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Ultraconserved Regions Encoding ncRNAs Are Altered in Human Leukemias and Carcinomas

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SUMMARY

Noncoding RNA (ncRNA) transcripts are thought to be involved in human tumorigenesis. We report that a large fraction of genomic ultraconserved regions (UCRs) encode a particular set of ncRNAs whose expression is altered in human cancers. Genome-wide profiling revealed that UCRs have distinct signatures in human leukemias and carcinomas. UCRs are frequently located at fragile sites and genomic regions involved in cancers. We identified certain UCRs whose expression may be regulated by microRNAs abnormally expressed in human chronic lymphocytic leukemia, and we proved that the inhibition of an overexpressed UCR induces apoptosis in colon cancer cells. Our findings argue that ncRNAs and interaction between noncoding genes are involved in tumorigenesis to a greater extent than previously thought.

INTRODUCTION

All malignant cells have specific alterations at DNA loci that encode genes for oncoproteins or tumor suppressors (Balmain et al., 2003; Wooster and Weber, 2003). This common feature has recently been expanded to include a large class of noncoding RNAs (ncRNAs) called microRNAs (miRNAs) (Ambros, 2004) that are also involved in

SIGNIFICANCE

Common features of the genomic architecture of malignant cells are specific alterations at loci harboring genes coding for oncoproteins or tumor suppressors. In the last few years, this dogma of molecular oncology has evolved with the inclusion of a large class of noncoding RNAs (ncRNAs) named microRNAs (miRNAs) in the ever-expanding list of genes involved in cancer initiation and progression. We demonstrate that another class of genes encoding ncRNAs, comprising hundreds of transcribed ultraconserved regions (T-UCRs), is consistently altered at the genomic level in a significant percentage of analyzed leukemias and carcinomas and that miRNAs may interact with T-UCRs. This offers the prospect of defining tumor-specific signatures of ncRNAs that are associated with diagnosis, prognosis, and response to treatment.

cancer initiation and progression (Calin et al., 2002; Croce and Calin, 2005; Berezikov and Plasterk, 2005a; Esquela-Kerscher and Slack, 2006; Calin and Croce, 2006a). MiRNAs affect the regulation of gene expression at both the transcriptional and posttranscriptional levels (Ambros, 2003, 2004).

The extent of involvement of miRNAs and the involvement of other classes of ncRNAs in human tumorigenesis is unknown. Research into these questions will offer new insights into the molecular mechanisms and signal transduction pathways altered in cancer and also present unexpected opportunities for the identification of new molecular markers and potential therapeutic agents. We focused our attention on the ultraconserved regions (UCRs) of the human genome (Bejerano et al., 2004b) because also miRNAs are almost completely conserved among various species (Berezikov et al., 2005b). For example, the active molecules of the *miR-16-1/miR-15a* cluster, shown to be an essential player in the initiation of chronic lymphocytic leukemia (CLL) (Calin et al., 2005), are completely conserved in human, mouse, and rat and highly conserved in nine out of the ten sequenced primate species (Berezikov et al., 2005b). Comparative sequence analysis has identified a number of highly conserved genomic sequences. Some of these regions do not produce a transcript that is translated into protein and are therefore considered to be nongenic. Various names have been applied to this class of sequences: conserved nongenic sequences (CNGs) (Dermitzakis et al., 2005), conserved noncoding sequences (CNSs/CNCs) (Meisler, 2001), multiple species conserved sequences (MCSs) (Thomas et al., 2003), or highly conserved regions (HCRs) (Duret et al., 1993).

UCRs are a subset of conserved sequences that are located in both intra- and intergenic regions. They are absolutely conserved (100%) between orthologous regions of the human, rat, and mouse genomes (Bejerano et al., 2004b). In contrast to other regions of conserved sequence, 53% of the UCRs have been classified as nonexonic (''N'', 256/481 without evidence of encoding protein), while the other 47% have been designated either exonic (''E'', 111/481, that overlap mRNA of known protein-coding genes), or possibly exonic (''P'', 114/481, with inconclusive evidence of overlap with protein coding genes).

A large portion of transcription products of the noncoding functional genomic regions have significant RNA secondary structures and are components of clusters containing other sequences with functional noncoding significance (Bejerano et al., 2004a). The UCRs represent a small fraction of the human genome that are likely to be functional but not encoding proteins and have been called the ''dark matter'' of the human genome (Bejerano et al., 2004a). Because of the high degree of conservation, the UCRs may have fundamental functional importance for the ontogeny and phylogeny of mammals and other vertebrates. This was illustrated by the recent finding of a distal enhancer and an ultraconserved exon derived from a novel retroposon active in lobe-finned fishes and terrestrial vertebrates more than 400 million years ago and maintained as active in a ''living fossil' coelacanth (Bejerano et al., 2006). Further experimental proof of the functional importance of UCRs is based on analysis of mice with targeted mutations. Megabase deletions of gene deserts that lack ultraconserved elements or highly conserved sequences resulted in viable mice that developed apparently without detectable phenotypes (Nobrega et al., 2004). By contrast, gene deserts containing several UCRs (such as the two gene deserts surrounding the *DACH1* gene on human chromosome 13q21.33) were shown to contain long-range enhancers, some of them composed of UCR sequences (Nobrega et al., 2003).

We present here a thorough genomic interrogation of the status of UCRs in a large panel of human leukemias and carcinomas. We investigated the genome-wide expression of UCRs in various normal and cancer samples, and we assessed the relationship between the genomic location of these sequences and the known regions involved in cancers. Furthermore, we identified a functional role for miRNAs in the transcriptional regulation of cancer-associated UCRs. Finally, we proved in cancer systems that a differentially expressed UCR could alter the functional characteristics of malignant cells. By combining these data with the much more elaborate model involving miRNAs in human tumorigenesis, we propose a model in which alteration in both coding and noncoding RNAs cooperate in the initiation and progression of malignancy.

RESULTS

Genome-wide Profiling Reveals Extensive Transcription of UCRs in Normal Human Tissues

To investigate the possible involvement of UCRs in human cancers, we analyzed 481 genomic regions longer than 200 bp (Bejerano et al., 2004b) by northern blot, quantitative PCR (qRT-PCR), and microarray. Both exonic (E) and possibly exonic (P) and nonexonic (N) UCR probes detected transcripts (in sense or antisense, A, orientation) over a large range of lengths from various normal tissues (Figure 1A and Figure S1 in the Supplemental Data available with this article online). The length of two of the transcripts was confirmed by cloning the cDNA by 5′- and 3′-RACE for the exonic *uc.246(E)* from normal human colon and the nonexonic *uc.269A(N)* from normal human bone marrow. Neither of these cDNAs contained open reading frames (ORFs) of significant length, confirming their likely nonprotein coding nature. These nonspliced full-length cDNAs, which we named noncoding ultraconserved genes, nc-UCGs, are of variable length (about 0.8 kb for the ultraconserved gene *UCG.246* and about 1.8 kb and 2.8 kb for the ultraconserved gene *UCG.269A*). Transcription of these nc-UCGs may be initiated from polyadenine rich genomic regions, as was recently proposed for several long ncRNAs from mouse (Furuno et al., 2006).

We compared the transcription levels of several UCRs from normal and disease tissue using microarray analysis followed by qRT-PCR and northern blot confirmation. The expression of *uc.291(P)* and *uc.73A(P)* was significantly

higher in normal CD5+/CD19+ lymphocytes than in CLL cells ($p < 0.05$) (Figure 1B). The data obtained with this microarray platform has been confirmed in various studies (Calin et al., 2005; Yanaihara et al., 2006; Volinia et al., 2006). The strength of our data is reinforced by the fact that two independent sets of normal CD5 cells were included in microarray and quantitative RT-PCR experiments. When both *uc.291(P)* and *uc.73A(P)* were investigated by qRT-PCR and microarray in two different sets of CD5/CD19 positive B cells and malignant B cells, the differential expression was statistically significant by both assays (Figure 1B). Furthermore, qRT-PCR and northern blotting for eleven and six UCRs, respectively, gave results that were concordant with microarray results (Figures S1 and S2).

Using microarray analysis, we found that the majority of transcribed UCRs (that we named here T-UCRs) were expressed in normal human tissues both ubiquitously and in a tissue-specific manner (Figure 1C). About 34% of putative T-UCRs (325/962) had hybridization signals with an intensity over background (calculated as average signal of blank spots + 2 SD) in all 19 tissue samples. The highest number of T-UCRs was found in B cells, while the lowest was in ovary. About 93% of the UCRs (890 of 962) were expressed over background in at least one sample, and therefore we considered these as T-UCRs. The three different types of UCRs were transcribed with similar frequencies: 41% of exonic UCRs, 33% of possibly exonic UCRs, and 30% of nonexonic UCRs.

