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Detection of Circulating Tumour DNA in Early-Stage NSCLC Patients by a New Lung Cancer Targeted Sequencing Panel

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Abstract

Circulating tumor DNA（ctDNA）derived from tumor cells carrying unique genetic information of tumor cells may enable a high specificity and noninvasive "liquid biopsy" for early diagnosis of cancer. Most previous studies have paid attention to ctDNA condition of advanced patients, and the dynamic changes of ctDNA during the treatment. Although small amount of research has focused on the early stage cancer patients, most of them used the next-generation sequencing panel, which include a lot of gene, and thousands of mutational hotspot site. It costs a lot and lack of pertinence. In this study, we constructed a small lung cancer targeted sequencing panel which only contains 13 genes, which consist of EGFR, KRAS, STK11, PDGFRA, CDKN2B-AS1, ALK, CTNNB1, RET, PTEN, NRAS, BRAF, TP53 and PIK3CA. Mutations in these genes are common in non-small-cell lung cancer (NSCLC) patients. In all NSCLC and early-stage patients, using our panel the mutation coverage was 84.1% and 85.5%, respectively. In 34 NSCLC patients, compare to the tumor biomarkers, the ctDNA is much sensitive (85.3% vs. 52.9%). For all of 64 patients, we conducted a 5-year follow-up. We found that the patients, who were diagnosed at an early-stage, would have a good prognosis. This study demonstrates that our panel has potential clinical utility in the diagnosis and prognosis of lung cancer.

Keywords: circulating tumour DNA; tumour biomarkers; lung cancer targeted sequencing panel; 5-years survival.

1. Introduction

Lung cancer is the most common cancer in the world and the leading cause of cancer death in men and the second leading cause in women worldwide (Torre, 2015). In 2018, in the United States (US) lung cancer accounts for a quarter of total cancer mortality (Siegel, 2018).

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Lung cancer is difficult to be cured, once the development of advanced (Bozic, I., 2013). Compared with stage IA NSCLC patient's 70%-80% five-year survival rate, the five-year survival rate of stage IIIA lung cancer is only 24% (Huang, 2017). Therefore, the most effective treatment of lung cancer requires that it can be diagnosed in the early stage of cancer. Thus, early detection has significant value for reducing the mortality of lung cancer (Groome, 2007).

Due to the limitations of conventional imaging methods, less than 40% of lung cancers are diagnosed at a localized or regional stage (Siegel, 2018). Although the tumour biomarkers have been applied in clinical, such as carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), cytokeratin 19 fragment (CYFRA21-1), squamous cell carcinoma antigen (SCCA), carbohydrate antigen (CA125) and carbohydrate antigen 19-9 (CA19-9). But the tumour biomarkers are low-sensitivity, and they are susceptible to other diseases (Molina, R., 2003).Recently, as a new type of liquid biopsy, circulating tumor DNA (ctDNA)[8] is attracting more and more attention. Study have shown that ctDNA are released into blood via tumour cell necrosis, apoptosis, and active release of DNA (Stroun, 2001). Therefore, ctDNA contains tumour cell-specific mutations (Snyder, 2016). Research demonstrates that ctDNA has the potential to diagnose, monitor cancer progress and track treatment response (Kimura, T., 2004). Compare to tissue biopsy, liquid biopsy is easy to be obtained. Second, it can be repeatedly taken during the treatment progress. The third, the ctDNA is constantly changing (Dawson, 2013). Thus, it can respond to the development of the disease and the response to treatment in real time (Mok, T., 2015). Finally, liquid biopsy can comprehensive response to cancer condition, such as, the spread, metastasis, and heterogeneity of cancer. Although ctDNA as one of the liquid biopsies has shown great potential clinical utility (Lebofsky, 2015) it still faces enormous challenges. The research shown that the cell free circulating DNA (cfDNA) deriving from normal cells is 10 times more than ctDNA(Garcia-Olmo, 2008). In addition, the ctDNA content of cancer patients varies greatly, accounting for less than 0.1% of cfDNA, to more than 10% (Diehl, 2008). Therefore, a more sensitive detection method is urgently needed. With the continuous development of high-throughput sequencing (van Dijk, 2014), the detection of ctDNA is becoming more and more accurate and easy. Now, the cancer panels that are applied in clinical or research always include a lot of gene, and thousands of mutational hotspot site are detected. The research (Krimmel, 2016) Show that healthy people have low frequency mutation. Therefore, the more site is be detected, the more mutation may be detected. However, it is difficult to verify whether one of the mutations related to the cancer. In addition, the test costs a lot and causes a greater financial burden on the patient. To overcome these challenges, this prospective study constructed a small lung cancer panel, which only contains 13 genes, and used a unique multiplex-PCR. This study evaluated the utility of ctDNA in the diagnosis of early stage non-small-cell lung cancer (NSCLC) and the clinical significance.

