

EGFR and K-ras mutation analysis in non-small cell lung cancer: Comparison of paraffin embedded versus frozen specimens

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Abstract. *Background:* Mutational analysis of the Epidermal Growth Factor Receptor (EGFR) and K-ras genes to select non-small cell lung cancer (NSCLC) patients for treatment with novel EGFR tyrosine kinase inhibitors is an appealing possibility currently under investigation. Although frozen tumor tissue would probably be the optimal source for analysis, the most common source of tumor material is fixed and paraffin embedded (FPE) archival specimens. Here, we evaluate how different procedures of tissue sample processing and preservation may affect the outcome of EGFR and K-ras mutation analysis. Furthermore, we compare the sensitivity of the analysis using genomic DNA (gDNA) versus RNA. *Methods:* We used PCR amplification and direct sequencing to analyze EGFR and K-ras genes in paired FPE and frozen tumor samples corresponding to 47 NSCLC patients. In frozen samples, the analysis was carried out using both gDNA and RNA extracted in parallel. *Results:* Whereas 100% of frozen samples were successfully amplified, the rate of successful PCR amplification in FPE samples was approximately 50%. We detected three previously described EGFR point mutations in 2 samples. In ten other samples, a K-ras mutation was observed. These mutations were detected in DNA extracted from frozen samples as well as in DNA obtained from FPE tissue. In addition, 10 nucleotide changes, were detected in FPE samples that were not detected in the frozen specimens. Upon re-analysis, these nucleotide changes could not be confirmed and were most likely the result of paraffin embedding and fixation procedures. All mutations found in gDNA were also detected in the corresponding RNA and, in two cases, the presence of the mutant allele was easier to identify by using RNA. *Conclusions:* Our results indicate that RNA extracted from frozen tissue is the preferred source for EGFR and K-ras mutation testing. When analyzing FPE samples, reducing the size of the amplified fragments would increase PCR success rate, and care should be taken to control for false-positive results.

Keywords: EGFR, K-ras, NSCLC, mutation analysis, frozen, paraffin, DNA, RNA

1. Introduction

The Epidermal Growth Factor Receptor (EGFR) plays an important role in a variety of tumor types, including non-small cell lung cancer (NSCLC) [7]. Inhibitors of EGFR tyrosine kinase activity, such as gefitinib and erlotinib, have definite activity in a subset of NSCLC patients [4]. Response rates vary between 10 to 20 percent when used as second- or third-line treat-

ment for advanced disease [3,22]. Higher responses to EGFR tyrosine kinase inhibitors (TKIs) have been seen in east-Asian patients, in women, in non-smokers and in patients with adenocarcinoma [10,17]. Tumors of the majority of the responsive patients possess somatic EGFR mutations in the kinase domain of the receptor [14,18,19]. These mutations are most commonly short deletions in exon 19 or the Leu858Arg point mutation in exon 21, and are present in 10% of Caucasian patients and 30% of NSCLC patients with east-Asian ethnicity [11]. Besides these predominant mutations, some less frequent mutations have been described, i.e. Glu709Gly, Gly719Ser, Ser768Ile and Leu861Gln [5, 9]. In vitro, these “rare” mutations were also shown to

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be related to higher sensitivity to gefitinib [2]. In contrast to EGFR gene defects, mutations in K-ras predicted primary resistance to EGFR TKIs [21]. Therefore, the use of EGFR and K-ras mutation analysis may potentially have an impact in the selection of patients to be treated with EGFR TKIs.

Although several approaches for analyzing EGFR and K-ras mutations have been described [1,9,16,20], the most commonly used approach is direct sequencing of PCR-amplified DNA from tumor specimens. Several studies have used frozen samples as a source of DNA and or RNA for mutational analysis of EGFR [5,12]. These studies used tumor tissue resected from patients with early stage NSCLC. However, in clinical practice, and particularly in patients with advanced disease, the main source of tumor samples is fixed and paraffin embedded (FPE) archival tissue blocks. Fixation procedures can result in DNA damage making these samples difficult to analyze and potentially affecting the results of the analysis [6].

