Genomic Heterogeneity of Multiple synchronous lung cancers (MSLCs) in Chinese population

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Abstract

Introduction

It is clinically challenging to infer the phylogenetic relationship between different tumor lesions of patient with multiple synchronous lung cancers (MSLC), whether these lesions are the result of independently evolved tumor or intrapulmonary metastases.

Methods

Using Illumina X10 platform, we sequenced a total number of 128 stage I lung cancer samples collected from 64 patients with MSLC. All samples were analyzed for mutation spectra and phylogenetic inference.

Results

We detected genetic aberrations within genes previously reported to be recurrently altered in lung adenocarcinoma including EGFR, ERBB2, TP53, BRAF and KRAS. Other identified putative driver mutations are enriched in RTK-RAS signaling, TP53 signaling and cell cycle. Also we found some interesting cases, two cases which carried EGFR L858R and T790M co-mutation in one tumor and another tumor with only EGFR 19del, and 1 case with two KRAS hotspots in the same tumor. Due to the short follow-up time and early stage, whether the special mutation profile will affect the PFS and OS of these patients need further investigation. For the genetic evolution, among 64 tumor samples, 50 of them display distinct mutational profile, suggesting these are independently evolved tumors, which is consistent with histopathological assessment. On the other hand, 7 patients were identified to be intrapulmonary metastasis as the mutations harbored in different lesions are clonally related.

Conclusion

In summary, unlike intrapulmonary metastases, patients with MSLC harbor distinct genomic profile in different tumor lesions and we may distinguish MSLC from intrapulmonary metastases via clonality estimation.

Introduction

Lung cancer is the leading cause of cancer death globally. In recent years, with the advancement of early detection and diagnostic techniques, more nodules are being found. Multiple primary lung cancers refers to the occurrence of multiple primary tumors at different area of the lung on the same patient and it can be synchronous or metachronous(Martini and Melamed 1975). Multiple synchronous lung cancers (MSLC) have been reported to account for 0.2-8% of lung cancers(Gazdar and Minna 2009; Wang et al. 2009; Ferguson et al. 1985; Mathisen et al. 1984). It is different from pulmonary metastases. Based on whether the tumors are MSLC or metastatic lung cancers and the TNM staging, different treatment plans will be used(Pairolero et al. 1984).

Currently, there is no gold standard for the diagnosis and treatment for MSLC. Most clinicians refer to the criteria published by Martini and Melamed in 1975(Martini and Melamed 1975). In 2003, the American College of Chest Physicians (ACCP) suggested that when the tumor foci are of the same histological subtype, the use of molecular markers can be helpful to distinguish their genomic features(American College of Chest, Health, and Science Policy 2003). These molecular markers changed with the development of study, from the microsatellite(Shen et al. 2014) and loss of heterozygosity(Wang et al. 2009), to mutation status of some genes such as TP53(van Rens et al. 2002), and now to the mutation status of large scale genes(YJ et al. 2019; Liu et al. 2016; Ma et al. 2017).

Coincidentally, lots of studies had reported differences in certain cancer gene mutations and chromosome aberrations between MSLCs(Murphy et al. 2014; Shimizu et al. 2000; Girard, Ostrovnaya, et al. 2009; Girard, Deshpande, et al. 2009). Ramsey Asmar et al found that oncogenic driver mutations (EGFR, KRAS, etc.) are concordant between primary tumors and metastasis(Asmar et al. 2017). Otherwise, Yu Liu et al. highlighted the genomic heterogeneity of MSLC(Murphy et al. 2014). In 2017, Snehal B. Patel et al. (SB et al. 2017) confirmed this view by 50 gene panel. They found that primary-metastatic pairs showed high mutational concordance and driver mutations were always concordant whereas MSLC showed distinct mutational characteristics. There are some similar studies illustrated this point(Liu et al. 2016; Ma et al. 2017; Murphy et al. 2019; Zehir et al. 2017).