2. Experimental procedure

2.1 Participants

Between September 2012 and December 2013, 62 lung cancer patients who were 26 to 80 years old were invited to participants in the study (Table 1), in the Shanghai Pulmonary Hospital. The 5ml blood were collected before surgery. All participants provided written informed consent. And the study had been agreed by the ethics committee.

2.2 Blood Sample DNA Preparation

The plasma samples were taken from lung cancer patients before surgery. 5ml blood samples in EDTA tubes were centrifuged for 10 min at 1,600g. Then the supernatants from these samples were further centrifuged for 10 min at 16,000g, and plasma was collected and stored at -80℃ until needed. Following the manufacturer's instructions, the Serum/Plasma Circulating DNA Kit (TIANGEN) was used to extract cfDNA from 2.5ml of plasma, which was collected in 30μl of eluent. The Qubit 3.0 Fluorimeter and Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA) were used to quantify cfDNA following the protocol.

2.3 Lung Cancer Panel Construction

With reference to published articles and cancer database (TCGA, COSMIC), we constructed a lung cancer detection panel, which only contained 13 genes. The panel included EGFR, KRAS, STK11, PDGFRA, CDKN2B-AS1, ALK, CTNNB1, RET, PTEN, NRAS, BRAF, TP53 and PIK3CA (Supplementary table 1).

Table 1. **Demographic Characteristics of the study Participants**

	Characteristic	Value
Sex,n(%)	Male	39（63%）
	Female	23（37%）
Age (%)	Mean (SD)	59.4（10.9）
	Median	60（26-80）
Lung cancer subtype (%)	Adenocarcinoma (AC)	31（50%）
	Squamous cell carcinoma (SCC)	28（45.2%）
	other	3（4.8%）
Tumour Stage,n(%)	Ⅰa	2（3.2%）
	Ⅱa	2（3.2%）
	ⅠB	29（46.8%）
	ⅡB	11（17.7%）
	Ⅲa	10（16.1%）
	ⅢB	3（4.8%）
	Ⅳ	5（8.1%）

According to the Ion Torrent sequencing standard segment, we designed two set of primers. The first one set of primer was used to enrich the ctDNA. The second set of primer (Figure. 1a) was used to enhance the amplification specificity and construct the sequencing library, which was consist of sequencing adapt, barcode and common primer, by the nested PCR. In addition, the second of primer was consist of 4 primers (Figure. 1b), which were used to distinguish the forward and reverse.

