Overexpression of Dicer in Precursor Lesions of Lung Adenocarcinoma

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Abstract
Differential microRNA (miR) expression is described in non–small cell lung carcinoma. miR biogenesis requires a set of proteins collectively referred to as the miR machinery. In the proposed multistep carcinogenesis model, peripheral adenocarcinoma of the lung develops from noninvasive precursor lesions known as atypical adenomatous hyperplasia (AAH) and bronchioloalveolar carcinoma (BAC). The gene array analysis of BAC and adenocarcinoma showed a transient up-regulation of Dicer (a key effector protein for small interfering RNA and miR function) and PACT along with down-regulation of most genes encoding miR machinery proteins. Immunohistochemically, Dicer was up-regulated in AAH and BAC and down-regulated in areas of invasion and in advanced adenocarcinoma. A fraction of adenocarcinomas lose Dicer as a result of deletions at the Dicer locus. Expanded immunohistochemical and Western blot analysis showed higher Dicer level in squamous cell carcinoma (SCC) of the lung when compared with adenocarcinoma. Other proteins of the RNA-induced silencing complex (RISC; SND1, PACT, and FXR1) were also present at higher levels in a SCC cell line when compared with an adenocarcinoma cell line. In conclusion, the stoichiometry of miR machinery and RISC depends on histologic subtype of lung carcinoma, varies along the AAH-BAC-adenocarcinoma sequence, and might explain the observed abnormal miR profile in lung cancer. The status of the endogenous miR machinery in various histologic subtypes and stages of lung cancer may help to predict the toxicity of and susceptibility to future RNA interference–based therapy.

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Introduction
Lung cancer is the leading cause of cancer death for both men and women. Recently, adenocarcinoma became the predominant histologic form of lung cancer (1). In the proposed multistep carcinogenesis model, peripheral adenocarcinoma of the lung develops from a precursor lesion referred to as atypical adenomatous hyperplasia (AAH). AAH transforms into nonmucinous bronchioloalveolar carcinoma (BAC), which progresses into invasive adenocarcinoma, just as colon adenomas progress into colon adenocarcinomas (2).

MicroRNAs (miR) are a class of small noncoding 18- to 24-nucleotide-long RNAs that were recently implicated in the development of lung carcinoma: 43 miRs are differentially expressed between lung cancer and benign lung tissue (3).

Production and function of miR require a set of proteins collectively referred to as the miR machinery (summarized in ref. 4). Altered balance of miR machinery proteins can contribute to the development of lung cancer in a miR-guided fashion and independently of the RNA interference (RNAi) pathway. In a miR-guided fashion, the miR machinery regulates the expression of multiple tumor suppressor genes and oncogenes (5). The list of miR with known cancer gene targets continues to grow (e.g., bcl-2, c-myc, and RAS; ref. 6). Independently of the RNAi pathway, Dicer controls checkpoints in response to mutagenic stress. Dicer has been shown to regulate G1 arrest in response to nitrogen-limiting conditions and initiate the Cdc2-dependent DNA replication and DNA damage checkpoints (7).

The reduced expression of Dicer mRNA in non–small cell lung carcinoma (NSCLC) is associated with shorter postoperative survival (8). In transgenic mice, lungs that lack Dicer in the epithelium fail to branch normally and show large, fluid-filled cavities with epithelium being detached from the underlying mesenchyma (9). Another RNA-induced silencing complex (RISC) subunit, PACT, is up-regulated in small-sized adenocarcinomas (10). Therefore, it is important to characterize expression of Dicer and other endogenous miR machinery proteins in human malignancies.

Using our screening tissue microarray set and NSCLC cell lines, we observed higher levels of several RISC proteins in squamous cell carcinoma (SCC) compared with lung adenocarcinoma. Next, we examined RISC proteins along the AAH-BAC-adenocarcinoma sequence. AAH and BAC are morphologically distinct noninvasive neoplastic lesions. We employed these morphologic entities to study the timing of changes in expression of Dicer and other components of the miR machinery in lung adenocarcinoma. Our gene array analysis done on eRNA prepared from manually microdissected formalin-fixed, paraffin-embedded (FFPE) histologic sections showed a dramatic change in expression of most genes encoding proteins of the miR machinery. The up-regulation of Dicer in AAH and BAC was confirmed by immunohistochemical analysis of clinical samples. Furthermore, we show decreased level of Dicer in invasive lung adenocarcinoma. Finally, a fraction of adenocarcinomas lose Dicer as a result of deletions at the Dicer locus.