The microarray platform contains putative T-UCRs in both sense and antisense orientation. Eighty-four of the 962 UCRs (9%) were bidirectionally transcribed, while 241 were transcribed only from one strand, in all the normal tissues analyzed (Figures 1D and 1E and Table S1). Since identification of bidirectional transcription by microarray analysis may be hindered by trace contamination with genomic DNA, we performed a comparison of microarray results with strand-specific qRT-PCR for *uc.269(N)*, *uc.233(E)*, and *uc.73(P)*. In all three instances the data were concordant, showing predominant transcription from one strand (Figure 1E). Of note, out of the 156 nonexonic T-UCRs expressed in all 19 tissues, 92 (~60%) are intergenic, while 64 are intronic. Of the latter, 37 are in the antisense orientation compared with the host gene, suggesting that about 83% (129/156) of the nonexonic T-UCRs did not represent intronic transcription of long precursor transcripts of known host genes but bona fide independent noncoding transcripts.

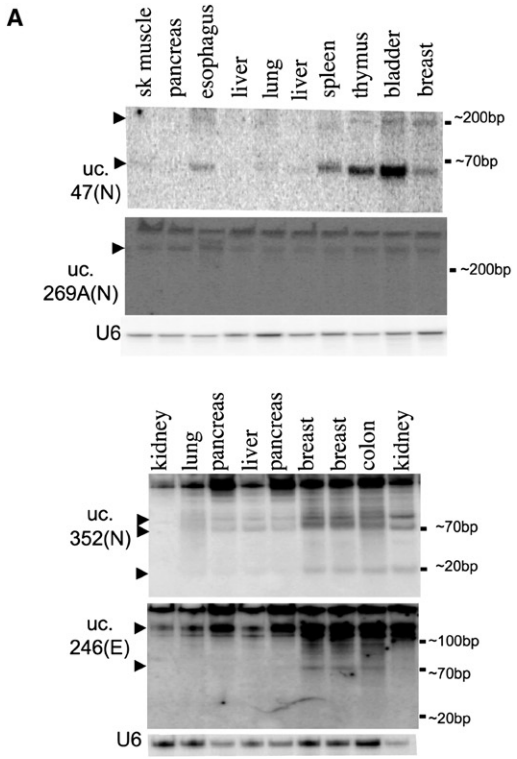
As with miRNAs (Liu et al., 2004), we performed a hierarchical clustering of T-UCR expression in hematopoietic tissues (represented by B lymphocytes, T lymphocytes, and mononuclear cells, each collected from two healthy individuals) and nonhematopoietic tissues. The same types of tissue from different individuals were clustered as closest neighbors (Figure 2A and Figure S3). These findings demonstrate that UCRs represent, in a significant proportion of cases, noncoding transcripts in normal human tissues and that the expression of these T-UCRs is tissue-specific.

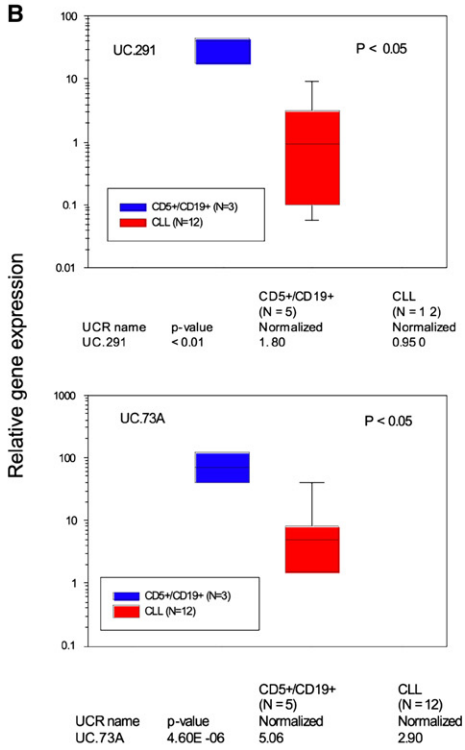
Distinct UCR Signatures in Human Leukemias and Carcinomas

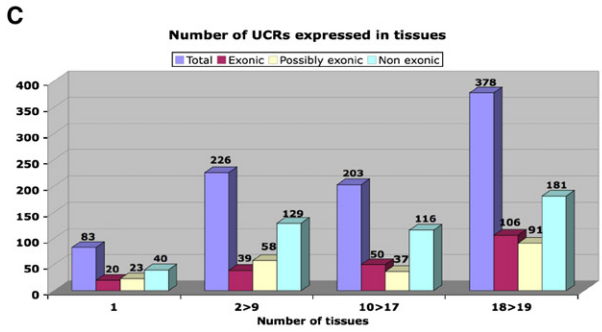
Since extensive gene expression alterations in cancer cells have been widely described for both protein-coding genes and miRNAs (Esquela-Kerscher and Slack, 2006; Calin and Croce, 2006a, 2006b; Lu et al., 2005), we investigated the expression of UCRs in a panel of 173 samples, including 133 human cancers and 40 corresponding normal tissues. Hierarchical clustering of the samples showed that various types of cancers clustered differently according to their developmental origins: the leukemias (CLL) and normal hematopoietic tissues were branched separately from the colorectal (CRC) and hepatocellular carcinomas (HCC) with their normal counterparts (Figure S4); moreover, specific groups of UCRs seemed to be differentially expressed in tumor types (Figure 2B). Since different tissues have specific UCR signatures, this clustering pattern could be the consequence of different tissue-specific origin of the tumors. Thus, we compared the expression of UCRs between the normal and tumor cells of the same origin. Out of 962 possible T-UCRs, 88 (9.1%) were differentially expressed at a highly statistically significant level ($p < 0.005$) in at least one type of cancer (Table 1 and Table S2). We found both downregulated and upregulated T-UCRs in cancers compared to the expression in corresponding normal tissues. By comparing each cancer type with the corresponding normal tissues, we found that the CLL signature was composed of 19 UCRs (8 up- and 11 downregulated), the CRC signature of 61 UCRs (59 up- and 2 downregulated), and the HCC signature of 8 UCRs (3 up- and 5 downregulated) (Table S2). Eighteen transcripts of the signatures were exonic UCRs (20%), 28 were possibly exonic UCRs (32%) and 42 were nonexonic UCRs (48%). Of the 18 exonic T-UCRs, 9 represented the anti-sense direction of the known host protein-coding gene transcripts. We therefore demonstrated that the T-UCR expression profiles can be used to differentiate human cancers.

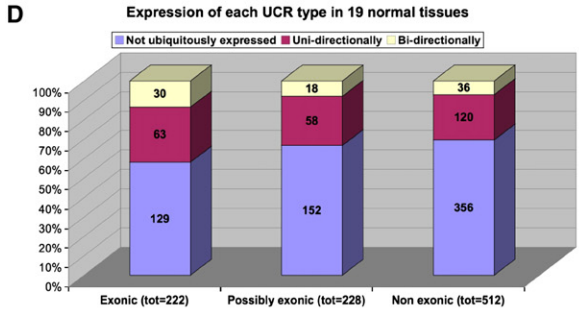
UCRs Are Frequently Located at Fragile Sites and Genomic Regions Involved in Cancers

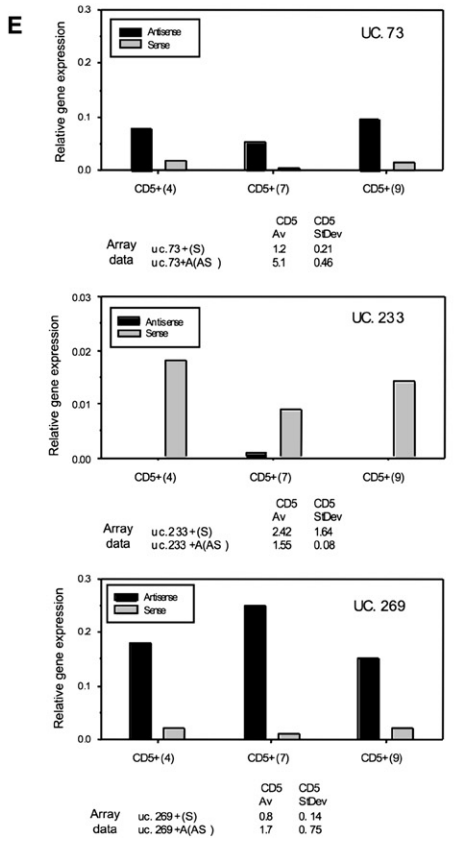
We compared the genomic location of UCRs with that of previously reported nonrandom genetic alterations identified in human tumors and cloned fragile sites (FRA) as described (Calin et al., 2004b). We used the set of 186 miRNAs previously reported (Calin et al., 2004b) and a set of 297 zinc finger protein-coding genes (ZNF) (http://genome.ucsc.edu/), a well known family of transcription factors shown to be associated with cancer (Huntley et al., 2006). We previously reported that miRNA genes are frequently located at FRA sites, HOX genes clusters, and genomic regions involved in cancer, such as minimal regions of loss of heterozygosity (LOH), and minimal regions of amplification, globally named cancer-associated genomic regions (CAGR) (Calin et al., 2004b). A recent study, using high-resolution array comparative genomic hybridization (aCGH), confirmed that miRNA loci exhibit genomic alterations at high frequency in human cancers (Zhang et al., 2006). Furthermore, by analyzing the miRNA


