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Utilization of cell-transferred cytologic smears in detection of *EGFR* and *KRAS* mutation on adenocarcinoma of lung

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Cell-transfer technique has been proven useful for performing immunocytochemistry on fine-needle aspiration smears. However, its utility for *EGFR* and *KRAS* molecular testing has not been validated. Molecular testing was performed using the cell-transfer technique on both Papanicolaou-stained ethanol-fixed and Hema 3-stained air-dried smears from 32 fine-needle aspiration samples that had diagnoses of adenocarcinoma of the lung, and then was compared to the results of the corresponding formalin-fixed paraffin-embedded tissues. The molecular testing was successfully performed on 32 of 32 ethanol-fixed and 31 of 32 air-dried samples. The molecular results on ethanol-fixed and air-dried smears showed 100% agreement. There is 100% (32/32) agreement for the *EGFR* and 97% (31/32) agreement for the *KRAS* between the cell-transfer technique and formalin-fixed paraffin-embedded tissues. One discrepant case was due to low percentage of tumor cells on the smears. Cell-transfer technique is a reliable alternative method for *EGFR* and *KRAS* testing if the cell blocks lack adequate cellularity.
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Lung cancer exacts a considerable toll within the United States, each year claiming approximately 160 000 lives, a figure that represents more than 25% of total annual US cancer mortality.[1] Non-small-cell lung cancers, which account for 85% of all lung cancers, are often diagnosed at an advanced stage and have poor prognosis.[2,3] Adenocarcinoma represents the most common type of lung cancer, and understanding of its molecular pathogenesis has led to the development of several novel chemotherapeutic agents that offer potentially effective therapy on the basis of targeting specific genetic alterations such as mutations in the *epidermal growth factor receptor (EGFR)* and the *Kristen-Rous sarcoma virus (KRAS)*. *EGFR* mutations are found in approximately 10–15% of non-small-cell lung cancers with the highest frequency occurring in adenocarcinoma.[4–8] Lung adenocarcinomas driven by *EGFR* mutations are sensitive to tyrosine kinase inhibitors such as gefitinib and erlotinib, and these patients will have longer progression-free survival than the patients whose tumors do not contain *EGFR* mutations.[9–12] *KRAS* represents a downstream effector of *EGFR* and lung adenocarcinomas with *KRAS* mutations are refractory to *EGFR* tyrosine kinase inhibitors. The occurrence of *EGFR* and *KRAS* mutations is mutually exclusive; hence performing both markers at the same time also serves an important quality control function.[13–16] Performing molecular testing on newly diagnosed advanced staged non-small-cell lung cancers in a timely fashion has become the standard of care.[17]

Fine-needle aspiration is a safe, minimally invasive and relatively inexpensive diagnostic method, which may be used for the evaluation of both primary and metastatic lung cancer.[18] Although a diagnosis can often be made primarily based on cytomorphologic features, the utilization of formalin-fixed paraffin-embedded cell blocks prepared from fine-needle aspiration specimens is also considered a reliable source of tumor cells for the assessment of molecular abnormalities.[19] Cell blocks, however, sometimes lack adequate cellularity even in the

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face of highly cellular aspirate smears. The use of direct cytologic smears as a resource for molecular testing has been described in several publications using a variety of different methods.[20–22] The cell-transfer technique has proven to be a reliable and useful method in our institution for performing immunocytochemistry using direct cytologic smears as a source of tumor cells when the cell block lacks adequate cellularity.[23,24] We therefore set out to establish the cell-transfer technique as a viable option for isolating tumor cells for subsequent *EGFR* and *KRAS* mutation analysis *via* the currently used polymerase chain reaction (PCR)-based platforms. To validate this technique, we tested corresponding formalin-fixed paraffin-embedded tissue in addition to the direct smears including both ethanol-fixed and air-dried smears for comparison.

