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Lung Cancer Xenografting Alters MicroRNA Profile but not Immunophenotype

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Abstract

Lung tumor xenografts grown in immunocompromised mice provide a renewable source of tumor tissue for research and a means to study individualized response to chemotherapy. Critical to this utility is verification that the xenograft cells retain core phenotypic characteristics of the original tumor. We compared eight non-small cell lung carcinomas with their corresponding xenografts grown in mice with severe combined immunodeficiency by way of histology, immunohistochemistry, and microRNA expression profiling. Six of the eight xenografts closely resembled their original tumor by light microscopy. The xenografts also largely retained key immunophenotypic features. With expression profiling of human microRNAs, however, xenografts clustered separately from the original tumors. While this may be partly due to contamination by non-neoplastic human and mouse stroma, the results suggest that miRNA expression may be altered in xenografts and that this possibility should be further evaluated.

Keywords

gene expression; immunophenotype; lung carcinoma; microRNA; xenografts

INTRODUCTION

Lung cancer is a major worldwide public health issue. In the United States alone, more than 159,000 deaths resulting from the disease were reported in 2005 [1], making it the leading cancer killer. The large number of deaths from lung cancer can be attributed to both its high incidence (196,687 in 2005 [1]) and generally poor outcome.

Animal models of cancer have been used for decades to study tumor biology and test new drugs. These *in vivo* models frequently use tumors grown from human tumor cells implanted in immunodeficient animals as xenografts. We have successfully used xenografts grown in mice with severe combined immunodeficiency (SCID) to study lung and other cancers [2–5]. Fundamental to these studies is the assumption that neoplastic cells retain their phenotype after engraftment. Prior reports have demonstrated histologic and limited immunophenotypic

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similarities between patient tumors and subsequent xenografts [6,7]. Whole genome messenger RNA (mRNA) expression analysis has also shown some concordance between lung tumors and matched xenografts [7,8]. In this study we compared a group of non-small cell lung carcinomas with subsequent xenografts by histology, immunohistochemistry (IHC) and microRNA (miRNA) expression profiling.

Three IHC markers were chosen for their utility in classifying pulmonary neoplasia. TTF-1 is a marker of pulmonary adenocarcinoma and some neuroendocrine carcinomas [9], CD56 is a sensitive marker for neuroendocrine differentiation, and expression of p63 is typically limited to squamous tumors in the lung [10]. Two additional markers (E-cadherin and β -catenin) were chosen to assess the possibility that xenograft tumors show advancement of epithelial-mesenchymal transition (EMT). EMT is the process by which carcinoma cells acquire some of the properties of mesenchymal or stromal cells, and is believed to contribute to tumor invasion and metastasis [11].

MicroRNAs (miRNAs) are a subset of small (19–25 nucleotide), non-coding, single-stranded RNAs that regulate mRNA translation by interacting with mRNA transcripts to repress translation or cause transcript degradation [12,13]. Altered miRNA expression has been described in several human tumors including lung cancer [14–16], and tumors can be classified by miRNA profile [15,17–19]. Although our understanding of miRNA in malignancy is still evolving, miRNA profiling may prove more robust for tumor classification and comparison than mRNA profiling. For this reason, and given the superior stability of miRNA in formalin-fixed, paraffin-embedded (FFPE) tissue [19], we compared xenograft miRNA profiles with those from corresponding human patient tumors.

METHODS

Mouse use and care

Our use of the SCID mouse-patient tumor xenograft model has been previously described [3, 4,20–23]. Patient lung tumors are received fresh, cut into pieces approximately 2 mm $\times$ 2 mm in size, and placed subcutaneously into anesthetized SCID mice. When tumors successfully engraft and reach approximately 1 cm in diameter, they are resected for re-passage and study. The Roswell Park Cancer Institute (RPCI) Institutional Animal Care and Use Committee approved all procedures carried out in this study.

Histology

Eight of the most recent xenografts (table 1) were chosen for inclusion based on the presence of significant epithelial tumor at the site of implantation. Portions of the harvested xenograft tumors were fixed in 10% neutral, buffered formalin and embedded in paraffin. Hematoxylin and eosin- (H&E) stained sections were prepared for microscopy. H&E slides from the primary human tumors were retrieved from the Department of Pathology slide archive for morphologic comparison by a pathologist (PNB). The proportion of viable neoplastic epithelium in both xenograft and matched patient tumor blocks was recorded.