However, the consistency of Histologic Prediction via CT imaging between genomic features is controversial because the accuracy of histologic comparison has not been benchmarked against molecular approaches. One study by Jason C. Chang et al. showed that 22% (17 of 76) patients showed discordant results between prospective histologic prediction and final molecular classification. Furthermore, the discordance rate was significantly higher for intrapulmonary metastasis (IPMs) (11/25, 44%) than MSLCs (6/51, 12%)(Chang et al. 2019). Till now, in clinical practice, gene markers have not been widely used in diagnosing MSLC. This is because there has not been reliable markers and algorisms to determine whether two or more tumor foci are of different origins. Here, we studied 64 patients including 128 samples by using NGS targeted gene sequencing to explore the mutation profile of the multiple synchronous lung cancer (MSLC) which were diagnosed by clinical criteria. By using the mathematic algorithm, we defined a clonality index (CI) to separate the MSLC from intrapulmonary metastasis.

Materials And Methods

Patients

This clinical trial was started in 2018. Between October 2018 and October 2019 we sequenced a total number of 128 stage I lung cancer samples collected from 64 patients with MSLC using Illumina X10 platform.Specimens which can be resected and matched peripheral blood samples were collected for High Throughput Sequencing (HTS). All patients were diagnosed with multiple synchronous lung adenocarcinomas by pathologists at the first visit. All patients were free of extrathoracic metastasis, and

none them received neoadjuvant therapies before surgery. Clinical data, including age, sex, smoking history, tumor pathological information and tumor biomarker information was collected.

This study was approved by the Local Ethics Committee at the First Affiliated Hospital of Dalian YJ-KY-FB-2019-18 . Written informed consent was obtained from the patient.

Sample collection and genomic profiling

Tumor tissues were collected, snap frozen and stored in liquid nitrogen. Tumor tissue samples were less than 1.5cm*1.5cm in size and the thickness is between 2-5mm. Genomic DNA was extracted from fresh-frozen samples using QIAamp DNA Mini kit (Qiagen, 51306, Valencia, CA, USA) according to the manufacturer' s protocol, and fragmented for constructing a library using KAPA Hyper Prep kits (KAPA, KK8504) and captured using the Agilent SureSelect XT Human All Exon v5 kit (Agilent Technologies, Santa Clara, CA, USA). Using Genetron's 509 cancer panel, exons of major cancer genes of all tumor tissue and corresponding WBC were sequenced on Illumina NovaSeq6000 which was provided by Genetron Health.

Statistical analysis

Both tumor DNA samples and their matched normal DNA samples were analyzed according to standardized computational workflow: Raw sequence data (FastQ format) were trimmed and filtered using Trimmomatic 0.33 using the following parameters:1.ILLUMINACLIP:TruSeq3-PE-adapter.fa:2:30:10:8:true; 2. TRAILING:3; 3. SLIDINGWINDOW:4:15; 4. MINLEN:36 (Bolger, Lohse, and Usadel 2014). Paired-end clean reads were mapped to the human reference genome (hg19) using the Burrows–Wheeler Aligner (BWA, version 0.7.10-r789) by defaults parameters (Heng et al. 2010). Duplicate removal, local realignment, and base quality recalibration were performed using PICARD (http://broadinstitute.github.io/picard/, version 1.103) and the Genome Analysis Toolkit (GATK, version 3.1-0-g72492bb) (DePristo et al. 2011). Somatic single nucleotide variations (SNVs) were called using Mutect (version 3.1-0-g72492bb) (Cibulskis et al. 2013), using the Catalogue of Somatic Mutations in Cancer (COSMIC) v54 and dbSNP138 as reference sets of known somatic and germline mutations, respectively. Small indels were called using strelka (version 1.0.14) by defaults parameters with BAM as input(Saunders et al. 2012). Effects of variants were annotated using ANNOVAR. DELLY2 was used to detect somatic structure variants (SVs), and somatic copy-number variants (CNVs) including loss of heterozygosity (LOH) were analyzed using Control-FREEC. Clonality index (CI) was calculate according to the previous methods(Schultheis et al. 2016).

Results

Patient

A total of 64 patients at the First Affiliated Hospital of Dalian Medical University between July 2018 and April 2020 were diagnosed with multiple primary lung cancer by clinical-pathologic criteria. Summary of

clinical information of the 64 patients in this study is listed in Table 1. All of these patients had two histologically separated tumor lesions. The median age was 56 years (range from 29-75 years), 53 of them were non-smokers. Among all these patients, 27 (42%) patients had both tumors in the same lobe, 37 (58%) in different lobe. For the 128 tumors, all of them were diagnosed as adenocarcinoma. None of the enrolled patients received neoajuvant chemotherapy or any other anticancer therapy before the operation.

