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Many commonly used siRNAs risk off-target activity ☆

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Abstract

Using small interfering RNA (siRNA) to induce sequence specific gene silencing is fast becoming a standard tool in functional genomics. As siRNAs in some cases tolerate mismatches with the mRNA target, knockdown of genes other than the intended target could make results difficult to interpret. In an investigation of 359 published siRNA sequences, we have found that about 75% of them have a risk of eliciting non-specific effects. A possible cause for this is the popular BLAST search engine, which is inappropriate for such short oligos as siRNAs. Furthermore, we used new special purpose hardware to do a transcriptome-wide screening of all possible siRNAs, and show that many unique siRNAs exist per target even if several mismatches are allowed. Hence, we argue that the risk of off-target effects is unnecessary and should be avoided in future siRNA design.

Keywords: siRNA; MicroRNA; RNA interference; Specificity

RNA interference (RNAi) is an ancient immune system on the genomic level that has been demonstrated to protect against viruses and transposons in lower animals and plants [1,2]. The active agents of RNAi are short RNAs with sequence complementarity to the target RNA. The potency and therapeutic potential of RNAi have been demonstrated by knockdown of mRNA in mammalian cells [3,4] and inhibition of disease-causing viruses like HIV [5].

Off-target activity by a small RNA can principally arise from two mechanisms: depletion on the mRNA level or translational suppression at the protein level. RNAi is generally believed to be exquisitely specific [6]. However, several groups have now observed that siRNAs can tolerate one mismatch to the mRNA target and at the same time retain good silencing capacity [7–14]. In some cases, siRNAs can tolerate several mismatches [7,11,12,15], or even tolerate mismatches while acting as a single-stranded antisense siRNA [16]. Furthermore, some domains of the siRNAs tolerate more of the mismatches than others [12,17]. A recent study also demonstrated tolerance for G:U wobble pairing between the RNA oligo and the target RNA [15]. While some microarray studies found a high specificity of siRNA effects [18,19], two other studies found large non-specific effects [20,21]. Large studies on siRNA mismatch tolerance have not yet been performed.

Another possible mechanism for off-target activity arises from the fact that the physical structure of siRNAs, ~21 nucleotide (nt) RNA oligomers, appears to be identical to the related class of microRNAs [22]. MicroRNAs are short endogenously transcribed RNAs that yield mRNA translation inhibition rather than mRNA degradation. MicroRNAs seem to have mismatches between RNA oligo and RNA target inherent in their structure. The rules regulating the functional structure of microRNA are not yet well known, but are under investigation [14,23,24].

Together the mechanisms of siRNA mismatch tolerance and microRNA translation inhibition create a risk of off-target activity when ~21 nt RNAs are introduced into human cells. We wanted to evaluate the risk of off-target activity in commonly used siRNAs in silico, and investigate the potential for finding oligomers without

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an inherent off-target risk in the set of all possible siRNAs in the human transcriptome.

Materials and methods

The collection of siRNAs was built by selection from publications in the most prestigious journals, and from the websites of commercial companies Qiagen (www.qiagen.com) and Ambion (www.ambion.com) with the rationale that the possible impact of off-target effects would be increased for siRNAs in common use. No complete collection of published siRNAs was attempted, and the reported mismatch incidence is thus only strictly valid for this subset of all published siRNAs.

A special purpose processor was used—the Pattern Matching Chip (Interagon; Trondheim, Norway; www.interagon.com)—to search for mRNAs with sequence similarity to published siRNAs. Moreover, we extracted 58,151,368 oligonucleotides of length 21 from the known cDNA in Ensembl's 17.33.1 release and screened these for mismatch similarity with the rest of the database. The Pattern Matching Chip's architecture is massively parallel and ideal for high throughput screenings such as this [25]. We consider only the number of mismatches in ungapped alignments between the siRNA probe and the mRNA target, but the Pattern Matching Chip's functionality is not by any means limited to this (information on applicability is available upon request to the authors). The total throughput of the accelerated workstation was equivalent to 512 GB/s per 21mer with unlimited mismatch sensitivity. The performance for pattern-matching purposes is thus several orders of magnitudes higher than what is generally achievable with known regular expression algorithms on ordinary processors, and enabled a screen of all 21mers against the transcriptome in just above 10 h—a task that has not been undertaken before because it would require orders of magnitude longer computing time.

