Abstract

Purpose: There is accumulating evidence that microRNAs may function like classic tumor suppressor genes but little is known about their mechanism of inactivation in cancer cells. We investigated whether somatic mutations are a common mechanism of inactivation of microRNA genes in ovarian cancer.

Experimental Design: Ten cancer-implicated microRNA genes were analyzed for somatic mutations in 90 ovarian epithelial cancers and matching normal DNA. High-resolution melt analysis and bidirectional sequencing was used to detect sequence variations.

Results: High-resolution melt analysis and direct sequencing did not identify any somatic mutations but did reveal numerous novel and previously reported germ line base substitutions, deletions, and insertions surrounding the mature microRNA sequences. The majority of variants were detected in the same proportion of non–cancer control individuals suggesting that they do not represent ovarian cancer–predisposing alleles.

Conclusion: The absence of somatic mutations in any of the 10 cancer-implicated microRNAs in our large cohort of ovarian tumors suggests that this may be an uncommon mechanism of inactivation of microRNAs in ovarian cancer.

Over the past decade, striking advances have been made in technologies that identify tumor-specific regions of genomic gain or loss. These aberrations are believed to be the hallmarks of important cancer-suppressing or cancer-promoting genes at those locations. To identify the responsible gene, many investigators have relied on identifying somatic activating or inactivating mutations, and more recently, epigenetic changes surrounding gene promoters. Although there have been successes, in many cases, the identity of the target gene has remained elusive. This outcome has presented a conundrum because in many instances there is strong evidence that such genomic aberrations should be reliable markers of cancer genes. Recently, a new paradigm in gene regulation has emerged which might explain, at least in part, the failure of past cancer gene mapping studies. Increasing evidences are emerging that microRNAs, which are small noncoding regulatory RNA products, are abnormally expressed in many cancer types (1). The fact that a large proportion of microRNA genes are also located in genomic regions that are altered in cancer (2, 3) suggests that they may behave as classic tumor suppressor genes and oncogenes. If this is true, then one would expect microRNA genes to accumulate somatic mutations in the same manner as conventional cancer-promoting genes. However, very few studies have included microRNA genes in mutational analyses, so the extent to which microRNA genes are targeted as classic tumors suppressors is unclear (4–7).

To gain a better understanding of the contribution to carcinogenesis of somatic mutation in microRNAs, we have investigated 10 key microRNA genes that have been implicated as having a tumor-suppressive role in one or more cancer types (Table 1; refs. 4, 8–20). These microRNAs were screened for somatic mutations that might affect the production of the mature transcript, including regions involved in the initiation of microRNA transcription and posttranscriptional processing. Ovarian cancers were studied as a model system because this is one of the cancer types in which extensive genomic alterations exist (21) but few of the target cancer genes have been identified.

Materials and Methods

Clinical samples and DNA extraction. Ovarian cancer biopsies were obtained from women undergoing surgery for primary ovarian cancer at hospitals in the south of England (22). Tumor DNA was extracted from fresh-frozen tissue. Representative sections were H&E-stained and all tumors were assessed to contain >60% tumor epithelium. Normal DNA was extracted from matching peripheral blood samples, as described previously (23). In total, 90 ovarian malignancies were analyzed and comprised 42 serous, 21 endometrioid, 15 mucinous, 2 clear cell,
Translational Relevance

MicroRNA expression is frequently altered in cancers, suggesting that they may represent a novel class of cancer-promoting gene. Classic tumor suppressor genes are frequently inactivated in cancer through somatic inactivating mutations but it is currently unclear if this is also true for tumor-suppressive microRNA genes. Understanding the molecular basis of aberrant microRNA expression in cancer will clearly affect the extent to which these genes can be used as clinically useful diagnostic or prognostic markers and/or novel therapeutic targets. In this study, we show that somatic mutations in microRNA genes are rare in ovarian cancers, and couple with data from other studies, it can be reasonably concluded that this is an uncommon mechanism of inactivation of microRNA genes in primary human cancers.

and 10 undifferentiated tumors. The controls (n = 90) represent the population from which the ovarian cases arose and consisted of Caucasian female volunteers who were either staff at the Princess Anne Hospital, Southampton, UK or patients attending for nonneoplastic disease conditions. The control and cancer groups were drawn from the same geographic area, which has a predominantly Anglo-Saxon population (24).

