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Novel mutation signatures in the prognosis of EGFR-TKIs targeted therapy for non-small cell lung cancer patients based on the 1000-gene panel sequencing

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The application of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer (NSCLC) may be affected by somatic mutations. The purpose of this study was to explore the effect of mutations on the prognosis and tumor markers of NSCLC patients treated with EGFR-TKIs. 21 NSCLC patients treated with EGFR-TKIs were selected, and the targeted sequencing of the tumor tissues or whole blood samples with the 1000-gene panel was conducted to screen mutations. Afterward, functional enrichment analysis was performed based on mutant genes. Subsequently, the correlation between mutations and clinical indicators, prognosis, and tumor markers were analyzed. Finally, the prognosis after taking osimertinib was compared between NSCLC patients with EGFR p.T790M positive and negative mutations, and the EGFR p.T790M concomitant and uncommon mutations were screened. A total of 485 mutations in 251 genes were identified, in which MTOR, AXIN2, AR, EGFR, NOTCH1, and HRAS mutations were significantly correlated with PFS and/or tumor markers. There was no significant difference in PFS, therapeutic effect, and prognosis between EGFR p.T790M positive and negative patients who received osimertinib treatment. Besides, we also found 80 concomitant mutations and 54 uncommon mutations of EGFR p.T790M. AR, HRAS, EGFR, AXIN2, NOTCH1, and MTOR might be key genes to the prognosis of NSCLC treated with EGFR-TKIs. Osimertinib has certain efficacy in EGFR p.T790M negative NSCLC patients.

Key words: non-small cell lung cancer, EGFR-TKIs, prognosis, EGFR p.T790M, targeted next-generation sequencing

Lung cancer is one of the fastest-growing malignant tumors globally, with non-small cell lung cancer (NSCLC) accounting for approximately 80% of all lung cancers. Although there is an increased number of clinically available treatment options, NSCLC is still the leading cause of cancer-related deaths worldwide, accounting for more than 85% of all lung cancers [1]. The clinical knowledge of the molecular status of epidermal growth factor receptor (*EGFR*) has led to a great change in the treatment mode for metastatic NSCLC. The detection rate of *EGFR* gene mutations in Caucasians is 10–15% and in East Asian patients as high as 50% [2, 3]. The overall molecular characteristics of lung cancer extend our understanding of the cellular origin and molecular pathways. Many of these genetic changes represent the potential therapeutic targets for drug development [4]. *EGFR* tyrosine kinase inhibitors (EGFR-TKIs) are a class of drugs targeting *EGFR* mutations. By targeting *EGFR*, they can inhibit its function, block the EGFR signaling pathway, finally suppress the proliferation of tumor cells, and perform an anti-tumor effect. Up to now, EGFR-TKIs such as erlotinib, gefitinib, afatinib, and osimertinib have been approved by the US Food and Drug Administration (FDA) as standard first-line treatment for patients with *EGFR* mutations [5]. Among them, osimertinib has a better effect on *EGFR* sensitive mutations and p.T790M resistant mutation. It is the first p.T790M mutant positive NSCLC target drug approved for the progression of disease during or after EGFR-TKI treatment and is the only third-generation EGFR-TKI approved for marketing in China [6].

The application of EGFR-TKI is affected by the mutations of *EGFR* [7]. Due to the large individual differences, patients carry a variety of gene mutations, and tumors with different mutation types have different sensitivities to drugs. In addition, with the development of next-generation sequencing (NGS) technology, the range of mutations detected has been greatly expanded, and more mutations that may affect the efficacy of drugs will be discovered.

Therefore, this study screened mutations of NSCLC patients who received 3 types of EGFR-TKIs treatment using

targeted NGS-based on 1000-gene panel and evaluated the clinical significance of the mutated genes. Furthermore, the efficacy of osimertinib for NSCLC patients with *EGFR* p.T790M mutation positive and negative was compared, as well as the concomitant mutation and uncommon mutation of *EGFR* p.T790M, exploring the effect of mutations on tumor markers and prognosis of EGFR-TKIs in NSCLC patients.

Patients and methods

Patient inclusion and exclusion. We collected patients who received gefitinib, icotinib, or ositinib in our hospital from 2017 to 2019. The inclusion criteria were: 1) age ≥18; 2) patients with non-small cell lung cancer confirmed by histopathology or cytology; 3) patients volunteered for 1000-gene panel test; 4) the curative effect can be evaluated after treatment, and clinical data can be obtained, such as tumor markers CEA, SCC, NSE, CA19-9, CYFRA21-1, and ProGRP before and during treatment.; 5) the group was enrolled according to the criteria of signing informed consent. Exclusion criteria: 1) unqualified sample collection; 2) unqualified gene test data; 3) lack of clinical data; 4) lack of follow-up information, and finally the remaining 21 patients with non-small cell lung cancer. This study was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University, and written informed consent was obtained from all patients prior to surgery.

Sample collection standard. Tumor tissue or whole blood samples were collected from each patient. Peripheral blood samples were collected with a Streck tube and the sample volume was ≥10 ml. 1–2 fresh surgical tissue samples were collected, 40–50 mg, accounting for more than 40% of tumor cells and ≤10% of necrotic cells. Fresh puncture tissue sampling ≥4 needles. Peripheral blood control samples were collected with an EDTA anticoagulant tube for 3–5 ml blood.

Targeted NGS gene panel sequencing. Tumor tissue or whole blood samples were targeted with 1000-gene plates to NGS, adjacent tissues, or leukocytes as controls (Supplementary Table S1). The detection types include gene point mutation, insertion deletion mutation, and related gene fusion mutation. The depth of tissue sequencing was more than 400×, the depth of ctDNA sequencing was more than 1000×, and the minimum detection frequency was 0.1%. Genomic DNA from a tissue sample and circulating free DNA (cfDNA) from the blood plasma sample was extracted using the Genomic DNA extraction kit (Qiagen, Hilden, Germany) and QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Size selection was performed using the Agencourt AMPure XP beads (Agencourt Biosciences, Beverly, MA, USA), followed by PCR amplification. SeqCap EZ MedExome Enrichment kit (Roche, Basel, Switzerland) was used to capture target sequences, subsequently targeted enrichment was performed using SeqCap EZ Prime Choice Probes (Roche, Basel, Switzerland), which captured a total of 1.1 Mb from 1,000 known cancer-related genes. The captured libraries were targeted sequenced using Illumina HiSeq Xten sequencer (San Diego, CA, USA).