Immunohistochemistry

IHC stains using antibodies against TTF-1 (Dako®, Carpinteria, CA; clone 8G7G3/1; 1:300 dilution), p63 (Dako®; clone 4A4; 1:50 dilution), CD56 (Biocare Medical®, Concord, CA; clone BC56C04; 1:50 dilution), E-cadherin (Novocastra®, Newcastle, UK; clone 36B5, 1:50 dilution) and β -catenin (Novocastra®; clone 17C2; 1.75 μ g/mL concentration) were performed. The blocks used were the same as those used for the H&E comparison. All staining was performed on a DAKO autostainer (Dako®). Primary antibody detection utilized Envision + (Dako®) for TTF-1, p63 and E-cadherin, and anti-mouse biotinylated secondary antibody

with either streptavidin (Invitrogen®, Carlsbad, CA) for CD56 or ABC reagent (Vector Labs®, Burlingame, CA) for β -catenin. Diaminobenzidine (DAB+) was used as a chromagen, and all slides were counterstained with hematoxylin. Stains were evaluated and scored by a pathologist (PNB). For TTF-1, p63 and CD56, the percentage of cells staining and the greatest intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong) were recorded. The percentage of cells showing cytoplasmic and membranous staining was recorded separately for E-cadherin (many cells showed staining in both compartments). A single intensity score combining both compartments was recorded. β -catenin intensity and percentage scores were recorded separately for both cytoplasmic/membranous and nuclear staining. Composite scores were generated by multiplying percentage by intensity scores, and were rounded to the first decimal point.

RNA extraction

Small RNA (<100 nucleotides) enriched in miRNA was extracted from deparaffinized and proteinase K-treated FFPE tissue cores with >70% tumor tissue using High Pure™ miRNA isolation kit (Roche®, Indianapolis, IN). Concentration and quality of RNA was assessed by absorbance spectrometry on a Nanodrop™ instrument (Thermo®, Waltham, MA).

Microarray hybridization

The microarray hybridization experiment using a common reference design was performed by Exiqon® (Woburn, MA). Sample and reference RNA (0.5 μ g), generated by pooling equal amounts of RNA from all 16 samples, were 3'- or 5'-end labeled with Hy3™ (Exiqon®) or Hy5™ (Exiqon®) dyes, respectively, using miRCURY™ miRNA Power Labeling Kit (Exiqon®), and hybridized on miRCURY™ locked nucleic acid (LNA™) [24] multi-species miRNA microarray slides (11.0; Exiqon®) on an HS4800 hybridization station (Tecan®, Australia) for 18 hours at 56 °C. After hybridization, slides were scanned on a G2565BA scanner (Agilent Technologies®) using ImaGene 8.0 software (BioDiscovery®, Los Angeles, CA). The arrays had quadruplicately-spotted LNA probes against 96% and 89%, respectively, of the 885 human and 689 mouse miRNAs in the miRBase 13.0 registry [25], and against twelve human non-miRNA small RNA and 327 proprietary human miRNA sequences (miRPlus™; Exiqon®). Besides 306 mouse-specific miRNA probes, 294 of the 1262 probes against human miRNAs were known to capture mouse miRNAs.

Microarray data analysis

Raw signal intensities from the 16 hybridizations were processed together using the limma [26] package (2.17) for Bioconductor (2.3) in R (2.8.1). After background correction using the normexp [27] method with an offset of 10, values were normalized with the global Loess algorithm [28] (smoothing value, 1/3), followed by limma's 'rquantile' quantile normalization method to achieve similar empirical distributions of Hy5™ intensities across all arrays. For summarization of Hy3™ and Hy5™ intensities, and their ratios (fold-change values), mean of four probe-spot values was used when the maximum was <150% of minimum; else, the median was used. Data from only the 1580 probes against human or mouse RNAs was considered from this point onwards. miRNAs detected by 1169 probes yielding Hy5™ intensities more than 50% of the within-array inter-quartile range in all samples were considered expressed. Wilcoxon rank sum (Mann Whitney) test was used to identify differentially-expressed miRNAs. Log₂-transformed fold-change values were used for further analyses using Prism™ (5.1; GraphPad®, La Jolla, CA) and MultiExperiment Viewer (4.3) [29] software. Un-centered Pearson correlation and average linkage distance metrics were used for clustering.