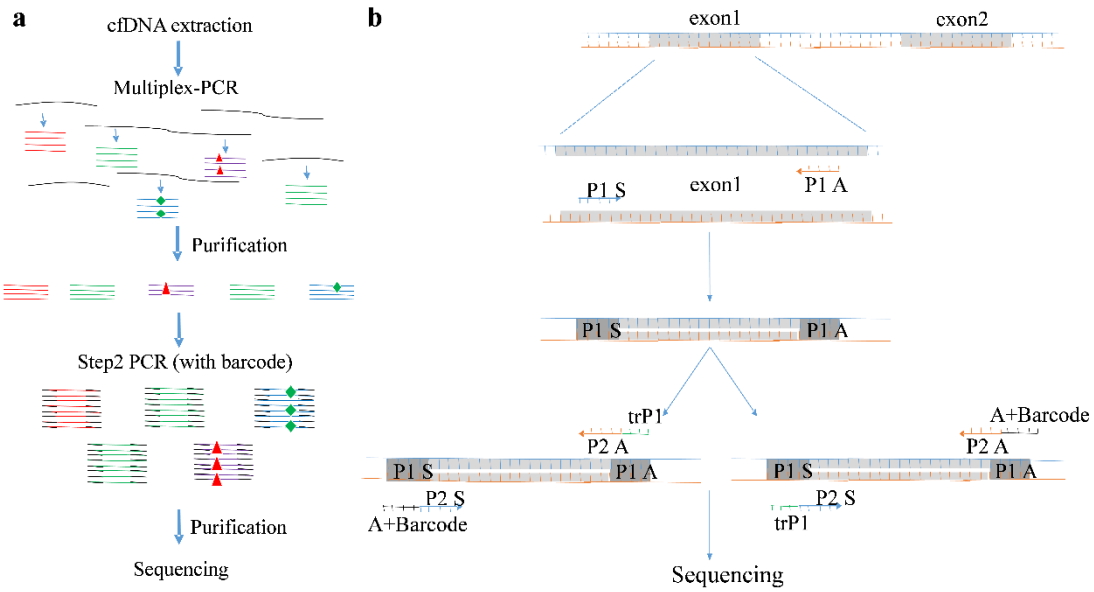

Figure. 1 **(a) The flow of library construction. (b) The PCR reaction of each pair primer. P1 represented the one of first one set primer; P2 represented the one of the second set primer; "A" and "trP1" were used to be recognized by the instrument.**

2.4 Sequencing Library Preparation and Sequencing

Limited the small amount of cfDNA, we carried out multiplex-PCR （Figure. 1a）using the first set of primer. The PCR product was purified by the Hieff NGSTM Smarter DNA Clean Beads (Yesen). Then the second set of primer was used to construct the sequencing library using the purified product as a template. Finally， pooled all the samples which were purified by the beads，and measured the sequencing library concentration using the Qubit ®3.0.

Before sequencing, a quality control of the library was conducted using the Agilent Bioanalyses 2100. Then the library was processed in series according the manufacture's instruction. Finally, the Ion Proton™ System was used to sequencing the library.

2.5 Variant Calling

To determine the minimum variant frequency threshold, we synthesized two base segments. One of them contain one mutation, while the other one was wild. The mutation segment was mixed into wild segment at a ratio of 0.01% and 0.1%, which was used as a positive control. The sequence results show that the mutation could be recognized when the threshold was 0.05%, while the false positive result was also called. Finally, taking account of the ultra-low frequency mutations in healthy people and the accuracy of Ion Proton™ System, a 0.5% threshold was set. The sequence data was filtrated by the Torrent Suite Software v3.0. Include the adapter sequences, short segments and the low-quality segments. Next, a "variant caller v4.0" plug was used to detect the mutation, which contains three conditions. The average coverage depth>10000, each variant coverage>20, p value<0.01; the frequency of each variant>0.5%; the frequency of forward and reverse mutations was similar to the total forward and reverse sequencing ratio. The third, the Integrative Genomics Viewer (IGV) software v2.3.97 (http://software.broadinstitute.org/software/igv/) was used to examine the sequencing error and find the missing mutations by manually.

3 Results

3.1 Sequencing result

62 blood samples were taken from the non-small-cell lung cancer (NSCLC) patients on the day of surgery. In addition, 9 healthy people were enrolled the study. All of the blood samples were extracted cfDNA, amplificated, constructed library, sequenced and analysed. Simultaneously, in order to determine the minimum detection limit of our sequencing method, 2 base segments were synthesized. One of the segments contained a positive mutation. The result showed that the sequencing length was in line with expectations, and most amplicons were sequenced more than 10000 times. The further analysis was conducted.

Finally, false positives, false negatives and low depth sequencing sites were filtered by manually using the IGV software. Overall, 53 patient samples (85.5%) were detected mutation, and more than two mutations were found in 29 of the 53 patient samples (53.7%). While, 37 (84.1%) of 44 early-stage (stage Ⅰ, Ⅱ) patient samples were detected mutation, and 16 (88.9%) of 18 advanced stage (stage Ⅲ, Ⅳ) patient samples were detected mutation. Nonsense mutation come from 4 patient samples (6.4%). 35 target regions were detected synonymous mutations. And most of the synonymous mutations were located on introns. Figure. 2 showed the detail information about the mutation of patient samples. In the 9 healthy people samples, we also detected low frequency mutations.

