].

On the other hand, in the function enrichment analysis, it was suggested that L858R tumors have MHC class 2 (antigen processing pathway is elevated) and that interferon gamma-dependent immune activity is increased, so that there is immune tolerance in the surrounding environment. It is suggested that the effect of ICIs, especially CTLA4 inhibitor, which is administered based on its effect on dendritic cells, may be effective for L858R tumors [13].

In addition, GSEA showed that the cell cycle was more active in L858R, suggesting the activation of RhoGTPase by formin and the activation of neuro-transmitter in the tumor, and the biological grade may be higher in L858R [14].

The formin family and Daams are expressed in tissues known to require Wnts and are consistent with Daams being effectors of Wnt signaling during vertebrate development [15]. The ectopic expression of a DAAM1 phosphodeficient mutant inhibited F-actin assembly and suppressed lung cancer cell migration and invasion [16].

Particularly under chronic stress, the continuous release of neurotransmitters from the neuroendocrine system can have a highly profound impact on the occurrence and prognosis of breast cancer [17].

In high pathological stages, L858R tumors may benefit from combination treatment with EGFR-TKIs, such as anti-CTLA, or in combination with anti-EGFR antibody. Also, regarding postoperative EGFR-TKI treatment, it may be better to treat L858R tumors more aggressively. Based on the increased activity of EGFR ligands, the therapeutic effect may be improved by devising targeted therapy, such as anti-EGFR antibody treatment. The results of the GE analysis also suggest that 19 deletion tumors may benefit from combined anti-VEGF therapy.

L858R and 19 deletion tumors are likely to have a slight difference in the time to recurrence. They suggest that even in *EGFR* driver tumors, which are treated as the same disease category, the biological characteristics of the tumors are different, which may leave room for innovations in postoperative treatment and treatment at recurrence.

Declarations

Conflict of interest statement

The authors declare no competing interests.

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Figures

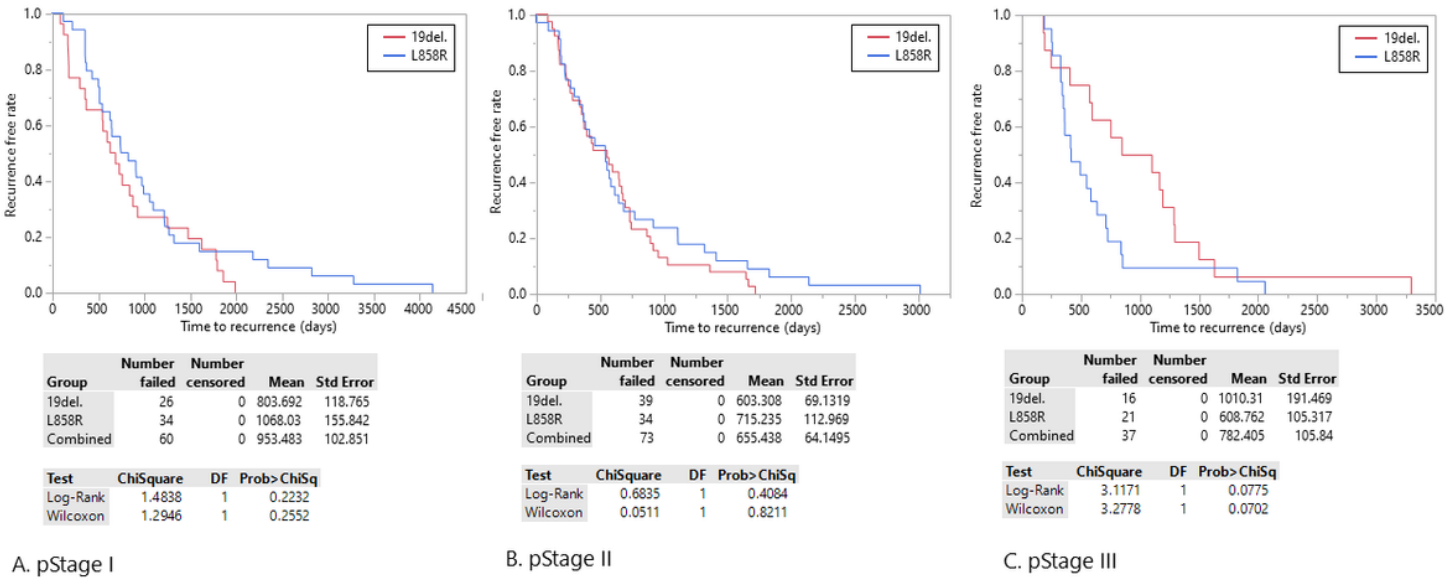

Figure 1.

Figure 1

(A) A Kaplan–Meier analysis of the time to recurrence of pstage I patients according to *EGFR* genotypes. (B) A Kaplan–Meier analysis of the time to recurrence of pstage II and (C) pstage III.

Figure 2

A heat map (A) and Volcano-plot of the differential expression according to *EGFR* genotype (B) of *EGFR*-mutated tumors.

