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Lung Cancer Transcriptomes Refined with Laser Capture Microdissection

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We evaluated the importance of tumor cell selection for generating gene signatures in non–small cell lung cancer. Tumor and nontumor tissue from macroscopically dissected (Macro) surgical specimens (31 pairs from 32 subjects) was homogenized, extracted, amplified, and hybridized to microarrays. Adjacent scout sections were histologically mapped; sets of approximately 1000 tumor cells and nontumor cells (alveolar or bronchial) were procured by laser capture microdissection (LCM). Within histological strata, LCM and Macro specimens exhibited approximately 67% to 80% nonoverlap in differentially expressed (DE) genes. In a representative subset, LCM uniquely identified 300 DE genes in tumor versus nontumor specimens, largely attributable to cell selection; 382 DE genes were common to Macro, Macro with preamplification, and LCM platforms. RT-qPCR validation in a 33-gene subset was confirmatory ($\rho = 0.789$ to 0.964, $P = 0.0013$ to 0.0028). Pathway analysis of LCM data suggested alterations in known cancer pathways (cell growth, death, movement, cycle, and signaling components), among others (eg, immune, inflammatory). A unique nine-gene LCM signature had higher tumor–nontumor discriminatory accuracy (100%) than the corresponding Macro signature (87%). Comparison with Cancer Genome Atlas data sets (based on homogenized Macro tissue) revealed both substantial overlap and important differences from LCM specimen results. Thus, cell selection via LCM enhances expression profiling precision, and confirms both known and under-appreciated lung cancer genes and pathways. *(Am J Pathol 2014, 184: 2868–2884; http://dx.doi.org/10.1016/j.ajpath.2014.06.028)*

Cancers are complex, even within traditional histopathological strata. Most lung tumors have supporting stromal cells, including infiltrating inflammatory cells, fibroblasts, and neovasculature. In nonmalignant lung, perhaps an even higher degree of cellular heterogeneity is present; normal lung has more than 42 identifiable cell types. The nonepithelial components, in both carcinomas and paired adjacent nonmalignant tissues, almost certainly contribute to the transcriptomes generated with traditional tissue-block homogenization. Thus, depending on the individual tumor characteristics, these other nonmalignant and nonepithelial cell types might contribute a very substantial fraction of the transcriptome that has been reported from studies with tissue-mincing homogenization. Of the handful of transcriptome studies of refined cell capture including microdissection for procurement of a verifiably enriched malignant cell fraction in lung cancers[1–3] or focused on coupled platforms,[4] most are limited by quite small sample size and/or nonpaired tumor and nontumor samples.

We hypothesized that lung cancer signature precision could be augmented by enhanced cell selection using laser capture microdissection (LCM) of morphologically malignant cells. Reduced contamination of tumor cells by admixed supporting stroma, combined with paired nonmalignant [ie, nontumor (NT)] lung parenchymal epithelium, alveolar (NTa) or bronchial (NTb), should help refine the features of the transcriptome unique to lung cancer. We therefore microdissected

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pairs of non−small cell lung cancers (T) and their far-adjacent NT tissue. In parallel, we also performed conventional macroscopic tissue-block homogenization (hereafter referred to as Macro) on the same tissue samples for comparison purposes. In addition, microaliquots of Macro T and NT lung sample extracts in a subset were preamplified using a LCM sample preamplification procedure, to control for bias inherent to the preamplification process itself.

Materials and Methods

Patient Recruitment and Sample Collection

The study population comprised 32 consenting individuals undergoing resectional surgery for clinically suspected non−small cell lung cancer (NSCLC) under a protocol[5] approved by the local institutional review boards.