In order to evaluate how different methods of tissue processing and preservation may influence the outcome of EGFR and K-ras mutation analysis, we used PCR amplification and direct DNA sequencing to analyze the mutational status of EGFR (exons 18–21) and K-ras (exons 1 and 2) in a series of paired FPE and frozen tumor samples corresponding to 47 NSCLC patients. In addition, we compared the influence on PCR success rate of sublimate formalin versus neutral buffered formalin in paraffin embedded specimens. Sublimate formalin is a mercury-containing fixative that has been used in some Pathology Departments to obtain a better morphology for immunohistochemistry purposes, but is nowadays commonly replaced by 4% neutral buffered formalin. In the frozen samples, we also compared the sensitivity of the analysis using genomic DNA (gDNA) versus RNA.

2. Materials and methods

2.1. Samples

Resected tumor samples from 47 NSCLC patients of which both frozen and FPE material was available were selected. Of these resected specimens, one piece of the tumor was snap-frozen and stored in liquid nitrogen and another piece was processed for fixation and paraffin embedding. 20 of the FPE tissues had been fixed in 4% neutral buffered formalin and the remaining 27 had been fixed using sublimate formalin,

a mercury-containing fixative. The collection date of the samples ranged from 1992 to 2002. The range for neutral buffered formalin samples is 1992–2002 and the range of the sublimate formalin is 1994–2000.

2.2. DNA and RNA isolation

For FPE samples, a 4 μm -thick section was stained with hematoxylin-eosin and examined by a pathologist (KG) to select areas of tissue containing at least 50% tumor cells. These areas were macro-dissected from 15 unstained consecutive sections of 10 μm , and DNA isolation was performed using the QIAamp DNA mini kit as indicated by the manufacturer (Qiagen, Venlo, The Netherlands). For frozen samples, cryo-sections were verified by the pathologist to contain at least 50% of tumor cells. Genomic DNA and RNA was extracted from each sample using Trizol (Life Technologies, Breda, The Netherlands). Tissue samples were homogenized in Trizol (1 ml per 50–100 mg of tissue), and chloroform (200 μl per ml of Trizol) was added. After incubation at room temperature for 3 minutes and centrifugation for 10 minutes at 12000g at 4°C, the aqueous layer containing the RNA was transferred to a new vial and the interphase was stored for subsequent DNA isolation. RNA was precipitated by addition of isopropanol and centrifugation. The pellet was washed with 1 ml 70% ethanol, air-dried, and dissolved in RNase-free water. For DNA isolation, 100% ethanol (0.3 ml per ml of Trizol) was added to the stored Trizol interphase. After mixing and incubation at room temperature for 3 minutes, samples were centrifuged for 10 minutes at 20000g at 4°C. The DNA pellet was washed twice in 1 ml 10% ethanol containing 0.1 M sodium citrate and once with 70% ethanol. Finally, the pellet was air-dried and dissolved in milliQ water.

2.3. PCR amplification and DNA sequencing

We used 100 ng of genomic DNA, derived from tumor cells, as template in nested PCR reactions to amplify DNA fragments corresponding to exons 18–21 of EGFR and exons 1 and 2 of K-ras. The PCR protocol and the sets of primers have been described in detail previously [8]. Additionally, total RNA extracted from frozen samples was used as template to amplify a single PCR fragment encompassing EGFR exons 18–21 using the primers designed by Kosaka et al. [12], FOR 5'-GGTGC GGAGAGAGGCCTGCT-3' and REV 5'-ATCTCAGAGTCCTTAACTC-3'. To this end, the Qiagen one-step reverse transcription PCR kit was

used according to the manufacturer's instructions. PCR products were purified using a presequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) and sequenced with both forward and reverse primers using the BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), with the ABI PRISMTM 3100 Genetic analyzer (Applied Biosystems). Mutations were confirmed by sequencing independent PCR products, on DNA derived from the tumor cells.

2.4. Statistical analysis

Logistic regression techniques were used to analyse the data. The generalized estimating equations (GEE) method [13] was applied to account for the variations in success rate of various DNA fragments derived from one sample.