RESULTS AND DISCUSSION

Recent evidence suggests that gene expression is altered when neoplastic cells are grown in cell culture, and that these changes are not seen in xenografts [8]. While a xenograft may be more stable than cell culture, it does not provide an exact replica of the original tumor. Depending on anatomic placement, the xenograft may be in an environment that is significantly different from its origin (our lung xenografts were grown subcutaneously). Furthermore, during engraftment and growth the xenograft is progressively infiltrated by stromal cells from the host animal. These changes in the microenvironment may alter the cancer phenotype.

The histology of our eight original patient tumors and their corresponding xenografts is detailed in table 1. Three of the xenografts were from at least the fifth passage (cases 1, 4 and 6). The remaining five xenografts were from the first passage. Six xenografts (75%) showed H&E histology that was very similar to corresponding human tumors (examples are provided in supplementary figure 1), and maintained the light microscopic features that aid classification into standard types of non-small cell carcinoma. Notably, all three of the xenografts passaged more than once still closely resembled their matched original tumors. Two xenografts (25%, cases 3 and 5) did not resemble their source tumors as closely and were difficult to subtype by H&E appearance alone. These two xenografts, however, showed IHC staining profiles that were nearly identical to those of their source tumors.

Overall, most xenografts were very similar to matched patient tumors by IHC staining (table 2 and supplementary figure 1). Most exceptions to the staining consistency were minor, since score differences of <1.0 are likely insignificant at this sample size. Case 5 showed minimal TTF-1 staining that disappeared in the xenograft. Case 4, a neuroendocrine carcinoma, showed a slight increase in xenograft staining for TTF-1, but consistent staining with CD56. Two of the adenocarcinomas (cases 2 and 6) showed weak CD56 xenograft staining that was absent in the original tumor. The most significant immunophenotypic discrepancy was seen in case 7, where the xenograft showed a loss of p63 staining.

E-cadherin and β -catenin showed generally weak staining (table 3). Most of the xenograft tumors showed increased membrane E-cadherin staining, but it is loss of membrane E-cadherin that is normally associated with EMT [11]. Little difference between original tumors and grafts was seen with β -catenin. Nuclear translocation of β -catenin is associated with EMT [11], and most of the tumors showed no nuclear staining. Case 3 was the only example demonstrating nuclear β -catenin, and that score was the same in both primary and graft tumors.

These findings essentially confirm the morphologic and immunophenotypic stability reported by other groups [6,7]. Any changes in the microenvironment do not seem to have significantly altered the core phenotypic characteristics we examined, and our IHC panel provided no evidence of EMT. While important, these features assess only a minute portion of the overall tumor phenotype. Genome-wide profiling such as that accomplished by mRNA or miRNA expression analysis provides a much broader assessment of cellular activity. To quantitate expression of miRNAs, small RNA was extracted from FFPE tissue. RNA sample absorbance ratios varied from 1.77 to 1.96 (mean, 1.86; SD, 0.06) at 280 nm and 260 nm, and from 1.16 to 2.19 (mean, 1.76; SD, 0.26) at 260 nm and 230 nm. Hy3TM-labeled RNA samples were hybridized on two-color, multi-species microarrays in a reference design using RNA pooled from all 16 samples as the Hy5TM-labeled reference RNA. Many of the array probes were not species or miRNA-specific.

As shown in figure 1A, data for the 224 miRNAs whose capture probes were designated mouse-specific by the array manufacturer demonstrates the presence of mouse RNA in the xenograft samples. Of the expressed human miRNAs, 708 had capture probes annotated by the manufacturer as not recognizing mouse miRNA. Unsupervised hierarchical clustering of the 16

samples based on expression levels for these 708 miRNAs is shown in figure 1B. Distinct separation of the primary tumors from the xenografts, and of paired primary tumors and xenografts, can be seen. To our knowledge, this is the first time a difference such as this has been reported in lung cancer xenografts.

Using the non-parametric Mann Whitney test, 121 of the 708 miRNAs (17%) were considered differentially expressed at a critical P value of 0.01; P values were <0.002 for 22 (table 4). A plot of the top two principal components (contributing to 91.1% and 2.4% of the variance) using median centering is shown in figure 1C. A heat map of the relative expression values for the 121 significant human miRNAs is provided in figure 1D. There was poor correlation between the primary tumors and their corresponding xenografts for expression levels of both the 22 most significant human miRNAs (Spearman correlation coefficient range, -0.754–0.241; mean, -0.295), and the 121 differentially expressed human miRNAs (Spearman correlation coefficient range, -0.776–0.362; mean, -0.325).

