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The Application of Molecular Diagnostics to Stained Cytology Smears

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Detection of mutational alterations is important for guiding treatment decisions of lung non—small-cell carcinomas and thyroid nodules with atypical cytologic findings. Inoperable lung tumors requiring further testing for staging and thyroid lesions often are diagnosed using only cytology material. Molecular diagnostic tests of these samples typically are performed on cell blocks; however, insufficient cellularity of cell blocks is a limitation for test performance. In addition, some of the fixatives used while preparing cell blocks often introduces artifacts for mutation detection. Here, we applied qClamp xenonucleic technology and quantitative RT-PCR to cells microdissected directly from stained cytology smears to detect common alterations including mutations and translocations in non—small-cell carcinomas and thyroid lesions. By using this approach, we achieved a 1% molecular alteration detection rate from as few as 50 cells. Ultrasensitive methods of molecular alteration detection similar to the one described here will be increasingly important for the evaluation of molecular alterations in clinical scenarios when only tissue samples that are small are available. *(J Mol Diagn 2016, 18: 407—415; http://dx.doi.org/10.1016/j.jmoldx.2016.01.007)*

Molecular analyses of genetic alterations are increasingly important for guiding treatment decisions. This is particularly important for guiding targeted therapies of lung adenocarcinomas that harbor epidermal growth factor receptor (EGFR) mutations, and echinoderm microtubule-associated protein-like 4 (EML4)—anaplastic lymphoma kinase (ALK) gene translocations with tyrosine kinase inhibitors (TKI),[1,2] as well as for making surgical decisions for thyroid nodules with atypical cytologic findings that possibly may harbor BRAF (B-Raf Proto-Oncogene, Serine/Threonine Kinase)-V600E mutation.[3]

Approximately 85% of lung carcinomas show glandular differentiation and more than 60% of them harbor somatic driver mutations, most commonly found in *EGFR* (23%) and the Kristen Rat Sarcoma (*KRAS*; 25%) gene.[4,5] The detection of these mutations is significant because adenocarcinomas with EGFR mutations are sensitive to single-agent TKI therapy such as gefitinib (Iressa; AstraZeneca London, London, UK) or erlotinib (Tarceva; OSI Pharmaceuticals, Farmingdale, NY), whereas carcinomas that harbor KRAS mutations do not respond to EGFR TKIs. In addition, most studies found that EGFR and KRAS mutations are mutually exclusive in patients, implying that standard chemotherapy would be the first line of treatment of choice for cancers harboring *KRAS* mutations. The most mutations in EGFR are found in exons 18 to 21, and can be classified roughly into three major categories: in-frame deletions in exon 19, insertion mutations in exon 20, and missense mutations in exons 18 to 21.[4] KRAS proteins acquire transforming potential when an amino acid at position 12, 13, or 61 is replaced as a result of a point mutation, resulting in constitutively active KRAS.[6] Several other mutations are encountered in lung carcinomas but with less frequency, such as: BRAF, approximately 3%; PIK3CA,

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approximately 3%; MET amplifications, approximately 2%; ERBB2 (Her2/neu), approximately 1%; MAP2K1, approximately 0.4%; and NRAS, approximately 0.2%.[7]

Approximately 6% of lung adenocarcinomas harbor an EML4−ALK translocation. ALK-fusion−positive lung cancers are resistant to the EGFR TKIs, gefitinib, and erlotinib, but they are sensitive to crizotinib, a small-molecule TKI against ALK.[7] The most common ALK translocations are the result of fusion between exons 13, 20, 6 of EML to exon 20 of ALK, representing more than 90% of the translocations. At least seven EML4−ALK variants (variants 1 to 7) have been identified in lung adenocarcinomas.[8] All seven variants are formed through fusion of the intracellular tyrosine kinase domain of ALK with a variably truncated *EML4* gene promoter.