Results

BLAST [26] is frequently used to determine if an siRNA is target specific. It is important to notice that BLAST is a search heuristic that sacrifices some sensitivity to gain speed, and that different search parameters may yield very different results. For example, the word size—often denoted w—is important because a region that does not contain at least w successively matching characters will be missed by BLAST [27]. The loss of sensitivity is negligible for most applications, but short query searching in general and siRNA screening in particular, require careful attention: a fraction of all potentially relevant alignments may be missed by BLAST depending on the length of the query and the positions of the mismatches.

Table 1 shows the fraction of alignments that will remain undetected by BLAST given different word sizes and number of mismatches for 19mer and 21mer queries. It has been suggested that the 3′ overhang nucleotides of the siRNA duplexes do not contribute to sequence specificity [3], and this would make 19mer targets viable. If the word size is seven—as is the recommended word size when searching for short, nearly exact matches with NCBI's BLAST—about 6% of all possible alignments with three mismatches between

Table 1
Fraction of short sequences that remains undetected by BLAST given word size, w, mismatch threshold, and oligonucleotide size

	1 mismatch		2 mismatches		3 mismatches		4 mismatches		5 mismatches		6 mismatches	
	19-mer	21-mer	19-mer	21-mer	19-mer	21-mer	19-mer	21-mer	19-mer	21-mer	19-mer	21-mer
$w = 6$	0.000	0.000	0.000	0.000	0.036	0.008	0.168	0.070	0.363	0.207	0.563	0.387
$w = 7$	0.000	0.000	0.018	0.000	0.154	0.063	0.374	0.222	0.586	0.425	0.762	0.615
$w = 8$	0.000	0.000	0.088	0.029	0.325	0.185	0.574	0.411	0.762	0.621	0.881	0.779
$w = 9$	0.000	0.000	0.211	0.100	0.505	0.343	0.729	0.586	0.870	0.766	0.946	0.881
$w = 10$	0.053	0.000	0.368	0.214	0.653	0.504	0.837	0.724	0.935	0.864	0.978	0.940
$w = 11$	0.158	0.048	0.509	0.357	0.769	0.639	0.910	0.825	0.971	0.926	0.993	0.972

The statistics are calculated assuming a random distribution of mismatches between 21mers in the transcriptome.

