Inherited polymorphisms in the RNA-mediated interference machinery affect microRNA expression and lung cancer survival

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MicroRNAs (miRs) are small non-coding RNAs that bind to the target transcript in the 3′-UTR and can inhibit the translation of proteins and destabilize their target mRNA (Baek et al, 2008). miRs are predicted to regulate ~30% of the human genome (Lewis et al, 2005) including genes in stress resistance, fat metabolism, cell proliferation and apoptosis pathways (Ambros, 2003). Polymorphisms in miR genes or in genes involved in miR biogenesis may affect miR-mediated cell regulation (Mishra and Bertino, 2009). Clague et al (2010). miR biogenesis includes generation of a primary transcript (pri-miR) under RNA polymerase II (PolR2A); excision of a stem-loop structure by the nuclear RNase III enzyme (Drosha) to generate the pre-miR; transportation of the pre-miR to the cytoplasm and processing by another RNase III enzyme (Dicer) into a ~22-base mature duplex RNA (Bartel, 2004). An alteration in any step during the maturation process could affect miR production. Impaired miR processing and maturation has been shown to enhance cellular transformation and tumourigenesis (Kumar et al, 2008). Given the mounting evidence implicating miRs in lung cancer development and progression (Yanaihara et al, 2006; Kumar et al, 2008; Landi et al, 2010), we investigated the role of single-nucleotide polymorphisms (SNPs) in the RNA-mediated interference machinery involved in miR maturation in lung cancer.

MATERIALS AND METHODS

We performed SNP genotyping and miR expression profiling using blood and tumour tissue samples from the Environment And Genetics in Lung cancer Etiology (EAGLE) study (Landi et al, 2010), including 2100 primary lung cancer cases and 2120 population controls, frequency matched on age, sex and residence, all Caucasians, enrolled in the Lombardy region of Italy in 2002–2005. Institutional review boards of the enrolling hospitals and National Cancer Institute approved the study and participating subjects signed an informed consent.

Genomic DNA was isolated from blood samples from 1984 cases and 2073 controls and used to genotype 12 SNPs (Table 1) covering...
Table 1 SNPs in the RNA-mediated interference machinery and correlation with miR expression

| Gene       | SNP       | Position | Alleles | MAF   | Ref. MA | miR P-value | AD samples | SQ samples |
|------------|-----------|----------|---------|-------|---------|-------------|------------|------------|
|            |           |          |         |       |         |             | All samples |            |
| RNASEN     | rs423328  | Chr5:5314363760 | G/A    | 0.21  | 168 108 20 0.125 | 101 59 15 0.205 | 67 49 10 0.365 |
| RNASEN     | rs3805516  | Chr5:456427 | T/C    | 0.22  | 163 111 25 0.032 | 97 62 11 0.293 | 66 49 17 0.155 |
| RNASEN     | rs4973760 | Chr5:4729779 | A/G    | 0.29  | 144 132 3 0.851 | 77 82 3 0.841 | 67 50 4 0.795 |
| RNASEN     | rs640831 | Chr5:495192 | CA     | 0.34  | 123 152 30 0.056 | 68 92 56 0.007 | 55 60 1 0.985 |
| RNASEN     | rs7735863 | Chr5:5229277 | CT     | 0.30  | 223 49 9 0.593 | 130 26 5 0.656 | 93 23 9 0.429 |
| RNASEN     | rs10520985 | Chr5:5365322 | CT    | 0.46  | 78 19 3 0.351 | 47 109 10 0.335 | 31 84 2 0.946 |
| Dicer1     | rs1209904 | Chr5:49634656 | GA    | 0.26  | 139 137 4 0.757 | 80 79 20 0.123 | 59 58 2 0.944 |
| Dicer1     | rs2297773 | Chr5:49464842 | AG    | 0.08  | 230 45 12 0.276 | 139 19 9 0.393 | 91 26 6 0.626 |
| Polr2a     | rs8065577 | Chr7:325898 | GC     | 0.24  | 165 112 2 0.929 | 100 60 3 0.840 | 65 52 3 0.877 |
| Polr2a     | rs7217258 | Chr7:336273 | AG    | 0.28  | 145 132 5 0.658 | 87 73 4 0.746 | 58 59 2 0.944 |
| Polr2a     | rs2071504 | Chr7:346661 | CT    | 0.10  | 215 58 3 0.852 | 123 34 2 0.928 | 92 24 9 0.420 |
| Polr2a     | rs6761   | Chr7:358387 | TC    | 0.33  | 123 151 1 0.982 | 75 83 2 0.927 | 48 68 2 0.945 |