Materials and Methods
Clinical profile of cases and screening paraffin-embedded tissue microarray. Approval for this study was obtained from the Institutional Review Board. Overall, 110 lung tissue specimens were analyzed. The tissue
cRNA was prepared from fifteen studies was done with Sigma Stat (SYSTAT, Point Richmond, CA). Total RNA were used in the first-strand cDNA synthesis with T7-d(T)24 process followed the manufacturer's recommendation. Five micrograms of synthesized cDNA. The cDNA was purified through phenol/chloroform and MgOAc at 95°C in a fluidic station with low-stringency buffer (6/C 2/C 2 for 14 to 16 h. After the hybridization, cocktails were removed, and the chips were then washed in low-stringency buffer and stained with streptavidin-phycoerythrin (SAPE). This was followed by incubation with biotinylated mouse anti-avidin antibody and restained with SAPE.

Results

Expression of Dicer, FXR1, Tudor-SN, and PACT and histologic type of lung cancer. Normal bronchial respiratory epithelium showed diffuse cytoplasmic Dicer expression (Fig. 1A) and was used as an internal positive control. Alveolar epithelium was non-reactive for Dicer. Bronchial respiratory epithelium was used as an internal positive control. Interestingly, all nine cases of SCC on the screening tissue microarray showed high Dicer level independent of stage (Fig. 1B). This finding raised the question as to whether Dicer and other RISC proteins are differentially expressed in NSCLC lines. We compared levels of Dicer and three other RISC proteins (FXR1, Tudor-SN, and PACT) in NCI-H520 and NCI-H11568 lung cancer cell lines. Of note, NCI-H520 is derived from SCC of the lung. The NCI-1568 cell line is derived from a human lung adenocarcinoma metastatic to the lymph node. Western blot analysis of these cell lines incubated under routine cell culture conditions showed higher Dicer expression in NCI-H520 compared with NCI-H11568. Likewise, Tudor-SN, FXR1, and PACT were detected at a higher level in squamous carcinoma cell lines when compared with adenocarcinoma cells (Fig. 1C). Next, we decided to examine expression of miR-associated genes along the AAH-BAC-adenocarcinoma sequence.

Gene array analysis of miR machinery in lung adenocarcinoma. Using Affymetrix HGU133 x3p chips, we analyzed expression of all genes encoding proteins involved in miR maturation. Maturation of miR is summarized in Fig. 2A. At a false discovery rate of 5%, most nuclear components involved in miR biogenesis (DGCR8, POL2R2A, and XPO5) showed down-regulation in BAC (n = 4) and stage I adenocarcinoma (n = 5).

Dicer showed an up to 10-fold up-regulation in stage I lung adenocarcinoma. In addition, PACT was up-regulated 9.4-fold in...
stage I lung adenocarcinoma. Similar alterations of PACT in early adenocarcinomas of the lung have been previously shown (10).

Most of the other genes encoding RISC proteins were down-regulated in BAC and stage I lung adenocarcinoma. Some were down-regulated in stage I adenocarcinoma only. For instance, EIF2C2, EIF2C1, SERBP1, and TRBP2 were down-regulated 3.4-, 5.1-, 2.1-, and 3.4-fold, respectively. However, TNRC6B and HSPCA were significantly down-regulated in both BAC and stage I

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**Figure 1.** A, Dicer immunoreactivity in normal lung. Dicer highlights normal epithelium of the terminal bronchioli and mucus glands. Original magnification, ×100 (immunohistochemistry). B, Dicer immunoreactivity in SCC of the lung. Original magnification, ×100 (immunohistochemistry). C, Western blot analysis of Dicer, Tudor-SN, FXR1, and PACT expression in NCI-H520 (SCC of lung) and NCI-H1568 (metastatic adenocarcinoma of lung) cell lines. PCNA is used as a loading control.