3. Results

3.1. Rate of successful PCR amplification using genomic DNA extracted from FPE versus frozen samples

Frozen and FPE tissue samples corresponding to 47 NSCLC patients were selected based on availability. Samples were collected between 1992 and 2002. Using genomic DNA extracted from either the FPE or the frozen tissue as template, nested PCR reactions were carried out to amplify 6 separate DNA fragments encompassing EGFR exons 18–21 and K-ras exons 1 and 2. DNA extracted from frozen samples was successfully amplified in 100% of all 47 samples for each exon. The success rate of PCR amplification for DNA extracted from FPE samples however, was much lower. Amplification of EGFR exon 18 (248 bp) was successful in 43%, in exon 19 (225 bp) the percentage of successful amplifications was 72%, PCR for EGFR exon 20 (325 bp) was successful in 45% and PCR products for EGFR exon 21 (301 bp) were formed in 42% of cases. For K-ras, PCR for exon 1 (253 bp) was successful in 40% and exon 2 (295 bp) in only 19% of cases (Table 1). Thus, rate of successful PCR amplifications appeared to be, at least partially, related to the size of the product, which is as expected. These results suggest that the use of shorter amplification products may partially circumvent a low PCR success rate when working with FPE samples, which likely contain fragmented DNA. To evaluate this possibility, we designed

Table 1

Success rate of PCR on DNA derived from frozen and sublimate formalin or neutral buffered formalin fixed paraffin embedded NSCLC specimens

	Frozen	Success rate of PCR (%)	
		Paraffin	
		SF	NBF
EGFR exon 18 (248 bp)	47 (100)	20 (43)	
		6 (30)	14 (70)
EGFR exon 19 (225 bp)	47 (100)	34 (72)	
		18 (53)	16 (47)
EGFR exon 20 (325 bp)	47 (100)	21 (45)	
		7 (33)	14 (67)
EGFR exon 21 (301 bp)	47 (100)	22 (42)	
		7 (32)	15 (68)
K-ras exon 1 (253 bp)	47 (100)	19 (40)	
		5 (26)	14 (74)
K-ras exon 2 (295 bp)	47 (100)	9 (19)	
		0 (0)	9 (100)

Frozen, number of samples on which PCR was successful in frozen specimens; Paraffin, number of samples on which PCR was successful in paraffin specimens; SF, samples fixed in sublimate formalin; NBF, samples fixed in neutral buffered formalin.

a new set of primers to reanalyze K-ras exon 2. Indeed, by reducing the size of the amplified DNA fragment from 295 bp to 235 bp the success rate was increased from 19% to 61%.

3.2. Effect of different fixatives on the rate of successful PCR amplifications using DNA extracted from FPE samples

27 of the 47 FPE tissue specimens used in this study had been fixed in sublimate formalin and 20 in 4% neutral buffered formalin. Success rates of PCR in sublimate formalin (SF) fixated samples versus samples fixed in neutral buffered formalin (NBF) are indicated in Table 1. To investigate the potential effect of fixative on the PCR success rate, a logistic regression analysis was performed. The generalized estimation equations method with exchangeable working correlation structure was used to take into account the dependencies between fragments amplified from the same sample. The analysis revealed that the success rate was significantly higher in samples fixed with neutral buffered formalin than in samples fixed with sublimate formalin (75% vs. 33%, $P = 0.001$). We furthermore evaluated the in-

fluence of storage time on rate of successful amplifications but no significant effect was observed, thereby also excluding any possible confounding between storage time and type of fixative used.

3.3. Detection of mutations and single nucleotide polymorphisms (SNPs) in DNA from FPE versus frozen samples

Three previously described EGFR point mutations [9], Pro848Leu, Ser768Ile and Leu861Gln, were detected in two frozen samples (one sample contained both Ser768Ile and Leu861Gln). No results were obtained in the matching FPE samples because of PCR failure. The EGFR single nucleotide polymorphism (SNP) Arg836Arg in exon 21 was found in 4 cases. In two of these patients, PCR was successful in the corresponding FPE samples and the Arg836Arg SNP was

detected. A second SNP (Gln787Gln) in exon 20 was detected in 42 out of 47 patients using frozen samples. The SNP was also detected in the corresponding FPE samples in those twenty cases for which a successful PCR amplification of exon 20 was possible. We identified 10 nucleotide changes initially thought to be mutations in FPE samples that were not detected in the corresponding frozen sample (Fig. 1). Upon re-analysis of these samples, a wild type sequence was observed, indicating that these nucleotide changes were most probably artifacts. As detailed in Table 2, all artifactual mutations resulted from C>T or G>A transitions. A total of 10 artifactual nucleotide changes were observed in 7 samples. 5 samples harboring the artifactual changes were fixed in sublimate formalin and each contained 1 nucleotide change. Two samples were fixed in neutral buffered formalin and contained 2 and 3 nucleotide changes per sample, respectively. Therefore, the type of fixative does not appear to have a clear influence on