The genetic landscape of MSLC

We analyzed the mutation pattern of 128 samples. A total of 292 genetic changes were identified, which include 290 somatic, non-synonymous mutations, 1 amplification and 1 fusion, with an average of 0.95 mutations per tumor (range from 0 to 15.08). At least one mutations were found in 118 tumors (92.2%), and no mutation was detected in 10 tumors (7.8%). The mutational landscape is shown in Fig. 1. Recurrently mutated genes include EGFR (n=70), ERBB2 (n=15), TP53 (n=14), BRAF (n=10), and KRAS (n=9). ALK, RET and ROS1 fusion were not found in these tumors, MET exon 14 skipping were detected in one samples. In the 70 tumors which had EGFR mutation, L858R mutation was found in 38 (54.3%) tumors, two samples accompanied with EGFR T790M mutation. Two samples carried both G719A (exon 18) and L861Q (exon 21) mutations and one sample carried only G719A (exon 18) mutation. 19 deletions were detected in 23 (32.9%) tumors, and another six samples detected exon 20 insertion (supplementary table 1). KRAS mutations were found in nine samples (9 were G12 mutation, 1 was Q61H), with one (Case 31) carried both G12D and G12V mutations. Also, all of the KRAS mutations were not associated with EGFR mutations. In the eleven samples which had BRAF mutation, two of them were BRAF V600E mutation. None of the tumors had co-drivers of EGFR, KRAS, BRAF, ERBB2 and MET. TMB was calculated and the median TMB was 0.79 (from 0 to 15.08).

Genomic heterogeneity of multiple synchronous lung cancers

We then compared the driver mutation profile of different tumor focus of the same individuals. Depending on whether it contains the same driver mutation among different tumors in the same individual, 64 patients were separated into 3 groups. Group 1 contains 10 patients who had one tumor with no mutations being detected, the individuals in group 2 had same driver mutation or with no driver mutation but had other same gene mutations between different tumors, and the samples in group 3 had different driver mutations or with no other same mutation genes in one individual. There are 10 (15.6%), 14 (21.9%) and 40 (62.5%) patients in group 1, 2 and 3 separately (Fig 2). In the group 1 and group 3, for there were no same mutations in the two separated tumors, this is consistent with the conclusion drew from pathology and imaging evidence. But the individuals in group 2, which carried the same driver mutation or other same mutation (with no driver mutation), the nature of their origin need to be determined.

Define clonality index to classification MSLC with intrapulmonary metastasis

For the 14 patients in group 2, which shared gene mutation between different tumors in one individual, we defined a clonality index (CI) to differentiate MSLC with intrapulmonary metastasis. Assessing the likelihood of two samples sharing mutations not expected to have co-occurred by chance, based on all somatic synonymous and nonsynonymous mutations. The result of these 14 patients and the cut-off for CI is shown in Table 2. Six patients have CI dramatically above the cut-off which means these tumors might be intrapulmonary metastasis. In these individuals, except the same driver mutations, there were other passenger mutations shared between different tumors in one patients. And the remaining eight patients, who have CI close to cut-off (cut-off is 7.77, table 2). The mutations shared in these patients are only hotspot of driver gene.

Discussion

In recent years, increasing number of multiple synchronous lung cancer (MSLC) cases are being diagnosed. MSLC generally have better prognosis. However, differentiating MSLC from metastatic lung cancer remains a challenge in clinical practice. Many clinical cases cannot be diagnosed solely based on tumor location and histological features. With the recent advancement in genomic technology, genomic markers are being used to facilitate diagnosis in many diseases. Here, we characterized the genomic features of individual tumor focus in MSLC and attempted to introduce genomic markers to facilitate the diagnosis of MSLC.