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**Figure 2.** A, schematic representation of miR maturation. Genes highlighted in green are down-regulated; genes highlighted in red are up-regulated; and genes in black showed no change. B, expression of genes encoding miR machinery in bronchioloalveolar carcinoma (n = 4) and stage I lung adenocarcinoma (n = 5), at false discovery rate of 5% (fold change). C, cluster analysis in normal lung tissue, BAC, and stage I and II adenocarcinoma of the lung (n = 8). A dendrogram generated by a cluster analysis showing the separation of BAC and stage I lung adenocarcinoma from normal tissues on the basis of miR-associated genes differentially expressed between normal lung tissue and BAC and stage I lung adenocarcinoma. Stage II lung adenocarcinoma samples were similar to normal lung samples.
adenocarcinoma (summarized in Fig. 2B). The alteration of two additional RISC components (MOV10 and Tudor-SN) was less clear due to a significant discordance in trends showed by different Affymetrix ID probes for these genes. This is usually due to the presence of uncharacterized alternatively spliced products.

Surprisingly, stage II lung adenocarcinoma samples (n = 8) were similar to normal lung samples (Fig. 2C).

**Dicer overexpression in precursor lesions of lung adenocarcinoma: AAH and BAC.** Variable Dicer expression along the AAH-BAC-adenocarcinoma sequence prompted us to examine the Dicer expression level on additional full histologic sections of AAH, BAC, and invasive adenocarcinoma. AAH was highlighted by Dicer expression with an average immunoreactivity score of 1.7. Fifty-five percent of all AAH cases showed Dicer immunoreactivity ≥2 (6 of 11). Even higher levels of Dicer were seen in BAC with the average immunoreactivity of 2.2. Eighty-three percent of BAC cases showed Dicer immunoreactivity ≥2 (14 of 17; Figs. 3 and 4). All three cases of BAC with Dicer immunoreactivity <2 were of mucinous subtype.

**Dicer down-regulation in invasive adenocarcinoma.** When compared with BAC, invasive adenocarcinoma showed lower Dicer expression with the average immunoreactivity for stage I adenocarcinoma being 1.3 (n = 23). BAC component with higher Dicer level was commonly found at the periphery of the invasive adenocarcinoma. Dicer expression was further reduced in invasive adenocarcinomas of stage II (average Dicer immunoreactivity, 1.1; n = 14); however, the difference between Dicer expression in stage I and stage II adenocarcinomas was not statistically significant.

In summary, in progression from AAH through BAC to adenocarcinoma, Dicer is first up-regulated in AAH and BAC (Fig. 3A–C). With invasion, Dicer expression decreases but not to the levels seen in the normal alveolar epithelium (Fig. 3D and Fig. 4).

**LOH of the Dicer region.** Human Dicer is mapped to the subtelomeric region of chromosome 14 (14q32.13). The 14q32.13 locus is one of the most commonly altered loci in lung adenocarcinomas both in smokers and nonsmokers (12). Here, we show increasing LOH rates at 14q32.32 (D14S272) and 14q32.31 (D14S118). The LOH in these regions is seen in 62% of AAH (8 of 13), 66% of BAC (4 of 6), and 83% of stage II adenocarcinoma (5 of 6). However, markers mapped closer to the Dicer locus (D14S78 and D14S51) showed LOH only in one of six tested informative cases of BAC. None of 20 informative AAH cases and six informative cases of stages I and II adenocarcinoma showed LOH for D14S78 and D14S51.

**Discussion**

Normal alveolar epithelium is non-reactive for Dicer. Accordingly, expression analysis of adult mouse organs showed lower levels of Dicer mRNA in lung when compared with kidney, brain, testis, spleen, and liver (13).

In our analysis of clinical samples and representative cell lines, SCC of the lung showed higher Dicer expression when compared with invasive adenocarcinoma. Like Dicer, other RISC proteins (FXR1, Tudor-SN, and PACT) are present at higher levels in the SCC cell line NCI-H520 when compared with the lung adenocarcinoma cell line NCI-1568. The differential expression of RISC proteins in histologic subtypes of lung carcinoma seems to correlate with the described general trends of alterations in miR profile. Specifically, in SCC, 10 of 16 differentially expressed miRs are up-regulated, and in adenocarcinoma, 12 of 17 differentially expressed miRs are down-regulated (3).