A
sk muscle
pancreas
esophagus
liver
lung
liver
spleen
thymus
bladder
breast
uc. 47(N)
~200bp
~70bp
uc. 269A(N)
~200bp
U6
kidney
lung
pancreas
liver
pancreas
breast
breast
colon
kidney
uc. 352(N)
~70bp
~20bp
uc. 246(E)
~100bp
~70bp
~20bp
U6


B
Relative gene expression
UC.291
P < 0.05
CD5+/CD19+ (N=3)
CLL (N=12)
UCR name p-value CD5+/CD19+ (N = 5) Normalized CLL (N = 12) Normalized
UC.291 < 0.01 1.80 0.95 0
UC.73A
P < 0.05
CD5+/CD19+ (N=3)
CLL (N=12)
UCR name p-value CD5+/CD19+ (N = 5) Normalized CLL (N = 12) Normalized
UC.73A 4.60E -06 5.06 2.90


C
Number of UCRs expressed in tissues
Total
Exonic
Possibly exonic
Non exonic
Number of tissues
1
2>9
10>17
18>19


D
Expression of each UCR type in 19 normal tissues
Not ubiquitously expressed
Uni-directionally
Bi-directionally
Exonic (tot=222)
Possibly exonic (tot=228)
Non exonic (tot=512)


E
Relative gene expression
Antisense
Sense
UC. 73
CD5+(4)
CD5+(7)
CD5+(9)
Array data
uc.73+(S) CD5 Av 1.2 CD5 StDev 0.21
uc.73+A(AS) 5.1 0.46
UC. 233
uc.233+(S) 2.42 1.64
uc.233 +A(AS) 1.55 0.08
UC. 269
uc. 269+(S) 0.8 0. 14
uc. 269+A(AS) 1.7 0. 75

expression in NCI-60 cell lines, another group found that the candidate tumor-suppressor and oncogenic miRNAs are located in CAGRs (Gaur et al., 2007).

Here, we show that the association between the genomic location of UCRs and the analyzed cancer-related genomic elements is highly statistically significant and comparable to that reported for miRNAs. The ZNF transcription factors did not show any significant association with any of the analyzed regions of interest (Table 2 and Table S3). There was a similar lack of association for the smaller family of protein-coding genes involved in RNA splicing (80 genes, data not shown). For example, the probability for the association of UCRs or miRNAs with minimal LOH regions versus nondeleted genomic regions was less than 0.001 in both instances (IRR of 2.02 and 4.08, respectively). As an internal control, we used the human papilloma virus 16 (HPV16) integration sites, which frequently occur in FRA sites. If UCRs are significantly associated with FRA, then we expected to find an association with the HPV16 integration site. This is exactly what we observed for both UCRs and miRNAs, but not for ZNF protein-coding genes (Table 2) or for the protein-coding genes involved in RNA splicing (data not shown).

Additional data illustrate the importance of the genomic location of UCRs. First, we found that the ubiquitously expressed T-UCRs (expressed in 18 or 19 normal tissues in Figure 1C) are significantly more frequently located in CAGRs ($p < 0.005$, Fisher's exact test) when compared with all other UCRs (97 out of 189 versus 71 out of 292). Second and more importantly, T-UCRs differentially expressed in human cancers are located in CAGRs specifically associated with that type of cancer. For example, the chromosomal region 13q21.33–q22.2 has been linked to susceptibility to familial CLL (Ng et al., 2007). No mutations were found in any of the 13 protein-coding genes screened within this interval. We identified a cluster of seven UCRs (*uc.347* to *uc.353*) located within this CAGR. Two of them, *uc.349A(P)* and *uc.352(N)* are among the T-UCRs that are differentially expressed between normal and malignant B-CLL CD5-positive cells. This suggests, at least in this case, that it is not the protein-coding genes but the UCRs that represent the "unknown" culprits located in the CAGR. Together these data provide evidence that the UCRs are located in genomic regions altered during the malignant process and suggest that T-UCRs could be candidate genes for cancer susceptibility.

Negative Regulation of T-UCRs by Direct Interaction with MicroRNAs

In order to begin to functionally characterize some UCRs involved in human cancers, we performed a genome-wide expression study in the same set of CLL samples investigated above. We found that a signature of five UCRs, *uc.269A(N)*, *uc.160(N)*, *uc.215(N)*, *uc.346A(P)*, and *uc.348(N)*, was able to differentiate between two main CLL prognosis groups previously differentiated by the expression of 70-kDa zeta-associated protein (ZAP-70). These five T-UCRs displayed variations in their expression level that was negatively correlated with the miRNA expression signature reported in CLL (Calin et al., 2005) (Table 3). This finding raises the possibility of complex regulatory mechanisms between miRNAs and T-UCRs. We identified, by sequence alignments, that three out of the 5 UCRs have significant antisense complementarity with 5 out of the 13 miRNAs from the signature, giving rise to six possible interacting pairs: *uc.160::miR-24*, *uc.160::miR-155*, *uc.160::miR-223*, *uc.160::miR-146a*, *uc.346A::miR-155*, and *uc.-348::miR-29b* (Figure 3A). In this analyzed set of miRNA::UCR pairs, the 5′- end "6 base seed" complementarity rule described for miRNA::mRNA interaction was valid; furthermore, the levels of 3′-end complementarity could be variable: more than 60% complementarity for *miR-24::uc160* or *miR-155::uc.346A* pairs to less than 25% for the *miR-155::uc.160* pair. As a control, when randomly generated combinations of five UCRs and 13 miRNAs were compared, the sense and antisense complementarity was not significant.

Negative correlations between the microarray expression values of specific T-UCRs and predicted interactor miRNAs was confirmed by qRT-PCR for selected T-UCRs and miRNAs from lymphocytes of an independent set of CLL patients and normal controls (Figure 3B). We performed in vitro assays of miRNA::UCR interaction

Figure 1. Transcriptional Characteristics of Various Types of UCRs

(A) Northern blots showing the expression of various UCRs in normal tissues. In the case of *uc.246(E)* and *uc.269A(N)*, the presence of the long transcript was confirmed by the RACE cloning experiments. For some tissues, duplicate samples were procured to confirm reproducibility. Normalization was performed with U6. The arrows on the left side show the identified transcripts.

(B) T-UCRs 291 and 73A expression (normalized to 18S rRNA) was confirmed by qRT-PCR (graphs) and microarray analyses (Normalized number under the graph) in normal CD5+/CD19+ lymphocytes and malignant CLL samples. p values were significant for both qRT-PCR and microarray data statistical comparison. Each box represents the distribution of expression measured for normals (blue) and CLL patients (red), ends of the boxes define the 25th and 75th percentiles, a line indicates the median, and bars define the 10th and 90th percentiles.

(C) Number of UCRs expressed in one or more of 19 tissues, as revealed by microarray analysis; UCR type (E, N, and P) numbers are indicated. Four types of transcription were found: ubiquitously expressed UCRs (in 18 or 19 out of 19 different tissues), UCRs expressed in the majority of tissues (10–17), UCRs expressed in a minority of tissues (2–9), and tissue specifically expressed UCRs.

(D) Percentage of each UCR type (E, N, and P) that is ubiquitously transcribed (both uni- and bidirectionally) in all the analyzed tissues; the absolute numbers for each UCR type are shown in the boxes.

(E) Expression of the sense or antisense strand UCRs 73, 133, and 269, relative to 18S rRNA, in CD19+ B cells from three different donors. Sense/antisense strand-specific real-time RT-PCR was used to validate the strand-specific expression of the UCRs observed with microarray analysis; the average ± standard deviation of microarray results for CD5+ samples is under each graph. Microarray probes are named as follows: the sense genomic probe is named "+," while the probe to the complementary sequence is named "A+."