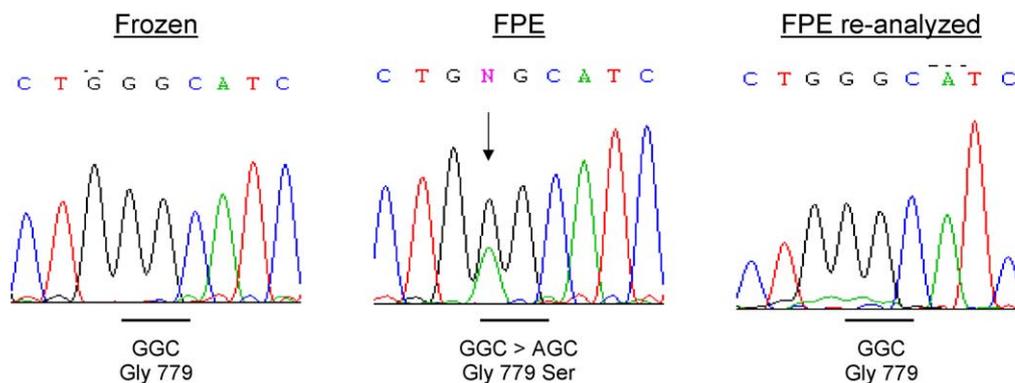


Fig. 1. Sequence chromatograms for sample #3977909 showing a wild type sequence when DNA from frozen tissue was used as template for PCR, whereas when DNA extracted from the fixed and paraffin embedded (FPE) sample was used as template for PCR, a mutation in exon 20 could be observed, Gly779Ser (arrow). Re-analyzing this fixed and paraffin embedded sample revealed a wild type sequence.

Table 2
Types of artifactual mutations detected in DNA from fixed paraffin embedded samples

#	Fixative	Artifactual mutation	Nucleotide change
7876980	Sublimate formalin	Ala 722 Val	C>T
1989395	Sublimate formalin	Cys 781 Cys	C>T
4987459	Sublimate formalin	Leu 792 Leu	C>T
0951411	Sublimate formalin	Leu 858 Leu	C>T
6123993	Sublimate formalin	Leu 858 Leu	C>T
5148421	Neutral buffered formalin	Pro 733 Ser	C>T
5148422	Neutral buffered formalin	Glu 746 Lys	G>A
3977909	Neutral buffered formalin	Gly 779 Ser	G>A
3977909	Neutral buffered formalin	Glu 804 Lys	G>A
3977909	Neutral buffered formalin	Ala 822 Thr	G>A

Ala 722 Val, Alanine to Valine amino acid change at position 722; Cys, Cysteine; Leu, Leucine; Pro, Proline; Ser, Serine; Glu, Glutamic acid; Lys, Lysine; Gly, Glycine; Ala, Alanine; Thr, Threonine; C, Cytosine; G, Guanine; T, Thymine; A, Adenine.