Levels of Dicer dramatically increase in AAH when compared with normal alveolar epithelium. Further Dicer up-regulation is seen in BAC. Hence, Dicer overexpression might be a very early event in the development of peripheral adenocarcinomas of the lung. A decrease in Dicer expression seems to correlate with stromal invasion. Like Dicer, PACT is expressed strongly and diffusely in

![Figure 3.](https://cancerres.aacrjournals.org/)

*Figure 3. Dicer immunoreactivity in non-invasive AAH, BAC, and invasive lung adenocarcinoma. A, increased Dicer in AAH. Original magnification, ×200 (immunohistochemistry). B and C, Dicer highlights BAC. Original magnification, ×100 and ×200, respectively (immunohistochemistry). D, Dicer expression in invasive adenocarcinoma, stage II. Original magnification, ×200 (immunohistochemistry).*
small adenocarcinomas of the lung but not in normal alveolar epithelium (10). Similar Dicer and PACT expression is expected because it has been shown earlier that PACT may contribute to the stabilization of Dicer protein (14). The Fragile Histidine Triad, a tumor suppressor, is another protein with a similar expression pattern along the AAH-BAC-adenocarcinoma sequence (15).

We described changes in miR-associated genes and Dicer in BAC and stage I adenocarcinomas compared with normal lung tissue. The clustering analysis showed that stage II lung adenocarcinoma samples are similar to normal lung samples. Lung cancer is genetically heterogeneous with numerous secondary genetic mutations being accumulated as it progresses. In our previous LOH analysis of different microdissected areas of the same tumor, we have found tremendous i.t. heterogeneity at the loci of major tumor suppressor genes (16). This increasing heterogeneity might have precluded the detection of significant changes in levels of miR-associated genes between normal lung and stage II adenocarcinoma. However, our immunohistochemical analysis also showed lack of Dicer expression in normal alveolar epithelium and a significant decrease in Dicer expression in advanced invasive adenocarcinoma. In invasive adenocarcinoma, Dicer immunoreactivity was lower than in adenocarcinoma precursors (AAH and BAC) but still higher than in normal alveolar epithelium. Together with the undetectable level of Dicer expression in our Western Blot analysis of NCI-H1568 (a metastatic lung adenocarcinoma cell line; 30 μg of total protein extract), these data suggest a transient up-regulation of Dicer in the earliest stages of lung adenocarcinoma. This view is further supported by an up-regulation of PACT in early adenocarcinomas (10). Wong et al. have used miRNA probe microarrays with two lung cancer cell lines (A549 and NCI-H157) were treated with 5-aza-20-deoxycytidine and/or Trichostatin A, no global change in miR expression was seen, disqualifying hypermethylation and histone deacetylation as major transcriptional mechanisms controlling miR expression in lung cancer (3). However, in a separate study by Scott et al., deacetylation was thought to be an important factor capable to alter global miR levels (20).

It has been recently shown that tissue-specific mammalian miR processing can be regulated by Dicer (21). In addition, failure at the Drosha processing step explains down-regulation of miR in the P19 teratocarcinoma cell line (22).

As shown by Grimm et al. (23), short hairpin RNA (shRNA) expression in hepatocytes after i.v. infusion resulted in fatal dose-dependent liver injury. Adverse effects were most likely caused by competition of exogenous siRNA pathway with the endogenous miR pathway and saturation of Exportin-5 and one or more other components of miR machinery (23). The status of Dicer and other proteins of the miR machinery in various histologic subtypes of lung cancer and different stages of lung carcinogenesis might explain abnormalities in the miR profile of NSCLC and may help predict the toxicity of and susceptibility to future RNAi-based therapy. With the development of RNAi-based treatments, it might be crucial to learn how the changes of RISC described here affect each branch of the small RNA pathway (endogenous miR pathway and experimental, exogenous, siRNA pathway) and what modality of RNAi delivery (e.g., chemically modified siRNA versus shRNA from viral or non-viral vectors) will be most promising in a specific cancer type. Further studies are needed to predict the effect of decreased Exportin-5 and lower levels of RISC proteins in adenocarcinoma of the lung shown here on susceptibility to RNAi-based therapy and rates of RNAi-induced adverse effects.

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