Mutation analysis. SOAPnuke was used to filter the sequencing data, remove low-quality data, and the sequence containing Adapter. Afterward, the sequencing data were mapped to the hg19 reference genome with Burrows-Wheeler Aligner software for tumor-specific somatic mutation detection. VarScan version 2.4.3, MuTect version 1.1.4, and Genome Analysis Toolkit (GATK) version 2.3.9 were used to identify single-nucleotide polymorphisms (SNPs) and insertion/deletion (Indel). CONTRA version 2.0.4 was adapted for gene copy number variations (CNV) estimation, and fusion mutations were analyzed with an independently developed program.

Functional enrichment analysis. Gene Ontology (GO) terms and pathway enrichment analysis were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/) and KEGG PATHWAY (http://www.genome.jp/kegg) databases. A p-value < 0.05 was the cut-off criterion.

Statistical analysis. SPSS statistics version 23 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were compared between groups by t-test, and categorized variables were compared with the χ^2 test or Fisher exact test and expressed by numbers and frequencies. Multivariate regression analyses were performed to compare the mutated percentage of each gene.

Results

Patient characteristics and mutations. The clinical characteristics of 21 NSCLC patients are shown in Table 1. For all patients, a total of 485 mutations in 251 genes were screened out. The distribution of mutation classifications was determined: missense (53%), frameshift (23%), CDS-Indel (18%), splicing (3%), nonsense (2%), and substitution (1%). Table 2 lists the top 20 mutations according to the mutation frequency. Among them, *AURKA* c.1075C>A and *EGFR* c.2573T>G were the two mutations with the highest mutation frequencies (80% and 55%). For all mutated genes, a total of 28 genes were significantly different in the number of patients treated with gefitinib with and without mutations, as well as 3 and 1 genes in patients treated with icotinib and osimertinib, respectively. Figure 1 shows the mutations of the 43 genes, including the above 32 genes and the top 15 genes with the highest mutation frequencies in all patients, with duplicates deleted.

Functional enrichment analysis. The 43 mutated genes in Figure 1 were uploaded into DAVID to identify the functional enrichment of the representative mutations. A total of 58 GO terms with p-value <0.05 were obtained (Figure 2A), containing 38 biological processes (BPs), 10 cellular components (CCs), and 10 molecular functions (MFs). The BPs were primarily enriched in positive regulation of transcrip-

Table 1. Clinical characteristics of NSCLC patients.

Characteristics	Group	n (%)	Characteristics	Group	n (%)
Sex	Males	10 (47.62)	N	0	2 (9.52)
	Females	11 (52.38)		1	0 (0.00)
Age	≥65	9 (42.86)		2	5 (23.81)
	<65	12 (57.14)		3	6 (28.57)
Weight (kg)	≥65	11 (52.38)		4	0 (0.00)
	<65	10 (47.62)		x	8 (38.09)
Smoke	No	14 (66.67)	M	0	4 (19.05)
	Yes	7 (33.33)		1	17 (80.95)
Drinking	No	16 (76.19)	Adverse effect	No	19 (90.47)
	Yes	5 (23.81)		Yes	2 (9.53)
Pathological types	Adenocarcinoma	21 (100.00)	Therapeutic effect	PD	2 (9.52)
Stage	III	4 (19.05)		SD	9 (42.86)
	IV	17 (80.95)		PR	5 (23.81)
Recurrence and metastasis	No	11 (52.38)	EGFR-TKIs	None	5 (23.81)
	Yes	10 (47.62)		Gefitinib	9 (42.86)
T	1	2 (9.52)		Icotinib	7 (33.33)
	2	7 (33.34)		Osimertinib	5 (23.81)
	3	0 (0.00)	OS (d)		665±293
	4	6 (28.57)	PFS (d)		601±326
	x	6 (28.57)			

Abbreviations: PD-progressive disease; SD-stable disease; PR-partial response

Table 2. Top 20 mutations with the highest frequency.

Mutation point	pHGVS	Chromosome	Variant Classification	Variant Type	Frequency (%)
AURKA c.[1075C>A]	p.L359I	chr20	Missense	SNV	80
EGFR c.[2573T>G]	p.L858R	chr7	Missense	SNV	55
TP53 c.[560-1G>A]	*	chr17	Splice-3	SNV	39.03
TP53 c.[742C>T]	p.R248W	chr17	Missense	SNV	38.26
SMAD4 c.[1051_1052delinsTT]	p.D351F	chr18	Substitution	Substitution	35.02
RB1 c.[1811A>G]	p.D604G	chr13	Missense	SNV	32.03
PTEN c.[399_400insATG]	p.V133delinsVM	chr10	CDS-Indel	Insertion	28.76
CHD4 c.[1111G>A]	p.D371N	chr12	Missense	SNV	25.5
HLA-A c.[453C>A]	p.N151K	chr6	Missense	SNV	25
TP53 c.[796G>C]	p.G266R	chr17	Missense	SNV	23.38
PIK3R2 c.[2049C>G]	p.Y683*	chr19	Nonsense	SNV	22
NOTCH1 c.[3369_3370delinsTT]	p.V1123_D1124delinsVY	chr9	Substitution	Substitution	21.73
POLD1 c.[199G>C]	p.D67H	chr19	Missense	SNV	14.37
CCN6 c.[1088G>C]	p.G363A	chr6	Missense	SNV	12.61
GNAS c.[697T>G]	p.F233V	chr20	missense	SNV	9.3
PARP4 c.[3176A>G]	p.Q1059R	chr13	Missense	SNV	9.03
KMT2D c.[13423A>C]	p.S4475R	chr12	Missense	SNV	8.07
SLC19A1 c.[334_336delCTG]	p.L112del	chr21	CDS-Indel	Del	7.5
FAT4 c.[12344T>G]	p.L4115R	chr4	Missense	SNV	7.31
SF3B1 c.[1838T>G]	p.M613R	chr2	Missense	SNV	6.99

tion from RNA polymerase II promoter (p=0.001), positive regulation of epithelial cell proliferation (p<0.001), and negative regulation of apoptotic process (p=0.002). The CCs results indicated that the 43 genes were mainly enriched in the nucleus (p=0.003), plasma membrane (p=0.036), nuclear chromatin (p<0.001), and nucleolus (p=0.008). The MFs were mainly enriched in ATP binding (p<0.001), chromatin binding (p<0.001), RNA polymerase II core promoter proximal region sequence-specific DNA binding (p=0.026), and so on.