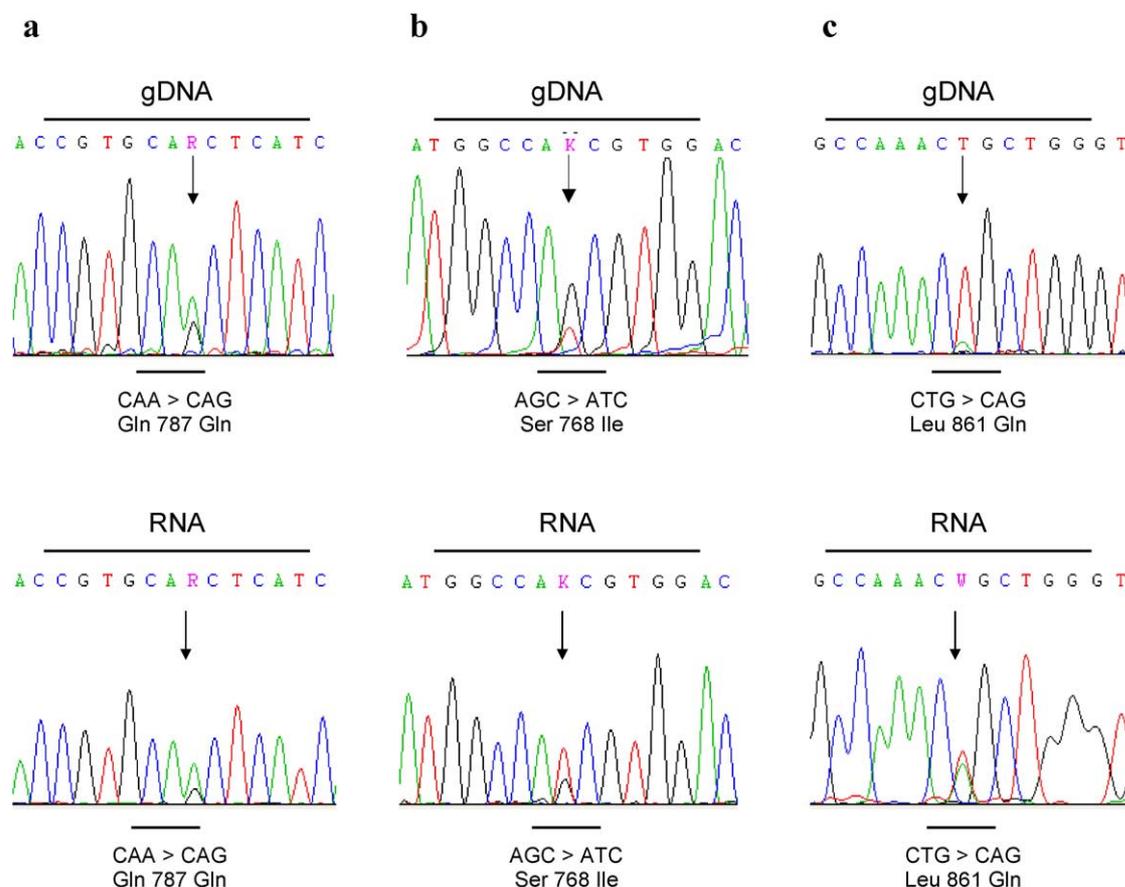


Fig. 2. Sequencing EGFR from gDNA and RNA in sample #8446712 showing the single nucleotide polymorphism Gln787Gln (a) and the EGFR point mutations Ser768Ile and Leu861Gln (b and c). Whereas the wildtype to mutant allele ratio in the polymorphism is comparable in the chromatograms from gDNA versus RNA (a – arrows), both EGFR point mutation peaks are elevated in the chromatograms from RNA compared to the chromatograms from gDNA (b and c – arrows).

presence of artifactual mutations, although the possibility of drawing statistically meaningful conclusions is hampered by the limited amount of samples. On the other hand, 7 codon 12 and 3 codon 13 K-ras mutations were detected using DNA extracted from frozen samples. These mutations were also detected in the corresponding FPE samples in the 4 cases for which a successful PCR amplification of K-ras exon 1 was possible. No artifactual mutations in K-ras were observed in DNA extracted from FPE samples.

3.4. Sensitivity of EGFR analysis using genomic DNA versus RNA

To determine whether the use of genomic DNA or RNA may influence the sensitivity of the analysis, we compared the sequencing results obtained using either

type of template. Genomic DNA and RNA were extracted in parallel from 30 frozen tissue samples using Trizol as described in the Materials and methods section. In every case, the EGFR point mutations (Pro848Leu, Ser768Ile and Leu861Gln) and SNPs (Arg836Arg and Gln787Gln) detected using genomic DNA as template were also detected using RNA. The chromatograms obtained using either template were similar in the case of the Pro848Leu mutation and the SNPs (see for SNP Gln787Gln, Fig. 2a). However, the peaks corresponding to the mutant allele for Ser768Ile and Leu861Gln were more evident in the sequencing chromatogram from RNA (Fig. 2b and c). This observation suggests that, at least in some cases, RNA would be a better template for EGFR mutation analysis.

4. Discussion

This study was conducted to compare the sensitivity and specificity of detecting EGFR and K-ras mutations in RNA and DNA from frozen and DNA from fixed and paraffin embedded (FPE) and frozen lung cancer specimens, using direct sequencing. In addition, two different types of fixatives were compared for the effect on success rate of PCR.