Appropriate institutional ethics committees approved the collection and use of tissues for this study, and ethics approval for this project was obtained from the Peter MacCallum Cancer Centre, where the molecular studies were carried out.

Detection of microRNA mutations. Primer pairs were designed to allow the analysis of two overlapping amplicons surrounding the mature microRNA sequence. Mutation detection was done using high-resolution melting analysis. PCR and melt profiling were done in a LightCycler480 (Roche Diagnostics) using a 96-well plate high-resolution melting analysis. PCR and melt profiling were done to allow the analysis of two overlapping amplicons surrounding the mature microRNA sequence. Mutation detection was done using high-resolution melting analysis. PCR and melt profiling were done in a LightCycler480 (Roche Diagnostics) using a 96-well plate format. Reactions (10 μL total volume) were prepared as follows:

PCR buffer containing 1.5 mmol/L of Mg2+ (Qiagen), 200 μmol/L of each deoxynucleotide triphosphate, 300 nmol/L of specific primers (Table 2), 2.5 μmol/L of Syto9 dye (Idaho Technology), 0.25 units of HotStarTaq DNA polymerase (Qiagen), and 15 ng of genomic DNA.

PCR was done at 94°C for 10 min, followed by 50 to 65 cycles of 94°C × 30 s, 65°C × 30 s, and 72°C × 30s, at the end of which the temperature was raised to 94°C for 10 s followed by rapid cooling to 40°C.

Melt analysis was done using LightScanner software (Idaho Technology). Melting profiles were generated when the specific PCR amplicons were heated from 70°C to 97°C. Melting data was normalized, temperature shifted, and displayed as melting curves and subtractive difference plots.

Sequence analysis. Genomic DNA was amplified by PCR (primer sequences in Table 2) from those ovarian tumor biopsies in which a shift in melt profiles was detected, and was subjected to bidirectional sequencing using the BigDye terminator sequencing method (Applied Biosystems) on an autosequencer (ABI 3130, Applied Biosystems).

Results and Discussion

Ninety epithelial ovarian cancers were assessed for somatic mutations in an ∼500 bp genomic region surrounding each mature microRNA, which includes the binding sites for posttranscriptional processing by the RNases, Drosha and Dicer, as well as covering potential upstream regulatory sequences. Matching normal DNA was available for all tumors, which enabled us to determine whether the genetic variation detected was somatic or germ line. Despite analyzing a large cohort of primary tumors, no somatic mutations were detected in the genomic region corresponding to the primary, precursor, or mature microRNA in any of the 10 cancer-implicated microRNA genes studied. If somatic mutation were a common mechanism of inactivation/down-regulation of microRNAs, one might have expected to have observed at least

Table 1. Genomic location and role of the candidate microRNA in cancer

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Genomic location</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7a-2</td>
<td>11q24.1</td>
<td>Ras regulation</td>
<td>(8)</td>
</tr>
<tr>
<td>let-7a-3</td>
<td>22q13.31</td>
<td>Ras regulation</td>
<td>(8)</td>
</tr>
<tr>
<td>let-7b</td>
<td>22q13.31</td>
<td>Ras regulation</td>
<td>(8)</td>
</tr>
<tr>
<td>mir-10b</td>
<td>2q31.1</td>
<td>Increased expression with increasing metastatic potential</td>
<td>(9)</td>
</tr>
<tr>
<td>mir-125b-1</td>
<td>11q24.1</td>
<td>Down-regulated in breast cancer and ovarian tumors</td>
<td>(11, 20)</td>
</tr>
<tr>
<td>mir-125b-2</td>
<td>21q21.1</td>
<td>Down-regulated in breast cancer</td>
<td>(11, 12)</td>
</tr>
<tr>
<td>mir-143</td>
<td>5q32</td>
<td>Down-regulated in colorectal cancer</td>
<td>(13)</td>
</tr>
<tr>
<td>mir-145</td>
<td>Down-regulated in colorectal cancer</td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>mir-200c</td>
<td>12p13</td>
<td>Implicated in epithelial to mesenchymal transition</td>
<td>(16)</td>
</tr>
<tr>
<td>mir-206</td>
<td>6p12.2</td>
<td>Somatic mutation in pre-microRNA in chronic lymphocytic leukemia</td>
<td>(4)</td>
</tr>
</tbody>
</table>
some mutations because the majority of the microRNA genes we analyzed have been specifically implicated in ovarian tumorigenesis (20, 25). In addition, many of these microRNA genes are located in genomic regions frequently showing loss of heterozygosity in ovarian and other cancers (2, 3), adding further weight to the expectation that some of these genes should harbor somatic mutations. For example, within our sample set, loss of heterozygosity at the let-7a-3/let-7b and mir-143/mir-145 loci was detected in 50% and 22% of cases, respectively (data not shown). The absence of somatic mutations is unlikely to be due to the mutation detection method because high-resolution melting is emerging as one of the more robust and sensitive methods available (26). In addition, we were able to detect many germ line base substitutions, deletions, and insertions in 5’ and 3’ sequences surrounding the precursor sequences (Table 3). A summary of the sequence variants detected by us and others, and their location relative to the pre-microRNA and mature microRNA sequence, is presented in Fig. 1.

