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Kallikreins as microRNA targets: an *in silico* and experimental-based analysis

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Abstract

microRNAs (miRNAs) are non-coding RNAs that target specific mRNAs. They have been shown to control many biological processes including cancer pathogenesis. Kallikreins (KLKs) are a family of serine proteases that are attracting interest as cancer biomarkers. The mechanism of regulation of kallikrein expression is largely unknown. We investigated the potential roles of miRNAs in regulating KLK expression. Using a bioinformatics approach, we identified 96 strong KLK/miRNA interactions. KLK10 is the most frequently targeted kallikrein, followed by KLK5 and KLK13. KLK1, KLK3, KLK8 and KLK12 do not have strongly predicted miRNA/KLK interactions. Ten miRNAs are predicted to target more than one KLK. KLK2, KLK4, KLK5 and KLK10 have multiple miRNA-targeting sites on their transcript. Chromosomes 19 and 14 harbor significantly more KLK-targeting miRNAs. Many KLK-targeting miRNAs have been shown to be dysregulated in malignancy. We experimentally verified our bioinformatics data for the let-7f miRNA in a cell line model. let-7f transfection led to a significant decrease in secreted KLK6 and KLK10 protein levels. Co-transfection of let-7f and anti-let-7f inhibitor was able to partially rescue these protein levels. We conclude that miRNAs play a role in the regulation of KLK expression. Further studies are needed to investigate whether this regulation is altered in cancer.

Keywords: cancer; kallikrein; KLK; microRNA; miRNA; RNAi; siRNA; tumor markers.

Introduction

A new class of small non-coding RNAs, named microRNAs (miRNAs), was discovered recently and shown to regulate gene expression at the post-transcriptional level, for the most part by binding through partial sequence homology to the 3′ untranslated region (UTR) of target mRNAs and causing block of translation and/or mRNA degradation (Esquela-Kerscher and Slack, 2006). miRNAs are 19–25-nt molecules cleaved from 70–100-nt hairpin pre-miRNA precursors. The precursor is cleaved by cytoplasmic RNase III Dicer into a nucleotide miRNA duplex: one strand of the short-lived duplex is degraded, whereas the other strand, which serves as mature miRNA, is incorporated into the RNA-induced silencing complex and drives the selection of target mRNAs containing antisense sequences (Wu et al., 2007).

Studies have shown that miRNAs play important roles in essential processes, such as differentiation, cell growth, and cell death (Shivdasani, 2006). Moreover, it has been shown that miRNAs are aberrantly expressed or mutated in cancers, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate (Iorio et al., 2005).

Tissue kallikreins are serine proteases that are encoded by highly conserved multi-gene family clusters in rodents and humans. The human locus at 19q13.4 contains 15 genes. Members of the tissue kallikrein (KLK) gene family are fast attracting clinical interest owing to their potential as cancer biomarkers, particularly for hormone-dependent cancers (Borgono and Diamandis, 2004; Borgono et al., 2004; Clements et al., 2004; Yousef et al., 2005).

Control of kallikrein expression is not fully understood. It is known that steroid hormones play a role (Yousef and Diamandis, 2002), and that many KLKs are expressed in parallel (Shaw and Diamandis, 2007). Apart from *KLK1–3*, no kallikrein gene promoters have been well characterized. Moreover, the relatively short distances between adjacent kallikrein genes (as short as the 1.5 kb between *KLK1* and *KLK15*; Yousef et al., 2000) and the absence of classic promoter motifs in many KLK genes raise the possibility of the existence of other mechanisms that simultaneously control expression of groups of kallikreins.

In this study we investigated the possible involvement of miRNAs as kallikrein post-transcriptional regulators. We provide the first detailed bioinformatic analysis of the predicted relationship between kallikreins and miRNAs. We also provide experimental validation of one such prediction.

Results

Bioinformatics target prediction analysis

Collectively, the four programs predicted 646 unique miRNA/KLK interactions, with 550 (85%) of these inter-

actions predicted by only one program, 85 interactions predicted by two programs and 11 interactions predicted by three programs (Figure 1). We defined 'strongly predicted interactions' as those predicted by two or more programs and further analyses are limited to this group. KLK10 was predicted to be the most targeted kallikrein, with 19 predicted targeting miRNAs. KLK5 and KLK13 were both predicted targets of 15 miRNAs (Table 1 and Figure 1). KLK1, KLK3, KLK8 and KLK12 do not have strongly predicted miRNA interactions. Ten miRNAs were predicted to target more than one KLK (Table 2). For example, let-7f was predicted to target KLK6 and KLK10; KLK10 and KLK15 were both predicted targets of miR-224; and the closely related miR-33a and miR-33b were both predicted to target KLK7 and KLK9. No miRNAs were predicted to target more than two KLKs.

miRNA target sites on KLK transcripts

In 83 of the 96 (86%) miRNA/KLK interactions, there was only one predicted target site on the 3′UTR of the KLK transcript for the corresponding miRNA. KLK2, KLK4, KLK5 and KLK10 have multiple miRNA-targeting sites on their transcript (Table 1). KLK4 had five and ten predicted target sites on its transcript for miR-637 and miR-765, respectively. There were four target sites on the KLK2 transcript for miR-502. There were three target sites on the KLK10 transcript for miR-516a and miR-516b.