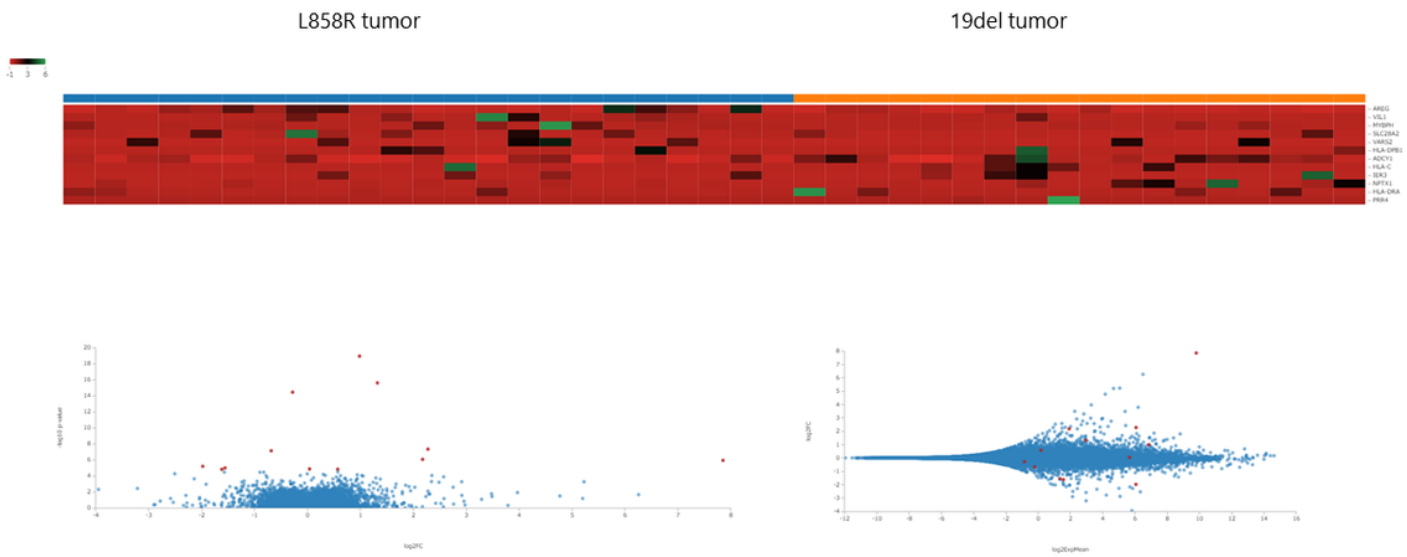

Figure 3.

Figure 3

A heat map of the differential expression of EGFR L858R tumors and 19 deletion tumors.

A heat map of the differential expression of the normal lung and KRAS-mutated tumors.

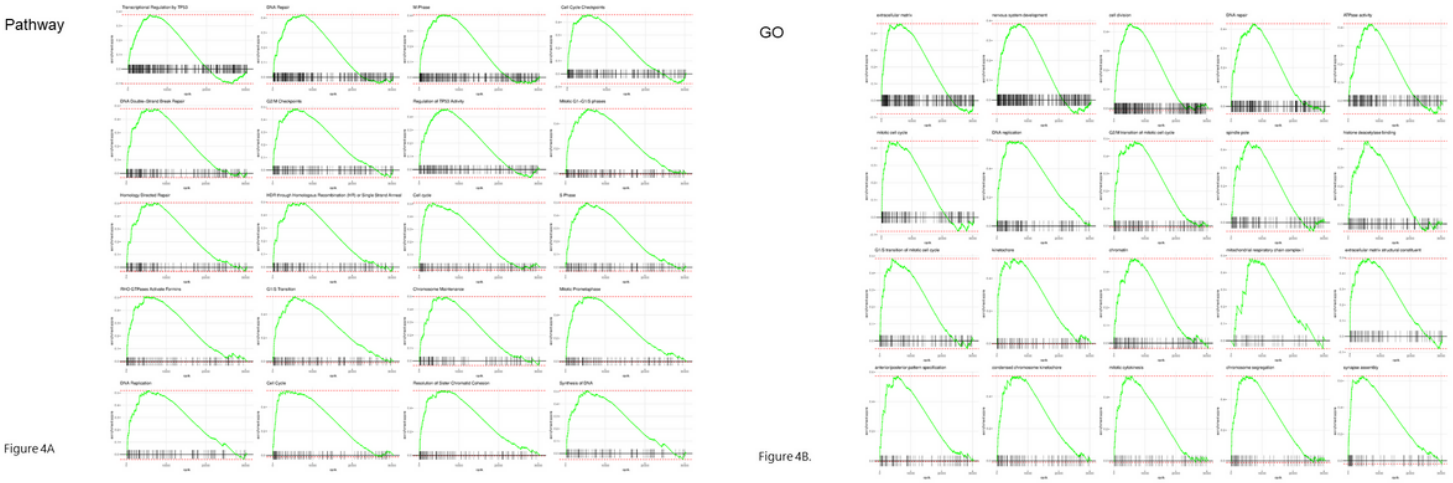

Figure 4A

Figure 4B.

Figure 4

(A) A gene set enrichment analysis of the EGFR L858R tumors in the pathway. (B) A gene set enrichment analysis of the EGFR L858R tumors in the gene ontology.