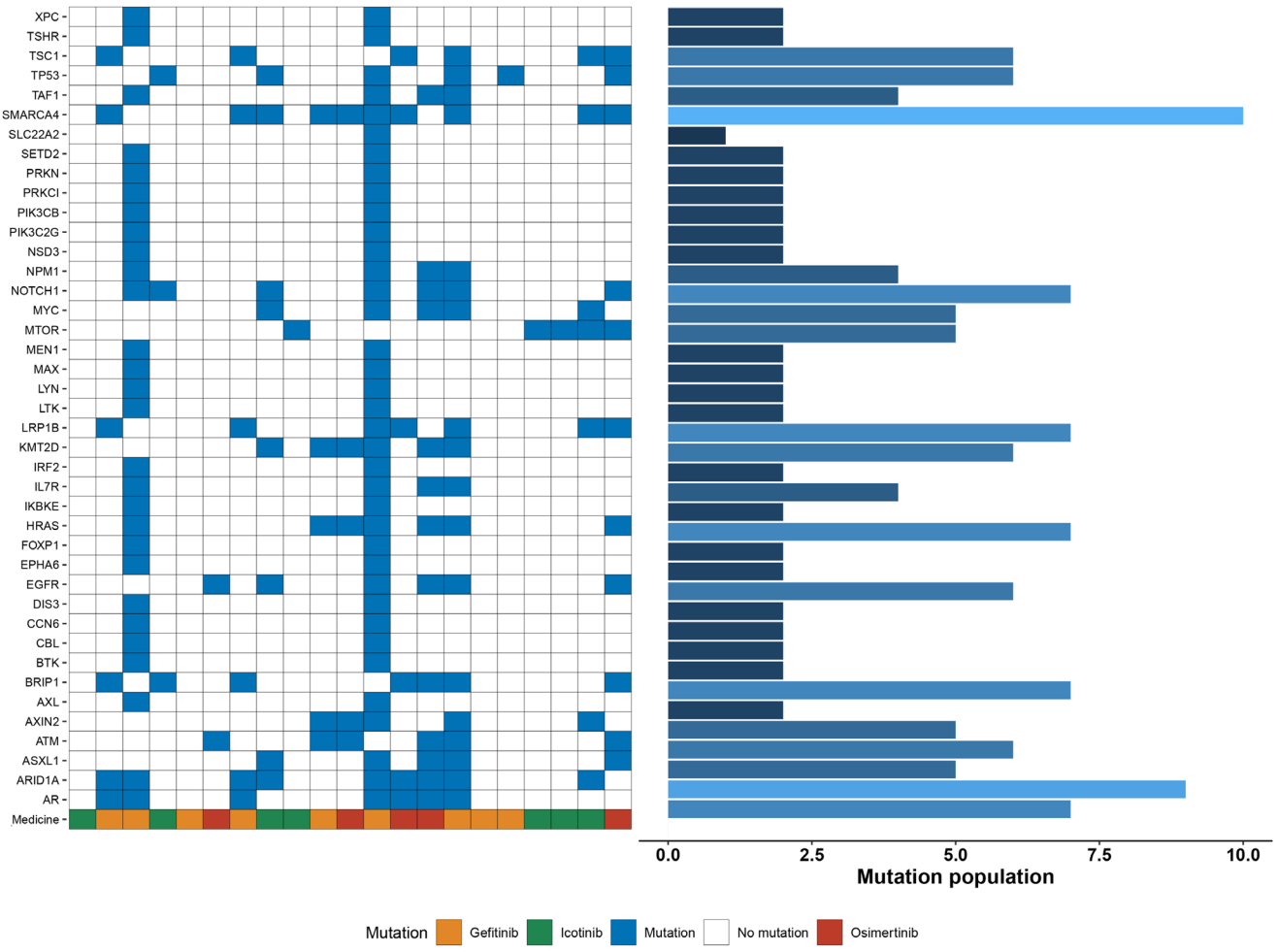

Figure 1. Mutation landscape of 32 genes with a significant difference in mutation state in three kinds of EGFR-TKI and 15 high-frequency mutation genes, 4 duplicates were deleted. The histogram on the right showed the proportion of mutations in 21 patients. The yellow, green, and red in the lower-left indicated treatment therapy of gifitinib, icotinib, and osimertinib, respectively.

KEGG pathway enrichment revealed that the mutated genes were significantly enriched in 39 pathways ($p<0.05$), including endometrial cancer ($p<0.001$), central carbon metabolism in cancer ($p<0.001$), pathways in cancer ($p<0.001$), PI3K-Akt signaling pathway ($p<0.001$), and ErbB signaling pathway ($p<0.001$), etc. Figure 2B exhibits all of the enriched pathways.

Clinical significance of the mutated genes. To explore the clinical significance of those mutated genes, we further analyzed the relationship between the mutation status of 43 genes and the prognostic indicators of tumor markers and progression-free survival (PFS). Pre-treatment CEA, NSE, Cyfra21-1, and ProGRP levels were significantly different between the mutation status of 49, 1, 3, and 1 mutated genes, respectively (Table 3). For all 43 mutated genes, the mutation status of *NOTCH1* and *MTOR* were significantly related to PFS (Figures 3A, 3B). Of all these genes, a total of 4 genes whose mutations were significantly correlated with more than two indicators. Among them, *MTOR* were related to the most indicators (NSE, Cyfra21-1, ProGRP before treatment, and PFS during treatment; $p<0.05$), followed by *NOTCH1* with CEA before treatment and PFS during treatment, *HRAS* with CEA and Cyfra21-1 before treatment, and *AR* with CEA before treatment and change of SCC during treatment ($p<0.05$). When these genes were mutated, the patients' tumor marker levels were significantly increased, while PFS was remarkably reduced. Therefore, they might be the keys to the prognosis of NSCLC patients.

Prognosis of NSCLC patients with EGFR p.T790M mutation positive and negative treated with osimertinib. Since osimertinib is the target drug for *EGFR* p.T790M mutation, we further compared the survival of patients with *EGFR* p.T790M positive and negative treated by osimertinib, as well as the concomitant and uncommon mutations of *EGFR* p.T790M. In this study, 5 patients were treated with osimertinib, and one appeared *EGFR* p.T790M mutation