100% of frozen samples could be successfully analyzed whereas only ~50% of PCRs on FPE samples succeeded, which was partly correlated with PCR product size. Previous reports identifying EGFR mutations have also used paraffin embedded diagnostic specimens and could successfully amplify PCR products with a size of approximately 400 bp, although the longest PCR product we obtained was 325 bp [14,18]. We show that type of fixative was a main reason for PCR failure. In neutral buffered formalin fixed samples, the rate of successful PCR amplifications was 75% whereas only 33% of samples fixed in sublimate formalin were successfully amplified. Another factor that could have influenced the PCR success is the time of fixation. As shown by Inoue et al. [6], fixation for 2–3 days resulted in a rate of PCR failure of 56%, whereas fixation for 1 day does not appear to damage the DNA. We were unable to determine if this has been of influence in our study, since no standard fixation time is applied in our Pathology department. Although one might also expect the time of storage could influence PCR success rate, this was not of influence in our sample set.

The EGFR point mutation Pro848Leu and SNPs Arg836Arg and Gln787Gln could be detected with the same accuracy in DNA and RNA. However, two EGFR point mutations, Ser768Ile and Leu861Gln were detected with greater accuracy in RNA than in DNA. In particular the Leu861Gln mutation was almost undetectable in DNA whereas the mutant:wildtype allele distribution was ~1:1 in RNA. Since the Gln787Gln SNP in this sample showed comparable distribution of mutant and wild type alleles in RNA and DNA, this is most probably the result of elevated expression of the EGFR mutant alleles. Indeed it has been described by others that the EGFR mutant allele can be selectively amplified [23]. Thus, RNA seems to be a better source for sequencing than DNA for the detection of certain EGFR mutations. In contrast to EGFR mutations, K-ras mutations were detected with the same accuracy using DNA and RNA. This observation may stem for the fact that whereas amplification of mutated

EGFR alleles has been previously described [23], mutated K-ras alleles are not usually amplified. Tsao et al. reported the presence of 24 novel mutations in 110 patients from the BR21 study [24]. Since these mutations were not confirmed by repeated analysis, the mutations found could have been the result of mutational artifacts due to fixation and paraffin embedding. In our study, 10 novel nucleotide changes were found in FPE samples that were not observed in matching frozen samples. Upon re-amplification and repeated sequencing, these 10 novel nucleotide changes could not be confirmed. As discussed by Marchetti et al. [15], fixation and embedding can result in deamination of cytosine and adenine, leading to the generation of uracil or hypoxanthine, respectively. This may result in the artifactual detection of C>T or G>A transitions, which were exactly the types of non-reproducible changes we detected in some FPE samples. In case these nucleotide changes are indeed the result of fixation, one would expect to detect an equal amount of such artifacts on other locations upon re-analysis. We re-analyzed the samples bearing the initial nucleotide changes and did not observe any additional artifacts on other locations, which is most likely due to the smaller chance on such changes in a smaller sample set. Since these nucleotide changes were only observed in the paraffin samples and could not be confirmed by an independent analysis we assume these nucleotide changes have been the result of paraffin embedding and fixation of the samples. The number of amplifiable templates decreases with increasing product size, making these templates more vulnerable to artifactual amplifications. Of 10 artifactual nucleotide changes that were observed, indeed 5 (50%) were found in the longest product (exon 20, 325 bp). However, 2 artifactual nucleotide changes were observed in the shortest product (exon 19, 225 bp), 2 in exon 21 (301 bp) and 1 in exon 18 (248 bp). A remarkable finding is the fact that these nucleotide changes were observed only in the EGFR templates and not in K-ras. Although the total length of DNA sequence analyzed is longer for EGFR (564 bp) than for K-ras (290 bp), this is unlikely to wholly account for the difference in artifacts. Thus, there appear to be other factors, besides the size of the PCR product, which may be related to the detection of sequencing artefacts.

In conclusion, this study highlights the technical challenges in detecting EGFR and K-ras mutations and the need for a standardized fixation protocol to allow molecular studies to be completed successfully. When working with DNA derived from FPE, primers should

be designed to amplify DNA fragments as short as possible and each nucleotide change should be confirmed using an independent PCR-amplified product. For some EGFR mutations, RNA seems to be a superior source for direct sequencing than gDNA.

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