RESEARCH ARTICLE

Comparison of Two Methods to Extract DNA from Formalin-Fixed, Paraffin-Embedded Tissues and their Impact on EGFR Mutation Detection in Non-small Cell Lung Carcinoma

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Abstract

Objective: Molecular pathology tests are often carried for clinicopathological diagnosis and pathologists have established large collections of formalin-fixed, paraffin-embedded tissue (FFPE) banks. However, extraction of DNA from FFPE is a laborious and challenging for researchers in clinical laboratories. The aim of this study was to compare two widely used DNA extraction methods: using a QIAamp DNA FFPE kit from Qiagen and a Cobas Sample Preparation Kit from Roche, and evaluated the effect of the DNA quality on molecular diagnostics. Methods: DNA from FFPE non-small cell lung carcinoma tissues including biopsy and surgical specimens was extracted with both QIAamp DNA FFPE and Cobas Sample Preparation Kits and EGFR mutations of non-small cell lung carcinomas were detected by real-time quantitative PCR using the extracted DNA. Results and Conclusion: Our results showed that DNA extracted by QIAamp and Cobas methods were both suitable to detect downstream EGFR mutation in surgical specimens. Howover, Cobas method could yield more DNA from biopsy specimens, and gain much better EGFR mutation results.

Keywords: DNA extraction - cobas - QIAamp - EGFR mutation

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Introduction

In recent years, molecular biological techniques is becoming one of the newest and fastest growing branch of biomedical applications. Currently, molecular pathology is widely used in clinicopathological diagnosis, making the pathological diagnosis from a morphological level depth down to the genetic level (DeMarzo et al., 2003; Miettinen and Lasota 2006; Gonzalez-Angulo et al., 2010). It is making tremendous contributions to uncover the genetic nature of the disease from the perspective of morphological level and the gene level (Roukos, 2010). However, during the course of clinicopathological diagnosis, millions of formalin-fixed, paraffin-embedded (FFPE) tissues are collected and archived in different hospitals (Shi et al., 2002). Being as a rich tissue repository, these FFPE tissue banks can represent a source of information, which can tell us genetic events involved in different clinical conditions (Roukos, 2010).

For clinical molecular applications, isolation of DNA from FFPE tissues is difficult and challenging, because the nucleic acid is degraded into small fragments and cross-linked with protein in FFPE samples (Feldman 1973). We need a method that can yield high quality DNA with optimum concentration and purity (Sam et al., 2012). In most PCR laboratories, QIAamp FFPE Tissue Kit from Qiagen is widely used for extracting DNA from FFPE sections. It is said that high quality nucleic acids can be extracted in one day by using this isolation method (Huijsmans et al., 2010; Sam et al., 2012). On the other hand, in the global diagnostics, Roche Diagnostics ranked first, it has developed a nucleic acid extraction kit named Cobas sample preparation kit, which is a clinical diagnostic kit.

Therefore, the aim of this study was to compare DNA extraction efficiency using these two commercial DNA isolation kits in the surgical specimens and biopsy specimens, to comfirm whether DNA can be used in routine downstream applications. We performed EGFR mutation detection using extracted DNA in the human non-small cell lung carcinoma (NSCLC) tissues. Our results showed that QIAamp and Cobas methods were both suitable to extract DNA from surgical specimens for downstream EGFR mutation detection. However, Cobas DNA isolation method had more advantage to extract DNA from biopsy specimens for EGFR mutation detection.

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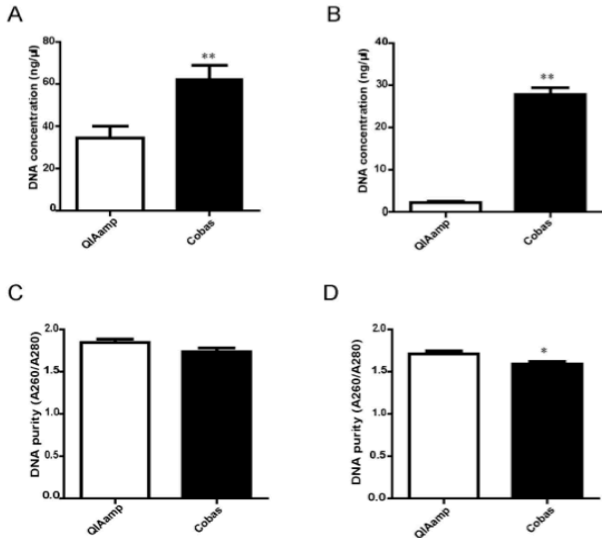