Table 2
Twenty published siRNAs with risk of off-target activity

siRNA primary target (Acc#/RefSeq)	siRNA name	siRNA off-target hit (Acc#/RefSeq) ⟨mismatches|wobbles⟩	Source article of siRNA
Lamin A/C (70 kDa lamin) (NM_170707)	Lamin A/C	NDUFS4 (NM_002495) <8|19> 5'-AACUGGAGUUCCAGAAGAGCA-3' ||||||| ||||||||||:|| 3'-UUGACCUGAAGGUCUUCUUGU-5'	Elbashir et al. [3]
Lamin B2 (NM_032737)	Lamin B2	CLSTN2 (NM_022131) <11, 21|> 5'-AAGAGGAGGAAGAAGCCGAGG-3' |||||||||| ||||||||| 3'-UUCUCCUCCUCCUUCGGCUCA-5' Plus four other double mismatch hits, and 63 triple mismatch hits (Supplemental Table S3).	Harborth et al. [29]
Caspase-1 (NM_033292)	Caspase-1	COP (NM_052889) <|> 5'-AAGGGGCACAGGCAUGCCAAA-3' ||||||||||||||||||| 3'-UUCCCCGUGUCCGUACGGUUU-5'	Lassus et al. [37]
Caspase-8 (NM_001228)	Caspase-8	TNRC9 (U80736) <14,16|> 5'-AAUCACAGACUUUUGCCAAAG-3' |||||||||||| | ||||| 3'-UUAGUGUCUGAAACCUGUUUC-5'	Chun et al. [30]
Serine/threonine E-protein kinase kist (NM_175866)	hKIS	SARS (NM_006513) <4,10|> 5'-AAGGAGUUCAUGCCGCCAGGA-3' ||| ||||| |||||||||| 3'-UUCGUCAAGAACGGCGGUCCU-5'	Boehm et al. [31]
β-Arrestin 2 (NM_004313)	ARRB2	PABPC4 (NM_003819) <3,15|> 5'-AAUGACCGCAAAGUAUUUGUG-3' || |||||||||| |||||| 3'-UUCCUGGCGUUUCACAAACAC-5' Plus O43713 (O43713) <3,15|>	Ahn et al. [38]
Suppressor of cytokine signaling 3 (NM_003955)	SOCS-3	ZNF323 (NM_030899)<8,9|> 5'-AAGACCCUAUCUGGGACCAAGAA-3' ||||||| |||||||||||| 3'-UUCUGGGUCAGACCCUGGUUCUU-5'	Leung et al. [32]
E1A-associated protein P300 (NM_001429)	P300	SLC1A2 (NM_004171) <4|1> 5'-GAUAGUGCUGAAGAGGAGGGG-3' :|| |||||||||||||||| 3'-UUACCACGACUUCUCCUCCCC-5'	Debes et al. [39]
HIF-prolyl hydroxylase 1 (NM_053046)	PHD1-II	SLC4A2 (NM_003040) <2|16> 5'-CCCCACCAUGGCCUUUGUGCG-3' | ||||||||||||| ||||| 3'-GCGGUGGUACCGGAACCACGC-5'	Berra et al. [40]
Serine Protease HTRA2 (NM_013247)	HtrA2-2	CRSP2 (NM_004229) <5,8|> 5'-AAUGCCAUCAACAAACUCCCC-3' |||| || |||||||||||| 3'-UUACCGUUGUUGUUUGAGGGG-5'	Martins et al. [48]
Tumor protein p53, TP53 (NM_000546)	p53	TP73 (NM_005427) <14|1,2> 5'-GGGCAUGAACCGGCGGCCCAU-3' ::||||||||||| ||||||| 3'-UUCGUACUUGGCCUCCGGGUA-5'	Martinez et al. [41]

Table 2 (*continued*)

siRNA primary target (Acc#/RefSeq)	siRNA name	siRNA off-target hit (Acc#/RefSeq) ⟨mismatches|wobbles⟩	Source article of siRNA
Aminopeptidase PILS (NM_016442)	ERAAP	NM_152418 (NM_152418) <21,11|6> 5'-AACGUGGUGACGGGACACCAG-3' |||||:|||| ||||||||| 3'-UUGCAUCACUACCCUGUGGUA-5'	Serwold et al. [42]
Serine/threonine protein kinase PLK (NM_005030)	Plk1	Q8N8U7 (Q8N8U7) <1|6,9> 5'-CAGGGUGGUUUUGCCAAGUGC-3' ||||:||:||||||||||| 3'-UUCCCGCCGAAACGGUUCACG-5'	Liu and Erikson [43]
RAS-related protein RAL-A (NM_005402)	RalA-II	CNTN4 (NM_175607) <5,21|2> 5'-AGAUACCACUGCUCAGCUCUC-3' |:|| |||||||||||||| 3'-UUUAAGGUGACGAGUCGAGAC-5'	Moskalenko et al. [44]
C–C chemokine receptor type (NM_000579)	CCR5-1	CCR2 (NM_000647) <4,7|18> 5'-AAGUGCUUGACUGACAUUUAC-3' ||| || ||||||||||:||| 3'-UUCUCGUACUGACUGUAGAUG-5'	Qin et al. [45]
Likely ortholog of *Caenorhabditis elegans* anterior pharynx defective 1A (APH-1A) (NM_016022)	APH-1a-2	IRS1 (NM_005544)<4,7|21> 5'-AACAGAAGGAGAUGGGUGAUU-3' ||| || |||||||||||: 3'-UUGCCUACCUCUACCCACUAG-5'	Lee et al. [28]
ATR interacting protein (NM_032166)	ATRIP	ARHGEF6 (NM_004840) <9|7,12> 5'-AAGAAGGGACCUAGAAAAGCU-3' ||||||:| ||:||||||||| 3'-UUCUUCUCCGGGUCUUUUCGA-5' Plus 4 other triple mismatch hits (Supplemental Table S3).	Cortez et al. [46]
Protein kinase C-delta (NM_006254)	PKC-delta	Q96I56 (Q96I56) <1,20|2> 5'-UGCUGAGCGCCUCCUUCAGC-3' :|||||||||||||||| | 3'-UUGACUCGCGGAGGAAGUAG-5' Plus 15 other triple mismatch hits (Supplemental Table S3).	Yoshida et al. [47]
Numa1 (NM_006185)	NuMa	FBXO21 (NM_015002) <10, 16|5> 5'-AAGGUGUGGAAGGAGCAGUUC-3' ||||:|||| ||||| ||||| 3'-UUCCGCACCGUCCUCUUCAAG-5' Plus 6 other triple mismatch hits (Supplemental Table S3).	Elbashir et al. [3]
Cylindromatosis (turban tumor syndrome) (NM_015247)	CYLD (19mer, from shRNA)	NM_021638 (NM_021638) <|13,14> 5'-CAAAGAGAACUGUGUGAGG-3' ||||||||||||::||||| 3'-GUUUCUCUUGACGUACUCC-5'	Kovalenko et al. [35]