Materials and methods

This study was approved by the Institutional Review Board of Indiana University (Protocol number 1205008629). A computerized search of the anatomic pathology information system was performed. Fine-needle aspiration cases diagnosed as primary or metastatic adenocarcinoma of the lung, which had corresponding formalin-fixed paraffin-embedded tissues, were identified over a 48-month period. The search yielded a total of 32 fine-needle aspiration specimens. The formalin-fixed paraffin-embedded tissues included 20 lobectomy specimens, 2 biopsies, and 10 cell blocks. The Papanicolaou-stained ethanol-fixed and Protol Hema 3-stained (Fisher Scientific, Kalamazoo, MI) air-dried direct smears from each case as well as the corresponding hematoxylin and eosin-stained slides from the corresponding formalin-fixed paraffin-embedded tissues were reviewed. One ethanol-fixed, one air-dried direct smear slide, and one representative formalin-fixed paraffin-embedded block containing tumor cells were selected for molecular testing. The areas on the direct smears containing tumor cells were marked by a pathologist and photographs were taken from these areas. Both *EGFR* and *KRAS* mutational studies were performed on cells obtained from ethanol-fixed, air-dried, and formalin-fixed paraffin-embedded samples of each case. The amount of DNA from each sample was quantitated. The number of tumor cells and the percentage of tumor cells were also documented.

Cell-Transfer Technique

The cell-transfer technique was performed using clean technique as follows: (1) the coverslip was removed using fresh histologic grade xylene (Fisher Scientific, Pittsburgh, PA, USA); (2) a thin layer of Mount Quick media (Daido Sangyo, Tokyo, Japan) was spread uniformly over the top of the cellular material; (3) the slide was then placed in a 60 °C heated oven for approximately 2–3 h (or until hardened to the touch); (4) a Sharpie marker was used on the surface of the dried media to divide the slide into multiple areas of interest; (5) the slide was then placed into a clean Coplin jar of deionized water and submerged into a warm water bath at 45 ± 3 °C for 30 min to 2 h, or until the media was soft enough to easily peel away from the slide; and (6) the media was cut along the marked areas, and each cut section was placed in an Eppendorf 2.0 ml safe-lock centrifuge tube and sent for molecular testing.

DNA Extraction

DNA extraction from formalin-fixed paraffin-embedded tissue and cytologic specimens was performed using the Qiagen QIAamp DNA Formalin-fixed Paraffin-embedded Tissue Kit (Qiagen, Valencia, CA, USA). A modification from the manufacturer's recommendations was made. Samples were incubated at room temperature for 5 min with 1 ml of xylene and were then centrifuged at 15 000 r.p.m. for 5 min. Xylene was removed from the pellet and ethanol wash was then performed as recommended by the manufacturer. DNA concentration was determined using the NanoDrop Spectrophotometer.

EGFR

After DNA concentration was measured, the DNA was adjusted to approximately 10 ng/μl in distilled water. For *EGFR* mutational analysis, PCR amplified products were analyzed on the Q24 Pyrosequencer with Qiagen *EGFR* Pyro kits (Qiagen). The pyrosequencing kit tests for mutations in the exon 18 codon 719 region, deletions in exon 19, mutations in the exon 20 codon 768 and codon 790 regions, and mutations in exon 21 codon regions 858 through 861. The resulting amplicons were purified, denatured, and sequenced using mutation adjacent primers. Pyrograms were generated by the software and interpreted for the presence of mutations in the corresponding codons.

KRAS

Samples were run using the Qiagen therascreen *KRAS* RGQ PCR on the Rotor-Gene Q MDx following the manufacturer's recommendations. Genomic DNA was used to detect seven somatic mutations in codons 12 and 13 of the *KRAS* oncogene using real-time PCR on the Rotor-Gene Q instrument using both Scorpions and Amplification Refractory Mutation System technologies (Qiagen).[25–27] The somatic mutations capable of being detected were 12ALA, 12ASP, 12ARG, 12CYS, 12SER, 12VAL, and 13ASP. The reaction mixes were duplex, containing reagents labeled with FAM to detect mutant targets and HEX to detect the internal control. Overall, this

is a two-step procedure with the first step being a control assay to assess the total DNA content in a sample and the second step being both the mutation and control assays to determine the presence or absence of mutated DNA. The threshold at which the signal is detected above background signaling is called the cycle threshold. Sample delta cycle threshold values are calculated as the difference between the mutation assay cycle threshold and wild-type assay cycle threshold from the same sample. Samples are subsequently classified as mutation positive if they give a delta cycle threshold less than the stated cutoff value for the assay and above this value the sample is not detected. The data were analyzed using Rotor-Gene Q series software. Appropriate positive and negative controls were run with each sample.