Abbreviations: miR = microRNAs; SNP = single-nucleotide polymorphism. Gene name, rsID, chromosome location, major: minor alleles and minor allele frequency (MAF) of the studied SNPs are reported in the first five columns. The remaining columns show the results for the analysis of correlation between each SNP and the expression of 199 human miRs using all samples and restricted to adenocarcinoma (AD) and squamous cell carcinoma (SQ) lung cancer tissue samples. For each SNP and analysis type, we reported the number of subjects homozygous for the most common allele and the number carrying one or two minor variant alleles. All samples AD samples SQ samples

RESULTS

None of the investigated polymorphisms in POLR2A, RNASEN and Dicer1 showed significant association with lung cancer risk or lung cancer survival either overall or by subgroups of histology or smoking status. Analyses based on additive and dominant models gave similar results (Supplementary Materials 1–8). However, we found that a RNASEN haplotype, GTAATC (frequency = 2%), was significantly associated with lung cancer-specific reduced survival compared with the most common haplotype GTACCT...
Figure 1  SNPs coverage for the Drosha gene. SNPs data available from the HapMap v3 database for the chromosomal region corresponding to the Drosha gene. The six SNPs studied in this report are shown in the insets, and linkage disequilibrium (r²) data from HapMap are compared with data in controls from the EAGLE population showing very similar patterns between the two datasets.

We validated the microarray results by qRT–PCR for 4 of the 56 miRs in lung cancer tissue for all samples and for AD and SQ separately (Table 1). In AD patients, RNASEN/rs640831, included in the GTACC'T haplotype, was associated with the expression of 56 miRs (global P = 0.007, Table 2). On average, for subjects who inherited this SNP, 37 miRs were upregulated and 19 miRs were downregulated in comparison to subjects with the consensus genotype. miRs with tumour suppressor potential (e.g., let-7 family) and miRs with oncogenic or metastatic potential (e.g., miR-21 (Zhu et al, 2008), miR-126 (Crawford et al, 2008) and miR-15a-Cimmino et al, 2005), were among those with altered expression in the carriers.

We further studied whether the 12 SNPs were associated with expression of mature miRs in lung cancer tissue for all samples and for AD and SQ separately (Table 1). In AD patients, RNASEN/rs640831, included in the GTACC'T haplotype, was associated with the expression of 56 miRs (global P = 0.007, Table 2). On average, for subjects who inherited this SNP, 37 miRs were upregulated and 19 miRs were downregulated in comparison to subjects with the consensus genotype. miRs with tumour suppressor potential (e.g., let-7 family) and miRs with oncogenic or metastatic potential (e.g., miR-21 (Zhu et al, 2008), miR-126 (Crawford et al, 2008) and miR-15a-Cimmino et al, 2005), were among those with altered expression in the carriers.

We validated the microarray results by qRT–PCR for 4 of the 56 miRs significantly associated with RNASEN/rs640831 in 49 EAGLE lung tumour samples. As shown in Supplementary Figure 1, the correlation was highly significant (P = 0.001, <0.0001, <0.0001, and 0.002 for let-7g, let-7f, miR-26a and miR-107, respectively). As expected, the correlation between the microarray and qRT–PCR data was inverse as qRT–PCR values are measured in terms of number of measurement cycles needed to reach a certain expression level: the lower the number of cycles the higher the detected expression level. In addition, the association between the expressions as measured by qRT–PCR and RNASEN/rs640831 was qualitatively concordant with the microarray-based results (inverse association in the 24 AD but not in the 23 SQ cases).

Finally, to further elucidate our finding of a correlation between the RNASEN/rs640831 and the miR expression profile among AD cases, we tested the association between RNASEN gene expression and the rs640831 polymorphism in non-involved lung tissue of 45 AD patients from EAGLE. The 25 AD patients carrying one or two rs640831 minor variants showed a significantly lower mRNA expression than the 20 AD patients homozygous with the more frequent allele (fold change = 0.87, P = 0.013).