Across all genes with mutations excepting the low depth sequencing sites and synonymous mutations, were found in $EGFR$ $(25/62, 40.3\%)$, $KRAS$ $(6/62, 8.1\%)$, $STK11$ $(15/62, 24.2\%)$, $PDGFRA$ $(4/62, 6.4\%)$, $CDKN2B-AS1$ $(8/62, 12.9\%)$, ALK $(3/61, 4.9\%)$, $CTNNB1$ $(2/62, 3.2\%)$, RET $(2/62, 3.2\%)$, $TPEN$ $(5/62, 8.1\%)$, $NRAS$ $(2/62, 3.2\%)$, $TP53$ $(13/62, 21.0\%)$, $BRAF$ $(1/18, 5.5\%)$, $PIK3CA$ $(3/41, 7.3\%)$,

Respectively (Figure. 3a). According to the lung cancer subtype, we divided the patient samples into AC and SCC and two groups. Three patient samples were removed.

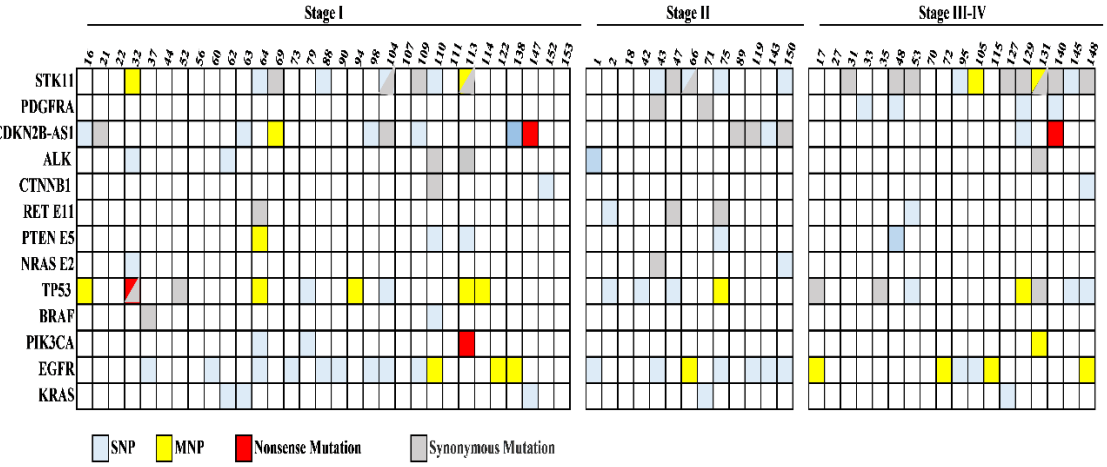

Figure. 2 **Mutation of the patient samples. Different colour square represented different mutations. Each column represented the mutation of different gene in one patient sample, and each row represented the mutation of a same gene in different patient samples.**

In different genes, the frequency of mutation (Figure. 3b) in two subtypes has changed more or less. The mutation frequency of KRAS, BRAF, PIK3CA, RET and NRAS has changed significantly. The mutation frequency of BRAF and PIK3CA, increased in AC group. On the contrary, mutation frequency of RET and NRAS increased in SCC group. The obvious change of BRAF and PIK3CA mutation frequency may be related to insufficient sample size, which contained 18 and 41, respectively. KRAS mutation was rare in SCC, but we found that the KRAS mutation frequency in SCC group was higher than that in AC group after grouping. Than we checked the pathological data of these SCC patients $(3/28,10.7\%)$ with KRAS mutation. We found that all of these patients were male and had a history of smoking. Perhaps the occurrence of KRAS mutation was related to smoking, but there was no direct evidence (Gerlinger, 2012).

This prospective study shown that it is feasible to use our lung cancer detection panel to analyse ctDNA for screening early stage NSCLC patients. In the 44 early-stage patients, $37(37/44, 84.1\%)$ patients were detected at least one mutation. Compare to the advanced stage (Ⅲ, Ⅳ) patients, although the mutation rate is lower than that of advanced patients (16/18, 88.9%), it is much higher than other indicators, such as imaging methods, and tumour biomarkers. Tumour biomarkers as the indicator are useful. But each of them is relatively low sensitive. It is not enough to diagnose and monitor the cancer (Hu, 2018). The results demonstrate that the mutation of ctDNA may be a good indicator of lung cancer; our lung cancer panel has the potential clinical application value.

In the 9 healthy people, we also found some low frequency mutations. Although most of the mutations are synonymous mutations, we need to construct a method to distinguish between mutations derived from tumour cells and mutations resulting from normal metabolism. The research (Hu, 2018) shown that some of the mutations come from clonal haematopoiesis, so it could affect the sequencing results. The best way is to detect ctDNA and the genome DNA of peripheral blood cells, simultaneously. However, it cost twice the cost of testing and more times in constructing library and analysing (Fisher, 2013). The establishment of gene mutation library of peripheral blood cells may be a good alternative.