The shown off-target hits, 9 with double-mismatches, 10 with triple mismatches, and 1 double-wobble mismatch from an shRNA, are excerpt of the full list of off-target hits available as Supplementary Table S3. Alignments of target areas of the most significant off-target hit are shown, with mRNA presented as the upper strand and the complementary siRNA strand being the lower strand. Unique accession numbers and Ensembl RefSeq numbers for the primary target mRNA and the possible off-targeted mRNA are given when available. In some cases dTT 3′ ends are presented as UU. A compressed presentation form is also utilized in the form $\langle X1, X2, \ldots, Xn|Y1, Y2, \ldots, Yn\rangle$, where Xn and Yn stand for mismatch positions and G:U wobble positions, respectively, relative to the 5′ end of the mRNA:siRNA alignment.

21mers will be missed. Moreover, the fraction of alignments that are missed increases to 15% if 19mers are used instead of 21mers. To summarize, increasing the word size or allowing more mismatches both contribute towards a higher rate of missed hits. Three or more mismatches may be biologically relevant since G:U

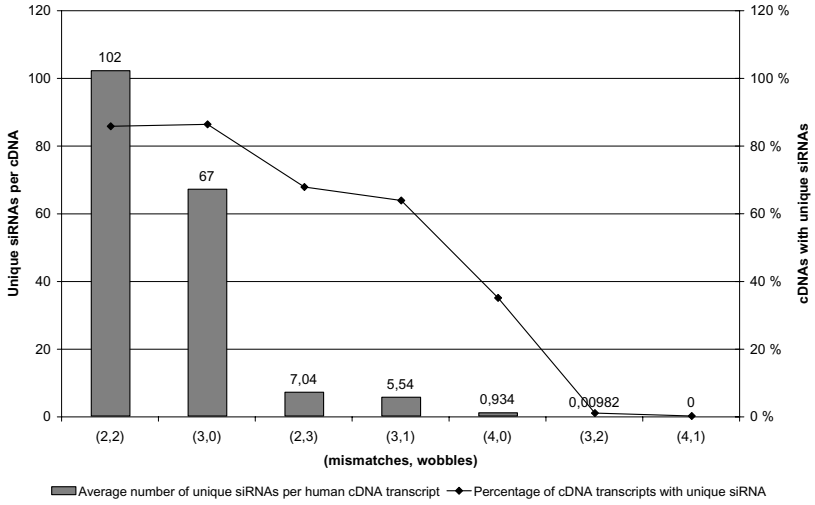

Fig. 1. siRNA uniqueness statistics for various dissimilarity thresholds in human cDNA. The bars relate to the left vertical axis and show how many unique siRNAs that exist given various mismatch and G:U wobble thresholds (G:U wobbles are not counted as regular mismatches). The line relates to the right vertical axis and shows the percentage of transcripts that contains at least one unique siRNA given the same threshold parameters.

mismatches seem to be more tolerable in the RNAi pathway than are regular mismatches [15].