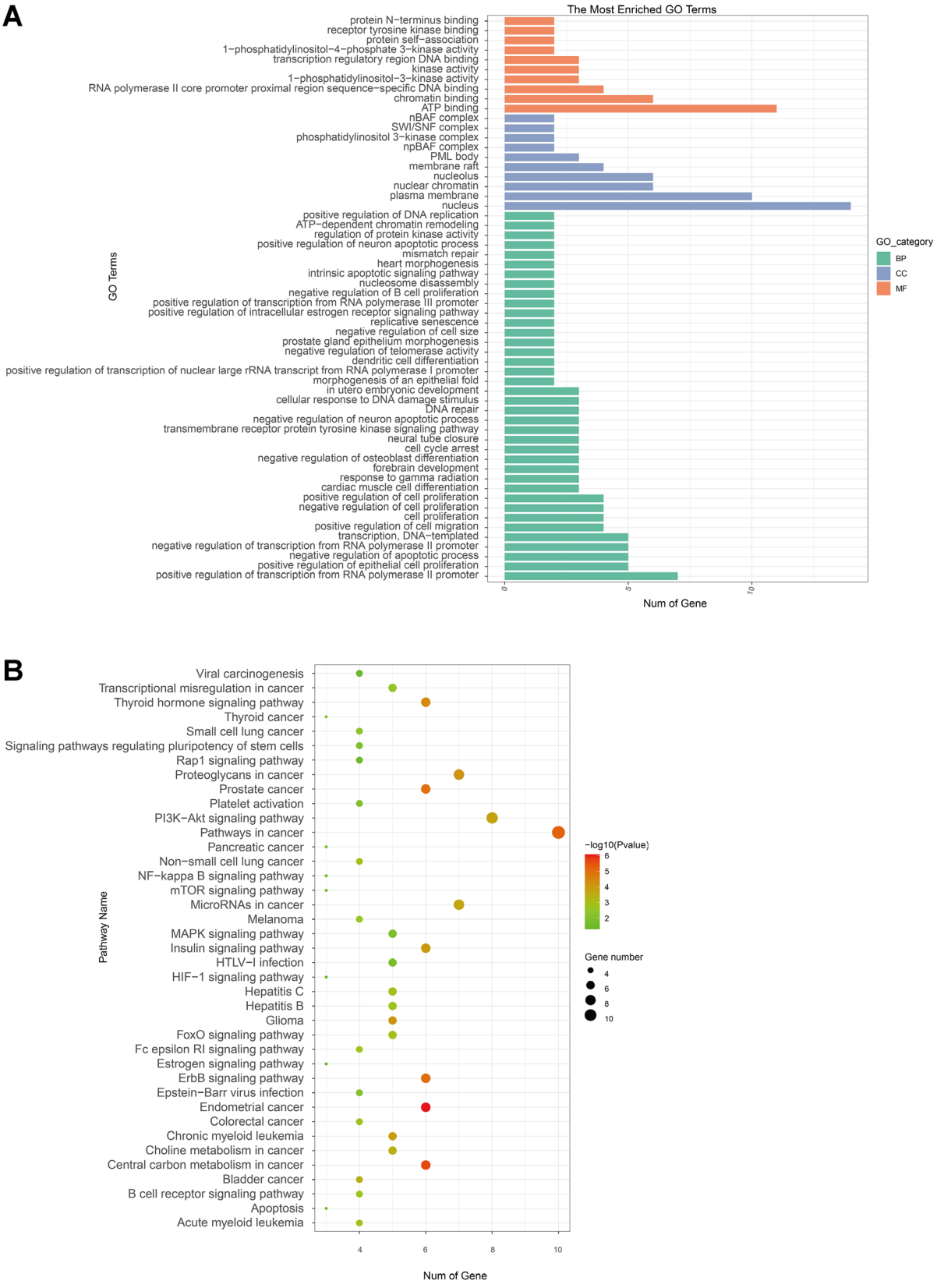

Figure 2. GO terms (A) and KEGG pathways (B) of 43 mutated genes with high proportion and mutation frequency in all patients.

Table 3. Top 20 mutations correlated with CEA and all mutations related to NSE, Cyfra211, ProGRP before treatment.

Tumor marker	Mutation point	Tumor marker levels in unmutated patients	Tumor marker levels in mutated patients	p-value
CEA (10 μg/l)	ASXL1 p.Q5_K6delinsQ	3.34 (0.94, 97.36)	115 (15.55, 719.7)	0.001
	AR p.C450_G451delinsC	3.34 (0.94, 719.7)	15.55 (9.1, 276.9)	0.005
	IL7R p.K265Kfs.30	3.36 (0.94, 719.7)	65.28 (15.55, 276.9)	0.006
	KMT2D p.Q3745_H3746delinsH	3.36 (0.94, 719.7)	65.28 (15.55, 276.9)	0.006
	NPM1 p.D175_D176delinsD	3.36 (0.94, 719.7)	65.28 (15.55, 276.9)	0.006
	SMARCA4 p.E1360del	3.46 (0.94, 719.7)	115 (9.1, 276.9)	0.026
	ARID1A p.G1848Gfs.35	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	AXIN2 p.E205K	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	AXL p.M25R	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	BRIP1 p.N1147Ilefs	5.84 (0.94, 719.7)	1.04 (1.01, 1.06)	0.031
	BTK p.K186Sfs.13	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	CBL p.H36del	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	CCN6 p.G363A	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	DIS3 p.I678Ffs.59	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	EPHA6 p.S914Y	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	FOXP1 p.Q58_Q59delinsQ	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	HRAS p.M182R	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	IKBKE p.P713L	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	LRP1B p.A2608T	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
	LTK p.G330del	3.55 (0.94, 719.7)	195.95 (115, 276.9)	0.031
NSE (10 μg/l)	MTOR p.F1751L	15.62 (8.96, 115.6)	51.79 (22.47, 53.02)	0.021
Cyfra211 (10 μg/l)	HRAS p.M182R	4.27 (1.81, 51)	12.58 (10.72, 16.27)	0.049
	MTOR p.R1896Q	4.27 (1.81, 20.99)	31.85 (12.69, 51)	0.046
	MTOR p.T314I	4.51 (1.94, 51)	2.08 (1.81, 3.06)	0.022
ProGRP (ng/l)	MTOR p.F1751L	41.10 (2.3, 3501)	684 (279.8, 1393)	0.018