Of the 10 microRNA genes investigated, let-7a-2, mir-145, and mir-200c did not harbor any detectable germ line variants in the cohort studied. Many of the variants detected are annotated single nucleotide polymorphisms, but three novel variations were identified in mir-10b and mir-143.

Table 3. Variations in the genomic sequences of microRNA genes in patients with ovarian cancer

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Previously reported variation*</th>
<th>Variant</th>
<th>Frequency in cases †</th>
<th>Frequency in controls †</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7a-3</td>
<td>rs738560</td>
<td>G-177A</td>
<td>15/90</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>rs731085</td>
<td>C+63G</td>
<td>2/90</td>
<td>nt</td>
</tr>
<tr>
<td>let-7b</td>
<td>Diederichs and Haber (5)</td>
<td>DelC-26</td>
<td>2/90</td>
<td>1/90</td>
</tr>
<tr>
<td></td>
<td>Diederichs and Haber (5)</td>
<td>G+29C</td>
<td>4/90</td>
<td>4/90</td>
</tr>
<tr>
<td>mir-10b</td>
<td>rs1867863</td>
<td>G-61T</td>
<td>41/90</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>Novel</td>
<td>T+7A</td>
<td>1/90</td>
<td>1/90</td>
</tr>
<tr>
<td>mir-125b-1</td>
<td>rs2081443</td>
<td>T+194G</td>
<td>8/90</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>Novel</td>
<td>T+175A</td>
<td>1/90</td>
<td>nt</td>
</tr>
<tr>
<td>mir-143</td>
<td>rs13158382</td>
<td>G-91A</td>
<td>18/90</td>
<td>25/90</td>
</tr>
<tr>
<td></td>
<td>Novel</td>
<td>C-54T</td>
<td>1/90</td>
<td>0/90</td>
</tr>
<tr>
<td>mir-206</td>
<td>rs17578796</td>
<td>C+77T</td>
<td>3/90</td>
<td>3/90</td>
</tr>
<tr>
<td></td>
<td>Novel</td>
<td>C+65T</td>
<td>3/90</td>
<td>nt</td>
</tr>
</tbody>
</table>

Abbreviation: nt, not tested.

*Single nucleotide polymorphisms in amplicons were identified from Ensemble Genome Browser (release 45).
†These numbers represent the number of individuals with the heterozygous or minor homozygous genotype.
Fig. 1. A summary of variations detected in the genomic sequence surrounding candidate microRNA. Novel variations detected in this study (red), alterations observed here that have been reported in previous studies (4–7) or SNP databases (Ensemble Genome Browser, release 45; blue), and variations that were not detected in this study (gray). Genes are represented in the 5′ to 3′ orientation and the positions of the variations are reported with respect to the precursor microRNA molecule. No variations have been reported or detected in this study in mir-200c. * These variations were detected in cell lines and therefore those not detected in this study might represent somatic mutations; ‡, reported as a somatic mutation in chronic lymphocytic leukemia (4).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

18. Adams BD, Furneaux H, White BA. The microribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERα) and represses ERα messenger RNA and protein expression in breast cancer cell lines. Mol Endocrinol 2007;21:1132–47.
Genetic Analysis of Cancer-Implicated MicroRNA in Ovarian Cancer


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