We collected and screened siRNAs from public sources to test the practical consequences of missed hits from BLAST. PubMed lists *p.t.* 467 articles with keyword *siRNA*, up from 180 in 2002 and 25 in 2001, illustrating the rapid and widespread application of siRNAs. A complete collection of published siRNAs was not possible, because the exact sequences of oligonucleotides were not always stated in the papers. Altogether, we screened 359 human siRNAs against all known cDNAs from Ensembl's 17.33.1 release.

Table 2 is an excerpt from the results (full listing in Supplementary Table S3) and contains the siRNAs that were perceived as most interesting based on the number of mismatches, the relative mismatch positions, and the prestige of the journals in which the siRNAs were published. We were surprised to see that many siRNAs were identical to other sequences in all but zero, one or two positions. About 20% of the collected siRNAs had two or fewer mismatches in off-target alignments (Supplementary Table S3). We will briefly discuss some illustrative hits in the Discussion.

Silencing activity with three mismatches between siRNA and mRNA was demonstrated only recently, where three G:U wobble positions were found to be tolerated [15]. If this phenomenon is widespread, then siRNA off-target activity might affect the results of 10 studies listed in Table 2 [28]. Furthermore, approximately 75% of the 359 siRNAs collected had off-target alignments with three or fewer mismatches (Supplementary Table S3). Hence, if triple mismatch tolerance is confirmed, the risk of off-target activity seems high with publicly available siRNAs.

To test our predictions of siRNA off-target activity, we re-analyzed the data of two microarray studies performed by Chi et al. and Jackson et al. [19,20]. These papers concluded differently on the specificity of siRNA. While Chi et al. found their siRNAs to elicit no secondary responses, Jackson et al. [20] found that their siRNAs were unspecific, despite having designed the siRNAs, using BLAST, to display fewer than 18 nucleotides of identity to known genes other than the targeted gene. However, we found 54 instances of 18 nucleotides of identity with other genes in the transcriptome (Supplementary Table S4). Furthermore, we also found that two of these off-target predictions resulted in very significant downregulation of the off-target genes, while several others had less significant downregulation (Supplementary Table S6). We note that Chi et al.'s siRNAs had only five instances of off-target hits with less than four mismatches (Supplementary Table S5). Thus, it seems that Chi et al.'s siRNAs were by design more unique than the siRNAs of Jackson et al. though both studies had siRNAs that were generally more unique than many of the other siRNAs in the literature (Supplementary Table S3). Taken together, these results indicate a partial explanation of the differing siRNA specificity results of Chi et al. and Jackson et al.

The probability of finding unique 21mers decreases as more mismatches are allowed. The possibility of screening every possible oligonucleotide is usually dismissed as impractical since it would take months of computing time to use BLAST to align the approximately 60 million 21mers that can be extracted from known cDNA. We completed the task in approximately 10 h with newly developed pattern matching hardware. Since genomic records are updated regularly, the screen must be repeated to account for differences between the versions; thus, speed is important from this perspective as well.

We found that most transcripts contain 21mers that are unique even if three mismatches are allowed. Fig. 1

shows the average number of unique 21mers that can be found per transcript (bars) and the fraction of transcripts that contain unique 21mers (line) at various levels of dissimilarity. A 21mer is considered unique if it matches the target sequence only, and if its reverse complement does not match any region in the full cDNA database. Note that many genes produce alternative transcripts that have several exons in common, which means that they are less likely to contain entirely unique oligonucleotides. If the distinction between transcripts and genes were not important, a higher fraction of genes would contain unique siRNAs at the different levels of dissimilarity. Nevertheless, about 90% of all transcripts contains at least one unique siRNA even if two mismatches and two G:U wobbles are allowed. Moreover, almost 40% of all transcripts have target specific oligos even if siRNAs were to have silencing activity with as many as four mismatches to their targets. Hence, very specific siRNAs exist for most targets in the human transcriptome.