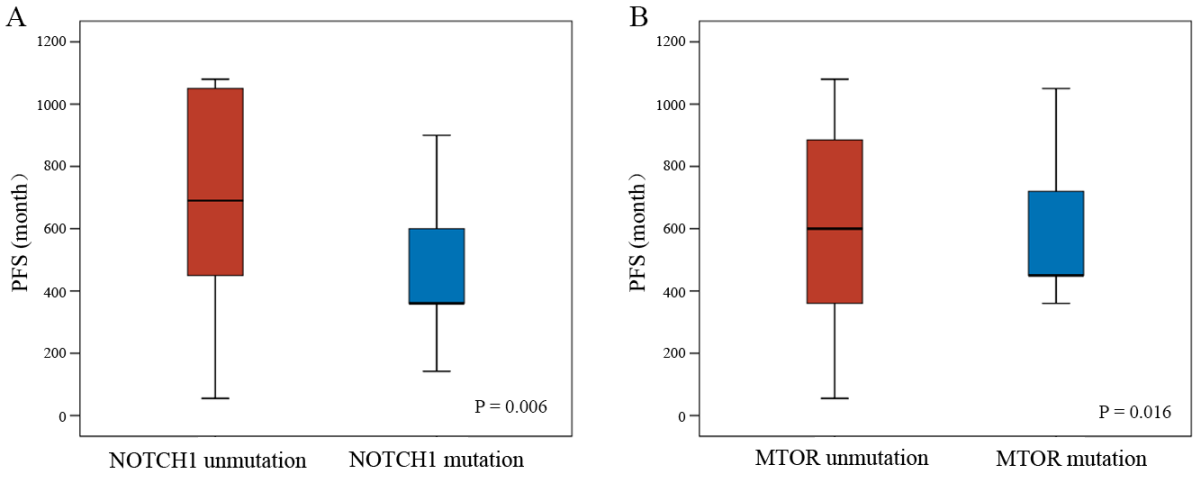

Figure 3. Boxplots of mutations with significant PFS during treatment under different mutation states. PFS time of patients with *NOTCH1* (A), *MTOR* (B) gene mutation were obviously lower than that of patients without mutations,

with the curative effect of partial response (PR). The curative effect of the other four *EGFR* p.T790M negative patients were one PR and three stable disease (SD). The PFS (360 vs. 750 ± 304.96 months, p=0.336), therapeutic effect (p=0.819) and prognosis (p=1.000) between *EGFR* p.T790M mutation positive and negative patients after taking osimertinib were no statistical differences, suggesting that osimertinib was also effective in the patients without *EGFR* p.T790M mutation.

Besides, in *EGFR* p.T790M positive patient, we found 80 *EGFR* p.T790M concomitant mutations, including *SMARCA4* p.K1390Q (2.23%), *DNMT3A* p.F755S (2.17%), *MTOR* p.Y1450* (2.15%), *TPMT* p.L182R (1.84%), *UGT1A1* p.L248W (1.74%), *C11orf30* p.H431P (1.46%), etc. (Table 4). For the rest of four *EGFR* p.T790M negative patients, one of them was PR and three were SD, accompanied with mutations such as *EGFR* p.L858R (55%), *ARID1A* p.P16del (2.83%), *TP53* p.R248W (21.73%), and *AR* p.Q60L (4.24%). In addition, among all the mutations, 54 uncommon mutations were detected, in which *NOTCH1* p.Y813S (1.35%), *NOTCH1* p.N109H (0.91%), and *MTOR* p. T314I (0.89%) were markedly associated with PFS.

Table 4. Top 20 EGFR p.T790M concomitant mutations in osimertinib-treated patients.

Mutation point	Frequency (%)	Mutation point	Frequency (%)
SMARCA4 p.K1390Q	2.23	MTOR p.M304L	0.96
DNMT3A p.F755S	2.17	SF3B1 p.M867Cysfs*	0.96
MTOR p.Y1450*	2.15	NTRK3 p.E179G	0.86
TPMT p.L182R	1.84	FAT1 p.D1757E	0.78
UGT1A1 p.L248W	1.74	ATM p.P1843L	0.76
C11orf30 p.H431P	1.46	BRCA1 p.K223Argfs*	0.71
TSC1 p.M880I	1.28	CDK4 p.A182S	0.66
GABRQ p.Q423*	1.27	ERBB4 p.P1080S	0.66
ZFHX3 p.Q1740E	1.13	CYP3A4 p.A150S	0.65
HLA-E p.Y134S	1.03	NTRK2 p.E70K	0.65

Discussion

Currently, the main principle of targeted therapy for NSCLC is to find and target mutations of a driver gene, such as EGFR-TKI, which has significant benefits in patients with *EGFR* mutations. However, there often exist mutations of other genes in tumors, which may significantly change the biological characteristics of tumors, affecting the therapeutic efficacy and prognosis of NSCLC patients. Compared with single gene detection, multi-gene panel detection reduces the cost and can have a faster turnaround time. Therefore, this study screened the mutations of NSCLC patients treated with EGFR-TKI by the 1000-gene panel. Ultimately, a total of 43 genes significantly correlated with therapeutic drug and/or with a higher mutation frequency were selected (Figure 1), and they were mainly enriched in the pathways of endometrial cancer, ErbB signaling pathway, PI3K-Akt signaling pathway, and pathways in cancer (Figures 2A, 2B).

Furthermore, in order to clarify the clinical significance of these genes, we analyzed the correlation of mutated genes with the PFS and tumor marker levels (CEA, NES, Cyfra21-1, ProGRP, and SCC). *AR*, *HRAS*, *NOTCH1*, and *MTOR* were the genes significantly related to at least two indicators, in which *NOTCH1* and *MTOR* were remarkably correlated with prognosis. Android receptor (*AR*) gene deletion or defect can lead to the loss of all or part of the normal biological effects of androgen and then result in the androgen insensitivity syndrome (AIS). At present, there are thousands of *AR* gene mutations reported in AIS (http://Android mcgill.ca/), including all or partial deletion, splicing region variation, frameshift variation caused by insertion or deletion, amino acid replacement or termination caused by missense or nonsense variation, etc. Xia et al. [8] found that the hemizygous variation of exon 2-8 deletion of the *AR* gene is the cause of complete AIS. Similarly, Wu et al. [9] detected a new mutation c.3864T>C in *AR* gene in prenatal diagnosis of twins, both of which were hemizygous of the mutation and had typical female external genitalia and bilateral testicles in the abdomen. However, no mutation of *AR* in cancer has been reported. *HRAS* belongs to the RAS gene family. By combining with GTP/GDP, RAS protein acts as a molecular switch to regulate RAF-MET-ERK, PI3K/AKT, and other signal pathways related to cell survival and proliferation [10, 11]. RAS is the most common mutant gene in human cancers. Scientific evidence shows that mutations of *KRAS*, *HRAS,* and *NRAS*, which are activated members of the RAS family, exist in 20–30% of human tumors [12]. The mutation of *HRAS* is closely related to the occurrence of many kinds of tumors. *HRAS* with p.V112A and p.Q61R mutations can induce colon cancer in mice [13]. Pandith et al. [14] reported that the SNPs of *HRAS* p.T81C increased the risk of bladder cancer (BC), and the rare allele (TC+CC) was a predictive marker of advanced BC. Sugita et al. [15] revealed that the expression of *HRAS* in BC was significantly up-regulated regardless of mutation. However, compared with *KRAS* and *NRAS*, *HRAS* mutations are usually found in BC and low incidence rate cancers such as Hurthle cell tumors or seminomas [16]. *NOTCH1* is a known prognostic biomarker of chronic lymphocytic leukemia (CLL). A previous study indicated that the mutation of *NOTCH1* showed poor survival, resistance to treatment, and disease progression [17]. The presence of *NOTCH1* gene mutation was associated with a 4.39-fold risk of CLL at stage III and has been reported in patients with CLL at moderate risk [18, 19]. Research also found *NOTCH1* mutation in 10–15% of the head and neck squamous cell carcinoma patients and was significantly related to HPV status (p=0.006) [20]. *MTOR* gene encodes mTOR protein, which belongs to the phosphatidylinositol kinase-related protein kinase family. As an important regulatory gene, *MTOR* plays an important physiological function by regulating the cell cycle, protein synthesis, cell energy metabolism, and so on. It also plays a central role in cell