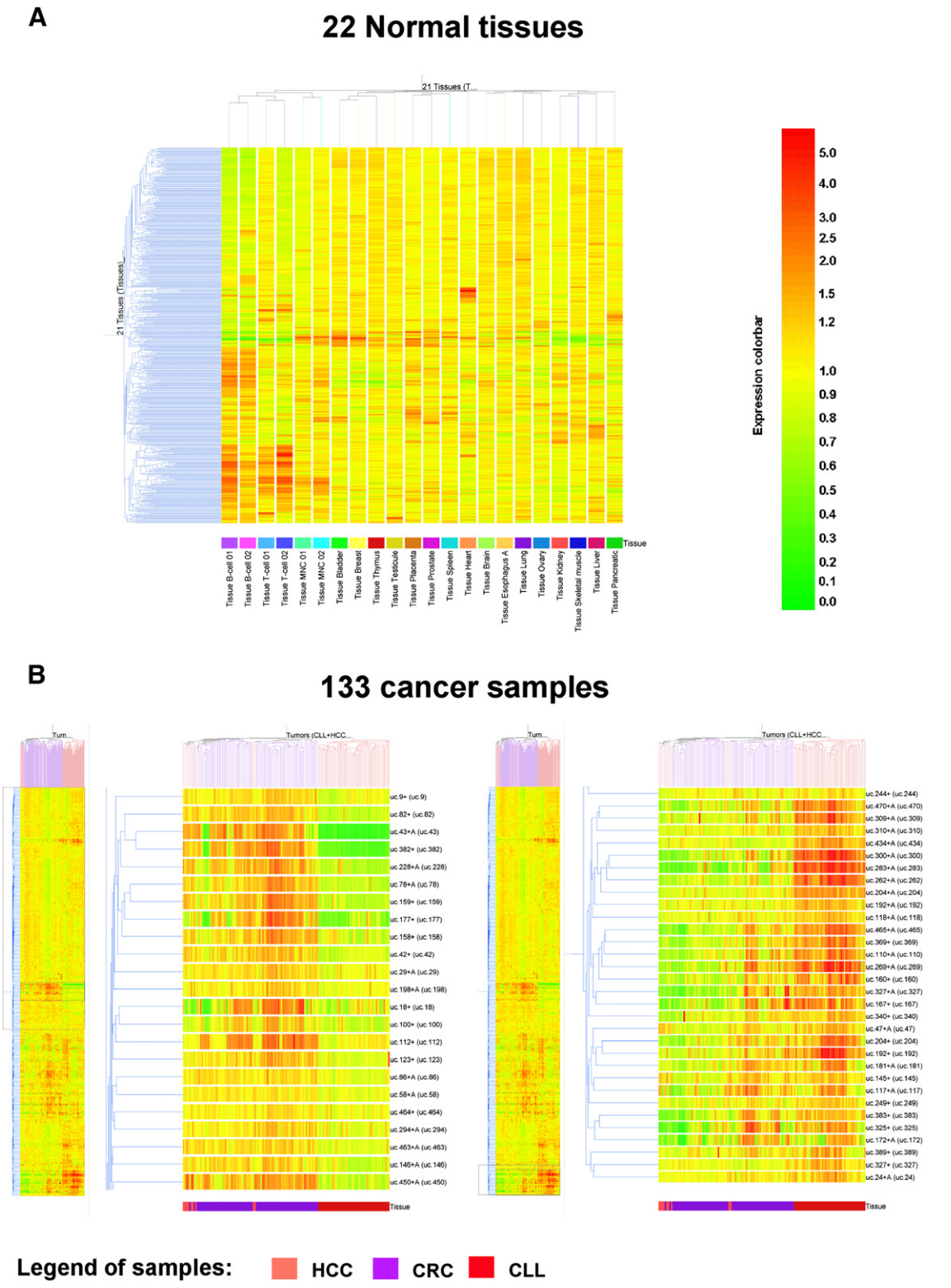


Figure 2. Hierarchical Clustering of Tissues and Tumors According to UCRs Expression

Unsupervised cluster of (A) 22 normal human tissues and (B) 133 leukemias and carcinomas made using the nonexonic UCRs of the chip. Some of the T-UCRs that well differentiate the tissue types (A) or carcinomas from leukemias (B) are expanded at the right. Samples are in columns, T-UCRs in rows. A green-colored gene is downregulated compared to its median expression in all samples, red is upregulated, and yellow means no variation. The complete UCRs profile of tissues and tumors can be found in Figures S3 and S4.

Table 1. Most Significant Differentially Expressed UCRs in Leukemias and Carcinomas*

UCR Name	Probe (T-UCR)	Type and Location	Significance	Upstream, Host, and Downstream Genes	CAGR Location and Host Gene Cancer Connection
uc.29	uc.29+A	nonexonic	high CRC versus normal	LMO4, \N AF118089	
uc.73	uc.73+A	possibly exonic	low CLL versus CD5; high CRC versus normal	AK126774, BC017741 ZFHX1B	
uc.111	uc.111+	possibly exonic	high CRC versus normal	AK128398, \N AB051544	yes
uc.112	uc.112+	nonexonic	high CRC versus normal	TBC1D5, \N SATB1	
uc.134	uc.134+A	possibly exonic	high CRC versus normal	AF257098, MGC12197, MLF1	
uc.135	uc.135+	exonic	low CLL versus CD5	GOLPH4, EVI1 ARPM1	yes in antisense with EVI-1 oncogene overexpressed by t(3;21)(q26;q22)
uc.206	uc.206+	nonexonic	high CRC versus normal	SP8, \N SP4	
uc.230	uc.230+	possibly exonic	high CRC versus normal	AK096400, \N TFEC	
uc.233	uc.233+	exonic	low CLL versus CD5	C7orf21, CENTG3 ASB10	in sense with CENTG3
uc.291	uc.291+	possibly exonic	low CLL versus CD5	AK024492, C10orf11 KCNMA1	
uc.292	uc.292+	exonic	high CRC versus normal	AF338191, MLR2 C10orf12	in sense with MLR2
uc.339	uc.339+	possibly exonic	high CRC versus normal	ATP5G2, \N KIAA1536	yes
uc.341	uc.341+	exonic	high CRC versus normal	HOXC11, HOXC10 HOXC9	yes in sense with HOXC10
uc.388	uc.388+	nonexonic	high CRC versus normal	BX641000, TCF12 FLJ14957	
uc.399	uc.399+A	nonexonic	high CRC versus normal	CYLD, \N SALL1	
uc.420	uc.420+A	exonic	high CRC versus normal	POLG2, DDX5 LOC90799	in sense with DDX5, downregulated in colon

* Note: Only UCRs at $p < 0.0001$ were selected. For full list of UCRs, see Table S2. CAGR, cancer associated genomic regions as reported by Calin et al. (2004b). \N, intronic location. Names of the genes as at www.ncbi.nlm.nih.gov/.

involving *miR-155*, which is overexpressed in the aggressive form of CLL (Calin et al., 2005) some lymphomas and carcinomas (Eis et al., 2005; Kluiver et al., 2005; Volinia et al., 2006), and *miR-24-1* and *miR-29-b*, which carry mutations in primary transcripts from CLL patients (Calin et al., 2005). We cloned the UCRs *uc.160(N)*, *uc.346A(P)*, and *uc.348(N)* in luciferase reporter vectors to assess the possible direct interaction with *miR-155*, *miR-24-1*, or *miR-29-b*. We observed consistent and reproducible reduction in luciferase expression with four miR::T-UCR pairings consistent with interactions taking place in vitro (Figure 3C). By contrast, *uc.348(N)* did not interact with *miR-155* as indicated by the luciferase assay, a result that is in concordance with the positive expression correlation of these two genes in CLL patients and the low sequence complementarity (Figure 3A).