The mutant allele frequencies of the *EGFR* base substitutions ranged from 0.6 to 10.7% (*EGFR* Pyro Handbook, Qiagen; September 2010; http://www.qiagen.com/knowledge-and-support). The mutant allele frequencies of the *KRAS* base substitutions ranged from 0.8% to 6.4% (therascreen *KRAS* RGQ PCR Kit, Instructions for Use (Handbook). QIAGEN. July 2012; http://www.qiagen.com/knowledge-and-support).

Results

There were 32 fine-needle aspiration specimens from 32 patients identified including 11 males and 21 females, age 36–84 years with an average of 64 years. All the cytological samples obtained using the cell-transfer technique contained more than 50-tumor cells with greater than 90% of cells being tumor cells, except for two cases: Case 1 (30–50 tumor cells, 75%) and Case 22 (<30 tumor cells, <10%). The DNA yield ranged from 0.2–77.8 μg (median, 2.8 μg) for ethanol-fixed and 0.8–60.2 μg (median 3.6 μg) for air-dried cytological samples (Table 1). In 32 of 32 (100%) ethanol-fixed and 31 of 32 (97%) air-dried cytologic samples, both *EGFR* and *KRAS* tests were successfully performed (Table 2). For *KRAS,* 15 mutations and 16 wild types, negative for mutation, showed correlation between the cytologic specimens using the cell-transfer technique and the formalin-fixed paraffin-embedded tissues. In one case, the formalin-fixed paraffin-embedded tissues showed *KRAS* mutations whereas the cell-transfer technique demonstrated wild-type result. Re-reviewing the original direct smears from this case (Case 22) showed only scant tumors cells (<10%) present in a background of

Table 1 Clinical data of EGFR mutational analysis on cytologic smears and formalin-fixed paraffin-embedded

Case no.	*Age*	*Sex*	*FNA site*	*Histology site*	*Tumor cell no.*	*% Tumor cells*	*DNA-EF (ng)*	*DNA-AD (ng)*
1	53	F	Lung RLL	Lobectomy	30–50	50–90%	10.4	7.6
2	81	M	Lung RML	Lobectomy	>50	>90	2.1	3.7
3	41	F	Lung LLL	Lobectomy	>50	>90	3.5	3.6
4	57	F	Lung LUL	Lobectomy	>50	>90	2.5	0.9
5	69	F	Lung LUL	Lobectomy	>50	>90	7.5	1.4
6	64	F	Lung RUL	Lobectomy	>50	>90	2.7	2.5
7	62	F	Lung LLL	Lobectomy	>50	>90	24.4	1.5
8	73	F	Lung LLL	Lobectomy	>50	>90	0.2	0.8
9	83	F	Lung LUL	Lobectomy	>50	>90	4.1	3.7
10	53	F	Lung RLL	Lobectomy	>50	>90	3.7	7.3
11	52	F	Lung RUL	Lobectomy	>50	>90	1.8	8.5
12	69	M	Lung RLL	Lobectomy	>50	>90	2.8	2.1
13	68	M	Lung RUL	Lobectomy	>50	>90	1.3	2.2
14	62	F	Lung RUL	Lobectomy	>50	>90	1.8	9.7
15	84	F	Lung LUL	Lobectomy	>50	>90	4.7	3.7
16	73	F	Lung RLL	Lobectomy	>50	>90	3.9	4.4
17	54	F	Lung LLL	Lobectomy	>50	>90	0.9	2.6
18	56	M	Lung RUL	Lobectomy	>50	>90	4.6	4.7
19	59	F	Lung RUL	Lobectomy	>50	>90	1.1	4.7
20	76	M	Lung LUL	Lobectomy	>50	>90	0.4	1.3
21	36	M	Lung RML	Biopsy	>50	>90	0.4	8.7
22	56	F	Lung hilar	Biopsy	<30	<10	12.9	3.4
23	53	M	Lung RLL	FNA cell block	>50	>90	1.9	1.8
24	74	M	Lung RUL	FNA cell block	>50	>90	1.4	5.2
25	45	M	LN subcarinal	FNA cell block	>50	>90	44.7	5.5
26	84	F	Lung LLL	FNA cell block	>50	>90	4.4	1.2
27	67	F	Lung RLL	FNA cell block	>50	>90	3.8	1.2
28	54	F	LN subcarinal	FNA cell block	>50	>90	2.4	0.8
29	62	F	Lung RUL	FNA cell block	>50	>90	14.8	19.7
30	59	F	LN subcarinal	FNA cell block	>50	>90	26	7.1
31	64	M	Lung RUL	FNA cell block	>50	>90	1.9	2.8
32	65	M	Lung RLL	FNA cell block	>50	>90	77.8	60.2