A recent study [7], found that 9 of 17 (53%) primary lung tumors clustered with corresponding xenografts in an mRNA expression analysis. Those 9 tumor samples all reportedly contained >25% tumor tissue. Of the 8 primary tumors that did not cluster with their respective xenografts, 5 had <10% tumor tissue. Dilution of neoplastic cells by stromal and inflammatory cells is a critical issue in expression analysis. In most methods harvested RNA comes from the entire pool of viable cells, and the profile from neoplastic cells can be obscured by other elements.

Our miRNA analysis attempted to minimize the influence of non-neoplastic cells in a number of ways. Only one primary tumor paraffin block (case 4) and one xenograft block (case 7) contained <25% viable tumor, and miRNA in our study was isolated from cores specifically chosen to optimize tumor yield. Despite these efforts, some non-neoplastic tissue (human and/or mouse) remained in our samples. This is illustrated by the increased expression of many known murine miRNAs in the xenograft tumors (figure 1A). To further minimize the impact of murine cells on our clustering analysis, we excluded known murine-reactive miRNA probes. Although we cannot be sure that we have identified all of these probes, analysis of xenograft samples could theoretically provide a purer image of the cancer phenotype since contaminating human stromal and inflammatory cells are eventually removed from the equation after engraftment and we are ignoring mouse miRNAs.

Although morphologic and IHC data suggest that non-small lung cancer xenografts maintain much of their original phenotype, we found that miRNA expression was altered in xenografts specimens. It seems likely that some of the separation seen in miRNA clustering analysis reflects variation in tumor stroma. Furthermore, many of the miRNA species in our study are of uncertain function, and it is possible that the differentially expressed miRNAs do not significantly impact carcinoma phenotype. Nonetheless, we cannot exclude the potential for miRNA mediated translational alterations that could impact the use of xenografts in research. Interestingly, the sole graft in our analysis that clustered close to its original patient tumor was case 1. This xenograft was from the fifth passage, suggesting that changes in miRNA expression are not necessarily increased by multiple passages. It is also worth noting that the case 1 tumor was well differentiated. Intuitively this suggests that less differentiated tumors may be more labile and unstable, and prone to changes in genome regulation. Case 1 also had a high percentage of viable neoplastic cells.

These findings highlight the need for interpretative caution when performing expression analysis with samples of low cellularity or those from xenografts. We must, however, recognize the possibility that lung cancer xenografts are not identical to their original source tumors. The significance, if any, of altered xenograft genome regulation via miRNA warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

[A] Heat-map showing mouse miRNA expression in RNA prepared from xenograft tissue. 53 (31%) of 224 mouse-specific miRNAs (Wilcoxon rank sum test, P value of 0.01) were differentially expressed between xenografts (X1–X8) and primary tumors (H1–H8). Log₂-transformed fold-change values with respect to reference RNA are depicted on bottom color scale. Dendrogram on the left shows miRNA hierarchical clustering. Because reference RNA contains xenograft RNA and there is possible cross-miRNA and cross-species reactivity on the arrays, some mouse miRNAs appear to be expressed in the primary tumors.

[B] Unsupervised hierarchical clustering of the samples based on the expression of 708 human miRNAs detected by probes annotated as human-specific.

[C] Principal component analysis of the primary tumors and xenografts based on fold-change values for the 121 human miRNAs differentially expressed between the two tumor-types.

[D] Heat-map showing differential expression of human miRNAs between primary tumors and xenografts. 121 (17%) of 708 miRNAs (Wilcoxon rank sum test, P value of 0.01) detected using human-specific probes were differentially expressed between xenografts and primary tumors.

Table 1
Primary tumor and xenograft characteristics. Degree of differentiation is well (WD), moderate (MD), moderate to poor (MD – PD) or poor (PD). Tumor types are adenocarcinoma (ACA), neuroendocrine carcinoma (NEC) and squamous carcinoma (SqCC).