In order to determine if these interactions occur in vivo, we transfected *miR-155* into MEG01 leukemia cells and assessed the levels of *uc.346A* and *uc.160* (both well-expressed in this cell line). At 24 hr after transfection,

Table 2. Mixed Effect Poisson Regression Results of Association of UCRs with Regions of Interest*

	Ultraconserved Regions			microRNAs			Zinc Finger Proteins		
Region of Interest	IRR	95% CI IRR	p	IRR	95% CI IRR	p	IRR	95% CI IRR	p
Fragile sites versus nonfragile sites	2.98	1.69, 5.07	< 0.001	9.12	6.22, 13.38	<0.001	1.36	0.76, 2.44	0.29
HPV16 insertion versus all other	5.07	3.37, 7.64	<0.001	3.22	1.55, 6.68	0.002	0.50	0.07, 3.60	0.49
Homeobox genes versus all other	2.39	1.69, 3.39	<0.001	2.95	1.63, 5.34	<0.001	0.16	0.02, 1.14	0.07
HOX cluster versus all other	7.09	3.61, 13.93	<0.001	15.77	7.39, 33.62	<0.001	–[a]	–	–
Amplified region versus nonamplified	3.05	1.99, 4.67	<0.001	3.97	2.31, 6.83	<0.001	0.39	0.12, 1.23	0.11
LOH versus other	2.02	1.62, 2.53	<0.001	4.08	2.99, 5.56	<0.001	0.54	0.14, 2.10	0.37

* The comparison with a "positive" and a "negative" control class of genes each, microRNAs and zinc finger proteins, respectively, is presented. IRR, Incident Rate Ratio.
[a] Too few events, likelihood-based models are numerically unstable.

miR-155 significantly reduced the expression level of both T-UCRs; after 48 hr, the reduction of exogenous *miR-155* levels was paralleled by an increase in T-UCR expression (Figure 3D). This reversible effect supports a regulation of T-UCR by specific miRNAs. As this interaction was proven for the genes of the "ZAP-70 signature," we investigated the correlations between the expression of all miRNAs and T-UCRs at the genome-wide level in all 50 CLL patients. Interestingly, we found a significant negative correlation (at a false detection rate [FDR] of less than 0.01) between 87 miRNAs (out of 235 spotted on the chip, 37%) and T-UCRs expression levels (Table S4). Furthermore, among the correlated genes we identified the *miR-24-1::uc.160* and the *miR-155::uc346A(P)* pairs, experimentally proven to interact (Figure 3E). Moreover, *miR-155* and *uc.348*, which did not interact experimentally, were not members of this list. Other pairs of possible interactors for which we identified positive luciferse assays were *miR-15-a::uc.78* and *miR-16::uc.78* (data not shown). Therefore, noncoding T-UCRs represent possible targets of miRNAs and these interactions may have biological and prognostic significance for cancer patients.

A T-UCR Could Act as an Oncogene

To expand the functional characterization of T-UCR, we examined the biological effects of *uc.73A(P)* in a cancer model. Since this is one of the most statistically significant upregulated T-UCRs in colon cancers ($p < 0.001$), we decided to investigate the effects of its downregulation in COLO-320 colorectal cancer cells that expressed high

Table 3. T-UCRs Whose Expression Inversely Correlates with Complementary miRNA Differentially Expressed in CLL Patients*

T-UCR Name	Type and Location	T-UCR Expression in ZAP-70 Positive versus Negative	Complementary miRNAs	MiRNA Expression in ZAP-70 Positive versus Negative	Upstream, Host, Downstream Genes
uc.269A	nonexonic, chr. 9	downregulated	no complementary sites		AK123000, KIAA1608 LHX2
uc.160	nonexonic, chr. 5	downregulated	miR-155, miR-146a, miR-24 and miR-223	upregulated downregulated	AK128395, \N AP3B1
uc.215	nonexonic, chr. 7	upregulated	no complementary sites		INHBA, GLI3 C7orf25
uc.346A	possibly exonic, chr. 12	downregulated	miR-155	upregulated	RPC2, \N RFX4
uc.348	nonexonic, chr. 13	upregulated	miR-29b, miR-29a, miR-29c, and miR-155, miR-24	downregulated upregulated	KLHL1, DACH FLJ22624 gene desert

* Among the UCRs differentially expressed between the 70 kD zeta-associated protein (ZAP-70) positive and negative CLLs, we selected only the UCRs identified by three different algorithms—GeneSpring ANOVA, PAM (Prediction Analysis for Microarrays), and SAM (Significance Analysis of Microarrays)—and that had a complementary site for microRNAs differentially expressed among the same groups of CLLs.

levels of *uc.73A(P)*. As a control we used the SW620 colon cancer cells in which the expression of this gene does not differ from normal colonic cells (Figure S5). Two small interfering RNAs (siRNA1 and siRNA3), as well as a pool of four siRNAs (siRNApool), were designed to target *uc.73A(P)* and transfected into COLO-320 and SW620 cells. There was significantly less expression of *uc.73A(P)* after 48 (Figure 4A and Figure S6), 72, and 144 hr (data not shown) in the COLO-320 cells treated with siRNAs 1, 3, and pool. The same was found also for SW-620 cells (Figure S6). The growth of COLO-320 cells was significantly reduced after 144 hr of treatment with specific siRNA compared to both untreated (null) or siRNA-treated control cells ($p < 0.05$ at 96 hr and $p < 0.01$ at 144 hr) (Figure 4B). In comparison, proliferation of the SW620 control cells was not significantly changed ($p = 0.83$ and $p = 0.23$ at 96 and 144 hr, respectively) (data not shown). Cell-cycle studies revealed an increase in the sub-G1 fraction of cells (suggesting the presence of apoptotic cells, data not shown) in COLO-320 cells, but not in SW620 cells, a finding confirmed by the apoptosis-specific AnnexinV assay (Figures 4C and 4D) and by caspase-3 assay (Figure S7). Furthermore, the intensity of effects on cell proliferation and survival were proportional to the degree of inhibition by siRNAs (Figure 4). These data suggest that in colorectal cancers, *uc.73A(P)* behaves like an oncogene by increasing the number of malignant cells as a consequence of reduced apoptosis.

DISCUSSION

According to the dogma of molecular oncology, cancer is a genetic disease involving tumor suppressor and oncogenic proteins (Bishop, 1991; Hunter, 1991; Weinberg, 1991). Recent findings strongly support the involvement of microRNAs in the pathogenesis of a majority of analyzed cancers and add a new layer of complexity to the molecular architecture of human cancers (Calin et al., 2002; Esquela-Kerscher and Slack, 2006; Calin and Croce, 2006a). MiRNAs could represent, however, just a particular group of ncRNAs involved in human cancers. It has been shown that antisense intronic ncRNA levels correlate with the degree of tumor differentiation in prostate cancer (Reis et al., 2005) and that MALAT-1 ncRNA expression predicts metastasis and survival in early stage non-small-cell lung cancer (Ji et al., 2003), suggesting a deeper link between ncRNAs and tumor biology.

To clearly address this question, we investigated at the genomic level a full new class of ncRNAs, namely the transcribed noncoding ultraconserved regions (T-UCRs). We used bioinformatics tools to demonstrate that the UCRs are located in genomic regions targeted during the malignant process indicative of a putative involvement in human tumorigenesis. Second, we were able to clone by RACE amplification cDNAs corresponding to *uc.246(E)* and *uc.269A(N)*, proving that, at least in these specific instances, the UCRs are bona fide genes (and we named these as nc-UCGs) that are expressed and can be cloned by standard methods. Various expression techniques including northern blot, qRT-PCR, and genome-wide microarray profiling proved that UCRs are frequently transcribed and that there are distinct signatures in human leukemias and carcinomas. We focused on chronic lymphocytic leukemia, the most frequent adult leukemia in the Western world (Chiorazzi et al., 2005), on colorectal carcinoma, one of the most common cancers in industrialized countries (de la Chapelle, 2004), and on hepatocellular carcinoma, the most rapidly increasing type of cancer in America (Wilson, 2005). We found that, for all the tumor types examined, the malignant cells have a unique spectrum of expressed UCRs when compared with the corresponding normal cells, suggesting that significant variations in T-UCR expression are involved in the malignant process.

Characterizing the functional significance of T-UCR alterations in human cancers is not a trivial task. A myriad of putative functions of T-UCRs can be hypothesized, including an antisense inhibitory role for protein coding genes or other noncoding RNAs or a role as ''aspecific'' miRNAs, meaning miRNAs with peculiarities such as very long precursors (e.g., *uc.339(P)*, which has a precursor length that is double the usual miRNA). This puzzle becomes more complicated by the fact that several UCRs do not act like genes and were found to have regulatory functions as enhancers (Nobrega et al., 2003; Pennacchio et al., 2006), while others represent exons of protein coding genes with known/unknown cancer connections. A particularly interesting region is the *DACH1* locus that contains 7 UCRs in about 700 kb (Bejerano et al., 2004b). Three of the UCRs from this region are differentially expressed in analyzed cancers, two of which are members of the CLL signature. The majority of scanned conserved regions from this locus in a mouse model are enhancers, including the *uc.351(N)* that was not expressed in any of the analyzed tissues in our study. Interestingly, the only two regions that failed to have enhancer function are *uc.348(N)* and *uc.352(N)*, both classified as noncoding and both differentially expressed in human cancers. Further increasing the interest in these specific T-UCRs, come the finding that this genomic region has been linked to susceptibility to familial CLL and that none of the known protein-coding genes were mutated (Ng et al., 2007).