Now, there is consistent view, still using mutation of tumour DNA as the gold standard to measure mutation of ctDNA. However, tumour DNA may not be detected completely, because of the spread and metastasis, and the heterogeneity of cancer. In addition, some of the tumour cell will enter the blood circulation. Therefore, a small part of tumour tissue may not reflect the genetic characteristics of the entire tumour (Fisher, 2013).

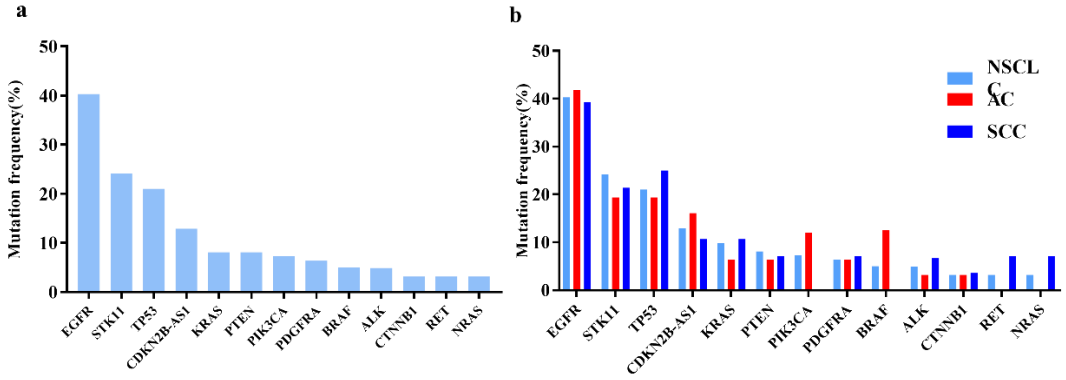

Figure. 3 (a) **Mutation frequency of each gene in all NSCLC patients; (b) Different colour bar represents different groups. Mutation frequency of each gene in different groups.**

3.2 Tumour biomarkers

Tumour biomarker of 34 NSCLC patients were detected before surgery. The positive of CYFRA21-1, SCC, CEA, NSE, CA19-9, CA125 is 13 (13/34, 38.2%), 4 (3/34, 8.8%), 3 (3/34, 8.8%), 3 (3/34, 8.8%), 3 (3/34, 8.8%) and 1 (1/33, 2.9%), respectively (Figure. 4). The positive rate of CYFRA21-1 was much higher than the others. It showed that CYFRA21-1 had highly sensitivity in patients with NSCLC. Overall, 18 (18/34, 52.9%) patients were positive for at least one biomarker. In addition, in the 34 patients, 29 (29/34, 85.3%) patient samples were detected mutations. Compared to tumour biomarkers, ctDNA was more sensitive

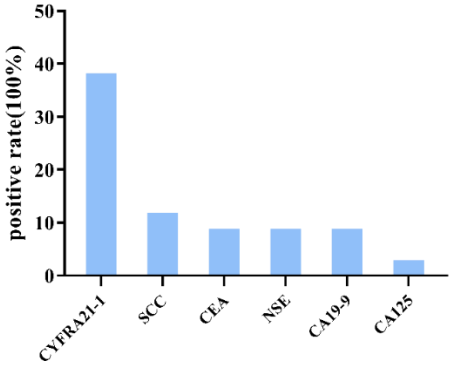

Figure. 4 **Positive rates of CYFRA21-1, SCC, CEA, NSE, CA19-9, CA125.**

3.3 Survival condition

For the patients who participated in the study, we conducted a follow-up of 5 years after the surgery. The 20 patients (20/62, 32.3%) were dead. After surgery, 16(16/20, 80%) of them have spread or metastasized to varying degrees. Among them, the number of deaths in stage I, stage II, and stage III-IV was 5 (5/31, 16.1%), 4 (4/13, 30.8%), and 11 (11/18, 61.1%), respectively. Obviously, patients in stage Ⅰ and Ⅱ had higher survival rates (Figure. 5a) than patients in advanced stage. The survival curves were significantly different between stage Ⅰ - Ⅱ and stage Ⅲ - Ⅳ (p=0.0007) by log-rank test. Interestingly, survival rates (Figure. 5b, c) varied widely among patients with different subtypes (AC, SCC). In AC patients, it was same to the survival curves of overall NSCLC patients in stage Ⅰ - Ⅱ or stage Ⅲ-Ⅳ. The