Case No.	Original Patient Tumor				Corresponding Xenograft Tumor			
	Degree of Differentiation	Tumor Type	Features	Viable Cancer (%)	Degree of Differentiation	Tumor Type	Features	Viable Cancer (%)
1	WD	SqCC		50	WD	SqCC		70
2	MD - PD	ACA	Solid and acinar patterns	40	MD	ACA		25
3	PD	ACA	Focal neuroendocrine	30	PD	Non-small cell		40
4	PD	NEC		20	PD	NEC		30
5	MD	ACA	clear cell	25	PD	c/w NSCLC		25
6	MD - PD	ACA		25	PD	ACA	Signet ring	80
7	MD	SqCC		25	MD	SqCC		15
8	MD - PD	ACA	Bronchoalveolar, signet ring	40	PD	ACA	Signet ring and mucinous	90

Expression of TTF-1, CD56 and p63 in primary tumors and xenografts. Composite expression scores reflect both staining intensity and fraction of positive cells.

Case No.	Original Tumor Type	Xenograft Passage No.	TTF-1		p63		CD56	
			Original Tumor Score	Xenograft Score	Original Tumor Score	Xenograft Score	Original Tumor Score	Xenograft Score
1	SqCC	5	0.0	0.0	2.7	2.9	0.0	0.0
2	ACA	1	0.6	1.2	0.0	0.1	0.0	0.7
3	ACA	1	2.7	2.7	0.0	0.0	0.0	0.2
4	NEC	5	1.4	2.4	0.0	0.0	2.7	2.7
5	ACA	1	0.3	0.0	0.0	0.0	0.1	0.2
6	ACA	7	0.0	0.0	0.0	0.0	0.0	0.7
7	SqCC	1	0.0	0.0	1.6	0.3	0.0	0.1
8	ACA	1	0.0	0.0	0.0	0.0	0.1	0.1

Expression of E-cadherin and β -catenin in primary tumors and xenografts. Composite expression scores reflect both staining intensity and fraction of positive cells.

Case No.	Original Tumor Type	E-cadherin			β -catenin		
		Membrane Score		Cytoplasmic Score		Cytoplasm/Membrane Score	
		Original Tumor	Xenograft	Original Tumor	Xenograft	Original Tumor	Xenograft
1	SqCC	2.9	2.9	0.2	0.2	1.9	3.0
2	ACA	0.8	2.1	0.5	0.2	1.8	1.8
3	ACA	0.9	0.6	0.1	0.3	1.4	1.6
4	NEC	0.1	0.6	1.2	2.1	1.8	2.7
5	ACA	0.4	0.6	0.1	0.1	0.7	0.7
6	ACA	0.1	0.8	0.0	0.1	0.7	0.4
7	SqCC	0.0	0.4	0.3	0.8	1.8	1.8
8	ACA	0.1	0.8	0.1	0.0	0.0	0.0

Table 4

Human miRNAs expressed differentially between primary tumors and xenografts.

miRNA ¹	Fold-change ²	P value ³
hsa-miR-1275	0.1	0.00078
hsa-miR-198	0.1	0.00078
hsa-miRPlus-C1115	0.2	0.00078
hsa-miRPlus-E1151	0.3	0.00078
hsa-miRPlus-F1050	0.3	0.00078
hsa-miRPlus-F1066	0.3	0.00078
hsa-miRPlus-F1202	0.1	0.00078
hsa-miR-147	1.2	0.00113
hsa-miR-337-3p	1.2	0.00113
hsa-miR-766	0.3	0.00113
hsa-miRPlus-E1075	0.3	0.00113
hsa-miRPlus-E1194	0.8	0.00113
hsa-miRPlus-F1160	0.9	0.00113
hsa-miRPlus-F1225	0.3	0.00113
hsa-miR-483-5p	0.1	0.00163
hsa-miR-525-5p	0.2	0.00163
hsa-miRPlus-A1015	2.7	0.00163
hsa-miRPlus-A1027	0.1	0.00163
hsa-miRPlus-A1056	0.3	0.00163
hsa-miRPlus-F1086	0.4	0.00163
hsa-miRPlus-F1136	0.3	0.00163
hsa-miRPlus-F1227	0.5	0.00163

¹ miRPlus miRNAs are not registered in miRBase.² Inter-group fold-change values are calculated from median fold-change values relative to a reference RNA.³ Wilcoxon rank sum (Mann Whitney) test.