Recently, it was found that short blocks of several tens of bp from the noncoding parts of the human genome named pyknons, occur within nearly all known protein coding genes (Rigoutsos et al., 2006). While the pyknons are distinct from the UCRs, the ultraconserved element containing the highest number of pyknons (four) was *uc.73(P)*, which we found to be one of the most differentially expressed T-UCRs in both CLL and CRC. These intriguing observations suggest a possible regulatory role for *uc.73(P)* on the coding genes with complementary sequences. Further expanding the involvement of this T-UCR in human cancers, we were able to prove an oncogenic function for *uc.73(P)* in colon cancer, as diminution of its overexpression induced apoptosis and had

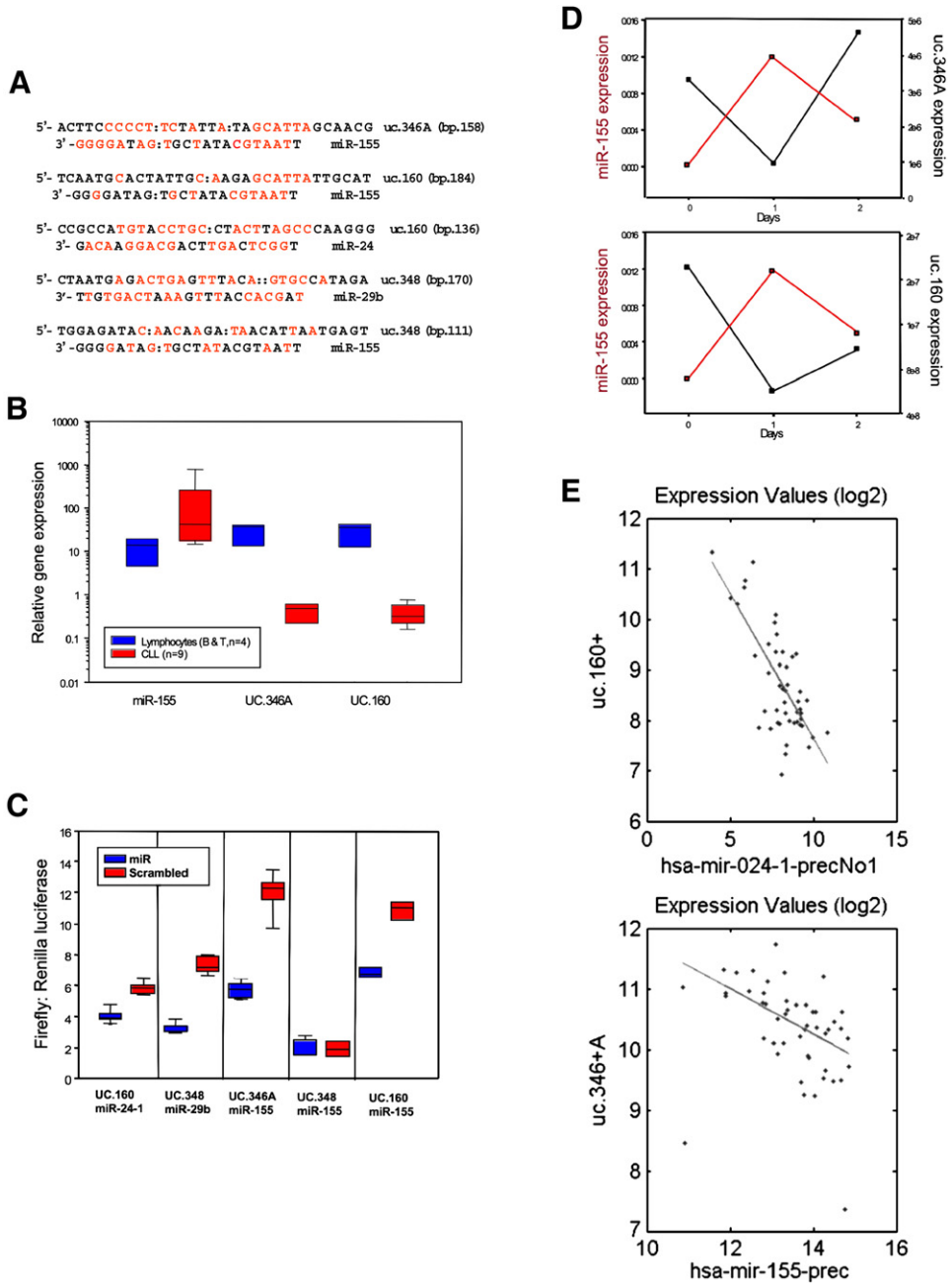


Figure 3. T-UCRs Represent Direct Targets of miRNAs

(A) Examples of sites of complementarity T-UCR::miRNA. The *uc.348::miR-155* pairing is shown as an example of low levels of complementarity in contrast with the other 4 interacting paired genes for which higher levels of complementarity are found.

(B) The correlation by qRT-PCR for miR-*155*, *uc.160*, and *uc.346A* expression in 9 CLL patients. Lymphocytes from four different individuals were used as normal controls. Each box represents the distribution of expression measured for normals (blue) and CLL patients (red), ends of the boxes define the 25th and 75th percentiles, a line indicates the median, and bars define the 10th and 90th percentiles.

(C) The direct miRNA::T-UCR interaction. Relative repression of firefly luciferase expression standardized to a transfection control, Renilla luciferase. pGL-3 (Promega) was used as the empty vector. All the experiments were performed four to six times in triplicate (n = 12–18). Each box represents the distribution of expression measured for miRNAs (blue) and scrambled oligos (red), ends of the boxes define the 25th and 75th percentiles, a line indicates the median, and bars define the 10th and 90th percentiles.

(D) The effects of *miR-155* transfection in MEG-01 cells on expression levels of *uc.160* and *uc.346A*. Effects were measured by qRT-PCR at 0, 24, and 48 hr posttransfection.

(E) Two scatter plots between expression values of *mir-24-1* and *uc.160* and of *miR-155* and *uc.346A* are presented. The regression line shows the negative correlation between these two genes. The name of the corresponding array probes are presented on the Y and X axes. Both probes recognize the mature form of the miRNA gene.

antiproliferative effects specifically in colon cancer cells abnormally expressing this T-UCR.

Our findings that another class of ncRNAs, the T-UCRs, is consistently altered at the genomic level in a high percentage of analyzed leukemias and carcinomas, support a model in which both coding and noncoding genes are involved and cooperate in human tumorigenesis (Calin and Croce, 2006b). Furthermore, correlations between the expression of UCRs and miRNAs in CLL patients raise the intriguing possibility of complex functional regulatory pathways in which two or more types of ncRNAs interact and influence the phenotype. We also demonstrated the existence of the miRNA::T-UCR interaction in which two different types of ncRNAs are interacting.

In conclusion, we found that nc-UCGs are consistently altered at the genomic level in a high percentage of leukemias and carcinomas and may interact with miRNAs in leukemias. The findings provide support for a model in which both coding and noncoding genes are involved in and cooperate in human tumorigenesis.

EXPERIMENTAL PROCEDURES

RACE Cloning and Expression Analysis by Microarray, qRT-PCR, and Northern Blot

RACE Cloning

The expression of six UCRs [*uc.47(N)*, *uc.110(N)*, *uc.192(N)*, *uc.246(E)*, *uc.269A(N)*, and *uc.352(N)*] was analyzed in brain, testis, bone marrow, small intestine, colon, and liver tissue using various combinations of PCR primers designed to amplify short products. These products included 40-mers used for probes in microarray analysis and the complete >200 bp UCR sequence. Two of the UCR products, one exonic, uc.246(E) and one nonexonic, uc.269A(N), were cloned by rapid amplification of cDNA ends (RACE) in both 5′ and 3′ directions. The sources of tissue from which sequences were cloned were bone marrow, leukocytes, fetal brain, and colon according to the manufacturer's protocol (Marathon-ready cDNAs, Clontech, Palo Alto, CA).

UCR Expression Study by Microarray

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) from 19 normal human tissues (Liu et al., 2004) and from 50 CLL samples from patients diagnosed with CLL. Informed consent was obtained from all patients at the CLL Research Consortium institutions in the USA. As controls, CD5+ B cells from 6 healthy individuals (four distinct samples, two being pools from two different healthy individuals) and mononuclear cells (MNC) from 3 individuals were used as reported in Calin et al. (2005). RNA was also extracted from 78 primary colorectal carcinomas, 21 normal colonic mucosas, 9 primary hepatocellular carcinomas, and 4 normal livers, collected at the University of Ferrara, University of Bologna, and University Tor Vergata, Rome (Italy). All samples were obtained with written informed consent according to institutional guidelines for the protection of human subjects.

Microarray chips were developed with a total of 481 human UCR sequences as in http://www.soe.ucsc.edu/~jill/ultra.html. For each UCR two 40-mer probes were designed, one corresponding to the sense genomic sequence (named "+") and the other to the complementary sequence (named "+A"). The design criteria were as described (Liu et al., 2004). Each oligo was printed in duplicate in two different slide locations, and therefore quadruplicate numerical values were available for analysis. Several thousand (3484) blank spots were used for background subtraction. RNA extraction and microarray experiments, consisting of the UCR microarray assembly, target preparation, and array hybridization, were performed as described in detail elsewhere (Liu et al., 2004; Calin et al., 2004a).