There have been several reports suggesting the heterogeneity of different tumor foci in MSLC(Arai et al. 2012; Liu et al. 2016; Ma et al. 2017; Sakai et al. 2018). Yu Liu et al analyzed genomic profiles of 15 lung adenocarcinomas and one regional lymph node metastasis from 6 patients and found all the 15 lung tumors demonstrate distinct genomic profiles(Liu et al. 2016). Another study used 22 hotspot genes to classify MSLC with intrapulmonary metastasis and found that combined histomolecular algorithm could help to classify multifocal lung cancers which also could guide adjuvant treatment decisions(Mansuet-Lupo et al. 2019). A recently study performed targeted sequencing of a 464 gene panel in 16 patients with multiple tumors and demonstrate that DNA sequencing can provide additional evidence in clinical practice when pathology in inadequate to make a conclusive diagnosis(Liu et al. 2020). However, these studies contain relatively small number of patients or used less genes. In our study, we used NGS sequencing to study the 509 genes of different tumors from 64 patients with multiple synchronous lung cancer at initial diagnosis. Our genomic study suggested that in 50 patients, different tumor foci do not have shared mutations, suggesting the tumor foci are of independent origin and are likely synchronous multiple primary lung cancers. The genotyping result is consistent with the conclusions drew from histology and imaging evidence. For patients with shared mutations between individual foci, we computed “Clonality Index” to infer whether the foci are synchronous or metastasis. Six patients have CI dramatically above the cut-off, suggesting the foci are likely metastasis. Eight patients have CI close to cut-off. In these cases, the individual may be of different origin initially but evolved in the same direction under similar selection pressure. Based on our data, we propose an flow diagram to classification of MSLC using next-generation sequencing and CI2(Fig. 3). Overall, retrospective histologic reassessment of

cases unambiguously classified by NGS will provide an unprecedented benchmark against which to refine histologic criteria for distinguishing MSLC and IPMs in future studies.

Also, there were some interesting cases in the study. We found 2 cases (Case 73 and Case 57) had primary EGFR T790M mutation. According to the previous report, which focused on stage III-IV patients, the results showed that de novo T790M mutation was more likely to carry concurrent EGFR L858R mutation(Tian et al. 2018; Wang et al. 2019), and showed good response to osimertinib treatment. In our cohort, the only 2 cases carried primary EGFR T790M mutation were co-mutated with EGFR L858R and the mutation frequency of these 2 loci were almost the same, which suggested that these two mutation sites occur simultaneously. Also, a meta-analysis evaluated the results from 22 clinical trials and reported that de novo EGFR T790M was an unfavorable predictive and prognostic marker in the late stage lung cancer(Liu et al. 2017). But there was no research focus on stage I lung cancer, and these early stage patients will not receive any treatment after surgery, whether the primary EGFR T790M will be an unfavorable prognostic marker need further investigate. Another special case (Case 31) is a 56y woman, one of the tumor carried two activation site (G12D and G12V), and the mutation frequency was 5.7% and 7.8% separately. We found no research reported this phenomena, so whether this tumor will be more aggressive still need follow up results.

Our study still had some deficiencies, for it was a single center study and the follow up data was not available at this time. The sample size is still need to enlarge and the intrapulmonary metastasis data will be further included.

Our results showed that the conclusions drew from genotyping results are fairly consistent with that drew from histology and imaging information, suggesting genomic markers can be used to facilitate diagnosis in clinical practice. In recent years, the advancement of genomic and sequencing technology has made the use of genomic markers more common in diagnosis. Genomic markers can be useful in diagnosis and guide treatment plan in many diseases such as breast cancer. Our study here provide evidence that the use of genome profiling in addition to histology and imaging approach can provide more comprehensive analysis for the diagnosis of MSLC.

Declarations

Author contributions

FC.Z and L.Z conceived and designed the study. J.W, YX.Z and P.W collected the data. L.Z , CS.L, SL.Z and G.T analysed and interpretated the data. FZ.L and FC.Z analyzed the statistical and wrote the paper. FC.Z , L.Z, CD.G and YT.Z reviewed and edited the manuscript. CD.G and YT.Z played a very important role in the revision of the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Tables

Table 1. Clinical Characteristics of 64 patients

Patients' characteristics	Value (%)
Total patients	64
sex	
Female	45 (70%)
Male	19 (30%)
Age at first diagnosis	
median	56
Range	29-75
Smoking history	
No	53 (83%)
Yes	11 (17%)
Stage	
IA	54 (84%)
IB	10 (16%)
Location	
RUL	45
RML	13
RLL	22
LUL	31
LLL	17

RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LUL: left upper lobe; LLL: left lower lobe.