survival curves were significantly different between stage Ⅰ - Ⅱ and stage Ⅲ - Ⅳ (p=0.0477), while there was no significantly different (p=0.0684>0.05) in SCC patients. Stage Ⅱ SCC patients (Figure. 5d) had a higher mortality rate (4/10,40%) than all the stage Ⅱ NSCLC patients' mortality rate (4/13, 30.8%).

Early diagnosis of lung cancer is of great significance for its treatment. Figure. 5a shown that the 5-year survival rate of patients with early NSCLC is significantly higher than that of patients with advanced NSCLC.

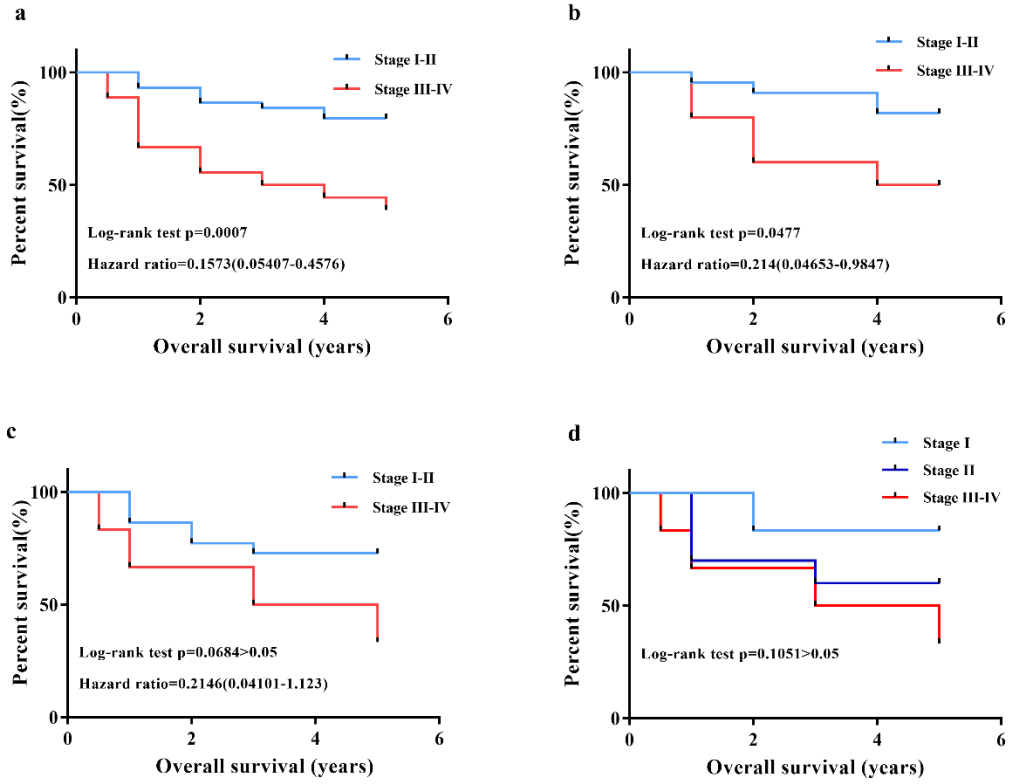

Figure. 5 **Survival Curves. (a) Overall survival curves of NSCLC patients in stage Ⅰ - Ⅱ or stage Ⅲ-Ⅳ. (b) Survival curves of AC patients in stage Ⅰ - Ⅱ or stage Ⅲ-Ⅳ. (c) Survival curves of SCC patients in stage Ⅰ - Ⅱ or stage Ⅲ-Ⅳ. (d) Survival curves of SCC patients in stage Ⅰ, stage Ⅱ or stage Ⅲ-Ⅳ.**

4. Conclusion

This study demonstrates that the ctDNA can be detected in early-stage NSCLC patients effectively by the Ion Proton™ System, and our lung cancer targeted sequencing panel is high sensitivity in early-stage NSCLC. Simultaneously, it has potential clinical utility in the diagnosis and prognosis of lung cancer.

5. Acknowledgements

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