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Analysis of paired primary lung and lymph node tumor cells: A model of metastatic potential by multiple genetic programs☆

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Abstract

Background: The current paradigm of metastasis proposes that rare cells within primary tumors acquire metastatic capability via sequential mutations, suggesting that metastases are genetically dissimilar from their primary tumors. We tested this hypothesis by examining the molecular differences, if any, between primary tumor cells and matched lymph node metastatic cells in human non-small-cell lung carcinoma specimens. *Methods:* We performed transcriptional profiling studies on malignant cells from 11 pairs of stage III tumors and their tumor-positive lymph nodes using multiple, complementary analytic techniques. To confirm the overall validity of microarray data, we used real-time polymerase chain reaction. *Results:* The molecular signature of nodal metastasis was a composite of two paradoxical, but not mutually exclusive, expression patterns: metastatic cells are: (1) different from their primary tumor cells based on a few genes and (2) genetically similar, overall, to their primary tumor cells. Consequently, we found a 27-gene subset sufficient to differentiate nodal metastatic cells from primary tumor cells. *Conclusions:* Thus, we concluded that a more accurate model of metastatic potential is based on a global primary tumor expression pattern along with the appearance of distinct metastatic variants. The 27-gene signature differentiating primary tumors from their metastatic cells may define non-small-cell lung carcinoma nodal metastatic potential.

Keywords: Non-small-cell lung carcinoma; Microarray; Expression profiling; Metastasis; Laser capture microdissection; Hierarchical clustering; Real-time quantitative RT-PCR; RNA isolation; NSCLC; Model; Metastatic potential; Lymph node metastatic cells; Multiple gene expression

1. Introduction

Lung cancer is the most common cause of cancer-related mortality in the United States. In 2003, it accounted for about 30% (163,700) of all cancer deaths, exceeding the next four cancers (breast, colon, prostate, and pancreas) combined [1]. Non-small-cell lung carcinoma (NSCLC), the most common histologic subtype, has high metastatic potential: over 70% of patients present with advanced disease [2,3]. Thoracic lymph node metastasis is a critical, independent negative prognostic factor that currently cannot be effectively treated nor prevented [4]. Yet, the molecular basis of this devastating process, whereby malignant cells from the primary tumor invade other tissues and perpetuate growth, remains poorly defined.

The current paradigm of metastasis is based on the hypothesis that most cells in a primary tumor have low

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metastatic potential, with only a very few cells acquiring enough somatic mutations to become metastatic cells [5]. Support for this classical model of metastasis is largely derived from experiments in murine models [5,6]. Highly metastatic variant cells could eventually be subcloned from human cell lines with low metastatic potential after: (1) undergoing repeated cycles of isolation from rare metastatic nodules, (2) expansion of these cells in vitro, and (3) injection of these selected cells into additional mice. However, this rare metastatic variant model has not been tested directly in human tumor tissues.

An alternative model of metastasis has been proposed on theoretical grounds: metastatic potential is encoded in the bulk of a primary tumor that has progressed to a pre-metastatic state, after which metastases may randomly occur without further gene expression changes [7]. This model of metastasis is based on the principle that only those cells with mutations conferring a growth advantage (altered oncogenes and tumor-suppressor genes) would be selected for and predominate in the primary tumor. Consequently, metastatic behavior would be governed by a global gene expression pattern shared by all cells of the primary tumor. An emerging body of microarray literature, namely in breast cancer [8,9], indirectly supports this global predisposition model, but has also introduced significant controversy.

To reconcile those two models, Hunter et al. [10] and Hynes [11] noted that they are not necessarily exclusive of one another, and proposed a more robust model of metastasis that may better explain the general mechanistic basis for the metastatic phenotype. Within the primary tumor, it is more likely that the rare metastatic gene signature be superimposed on the global predisposition gene signature in order for metastasis to proceed. It is assumed that the variant cells (which spawn metastases) share the global pattern of gene expression; they should not differ greatly from the other primary tumor cells. But currently, evidence fully supporting this third model is sparse, despite some animal data [12].

One method to directly characterize the molecular basis of cancer metastasis is to compare the genome-wide signature of metastatic cells with their corresponding primary tumor cells. Comparative analysis of lymph node metastases versus primary tumors may be confounded by the high background of lymphocytes in nodes and by the cellular heterogeneity of whole-tumor specimens. To circumvent this potential experimental bias, we employed laser capture microdissection [13] to isolate pure cell populations for further molecular studies. Our primary objective was to determine which model of metastasis was most likely valid in vivo and to better characterize NSCLC metastatic potential at the transcript level. This specific interest led us to select tissue specimens from stage III NSCLC patients. We isolated malignant cells from primary tumors and patient-matched lymph nodes for subsequent gene expression profiling. As a secondary objective, we also identified certain component genes that implied novel metastatic mechanisms.

2. Materials and methods

2.1. Tumor specimens

With approval from the institutional review boards at both the University of Minnesota and the Minneapolis Veterans Affairs Medical Center, we collected surgically resected tumor and lymph node specimens from a tumor tissue bank at our institution. Patients gave written informed consent beforehand to have their specimens stored. None of the patients had a past history of lung cancer or a concurrent malignancy, nor had they been exposed to any chemoradiotherapy. Tumor and lymph node specimens were immediately snap-frozen in liquid nitrogen. Histopathologic testing verified that all surgical tumor specimens contained malignant cells. In all, we examined a total of 22 specimens (11 tumor–lymph node pairs) from stage III NSCLC patients for our study. While this number of specimens may be considered somewhat small, we sought to maximize the relevance of the data derived from this representative group by examining only the tumor cells within each specimen, excluding contaminating stromal tissues.

2.2. Laser capture microdissection and RNA isolation

Frozen sections (8 μm thick) were prepared from tumor and lymph node specimens by standard pathologic techniques. For each specimen, prior to further processing, total RNA was isolated from one tissue section, purity was determined by spectrophotometry, and integrity was verified on ethidium bromide-stained 2% agarose-formaldehyde gels. Then, other tissue sections were stained with hematoxylin & eosin (H&E) and allowed to briefly air-dry just before microdissection. The PixCell II Laser Capture Microdissection (LCM) System (Arcturus Engineering, Mountain View, CA) was used according to the manufacturer's protocol. A board-certified surgical pathologist (S.H.T.) helped differentiate malignant cells. Areas of tumor necrosis were identified and excluded from harvesting. About 500–1000 laser pulse firings (30 μm beam diameter) were applied onto each specimen. When indicated, LCM from serial lymph node tissue sections were combined to reach the minimum required number of laser pulses. Total RNA was extracted from LCM tumor samples using the PicoPure RNA isolation kit (Arcturus Engineering), as outlined by the manufacturer.

2.3. Microarrays

The RiboAmp OA RNA amplification kit (Arcturus Engineering), based on a T7 RNA polymerase-catalyzed linear amplification method, was used in two successive rounds on all LCM-isolated total RNA samples, as outlined by the manufacturer. During the second amplification round, transcript targets were simultaneously labeled with biotin using the ENZO BioArray HighYield RNA Transcript