proliferation, growth, and differentiation [21]. A total of 559 *MTOR* mutations were recorded in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (https://cancer.sanger.ac.uk/). Although there are relatively few studies on these gene mutations in NSCLC, our study proved that they had certain clinical significance and deserve further study.

In addition, we compared the efficacy of osimertinib in *EGFR* p.T790M positive and negative NSCLC patients and analyzed the *EGFR* p.T790M concomitant mutations and uncommon mutations. As a third-generation EGFR inhibitor, osimertinib has IC50 of 11.4 nM and 12.9 nM for T790M/L858R mutation and exon 19 deletion of *EGFR*, respectively. Osimertinib is an irreversible inhibitor of *EGFR*, which can form a covalent bond with molecular targets, leading to a longer response duration and reduced drug resistance. Studies have found that *EGFR* p.T790M negative patients can benefit from osimertinib. For example, Jänne et al. [22] observed 88% of *EGFR* p.T790M positive patients had a median PFS of 9.6 months, meanwhile with no detectable EGFR T790M, 69% of the patients had a median PFS of 2.8 months. In addition, recent research suggested 10.8 and 5.1 months (p=0.007) of PFS in patients with p.T790M mutation positive and negative, as well as 22.5 and 13.4 months (p=0.002) of OS, respectively [23]. However, our results showed no significant difference in OS and PFS between *EGFR* p.T790M positive and negative NSCLC patients (360 vs. 750 ± 304.96 months, p=0.336), the reason may be as follows: 1) our study involved fewer patients treated with osimertinib, and there was only one EGFR T790M positive case, which might cause meaningless statistical significance; 2) *EGFR* p.T790M concomitant mutations and uncommon mutations may have impacts on the survival of patients. In this study, we detected 80 concomitant mutations in *EGFR* T790M positive patients, as well as 54 uncommon mutations, including *BRAF*, *MET*, *CDK4*, *CDK6*, etc. Blakely et al. [24] found that over 92.9% of lung cancer patients with *EGFR* mutation have more than one co-occurring mutation in other genes, and 10.2% of them were considered as concomitant mutations. The functional driver genes of *EGFR* co-mutations included *PIK3A*, *BRAF*, *MET*, *MYC*, *CDK6,* and *CTNNB1*. The occurrence of these co-mutations would affect the Wnt/β-catenin pathway and cell cycle-related genes *CDK4* and *CDK6* mutations, further synergistically promoting tumor metastasis or EGFR inhibitor resistance. Hong et al. [25] revealed that in NSCLC, *EGFR* concomitant mutations were significantly associated with reduced objective response (44% vs. 77%; p=0.01), shorter PFS (6.20 months vs. 18.77 months; HR, 3.51; p<0.001), and shorter OS (22.70 months vs. not achieved; HR, 4.65; p<0.001), and concomitant mutations were proved as an independent factor of poor prognosis in multivariable analysis. Uncommon mutations can co-occur with *EGFR* mutations. It has been reported that patients with *HER2* mutation and *MET* exon 14 skipping co-alteration with *EGFR* mutations responded well to EGFR-TKIs [26]. However, mutations in *TP53*, *PIK3CA,* and other genes may also occur simultaneously with *EGFR* changes, leading to a reduction of the therapeutic effect of EGFR-TKIs [27–29]. Collectively, these data highlighted the importance and necessity of studying *EGFR* T790M concomitant and uncommon mutations, but the sample size of this study is small, which is a disadvantage of this paper. Therefore, follow-up research can be carried out from this perspective in depth.

In conclusion, this study identified mutations in NSCLC patients treated with EGFR-TKIs. *AR*, *HRAS*, *EGFR*, *AXIN2*, *NOTCH1*, and *MTOR* were significantly related to the prognosis and tumor marker levels of patients, which may be potential prognostic markers of EGFR-TKIs in NSCLC. In addition, osimertinib has certain efficacy in *EGFR* p.T790M negative patients. However, a large sample size and further studies are needed to assess the outcome of treatment in patients with *EGFR* p.T790M concomitant and uncommon mutations.

Supplementary information is available in the online version of the paper.

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Novel mutation signatures in the prognosis of EGFR-TKIs targeted therapy for non-small cell lung cancer patients based on the 1000-gene panel sequencing

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Supplementary Information

Supplementary Table 1. Tumor tissue or whole blood samples targeted with 1000-gene plates to NGS, adjacent tissues, or leukocytes as controls.