Figure 1. Comparison of Genomic DNA Quality Extracted by Cobas and QIAamp Methods. Our results showed that there was significant difference of DNA yield between QIAamp and Cobas methods. Cobas method can yield more DNA than QIAamp method in the surgical specimens (A, $P<0.01$) and biopsy specimens (B, $P<0.01$). DNA purity by two methods was almost same in the surgical specimens (C, $P>0.05$), but which by Cobas method was poorer than that by QIAamp method in the biopsy specimens (D, $P<0.05$)

Materials and Methods

Tumor specimens and tissue processing

32 cases of NSCLC FFPE samples from the First Clinical Medical College of China Three Gorges University were randomly selected, which including 14 biopsy specimens and 18 surgical specimens, 23 cases of adenocarcinoma and 9 cases of squamous cell carcinoma. The tumor content of each specimen is ≥10%, which was assessed by two experienced pathologist (Liu YF and Chen HL). Approval for this study was obtained from the ethics committee of China Three Gorges University. For all experiments, two consecutive 5μm thick sections for surgical specimens and five consecutive 5μm thick sections for biopsy specimens were obtained from the paraffin blocks. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and rinsed using distilled water. Sections were then air-dried for 5-10 min. At last, we used sterile scalpel blade to scrape the unstained tissue from sections and collected into sterile 1.5 ml micro-centrifuge tubes.

QIAamp DNA FFPE Tissue Kit isolation method

Experiment was performed according to QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA, 'QIAamp method') manufacturer's handbook. The tissue pellet was resuspended in optimum amount of ATL (tissue lysis) buffer and proteinase K, then incubated at 56°C for 1h, in order to partially reverses formaldehyde modification of DNA, the samples were then incubated at 90°C for 1h. After briefly centrifugation, we added 2μL RNase A (100mg/ml) and incubate samples at room temperature for 2 min to avoid RNA contamination. In the following two steps, AL buffer and ethanol were consecutively added to samples and vortexed thoroughly. Next, we transferred the entire lysate to the QIAamp MinElute column placed in a 2ml collection tube. After centrifugation, we placed the QIAamp MinElute column in a clean 2 ml collection tube. The nucleic acid was adsorbed to the membrane of the QIAamp MinElute column, and then washed by AW1 and AW2 buffer. Finally, 50 μL ATE buffer was added to the center of membrane. After incubating at room temperature for 5 min, the samples were centrifuged at 14000 rpm for 2 min, and the DNA was collected into new sterile 1.5 ml micro-centrifuge tubes.

Cobas sample preparation kit isolation method

The whole experiment was also performed according to Cobas sample preparation kit (Roche Molecular Systems, Branchburg, NJ, USA, 'Cobas method') manufacturer's instruction. Except the reagents used were different, the main steps are the same as QIAamp method. Moreover, while resuspend the tissue pellet in 180 μL DNA Tissue Lysis Buffer, we added 70 μL of reconstituted proteinase K, and in the last step, 100 μL DNA EB (elution buffer) was added to the center of each membrane, after incubating the elution tube at 15°C to 30°C for 5 min. The elution tube was centrifuged at 8000×g for 1 min to collect eluate into the elution tube. During the whole process, we did not add any RNase A.

DNA Quantification and Cobas EGFR mutation test

The total amount of DNA from the above two isolation methods was spectrophotometrically determined by measuring the absorbance at 260 nm (A260), and DNA purity was assessed by detecting the A260/A280 ratio using the Varioskan Flash (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. After that, the DNA concentration was diluted to a final concentration 2 ng/μL, which was stored at 2°C to 8°C for up to two weeks or at -20°C for long term storage.

The Cobas® EGFR Mutation Test (Roche Molecular Systems, Branchburg, NJ, USA) is a real-time quantitative PCR test, which is also a CE-IVD-marked allele-specific PCR test. It is designed to detect the presence of 41 mutations in EGFR exons 18, 19, 20 and 21 in the NSCLC FFPE specimens. The test needs 150 ng total input DNA per sample. In order to confirm the validity of each run, a mutant control and a negative control are indispensable. The Cobas EGFR Mutation test system is highly automated, which the mutation data and results are automatically displayed in the cobas 4800 System Software (version 2.0.0.1028). EGFR mutation test was performed in the 32 cases of FFPE NSCLC specimens following the instructions in the package insert.

Statistical analysis

All data were analyzed by SPSS 17.0 software (Chicago, IL, USA). Chi-square and Fisher's exact tests were used to compare different rates. Analysis of variance (ANOVA) and paired samples t test were used to evaluate the statistical significance of differences in the DNA concentration and purity using two methods. Two-tailed P values less than 0.05 was considered statistically significant.