DISCUSSION

We have observed (i) an association between lung cancer survival and a haplotype in RNASEN, particularly, among early stage patients and (ii) a differentially expressed miR profile and RNASEN gene expression by RNASEN/rs640831 status in lung tissue. Carrying the minor variant A vs the common variant C in RNASEN/rs640831 contributed to the survival association for the RNASEN haplotype GTACCT compared with the haplotype GTACCT. Our results are consistent with the combined effect of multiple genetic markers within a haplotype as better representing the impact of the genetic locus on disease progression than individual markers (Johnson et al, 2001; Crawford and Nickerson, 2005). This is the first evidence that inherited variation in the miR-processing machinery, more specifically in RNASEN, might affect survival from lung cancer. Previous studies have shown that low RNASEN gene expression was associated with survival in oesophageal cancer patients (Sugito et al, 2006) and, suggestively, with reduced survival in non-small-cell lung carcinoma patients (Karube et al, 2005). Our findings provide a possible genetic basis for the previous reports. The most frequent variant in RNASEN
was associated with miR expression changes and with lower RNASeN mRNA expression in AD. Several of these miRs have been previously reported to be associated with lung cancer survival in the EAGLE study (Landi et al, 2010) and other lung cancer studies (Markou et al, 2008; Yu et al, 2008; Raponi et al, 2009). An analogous global modification of miR profile due to changes in