Briefly, 5 μg of RNA from each tissue sample was labeled with biotin by reverse transcription using random octomers. Hybridization was carried out on the second version of our miRNA-chip (ArrayExpress accession number A-MEXP-258), which contained the 962 UCR probes, 238 probes for mature miRNA, and 143 probes for precursor miRNAs. Each oligo was printed in duplicate in two different slide locations. Hybridization signals were detected by biotin binding of a Streptavidin-Alexa647 conjugate (one-color signal) using a GenePix 4000B scanner (Axon Instruments). Images were quantified using the GenePix Pro 6.0 (Axon Instruments).

Raw data were normalized and analyzed in GeneSpring GX 7.3 (Agilent Technologies, Santa Clara, CA). Expression data of the 22 tissue samples were normalized with Lowess function in Bioconductor (Limma package) and then were median centered using GeneSpring normalization; the threshold used to determine the level of UCR expression was calculated as the average of blank spots + 2 SD (standard deviation). Tumors were normalized using the on-chip and on-gene median normalization of the GeneSpring software. Hierarchical cluster analysis was done using average linkage and Pearson correlation as measures of similarity. Statistical comparisons of tumors and normal tissues were performed by filtering on fold change and then using the ANOVA (Analysis of Variance) statistic of the GeneSpring software and the Benjamini and Hochberg correction for reduction of false positives. The filter on fold change was set on 1.2 because this threshold, already used for microRNAs analyzed with the same chip (for examples see Calin et al., 2005; Cimmino et al., 2005; Iorio et al., 2005), was demonstrated to reflect a real biological difference. The T-UCRs differentially expressed among CLL patients, grouped in accordance to 70-kDa zeta-associated protein (ZAP-70) expression, were identified by combining the ANOVA results with the SAM (Significance Analysis of Microarray) and PAM (Prediction Analysis of Microarrays) analysis. Their expression was compared to that of microRNAs (Calin et al., 2005). All data were submitted using MIAMExpress to the ArrayExpress database and can be retrieved using the accession number E-TABM-184.

Quantitative RT-PCR for UCRs

Quantitative RT-PCR was the first method we used to confirm the microarray results. We validated the microarray data for eleven UCRs, including *uc.73(P)/73A(P)*, *uc.135(E)*, *uc.160(N)*, *uc.233(E)/233A(E)*, *uc.269(N)/269A(N)*, *uc.289(N)*, *uc.291(P)*, and *uc.346A(P)* in various combinations of samples, including 15 to 17 randomly selected CLL samples from the array set of 50, and various normal CD19+/CD5+ B cells and B and T lymphocyte controls by qRT-PCR. An additional set of 3 normal CD19+/CD5+ positive B cells, not used for microarray studies, was purchased from AllCells (Berkeley, CA), and used as an independent confirmation set. In all instances the qRT-PCR data confirmed the microarray data. RNA was treated with RNase-free DNase I and reverse transcribed to cDNA using random primers and SuperScript II reverse transcriptase. To determine if the sense or antisense UCR transcript was expressed, total RNA was reverse transcribed using Thermoscript RT and a gene-specific (i.e., sense or antisense) primer. RT conditions were as described (Schmittgen et al., 2004). cDNA was amplified using real-time PCR and SYBR green detection using PCR primers designed to amplify the same 40 bp regions as the oligo probe on the microarray. The relative amount of each UCR to 18S rRNA was determined using the Equation $2^{-dC}T$, where $dC_T = (C_{TUCR} - C_{T18S\ rRNA})$. Relative gene expression data were multiplied by 10^6 to simplify the presentation.

Northern Blot Analysis of T-UCRs

We analyzed five UCRs, *uc.110(N)*, *uc.192(N)*, *uc.246(E)*, *uc.269A(N)*, and *uc.352(N)*, by northern blot, two of which were then cloned by RACE experiments. For a sixth one, the *uc.47(N)*, the data are not shown. Total RNA was electrophoresed on 15% PAA-urea gels (Calin et al., 2002). RNA sources included 11 normal tissues (breast, liver, lung, kidney, and pancreas) in duplicate or triplicate (purchased from Ambion and Clontech) and 4 normal MNC samples and 16 CLL samples prepared in the laboratory. As this represents the investigation

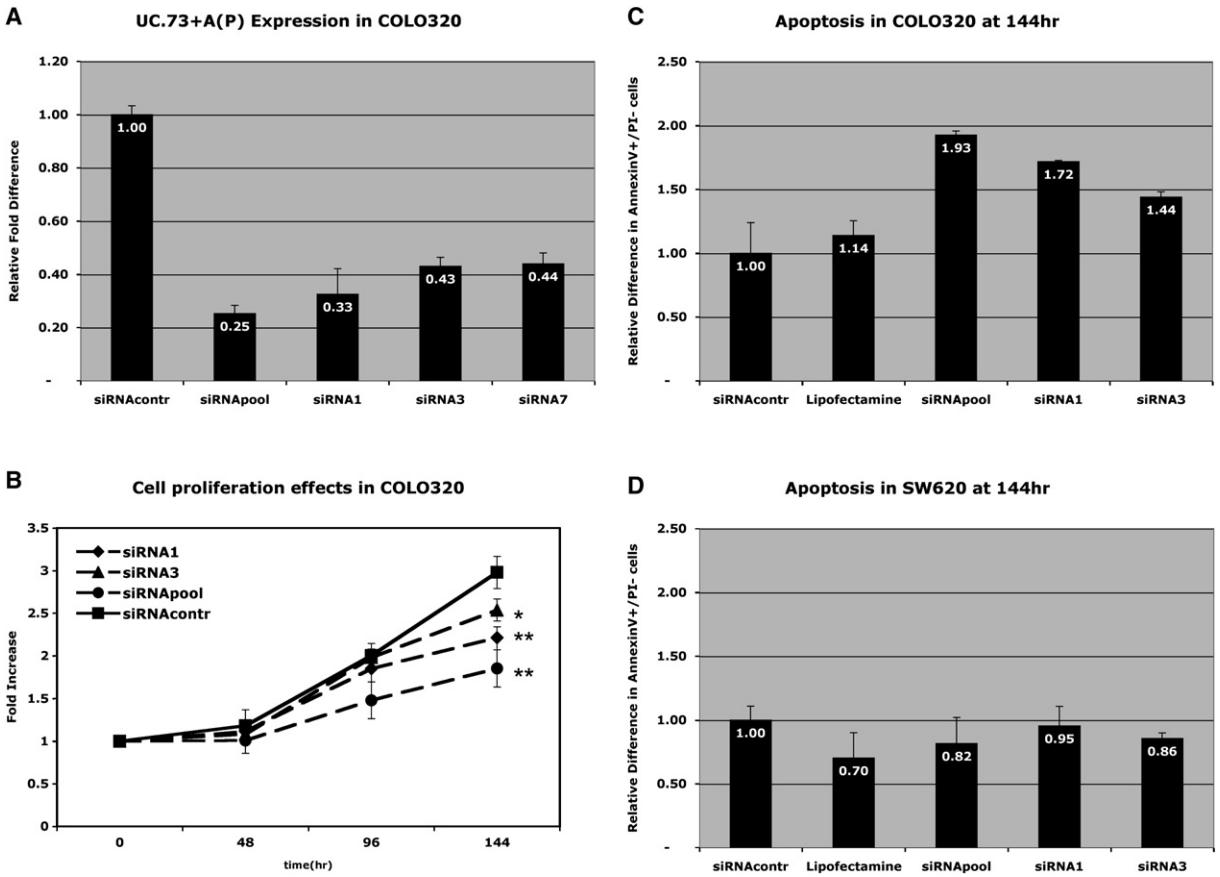


Figure 4. T-UCR 73A(P) Is Acting as an Oncogene in Colon Cancer Cells

(A) The expression inhibition by various siRNAs in COLO-320 cells. As reference value we used a siRNA control from Dharmacon. The most effective two siRNAs and a pool of four different siRNAs, including these two, were used.

(B) The antiproliferative effects of reduction in *uc.73A(P)* gene expression using siRNA-uc73A in COLO-320 colorectal cancer cells. All the results represent the median of three independent triplicate experiments. The levels of *uc.73A(P)* expression were measured by RT-PCR. Two asterisks indicate a statistically significant effect at $p < 0.01$, while one at $p < 0.05$.