Table 1. The Extraction Data of DNA Using QIAamp Method and EGFR Mutation Detection in the FFPE NSCLC Tissues

Sample	Concentration (ng/μl)	Purity (A260/A280)	Cobas EGFR Mutation Test	Flags
***6915	65.56	2.2	Exon 21 L858R	
***4488	48.54	1.94	Wild Type	
***4596	14.09	1.8	Exon 19 Deletion	
***0070	33.2	1.87	Exon 19 Deletion	
***0650	30.3	2.07	Wild Type	
***1020	14.15	1.72	Exon 20 Insertion	
***1066	37.5	1.68	Wild Type	
***1126	28.06	1.95	Wild Type	
***1447	77.25	1.72	Wild Type	
***1635	98.8	2.01	Exon 19 Deletion	
***1797	20.8	1.78	Wild Type	
***2064	43.57	1.97	Exon 21 L858R	
***3627	12	1.9	Exon 19 Deletion	
***3660	10.9	1.65	Exon 21 L858R	
***4350	24.66	1.9	Exon 19 Deletion	
***4894	32.89	1.87	Exon 21 L858R	
***6860	16.74	1.76	Exon 19 Deletion	
***7965	43.5	1.78	Wild Type	
***4578	2.11	1.51	Wild Type	
**5661	1.62	1.79	Invalid	R433
**6222	1.76	1.85	Invalid	R433
**6307	4.95	1.57	Invalid	R427
**6706	1.81	1.65	Exon 21 L858R	
**6707	1.51	1.67	Invalid	R427
**7085	1.52	1.55	Invalid	R433
**7149	2.34	1.54	Exon 21 L858R	
**7507	0.95	1.76	Invalid	R427
**7873	2.34	1.72	Wild Type	
**7880	1.21	1.81	Wild Type	
**8122	3.97	1.81	Wild Type	
**8622	1.61	1.89	Invalid	R427
**9266	2.61	1.63	Invalid	R451

***Surgical specimens; **Biopsy specimens

Table 2. The Extraction Data of DNA Using Cobas Method and EGFR Mutation Detection in the FFPE NSCLC Tissues

Sample	Concentration (ng/μl)	Purity (A260/A280)	Cobas EGFR Mutation Test	Flags
***6915	78.4	1.64	Exon 21 L858R	
***4488	44.48	1.52	Wild Type	
***4596	43.86	1.48	Exon 19 Deletion	
***0070	47.8	1.48	Exon 19 Deletion	
***0650	54	1.96	Wild Type	
***1020	93.6	1.64	Exon 20 Insertion	
***1066	11	1.95	Wild Type	
***1126	43.37	1.61	Wild Type	
***1447	72.8	1.58	Wild Type	
***1635	79.2	1.98	Exon 19 Deletion	
***1797	94.2	1.96	Wild Type	
***2064	109.1	1.8	Exon 21 L858R	
***3627	25.9	1.93	Exon 19 Deletion	
***3660	47.5	1.99	Exon 21 L858R	
***4350	99.1	1.81	Exon 19 Deletion	
***4894	54.6	1.59	Exon 21 L858R	
***6860	64.25	1.75	Exon 19 Deletion	
***7965	94.67	1.68	Wild Type	
***4578	29.72	1.62	Wild Type	
**5661	15.97	1.69	Wild Type	
**6222	25.35	1.72	Wild Type	
**6307	25.42	1.34	Wild Type	
**6706	30.92	1.62	Exon 21 L858R	
**6707	21.86	1.56	Invalid	R433
**7085	38.06	1.51	Exon 19 Deletion	
**7149	25.56	1.65	Exon 21 L858R	
**7507	28.41	1.62	Invalid	R433
**7873	30.4	1.67	Wild Type	
**7880	30.66	1.65	Wild Type	
**8122	23.69	1.37	Wild Type	
**8622	35.14	1.63	Wild Type	
**9266	30.02	1.61	Wild Type	

***Surgical specimens; **Biopsy specimens

Table 3. EGFR Mutation Test Data Using DNA Extraction from Biopsy Specimens by Two Methods

Different method	EGFR mutation test success	EGFR mutation test failure	*P* value
QIAamp	6	8	0.046*
Cobas	12	2	

*Fisher's Exact Test

Results

Compared to the QIAamp method, the Cobas method could yield more DNA from surgical specimens and biopsy specimens of NSCLC tissues. The concentration of DNA by Cobas method was significantly higher than QIAamp method (Figure 1A, B), while the purity of DNA extracted by Cobas method and QIAamp method in the surgical specimens was 1.74±0.18, 1.85±0.16, respectively, there was no significant difference (Figure 1C). However, in biopsy specimens, the purity of DNA extracted by Cobas method was 1.58±0.11, significantly lower than 1.71±0.12 of QIAamp method (Figure 1D).