Table 2 The clonality index of 14 individuals

Case	Driver gene alterations	clonality index (CI)	Final Class
7	**ERBB2 (Tyr772_Ala775dup)**	**19.86**	**IPM**
17	**None**	**31.93**	**IPM**
18	EGFR (L858R)	7.77	MSLC
24	**EGFR (L858R)**	**29.76**	**IPM**
27	EGFR (L858R)	7.77	MSLC
39	EGFR (L858R)	7.77	MSLC
49	EGFR (p.Glu746_Ala750del)	8.37	MSLC
64	**None**	**10.45**	**IPM**
65	**EGFR (p.Ala767_Val769dup)**	**10.45**	**IPM**
68	EGFR (L858R)	7.77	MSLC
69	KRAS (p.G12C)	6.69	MSLC
71	EGFR (L858R)	7.77	MSLC
74	EGFR (L858R)	7.77	MSLC
78	**ERBB2 (Tyr772_Ala775dup)**	**9.06**	**IPM**

Cut-off is 7.77

ADC: adenocarcinoma;

IPM: Intrapulmonary metastasis

MSLC: multiple synchronous lung cancers

Figures

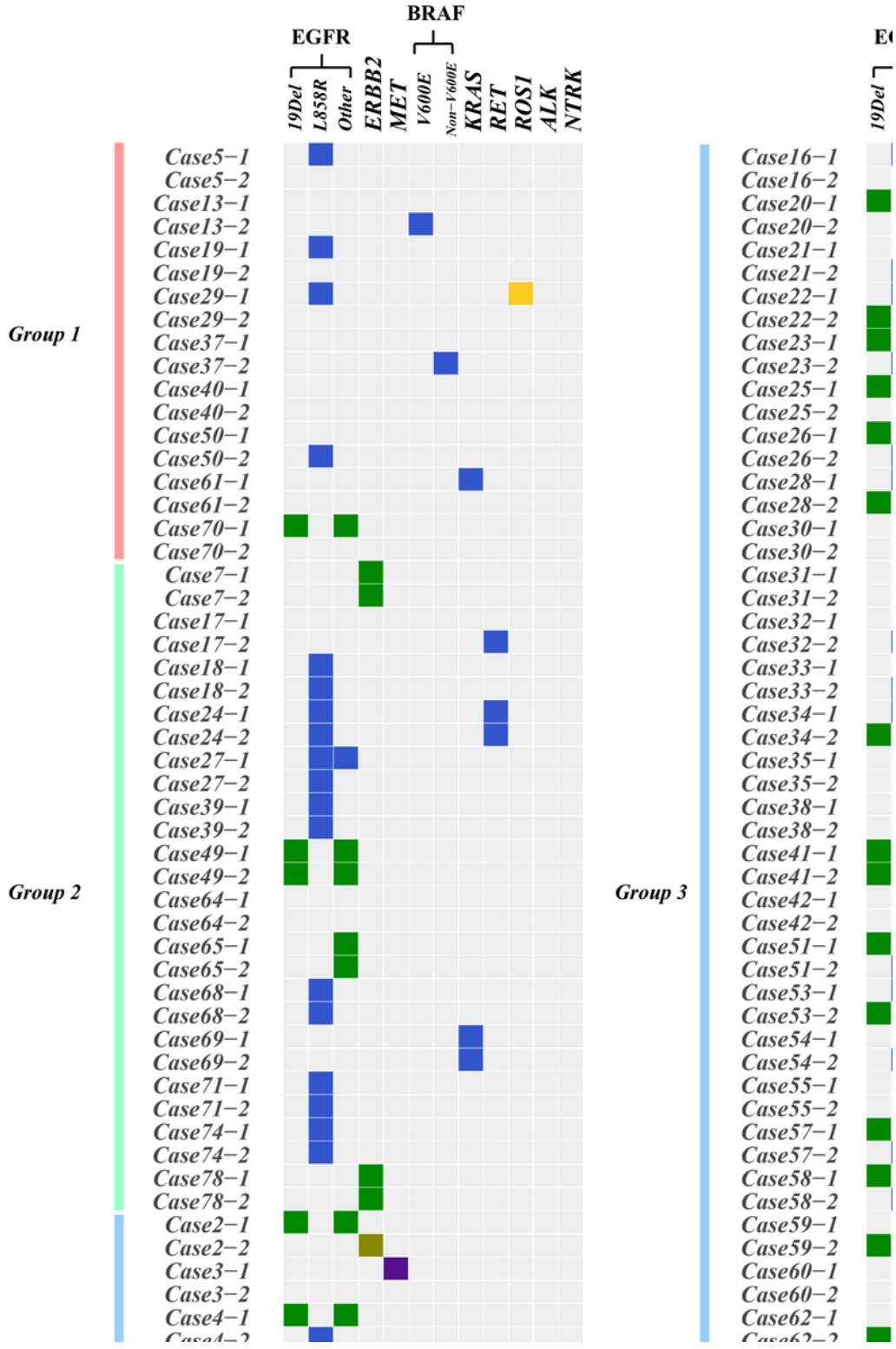

Figure 1

The mutation landscape of 128 samples

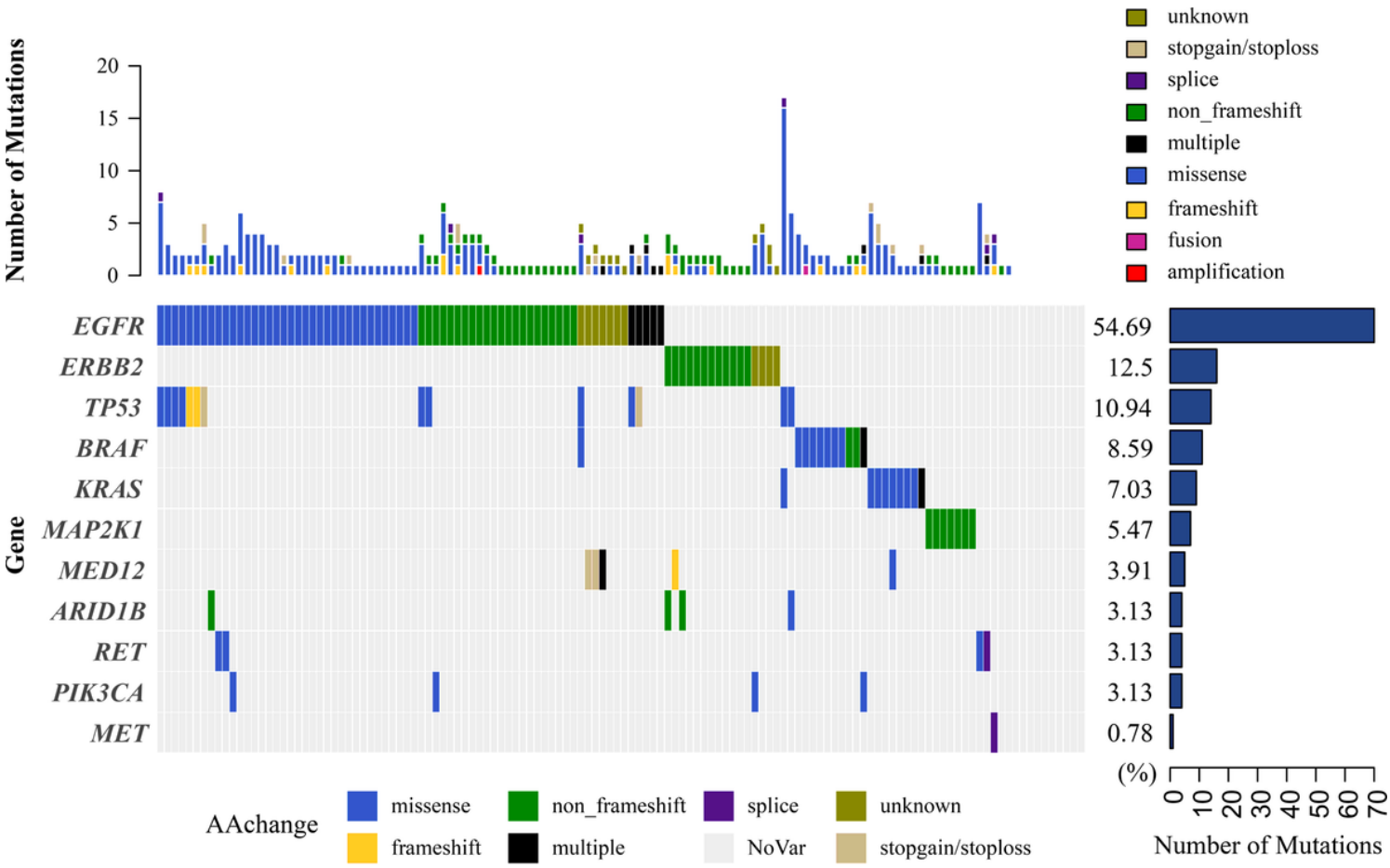

Figure 2

Somatic mutations spectra in different groups of MSLC.

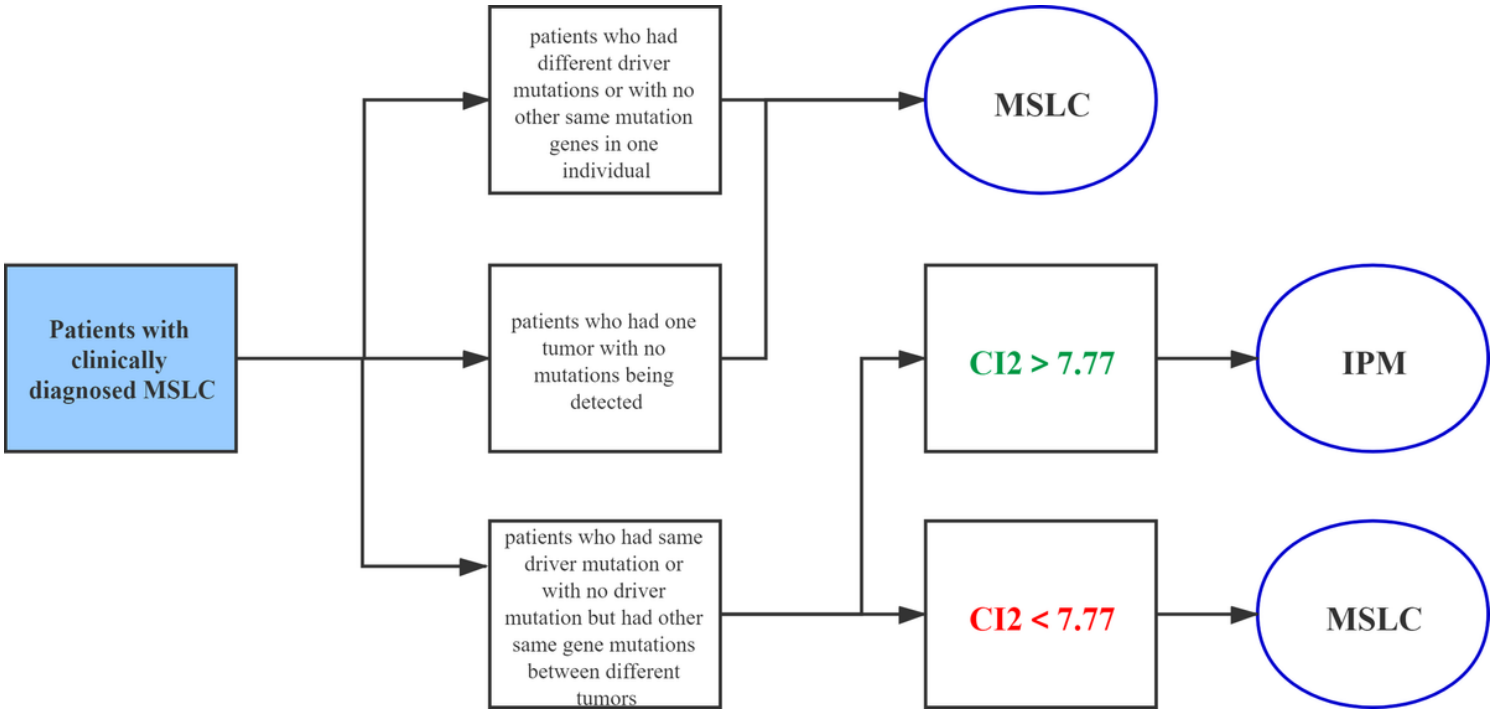

Figure 3

Flowchart for classification of MSLC using next-generation sequencing.