(C) Reduced levels of *uc.73A(P)* (using various siRNAs) results in enhanced apoptosis as shown by the Annexin-V staining assay in COLO-320 cells. As reference value we used a siRNA control from Dharmacon.

(D) Inhibition of uc.73A(P) by various siRNAs did not influence SW620 colon cancer cell survival. All the results represent the median of three independent triplicate experiments.

All the results represent the average ±SD of three independent triplicate experiments.

by northern blot of UCR expression, we used multiple samples from the same tissues to confirm data reproducibility. The probes were designed to be identical with the oligonucleotides on the chip in order to detect the same transcripts as the microarray, and the hybridization was done as described (Calin et al., 2002).

Databases and Statistical Analyses

Databases for Genomic Locations

The UCR databases used for all the studies reported here are as published (Bejerano et al., 2004b). We restricted our analyses to 481 segments longer than 200 base pairs (bp). The Fragile site (FRA) database and the cancer-associated genomic regions (CAGR) databases are as previously published (Calin et al., 2004b).

Statistical Analyses for Genomic Locations

To test hypotheses associating the incidence of ultraconserved regions (UCRs) with fragile sites, amplified regions in cancer, and deletion regions in cancer, we utilized random effect Poisson and negative binomial regression models. Under these models, "events" were defined as the number of UCRs, and exposure "time" (i.e., fragile site versus nonfragile site) was defined as nonoverlapping lengths of the region of interest. The "length" of a region was exact, if known, or estimated as 1 Mb if unknown. For example, for each chromosome the total length of all nonoverlapping fragile sites was computed and was used as the exposure time for fragile sites. We then counted the number of UCRs occurring within fragile sites for each chromosome. The remaining length of each chromosome (total Mb – fragile sites Mb) was assumed to be nonfragile, and the remaining UCRs in each chromosome were assumed to occur in the nonfragile region. Then for each region, alternative random effects models, the zero-inflated Poisson and the zero-inflated negative binomial models were fitted, and, of the three, the best model was selected using the Akaike's Information Criteria (based on the log likelihood and number of parameters). This same approach was used for analysis of the data from expression of zinc finger proteins. The best fitting model for fragile sites with UCRs and LOH with zinc finger proteins was the zero-inflated negative binomial. For all other cases, the Poisson model is reported. When the number of categories with zero events was more than expected for a Poisson distribution, the zero-inflated negative

binomial model was preferred. When the total number of events was too small for a region, the model likelihoods were unable to converge and results are not reported. The random effect in the Poisson, zero-inflated Poisson, and zero-inflated negative binomial regression models was the individual chromosome in that data within a chromosome was assumed to be correlated. The fixed effect in each model consisted of an indicator variable(s) for the type of region being compared. We report the incidence rate ratio (IRR), 2-sided 95% confidence interval of the incidence rate ratio, and 2-sided p values for testing the hypothesis that the incident rate ratio is 1.0. An IRR significantly > 1 indicates an increase in the number of UCRs within a region over that expected by chance.

The proportions of clustering of miRNAs and zinc finger proteins were compared using an asymptotic test of the difference in two independent proportions, where we report the difference, 95% confidence interval of the difference, and p value. Of note, the ZNF transcription factor class of genes showed a significantly lower clustering (a cluster defined as the location of at least two genes from the same class at less than 50 kb genomic distance) when compared with the microRNAs [32%, 95/297 clustered ZNF genes versus 48%, 90/186 clustered miRNAs, difference = 16.4%, 95% CI = (7.5%, 25.2%), $p < 0.001$]. All computations were completed using STATA v7.0 and StatXact v7.0.

Statistical Analyses for Negative Correlations between Microarray Expression of UCRs and miRNAs

A detailed description is provided in the Supplemental Data. Briefly, the input data was constituted by a list of T-UCRs and by a list of miRNAs (the "seeds") and the corresponding matrix of expression values. We calculated r, the Spearman rank coefficient of correlation for each pair of (miR, UC) genes; namely, we evaluate the p values of the correlation tests and select the genes whose correlation value is significant at a given value of rejection. Given the high number of correlation tests performed, p values were corrected for multiple testing by using the false detection rate (FDR), as in Benjamini and Hochberg, 1995. In this way, p values control the number of false positive over the number of truly null tests, while FDR controls the number of false positive over the number of significant tests.

Functional Studies

UCR's Downregulation by Direct Interaction with MicroRNAs

The genomic sequences of *uc.160*, *uc.346A*, and *uc.348* were cloned into pGL3-control vector (Clontech) using the XbaI site immediately downstream from the stop codon of luciferase. Human megakaryocytic MEG-01 and the cervical carcinoma HeLa cell lines were grown as recommended by the ATCC. Cells were cotransfected in 12-well plates using siPORT neoFX (Ambion) according to the manufacturer's protocol using 0.4 μg of the firefly luciferase reporter vector and 0.08 μg of the control vector containing Renilla luciferase, pRL-TK (Promega). For each well, 10 nM of miRNA-sense precursor and scrambled oligonucleotides (Ambion) were used. Firefly and Renilla luciferase activities were measured consecutively using the Dual-luciferase assays (Promega) 24 hr after transfection. All experiments were performed in triplicate on 4 to 6 different days (n = 12–18).

Expression of both the ultraconserved RNA and the mature miRNA was analyzed using real-time PCR. Expression of the UCR RNA was determined by real-time PCR as described above. Expression of the mature miRNA was performed using TaqMan looped primer assays to *miR-155* (Applied Biosystems) as described (Chen et al., 2005). Mature miRNA expression was presented as 2^{-dCT} where $dCT = C_{TmiRNA} - CT_{18S\ rRNA}$); data was multiplied by 10^6 to simplify presentation.

For the patient correlation a set of 13 samples was used (including 9 CLL patients and 4 normal lymphocyte samples) and *miR-155*, *uc.346A*, and *uc.160* levels were analyzed as described above. For the identification of the "in vivo" effects in MEG01 of *miR-155* transfection, the levels of uc.346A and uc.160 were measured by qRT-PCR as described at 0, 24, and 48 hr posttransfection with the pre-miRNA 155 (Ambion) using Lipofectamine reagent.

Effects on Cancer Cell Proliferation by uc.73A(P) Inhibition

The siRNA against the *uc.73A(P)* were designed using the Dharmacon algorithm (Dharmacon siDESIGN http://www.dharmacon.com/sidesign/) entering the complete sequence of the UCR. The eight highest rank target sequences were tested. The performance was assessed after 48, 72, and 144 hr posttransfection by semiquantitative RT-PCR. The most effective two siRNAs and a pool of four different siRNAs, including these two, were used. We named these as siRNA1, siRNA3, and siRNApool.

For the cell growth assay, the human colon cancer cell lines COLO-320 and SW620 were grown in RPMI1640 medium supplemented with 10% FBS, and 1×10^4 cells were plated in 96-well plate a day before transfection. The cells were transfected with siRNA-uc.73A(P) at a final concentration of 200 nM by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The siCONTROL Non-Targeting siRNA Pool (Dharmacon Research, LaFayette, CO, USA) was used as negative control. The transfection was repeated under the same conditions every two days at 48 and 96 hr. To evaluate the cell number the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega U.S., Madison, WI, USA) was used. The readings were performed at 0, 48, 96, and 144 hr, respectively, measuring the absorbance at 490 nm using an ELISA plate reader (Spectra MAX, Molecular Devices, Sunnyvale, CA, USA). The cell growth assay was performed three times in triplicate for each treatment. The statistical differences between the number of cells at various time points with respect to time 0 was calculated using the Student's t test.

For both cell-cycle and apoptosis assays, cells were plated in 6 well plates at 6×10^5 cells per well. The day after and then every 48 hr, the cells were transfected with 200 nM siRNA. The cells were collected and fixed in cold 70% ethanol for at least 30 min. The Propidium Iodide (PI) staining was performed at 48, 96, and 144 hr in a 50 μg/ml PI (Sigma Aldrich, St. Louis, MO) and 5 μg/ml RNase DNase free (Roche Diagnostics, Indianapolis, IN, USA) PBS Solution. The apoptosis staining was performed with the Annexin V-FITC Apoptosis Detection Kit (BD PharMingen, San Jose, CA, USA) and with the PE-conjugated monoclonal active Caspase-3 antibody apoptosis kit (BD Biosciences) at 0 and 144 hr according to the manufacturer's procedure using an FACS Calibur (BD Biosciences, San Jose, CA, USA) to acquire the data. Each experiment was performed three times.

Supplemental Data

The Supplemental Data include seven supplemental figures, four supplemental tables, and Supplemental Experimental Procedures, and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/3/215/DC1/.

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Accession Numbers

All data were submitted using MIAMExpress to the ArrayExpress database and can be retrieved using the accession number E-TABM-184.