ABL1	ABCB1	ABL2	ABCF2	ACE	ACER2	ACOT11
ACSL1	ACSM5	ACSS3	ACTL6B	ACVR1B	ADAM23	ADAM33
ADAMTS16	ADAMTS19	ADAMTS20	ADAMTS5	ADAMTSL1	ADD2	AGMAT
AGTPBP1	AHCTF1	AK5	AKR1B10	AKR1C1	AKT1	AKT2
AKT3	ALDH1A3	ALDH2	ALG5	ALK	ALX4	AMOT
ANK2	ANKRD13D	ANKRD20A4	ANKRD27	ANKRD28	ANKRD30A	ANKRD30B
ANKRD36B	ANO2	AP1B1	AP1G2	AP3B1	APAF1	APC
APLP2	APMAP	APPL2	AQP12A	AR	ARAF	ARFGAP1
ARFRP1	ARHGAP35	ARHGAP40	ARHGEF1	ARID1A	ARID1B	ARID2
ASTN1	ASXL1	ASXL2	ATAD2B	ATIC	ATM	ATP10B
ATP12A	ATP2C1	ATP6V0A2	ATP8B2	ATR	ATRX	ATXN2
AURKA	AURKB	AXIN1	AXIN2	AXL	B2M	BAP1
BARD1	BCL2	BCL2L1	BCL2L11	BCOR	BLM	BMPR1A
BRAF	BRCA1	BRCA2	BRD4	BRIP1	BSG	BTK
BTRC	C11orf30	C12orf5	C19orf38	C1orf112	C1orf35	C1QA
C1S	C20orf112	C2orf47	C2orf62	C7orf53	C8orf34	C9orf43
CACNA1A	CACNA1D	CACNA1E	CADM2	CAMKK1	CAPRIN1	CARS
CARS2	CASC4	CASP8	CASP8AP2	CASQ2	CATSPER2	CBFB
CBFB	CBL	CBR3	CCDC155	CCDC159	CCND1	CCND2
CCND3	CCNE1	CCT3	CCT6B	CD1E	CD274	CD300LF
CD5L	CD74	CD9	CD97	CDA	CDC73	CDH1
CDH26	CDK11A	CDK12	CDK13	CDK14	CDK18	CDK19
CDK4	CDK6	CDK8	CDKN1A	CDKN1B	CDKN2A	CDKN2B
CDKN2C	CDS1	CEACAM20	CEBPA	CECR2	CELA2B	CGN
CHD6	CHEK1	CHEK2	CIC	CISD3	CLCN7	CLEC16A
CNGB3	CNKSR2	CNOT3	CNOT4	CNTN1	CNTN4	CNTN5
CNTNAP3B	CNTNAP5	COASY	COL14A1	COL16A1	COL19A1	COL1A1
COL25A1	COL4A5	COL4A6	COL5A1	COL5A2	COL5A3	COL6A5
COL6A6	COL9A1	COPA	COPG1	CPA1	CPSF3	CPSF6
CREBBP	CRKL	CRTAM	CRTAP	CRYBG3	CSF1R	CSMD1
CSN3	CSNK1E	CSPP1	CTCF	CTIF	CTNNA1	CTNNB1
CTSF	CUL3	CYLD	CYP19A1	CYP2A13	CYP2D6	DAXX
DDB1	DDR1	DDR2	DDX3X	DEPDC4	DGKK	DHCR24
DHDDS	DHX9	DICER1	DKC1	DLST	DMD	DMXL1
DMXL2	DNAH10	DNAH5	DNAH9	DNAJC11	DNAJC9	DNMT3A
DOCK11	DOCK3	DOT1L	DPP10	DPYD	DRGX	DUOX1
DYSF	DZANK1	ECHDC1	EDN1	EEF1A1	EFCAB5	EFCAB6
EFCAB7	EFHA2	EFNA5	EGFR	EIF2B5	EIF2C2	EIF3E
EIF3I	EIF4ENIF1	ELAC2	EME2	EMID2	EML4	ENPP2
EP300	EPAS1	EPCAM	EPHA2	EPHA3	EPHA5	EPHB2
EPHB6	ERBB2	ERBB3	ERBB4	ERCC1	ERCC3	ERG
ERRFI1	ESR1	ETV6	EXT1	EXT2	EZH2	EZR
F8	F9	FAH	FAM123B	FAM131B	FAM135B	FAM13C
FAM157B	FAM175A	FAM21A	FAM3A	FAM49A	FAM49B	FAM5C

Supplementary Table 1. ***Continued ...***

FANCA	FANCC	FANCD2	FANCG	FANCM	FAS	FAT1
FAT2	FBXW7	FCGR2A	FCGR3A	FGFR1	FGFR2	FGFR3
FGFR4	FH	FLCN	FLNC	FLT1	FLT3	FLT4
FMN2	FMNL3	FNDC4	FNIP2	FOLH1	FOXA1	FOXJ2
FOXL2	FOXP1	FRMD4A	FRMPD2	FRMPD4	FSD2	FSHR
FUBP1	FUNDC1	GAB2	GAB3	GABRD	GAD2	GALNT12
GALNT14	GATA3	GFRAL	GIGYF1	GINS4	GIPR	GKN2
GLB1L3	GLYR1	GMDS	GNA11	GNAQ	GNAS	GNPTAB
GOLGA4	GPAT2	GPATCH2	GPR114	GPR125	GPR133	GPR144
GPS2	GRIN2A	GRIK2	GSTP1	GUCY2C	GYLTL1B	HAAO
HAP1	HAUS5	HAUS6	HCN1	HDAC1	HDAC4	HDAC6
HEATR7B2	HECTD4	HECW1	HECW2	HGF	HIST1H3B	HLA-DRB1
HLA-DRB5	HMCN1	HNF1A	HNF4A	HOXB13	HPS3	HPS4
HRAS	HSP90AA1	HSPD1	HYDIN	IBSP	IDH1	IDH2
IFNG	IFNGR1	IFT172	IGF1R	IGSF9	IKBKAP	IKBKE
IL1RAPL1	IL27RA	IL7R	IMPG1	INHBA	INPP4B	INPP5J
IRF2	IRS2	ITFG2	ITGA8	ITGA9	ITIH1	ITLN2
ITM2A	ITPKB	ITPR1	JAK1	JAK2	JAK3	KCNAB2
KCNH6	KCNQ2	KDM5A	KDM5C	KDM6A	KDR	KEAP1
KIAA0226	KIAA0319	KIAA0922	KIAA1191	KIAA1199	KIAA1211L	KIF13A
KIF1B	KIF26B	KIF5B	KIFC1	KIR2DL3	KIR3DL3	KIT
KLHL1	KLHL14	KLK1	KMT2B	KMT2C	KRAS	KRT2
KRT9	KRTAP5-5	KTN1	L3MBTL1	LARP1	LCN10	LCT
LCTL	LETM1	LGALS13	LILRB3	LILRB4	LIPN	LMAN1L
LMBR1L	LPCAT4	LPHN3	LRBA	LRP1B	LRP2	LRP4
LRRC16B	LRRC2	LRRC7	LRRC72	LRRD1	LRRFIP2	LRSAM1
LTBP1	LUC7L2	LUZP4	MAEL	MAGI1	MAML2	MAP2K1
MAP2K2	MAP2K4	MAP3K1	MAP4K1	MAPK1	MAPK3	MAPKAPK3
MAPRE3	MAX	MBIP	MBTPS2	MCF2L2	MCL1	MCOLN2
MDGA2	MDM2	MDM4	MDN1	MED12	MED23	MEFV
MEN1	MET	METTL5	MGAM	MICALL1	MID1	MIER2
MITF	MLH1	MLH3	MLL	MLL2	MLL3	MORN1
MPL	MRE11A	MRPL1	MRPS18B	MS4A1	MSH2	MSH3
MSH6	MSI1	MTA2	MTHFR	MTOR	MTR	MTRR
MUC5B	MUTYH	MYC	MYCBP2	MYCL1	MYCN	MYD88
MYH15	MYH2	MYH4	MYH8	MYH9	MYL5	MYL6
MYLK2	MYO3A	MYOD1	NACAD	NARF	NAT10	NAV3
NBN	NBPF10	NCF2	NCKAP1	NCOA4	NCOR1	NDUFA13
NELL1	NF1	NF2	NFE2L2	NIPBL	NLGN3	NLRC3
NLRP4	NMI	NOP2	NOS1	NOS2	NOTCH1	NOTCH2
NOTCH3	NOTCH4	NPM1	NRAS	NRXN2	NSD1	NTHL1
NTRK1	NTRK2	NTRK3	NUP205	NUP210	NUTM1	NWD1
NXF1	NXF5	OBP2A	OBP2B	OCA2	ODZ3	OR2T4
OR4A15	OR4C6	OR5L2	OR6F1	OSBPL10	OTOA	OTOGL
OVCH1	P4HB	PABPC4	PACS2	PAEP	PAGE1	PALB2
PARK2	PARP4	PAX5	PBRM1	PCK1	PCNXL2	PCSK5
PCYT1A	PDCD1LG2	PDE1C	PDE2A	PDE4DIP	PDGFRA	PDGFRB
PDIA5	PDILT	PDK1	PDRG1	PEX6	PGAP1	PHACTR3
PHF20L1	PHF6	PIK3CA	PIK3CB	PIK3CG	PIK3R1	PIK3R2
PIP4K2C	PIP5K1C	PIWIL1	PKD1L2	PKHD1	PKLR	PLAC8
PLCB4	PLCZ1	PLEC	PLK2	PLOD3	PLXNA1	PMS1
PMS2	POLD1	POLE	POLR2J	POLR3B	POLR3GL	POLRMT
POT1	POTEG	PPA1	PPARG	PPEF1	PPFIBP2	PPIL2
PPM1D	PPP4R4	PQBP1	PREB	PREX2	PRKAA1	PRKACA