| MicroRNAs | Unique ID | P-value | Fold change | Mean in Ref. (log2) | Mean in MA (log2) |
|-----------|-----------|---------|-------------|---------------------|------------------|
| Upregulated | | | | | |
| hsa-miR-30b | MIMAT0000420 | 0.0003 | 1.72 | −2.60 | −1.82 |
| hsa-miR-25 | MIMAT0000081 | 0.0011 | 1.57 | −2.07 | −1.41 |
| hsa-miR-92 | MIMAT000092 | 0.0013 | 1.51 | −4.07 | −3.48 |
| hsa-miR-7g | MIMAT0001414 | 0.0016 | 1.68 | −2.81 | −2.06 |
| hsa-miR-21 | MIMAT000076 | 0.0016 | 1.89 | −3.39 | −2.47 |
| hsa-miR-200c | MIMAT000617 | 0.0020 | 1.47 | 0.90 | 1.46 |
| hsa-miR-106a | MIMAT000103 | 0.0020 | 1.45 | −4.02 | −3.48 |
| hsa-miR-30c | MIMAT000244 | 0.0022 | 1.68 | −2.65 | −1.90 |
| hsa-miR-30a-5p | MIMAT000887 | 0.0025 | 1.48 | −1.18 | −0.62 |
| hsa-let-7b | MIMAT000063 | 0.0025 | 1.50 | −1.22 | −0.64 |
| hsa-let-7f | MIMAT000067 | 0.0031 | 1.65 | −2.74 | −2.02 |
| hsa-miR-181a | MIMAT000256 | 0.0031 | 1.38 | −0.90 | −0.43 |
| hsa-miR-20b | MIMAT000143 | 0.0036 | 1.38 | −3.52 | −2.96 |
| hsa-miR-103 | MIMAT000101 | 0.0036 | 1.37 | −1.60 | −1.14 |
| hsa-miR-98 | MIMAT000096 | 0.0038 | 1.62 | −2.33 | −1.63 |
| hsa-let-7c | MIMAT000064 | 0.0040 | 1.53 | −1.76 | −1.15 |
| hsa-miR-20a | MIMAT000075 | 0.0045 | 1.45 | −4.64 | −4.11 |
| hsa-miR-26a | MIMAT000082 | 0.0070 | 1.65 | −1.01 | −0.29 |
| hsa-miR-29a | MIMAT000086 | 0.0071 | 1.52 | −1.82 | −1.22 |
| hsa-miR-126 | MIMAT000445 | 0.0074 | 1.55 | 2.22 | 2.86 |
| hsa-miR-17-5p | MIMAT000104 | 0.0096 | 1.33 | −3.71 | −3.30 |
| hsa-miR-107 | MIMAT000074 | 0.0104 | 1.30 | −1.54 | −1.16 |
| hsa-miR-19b | MIMAT000145 | 0.0199 | 1.39 | −2.15 | −1.68 |
| hsa-miR-26b | MIMAT000038 | 0.0205 | 1.39 | −1.46 | −0.98 |
| hsa-miR-15a | MIMAT000068 | 0.0244 | 1.33 | −2.04 | −1.62 |
| hsa-miR-30d | MIMAT000245 | 0.0281 | 1.28 | −0.79 | −0.44 |
| hsa-miR-93 | MIMAT000093 | 0.0285 | 1.30 | −3.36 | −2.98 |
| hsa-miR-23a | MIMAT000078 | 0.0348 | 1.26 | 0.36 | 0.69 |
| hsa-miR-125a | MIMAT000443 | 0.0377 | 1.21 | 0.68 | 0.96 |
| hsa-miR-22 | MIMAT000077 | 0.0415 | 1.30 | 0.55 | 0.93 |
| hsa-miR-146b | MIMAT0002809 | 0.0422 | 1.36 | −4.46 | −4.02 |
| hsa-miR-429 | MIMAT0000736 | 0.0432 | 1.36 | −0.43 | 0.02 |
| Downregulated | | | | | |
| hsa-miR-452 | MIMAT0001635 | 0.0003 | 0.88 | 0.18 | 0.00 |
| hsa-miR-370 | MIMAT000722 | 0.0024 | 0.90 | 0.41 | 0.26 |
| hsa-miR-122a | MIMAT000421 | 0.0037 | 0.89 | 0.14 | −0.03 |
| hsa-miR-30b | MIMAT000691 | 0.0044 | 0.92 | −0.11 | −0.23 |
| hsa-miR-510 | MIMAT0002882 | 0.0047 | 0.91 | 0.22 | 0.08 |
| hsa-miR-188 | MIMAT000457 | 0.0065 | 0.91 | 0.18 | 0.04 |
| hsa-miR-509 | MIMAT0002881 | 0.0117 | 0.86 | −0.40 | −0.62 |
| hsa-miR-198 | MIMAT000228 | 0.0134 | 0.90 | 0.43 | 0.28 |
| hsa-miR-485-5p | MIMAT0002175 | 0.0134 | 0.90 | 0.81 | 0.65 |
| hsa-miR-518c* | MIMAT0002847 | 0.0145 | 0.89 | −0.01 | −0.17 |
| hsa-miR-610 | MIMAT000378 | 0.0162 | 0.83 | 1.01 | 0.73 |
| hsa-miR-488 | MIMAT0002804 | 0.0166 | 0.91 | −0.76 | −0.90 |
| hsa-miR-453 | MIMAT0001630 | 0.0353 | 0.93 | 0.38 | 0.28 |
| hsa-miR-628 | MIMAT0003297 | 0.0405 | 0.89 | −0.14 | −0.30 |
| hsa-miR-432 | MIMAT0002814 | 0.0420 | 0.89 | −0.02 | −0.20 |
| hsa-miR-623 | MIMAT0003292 | 0.0429 | 0.88 | −0.05 | −0.24 |
| hsa-miR-299-3p | MIMAT0000687 | 0.0473 | 0.92 | 0.05 | 0.07 |
| hsa-miR-524* | MIMAT0002849 | 0.0473 | 0.93 | 0.07 | −0.03 |
| hsa-miR-383 | MIMAT000738 | 0.0498 | 0.91 | 0.83 | 0.70 |

Abbreviations: AD = adenocarcinoma; miR = microRNA; SNP = single-nucleotide polymorphism. The 56 miRs significantly correlated with RNASeN/rs40834 in AD patients are listed ranking by P-value of each SNP-miR correlation. The analysed miR data is a miR expression intensity ratio between the examined miR and the reference EBV cell line, followed by median normalisation and log2 base transformation (i.e., a negative value indicates a ratio between 0 and 1). For each miR we also reported the fold change of the expression ratio for minor allele carriers (indicated with ‘MA’) compared with major allele homozygotes (indicated with ‘Ref.’), and the expression ratios means in the two compared groups. miRs whose expression has been associated with lung cancer in previous studies are shown in bold. The asterisk (*) symbol after a miR label designates a complementary miR.
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