Abbreviations: AD, air-dried cytologic samples; EF, ethanol-fixed cytologic samples; F, female; FNA, fine-needle aspirate; LLL, left lower lobe; LML, left middle lobe; ; LN, lymph node; LUL, left upper lobe; M, male; RLL, right lower lobe; RML, right middle lobe; RUL, right upper lobe.

Table 2 Mutational results on correlated formalin-fixed paraffin-embedded tissue and cytology smears

Case no.	*EGFR (FFPE)*	*EGFR (EF)*	*EGFR (AD)*	*KRAS (FFPE)*	*KRAS (EF)*	*KRAS (AD)*
1	L858R	L858R	L858R	Neg	Neg	Neg
2	Neg	Neg	Neg	G12S	G12S	G12S
3	Neg	Neg	Neg	Neg	Neg	Neg
4	Neg	Neg	Failed	G12V	G12V	Failed
5	Neg	Neg	Neg	G12C	G12C	G12C
6	Neg	Neg	Neg	Neg	Neg	Neg
7	Neg	Neg	Neg	G12C	G12C	G12C
8	Neg	Neg	Neg	Neg	Neg	Neg
9	Neg	Neg	Neg	G12D	G12D	G12D
10	Neg	Neg	Neg	Neg	Neg	Neg
11	Neg	Neg	Neg	G12A	G12A	G12A
12	Neg	Neg	Neg	Neg	Neg	Neg
13	Neg	Neg	Neg	G12D	G12D	G12D
14	Neg	Neg	Neg	G12V	G12V	G12V
15	Neg	Neg	Neg	G12C	G12C	G12C
16	Neg	Neg	Neg	G12V	G12V	G12V
17	Neg	Neg	Neg	Neg	Neg	Neg
18	Neg	Neg	Neg	Neg	Neg	Neg
19	Neg	Neg	Neg	Neg	Neg	Neg
20	Neg	Neg	Neg	Neg	Neg	Neg
21	Neg	Neg	Neg	Neg	Neg	Neg
22	Neg	Neg	Neg	G12R	Neg	Neg
23	19 del	19 del	19 del	Neg	Neg	Neg
24	19 del	19 del	19 del	Neg	Neg	Neg
25	Neg	Neg	Neg	G12C	G12C	G12C
26	Neg	Neg	Neg	Neg	Neg	Neg
27	Neg	Neg	Neg	G12C	G12C	G12C
28	Neg	Neg	Neg	Neg	Neg	Neg
29	Neg	Neg	Neg	G12A	G12A	G12A
30	Neg	Neg	Neg	G12C	G12C	G12C
31	Neg	Neg	Neg	G12C	G12C	G12C
32	Neg	Neg	Neg	Neg	Neg	Neg

Abbreviations: AD, air-dried smears; EF, ethanol-fixed smears; FFPE, formalin-fixed paraffin-embedded tissue; Neg, negative.

abundant benign bronchial cells. For *EGFR*, 3 mutations and 29 wild types showed complete agreement between the cell-transfer technique and the formalin-fixed paraffin-embedded technique (Table 2). When the molecular tests were successfully performed, the cell-transfer technique showed 100% agreement between ethanol-fixed and air-dried cytologic specimens. Correlation between the molecular analysis on the cell-transfer technique and the corresponding formalin-fixed paraffin-embedded resection specimen revealed a positive correlation in 15/16 *KRAS* and 3/3 *EGFR* cases and a negative correlation in 16/16 *KRAS* and 29/29 *EGFR* cases with a sensitivity rate of 95% and specificity rate of 100%.