Lung tissue resection samples were visually divided into T and NT (far-adjacent to remote) in a room adjacent to the operating room and used for preparing frozen sections. The samples were snap-frozen in liquid isopentane within 15 minutes of surgical resection and were stored in a tissue bank (in liquid nitrogen at −180°C) until analysis. The assigned clinical surgical pathologist confirmed the diagnosis of lung cancer in all cases, per clinical routine, and classified the samples as adenocarcinoma, squamous cell carcinoma, or mixed adenosquamous NSCLC, according to the 1999 World Health Organization histological classification of lung and pleural tumors[6] and recent updates.[7,8] In addition, all 32 selected cases were independently reviewed again by two other pathologists (J.L. and C.Z.), each masked to prior histological diagnosis, clinical, and transcriptome data.

Tissue Accession Sets

A set of 31 paired and 2 individual unpaired samples of macroscopic homogenized NSCLC T and NT (alveolar predominant) tissue were selected for bulk macroscopic tissue sampling and microarray analyses: 19 T−NT adenocarcinoma pairs and 1 nonpaired adenocarcinoma sample; 10 T−NT squamous cell carcinoma pairs and 1 nonpaired squamous cell carcinoma sample; and 2 T−NT pairs of adenocarcinoma−squamous cell carcinoma. Of these, 17 T−NT pairs (with alveolar cells selected for NT) and 9 unpaired samples that underwent LCM met RNA quality criteria for microarray analyses: 7 pairs and 7 nonpaired samples of adenocarcinomas; 8 pairs and 1 nonpaired sample of squamous-cell carcinoma; and 2 pairs and 1 nonpaired sample of mixed adenosquamous carcinoma. Additionally, NTb samples were microdissected from six specimens.

RNA Isolation from Macro Tissue Samples

Approximately 50 to 80 mg of snap-frozen lung tissue was added to a tube containing 1 mL of extraction buffer and was completely homogenized. Further total RNA extraction procedures were performed using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations, including an optional 30 minutes DNase I treatment. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and quality was confirmed on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). An RNA integrity number (RIN) of 7 was considered the threshold for proceeding.

Preparation of Macro Homogenized Samples for Microarray

Approximately 100 ng total RNA was amplified using an Ambion WT expression kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions; 5.5 μg of amplification product was labeled with an Affymetrix (Santa Clara, CA) WT terminal labeling kit and then hybridized to Affymetrix HG Gene 1.0ST chips using Affymetrix hybridization and wash and stain kits according to the manufacturer's instructions.

LCM

For every paired (T and NT) tissue block obtained from each resection subject, a pathologist (J.L. and C.Z.) assessed a digitized hematoxylin and eosin−stained (scout) slide to determine and outline tumor nests, thereby mapping the section and directing the laser capture microscopist toward irrefutably high tumor cell content on the adjacent frozen sections. The same procedure was used for the NT block. Distinguishing tumor cells from two different types of NT lung tissue compartments [ie, NTa or NTb epithelium] could be achieved with confidence in these snap-frozen samples.

Once the hematoxylin and eosin−stained scout section was mapped, we accessed corresponding adjacent frozen lung tissue (T or NT) and re-embedded it in cold Tissue-Tek OCT optimal cutting temperature medium (Sakura Finetek USA, Torrance, CA). Three sections (12 μmol/L thick) from each frozen T and NT block were cut in a cryostat at −25°C and placed on nuclease-free and human nucleic acid−free membrane slides (Leica Microsystems, Wetzlar, Germany). The sections were stored at −80°C until use or were immediately stained with a rapid hematoxylin and eosin ethanol-based staining protocol.[5,9] Within 30 minutes of air drying, the slide was placed on the LCM stage of a Leica AS LMD instrument for microdissection. The desired cells (either alveolar or bronchial, separately) were microdissected into the cap of 200-μL PCR tubes filled with 20 μL RLT/β-mercaptoethanol buffer (Qiagen). This procedure was repeated on the next slide until a total of 1000 cells of interest had been captured for that case. The number of (adjacent) slides used per case to reach this 1000-cell threshold varied from 1 to 3, because tumor proportion varied across samples and donors.