Supplementary Table 1. ***Continued*** **...**

PRKAR1A	PRKCD	PRKDC	PRKX	PRRX1	PRSS1	PRUNE
PSG2	PSG5	PSIP1	PSMB1	PSMB5	PSMC4	PSMC6
PSTPIP1	PTBP3	PTCD3	PTCH1	PTCH2	PTEN	PTGS2
PTGES3L-AARSD1	PTPLAD1	PTPN11	PTPN13	PTPRA	PTPRD	PTPRM
PYHIN1	RAD50	RAD51	RAD51B	RAD51C	RAD51D	RAF1
RALBP1	RAPGEF2	RARA	RARB	RASEF	RB1	RBM10
RBMX	RCC1	REC8	REG1B	RELN	RERE	RET
RFWD2	RHEB	RHOA	RICTOR	RINT1	RNASEL	RNF43
RNF43	ROCK1	ROS1	RPL22	RPL36A	RPS5	RPS6KA1
RPS6KB1	RPTOR	RPUSD4	RREB1	RRM1	RSPO2	RUNX1
RYR2	RYR3	SAFB2	SAG	SAGE1	SAMD8	SCN10A
SCN3A	SCN7A	SDHA	SDHAF2	SDHB	SDHC	SDHD
SELP	SEMA6A	SEPT12	SERPINB3	SERPINB4	SETD2	SF1
SF3B1	SF3B14	SF3B3	SGCZ	SGIP1	SGK1	SGPL1
SH2D3A	SH3BGR	SH3PXD2A	SHISA4	SI	SIDT2	SIK3
SIM1	SIM2	SLC13A3	SLC17A6	SLC17A8	SLC25A1	SLC25A30
SLC26A3	SLC2A2	SLC34A2	SLC35B2	SLC35B4	SLC38A4	SLC38A5
SLC43A1	SLC45A1	SLC4A10	SLC4A4	SLC5A1	SLC6A5	SLC8A1
SLCO1B7	SLIT1	SLX4	SMAD2	SMAD4	SMARCA4	SMARCB1
SMARCE1	SMO	SOD2	SOX2	SOX9	SPAG16	SPATA13
SPRED1	SPTA1	SRC	SRRT	SSBP3	SSH2	SSPO
ST18	ST6GALNAC1	STAG2	STAT1	STAT3	STAT4	STAT6
STK11	STK11IP	STK31	STX3	SUFU	SUPT5H	SUPT6H
SYCP2L	SYK	SYNE1	SYNE2	SYNJ2	TAF1B	TAF6
TARBP1	TBC1D1	TBC1D21	TBC1D3	TBC1D5	TBL1X	TBP
TBX15	TBX22	TBX3	TCF20	TCF7L2	TCP10	TCP11
TEK	TERT	TET2	TEX35	TFE3	TGFBR2	TGM2
TGM5	THBS2	THEM5	THOC1	THSD7A	THSD7B	TIMD4
TIMM44	TIMP3	TJP3	TLE1	TLL1	TMC2	TMED8
TMEM104	TMEM127	TMEM132D	TMEM145	TMEM247	TMEM80	TMEM87A
TMPRSS2	TMTC4	TMX3	TNFAIP3	TNFSF4	TNN	TNNT1
TNR	TNS3	TOP1	TOP2A	TP53	TP73	TPH2
TPM3	TPTE	TRIM33	TRIM51	TRIM58	TRIML1	TRIO
TRIP11	TRMT112	TRPC5	TRUB1	TSC1	TSC2	TSGA10
TSKS	TSPAN12	TSR2	TTF2	TTLL3	TTN	TUBA3C
TUBGCP4	TUBGCP5	TYK2	TYRP1	U2AF1	U2AF2	UBASH3A
UBE2Q1	UBE4B	UCHL3	UCK2	UGT1A1	ULK3	UMPS
UNC13A	UNC13D	UNC5D	USP12	USP34	USP39	USP45
USP48	VAV1	VEGFA	VEZF1	VHL	VILL	VIT
VPS13A	VPS33B	VSIG4	WAS	WASL	WDR44	WDR52
WDR62	WDR66	WDR72	WDTC1	WLS	WT1	WWP2
XBP1	XPC	XPO1	XRCC1	XRCC2	XRCC3	ZBTB8OS
ZC3H13	ZC3H7B	ZDHHC11	ZFHX3	ZFR	ZMAT3	ZNF143
ZNF350	ZNF385A	ZNF414	ZNF512B	ZNF541	ZNF563	ZNF614
ZNF687	ZNF705B	ZNF705G	ZNF711	ZNF804B	ZSWIM8	