Discussion

The cell-transfer technique is a feasible method for obtaining cellular material from fine-needle aspiration direct smears for immunocytochemical stains.[23] In this study, we also confirmed its utility to yield an adequate quantity of DNA from tumor cells for the molecular testing to identify *EGFR* and *KRAS* mutations. There is high agreement rate (97%) between the fine-needle aspiration smears using the cell-transfer technique and the formalin-fixed paraffin-embedded tissue. The selection of adequately cellular areas with tumor cells is very important for assessing the correct mutational status. The one case we encountered with discordant cell-transfer technique and formalin-fixed paraffin-embedded results (Case 22), which can be explained by inadequate tumor quantity on the direct smears. Upon re-review of the photographs taken before the cell transfer, only rare malignant cells could be identified on both wet-fixed and air-dried slides. An overabundance of benign bronchial epithelial contaminant was noted in the background, most likely the source of the spurious wild-type karyotype, whereas abundant tumor in the formalin-fixed paraffin-embedded sample provided an adequate sample for detecting the G12R mutation. Excluding this case, the agreement reached 100% between the cell-transfer technique and the gold standard formalin-fixed paraffin-embedded. At least 30 tumor cells and greater than 50% of the cells submitted being tumor cells are required for the adequate materials for the molecular testing in order to prevent a false negative result.[17] All the cases in our study that met the above criteria showed 100% agreement with the corresponding formalin-fixed paraffin-embedded tissues. The higher the number of tumor cells the more accurate molecular results can be achieved. For the cases with greater than 50% of tumor cells, the sensitivity to identify mutations is 100% for both air-dried and ethanol-fixed smears. Both air-dried and ethanol-fixed smears also showed 100% correlation with the formalin-fixed paraffin-embedded cell block tissue in 10 cases. Additional study needs to be done to see how sensitive this assay can be. For clinical practice, we will perform the assay even if the concentration of tumor cells is less than 50%; however, a comment regarding the sensitivity issue will be added for the negative results. The ability to visualize and select the tumor cells through the cell-transfer technique provides an advantage over the recut of the formalin-fixed paraffin-embedded tissue, especially when the tumor cells are fewer and the surrounding normal tissues are abundant in the block, factors that may cause a false-negative result for the formalin-fixed paraffin-embedded tissue. Both air-dried and ethanol-fixed direct smears are useful as the resource for the molecular testing as long as they are adequately cellular. In one of the 32 cases (Case 4), the air-dried smears failed to perform the molecular testing through the cell-transfer technique. However, for the rest of the 31 cases in our study, both ethanol-fixed and air-dried smears show 100% agreement between these two methods for the *EGFR* and *KRAS* mutational results.

The cell-transfer technique is a very useful method for obtaining cellular material for immunostaining

and molecular testing if the conventionally prepared cell blocks lack adequate cellularity. Technically, the processing steps are not complex and either a histology or cytology technologist can easily be trained to perform the procedure. Cell transfer can be performed in any laboratory and no special equipment is necessary. Furthermore, the additional cost is relatively low and multiple immunostains as well as multiple molecular mutational tests can be performed from a single cellular smear using the cell-transfer technique. The cost of the MountQuik is approximately $20.00 per tube, which can be used for 30–50 cases and the cost of scalpel blades, pipette and slides are minimal. After the selected areas have been removed, the rest of the smears can be re-coverslipped and kept in the file for future review.

In this study, the strong correlation between molecular assays performed on fine-needle aspiration material by utilizing the cell-transfer technique, and molecular assays performed on traditional unstained recut sections from formalin-fixed paraffin-embedded tissue samples indicate that the cell-transferred cytologic smears are a reliable alternative resource for assessing *EGFR* and *KRAS* mutations of both the primary and metastatic non-small cell carcinoma of the lung. The limitations of this study are retrospective, single center study, and small numbers of the cases. A larger scale, prospective, multi-institutional study is needed for further validation of this novel, cost-effective cell-transfer technique for molecular testing on the cytologic smears.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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