The most common genetic alterations in thyroid carcinoma involve point mutations in *BRAF*, *RAS*, and *RET/PTC* translocation.[2] *BRAF V600E* point mutation, found in approximately 40% to 45% of papillary thyroid carcinomas, involves nucleotide 1799 and results in a valine-to-glutamate substitution at residue 600 (V600E).[9] Not all variants are affected equally; 60% of classic papillary, 80% of tall-cell variant, and 10% of follicular variant harbor this mutation. Its detection is clinically significant because it represents a prognostic marker for thyroid papillary carcinoma.[9] The presence of the BRAF-V600E mutation is associated with extrathyroidal extension, advanced tumor stage at presentation, and lymph node or distant metastases. BRAF V600E point mutation is also an independent predicator of treatment failure and tumor recurrence, even with patients with low-stage disease.[2]

Both lung and thyroid lesions frequently are diagnosed using only fine-needle aspiration (FNA) biopsy−obtained material. FNA is a preferred method of tissue acquisition for lung cancer patients who present with advanced disease when surgical excision of the tumor is not recommended. Thyroid lesions are sampled by FNA because they often are very vascular, and a more aggressive method of tissue collection frequently results in tissue hemorrhage. Molecular diagnostic tests of cytologic samples are performed most commonly using cell blocks.[10] However, insufficient cellularity of cell blocks often represents a limitation to the performance of these tests.[11] In addition, errors introduced by formalin fixation, which sometimes is used for the preparation of cell blocks, may interfere with accurate detection of actionable mutations. A number of strategies have been studied to minimize the effect of these artifacts on the final results.[12] Several studies have reported successful molecular diagnostic results from cytologic direct smears stained with Diff-Quick (Thermo Fisher Scientific Waltham, MA), which are air dried and fixed in alcohol.[11,13] This approach ensures that material sent for molecular testing is representative of the lesion, the mutations are accurately detectable, and there is less delay in result reporting than testing performed on cell blocks.

The increased demand for molecular testing and frequent insufficient cellular cell blocks prompted us to assess whether commonly encountered actionable mutations and translocations in non−small-cell lung and mutations associated with biological behavior in thyroid lesions could be detected using cytologic direct smears and thus improve patient care.[1] DNA and RNA were isolated successfully, directly from Diff-Quick− and Papanicolaou (PAP)−stained smears obtained from lung adenocarcinomas and thyroid papillary carcinomas, and using qClamp xenonucleic acid (XNA; Diacarta, Richmond, CA) technology detected most common mutations in *EGFR*, *KRAS*, and *BRAF* genes. By using RNA obtained from cytologic smears and a quantitative RT-PCR (RT-qPCR) approach, *EML−ALK* translocations also were detected successfully. Both of these ultrasensitive technologies are capable of detecting the presence of mutations and translocations with a 1% molecular alteration rate from as few as 50 cells, however, they cannot detect the specific base change, the insertion or deletion sequence, or the site of translocation.

Materials and Methods

Case Selection

The approval for study design and specimen utilization was obtained from the Montefiore/Einstein Institutional Review Board. The cases, represented by cytologic smears and cell blocks, as well as by tissue excisions, were selected from the archives of the Department of Pathology at Montefiore Medical Center/Albert Einstein College of Medicine. Only cases with multiple smears were included in the study to ensure that sufficient diagnostic material remained for each case after research material was microdissected from a slide. The smears were reviewed by two pathologists and the areas containing at least 50 cancer cells without necrosis and/or inflammation were marked for analysis. The tumor cells from marked areas were microdissected selectively using RNA/DNA co-purification solution (Zymo Technologies, Irvine, CA), while leaving the majority of the smear with diagnostic cells still on the slide as described.[7,8] Material was collected from 31 lung adenocarcinomas, 24 papillary thyroid carcinoma (17 classic and 7 follicular variant), and 2 follicular thyroid carcinomas.

Mutations Tested

The following mutations and translocations in lung adenocarcinomas were tested: *EGFR* point mutations in exons 20 and 21, and in-frame deletions in exon 19; *KRAS* point mutations in codons 12, 13, and 61; and *EML-4−ALK* translocation. Thyroid carcinomas were tested for the *BRAF V600E* point mutation.

RNA and DNA Purification from Smears

Smears are examined microscopically and the areas of interest containing cancer cells are microdissected from