Although there were significant differences of DNA concentration and purity between two methods. DNA isolated from surgical specimens using the Cobas and QIAamp methods had good performance in the real-time PCR experiment to detect EGFR mutation of NSCLC patients (Table 1&2). But for biopsy specimens, Table 3 showed that Cobas method was better than QIAamp method for DNA extraction, which was used to detect EGFR mutation of NSCLC tissues. All the flags demonstrated in the table 1&2 that the Cobas and QIAamp methods could not detect any amplification signal. We found the positive rates of EGFR mutation using DNA extracted by Cobas method was 46.7% (14/30), except for two cases invalid amplification. EGFR mutation results using DNA extracted by QIAamp method were almost the same with Cobas method, only one case had no amplification signal in biopsy specimens.

In this study, Cobas method and QIAamp method are both using proteinase K to handle tissue at 56°C with tissue digestion buffer for 1h, in order to digest crosslinked mixture to release DNA, While incubating at the same time, the concentration of proteinase K in QIAamp method was 20 mg/ml, we added 20μL, a total of 0.4 mg; however, the concentration of proteinase K in Cobas method was 22.22 mg/ml, we added 70μL, a total of 1.56 mg, the amount of which was more than three times than that in QIAamp method.

Discussion

With the development of pathology, molecular pathology has become an indispensable method in clinical diagnosis and in personalized medicine (Weston and Hood, 2004), moreover, only high-quality nucleic acid can meets those requirements to be used for downstream routine molecular application (Turashvili et al., 2012). Nowadays, Tyrosine kinase inhibitors (TKIs) such as Irresa is the first-line drugs for the treatment of NSCLC, and EGFR mutation status is a prerequisite to determine whether NSCLC patients (Tsao et al., 2005; Sequist et al., 2008; Keedy et al., 2011), especially in patients with adenocarcinoma can be applied for EGFR-TKI drugs or not (Thatcher et al., 2005). However, most of pathology departments in the world, almost all of specimens are formalin-fixed, paraffin-embedded tissues. Although nucleic acids of FFPE samples are degraded into small fragments (Gilbert et al., 2007), it is essential for us to extract high-quality nucleic acids with optimum concentration and suitable purity from FFPE tissues (Lewis et al., 2001).

Since DNA molecules of FFPE samples crosslink with each other, and DNA is also crosslinking with RNA and protein. These crosslinking must be broken to release the DNA for the subsequent purification. If possible, the modification caused by chemical crosslinking should also be reversed, as the chemically modified DNA can not be recovered in the purification process, and it is also a poor substrate in other enzymatic or PCR analysis (Gilbert et al., 2007). In the surgical and biopsy specimens, we found that the amount of nucleic acid extraction by Cobas method was much more than that by QIAamp method to some extent. Because proteinase K digestion plays a major role in the DNA release, our results showed the amount of proteinase K in Cobas method was more than three times than that in QIAamp method, which maybe the main reason of Cobas method yielded more DNA, but this may aslo affect the nucleic acid peak absorption at A280 nm, resulting in a poor purity. DNA extracted by Cobas method is used to detect herpes simplex virus type 2 and chlamydia trachomatis infection in the cervical cancer, the results showed that there are no associations between herpes simplex virus type 2, or chlamydia trachomatis infection and cervical cancer (Farivar and Johari 2012; Farivar et al., 2012).

While processing tissue samples for downstream applications, we consider the methods that can yield high quality DNA and require less hands-on-time as appropriate way to isolate DNA. We performed all the experiments according to the operating instructions provided by the manufacturer without any modification. Based on our observations, both methods require nearly 3h to complete DNA extraction. In the surgical specimens, EGFR mutation using DNA extraction by both methods in the FFPE NSCLC tissues have the same results. The DNA yield from biopsy specimens using the QIAamp method was insufficient to perform the EGFR mutation assay in most of NSCLC patients, on the contrary, Cobas method performed very well in EGFR mutation test.

In summary, both Cobas and QIAamp methods can yield optimum DNA from surgical specimens for downstream applications, however, there is significant difference on purifying DNA from biopsy specimens, Cobas method is better than QIAamp method to perform EGFR mutation test. This difference should be considered in routine application of molecular pathology diagnosis.

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