Discussion

In some cases, short interfering RNAs (siRNAs) can tolerate mismatches [7–16], while microRNAs have been reported to be able to act as siRNA [14,24] and vice versa [23]. The phenomena of mismatch tolerance and microRNA silencing thus creates a risk of knockdown of other genes besides the intended target. A limited number of illustrative cases from influential publications will serve as examples (Table 2). The first two examples of siRNAs are chosen from two of the founder articles of the siRNA field. The Lamin A/C and the NDUFS4 mRNA are identical in 19 of 21 positions (Table 2): one is a G:U wobble near the 3′ end of the alignment, and the other is a mismatch in the less sensitive 5′ end of the siRNA [12,17]. This kind of off-target hit could theoretically carry a high risk of biological off-target activity.

More serious off-target hits are generated by the siRNA against Lamin B2, a gene that induced cell into apoptosis when it was downregulated. The gene was therefore concluded to be essential [29]. If there is significant mRNA depletion from only one of the four other genes that the Lamin B2 siRNA have only two mismatches with, or from the 63 other genes that this siRNA have three mismatches with (Table 2 and Supplementary Table S3), then the conclusions of these particular siRNA-experiments seem weaker.

Other significant examples from Table 2 should also be briefly mentioned. Apoptosis is a complex cascade of events where an initial difference in parameters might give a completely different outcome. Two siRNAs used in apoptosis-studies, Caspase-1 and Caspase-8, have significant similarity with other genes (Table 2), and might thus theoretically affect some of the conclusions of these works [30]. Cell signaling is another field with complex chains of effects where off-target activity by siRNA might influence the results. Illustrative examples include siRNAs against β-arrestin, KIST, and Suppressor of Cytokine Signaling 3 (Table 2) [31,32]. Furthermore, cancer studies, as in the case of the siRNA against p300 (Table 2), are yet another field where mismatch effects might cause confusion, both in laboratory experiments and later in clinical studies. The double mismatches that have been discussed so far would have been detected by BLAST (Table 1). Nevertheless, about 20% of the 359 siRNAs that was screened are not specific if two mismatches are allowed.

Tolerance of three mismatches between siRNA and mRNA was reported recently [15]. If triple mismatch tolerance is confirmed in other studies, the risk of off-target activity is rather common, with approximately 75% of the 359 siRNAs collected having off-target alignments with three or fewer mismatches (Supplementary Table S3). As many scientists seem to prefer to use established siRNAs from existing publications rather than designing siRNAs de novo, the risk of off-target activity is propagated to many new studies.

The use of siRNA from hairpin siRNA constructs provides additional complexity and an additional risk factor. Although both Brummelkamp et al. [33] and Paddison et al. [34] have demonstrated production of both sense and antisense strands from hairpin siRNA transcripts, the exact position of double-stranded hairpin cleavage has not been studied to our knowledge. Furthermore, the exact hairpin RNA transcript has only been predicted [33]. Possibly, several siRNAs differing only slightly in sequence are produced, thus increasing the uncertainty of specific targeting when using hairpin siRNA as compared with synthetic siRNA. The siRNA against CYLD [35] has a double-wobble mismatch in the central 19-mer (Table 2), which might cause off-target activity depending on the exact transcript, hairpin RNA cleavage, and the role of the siRNA overhangs in target recognition [3].

Thus, in conclusion, siRNA design should take into account that:

(1) BLAST may miss important alignments for such short oligos as siRNAs;
(2) many commonly used siRNAs that have been published are (therefore) not sufficiently unique to avoid risk for off-target activity; but
(3) oligos that are more unique do exist, and can be found using algorithms with higher sensitivity such as Smith and Waterman [36].

Finally, we stress that this in silico study merely points to possible targets—none of the siRNAs mentioned in this study have yet directly been tested experimentally. When computing the distribution of unique siRNAs in the transcriptome, we find that it is possible to avoid siRNA candidates with three or fewer

mismatches. Thus, we would argue that when designing experiments taking months of effort and high costs, not least of them the cost of the siRNAs themselves, and especially in the likely event of siRNAs going onward to animal and clinical trials, the risk of off-target activity is unnecessary and should be avoided.

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