Chromosomal distribution of KLK-targeting miRNAs

Most chromosomes (14/23) harbor between two and four KLK-targeting miRNAs. There were no KLK-targeting miRNAs on chromosomes 2, 4 and 16. Chromosome 19 (19 miRNAs) and 14 (14 miRNAs) had significantly more KLK-targeting miRNAs than other chromosomes, followed by the X chromosome (13 miRNAs). miRNAs appear to be in clusters on these chromosomes. Most of the KLK-targeting miRNAs on chromosome 14 were within the chromosomal band 14q32 (93%) and those on chromosome 19 were clustered around band 19q13 (74%) (Figure 2).

The KLK locus is located on chromosomal band 19q13, close to the largest KLK-targeting miRNA cluster. Figure 2 shows the relative locations of KLK-targeting miRNAs on chromosome 19. miRNAs within the 19q13 band target a range of KLKs, including KLK5, KLK7, KLK10, KLK13 and KLK15 (Table 2). The co-localization of the KLK cluster and their targeting miRNAs may be of biological significance.

Expression of KLK-targeting miRNAs in cancer

We compiled experimental results from 39 published studies on miRNA expression in cancer. Table 3 shows that many KLK-targeting miRNAs have been shown to be dysregulated in different cancers. For example, miR-125a and miR-125b were shown to be dysregulated in seven and eight cancers, respectively, including breast cancer. A recent study showed that transfection of a breast cancer cell line with either miR-125a or miR125b effectively suppressed ERBB2 and ERBB3 activities (Scott et al., 2007).

The correlation between locations of KLK-targeting miRNAs and cancer fragile sites of various cancers was also investigated. Table 4 shows a partial list of the KLK-targeting miRNAs located within reported fragile sites of breast, prostate and ovarian cancers. A total of 81 KLK-targeting miRNAs are located in reported breast cancer fragile sites, 9 in prostate cancer fragile sites, and 51 in ovarian cancer hotspot regions.

The let-7 family and KLK regulation

Bioinformatics analysis provided strong indications that the let-7 miRNA family interacts with KLKs. Seven members within this family are predicted to target KLK10; five of these interactions were predicted by three programs. In addition, KLK6 was predicted as a target of hsa-let-7f by three programs. The let-7 family is known to be differentially expressed in diverse cancers (Table 3). A recent report showed that reduced expression of let-7 miRNA in human lung cancers relative to normal tissues is an independent indicator of poor prognosis (Takami-

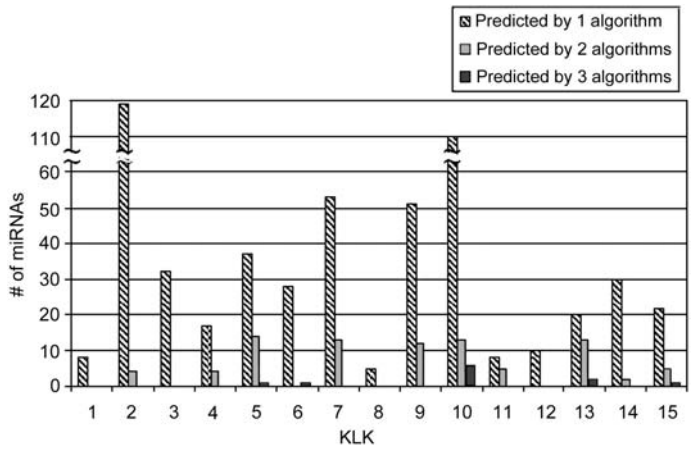

Figure 1 Predicted KLK-targeting miRNAs.
Kallikrein genes are represented by their numbers on the horizontal axis and the numbers of their targeting miRNAs are displayed on the Y-axis. Four different algorithms were used for target prediction. The patterned bar represents interactions that are predicted by only one algorithm and were not included in subsequent analyses. Interactions predicted by two programs are represented by the white bars and those by three programs by the solid bars.

Table 1 Predicted KLK-miRNA interactions and number of predicted target sites on KLK transcripts.

KLK	miRNA	Programs[a]	Target sites[b]
KLK2	hsa-mir-211	2	1
KLK2	hsa-mir-324	2	1
KLK2	hsa-mir-337	2	1
KLK2	hsa-mir-502	2	4
KLK4	hsa-mir-422a	2	1
KLK4	hsa-mir-548d	2	1
KLK4	hsa-mir-637	2	5
KLK4	hsa-mir-765	2	10
KLK5	hsa-mir-106a	2	1
KLK5	hsa-mir-106b	2	1
KLK5	hsa-mir-122a	2	1
KLK5	hsa-mir-125a	2	2
KLK5	hsa-mir-125b	2	2
KLK5	hsa-mir-143	2	1
KLK5	hsa-mir-17	2	1
KLK5	hsa-mir-185	3	1
KLK5	hsa-mir-20b	2	1
KLK5	hsa-mir-299	2	1
KLK5	hsa-mir-326	2	1
KLK5	hsa-mir-491	2	2
KLK5	hsa-mir-519d	2	1
KLK5	hsa-mir-519e	2	1
KLK5	hsa-mir-625	2	1
KLK6	hsa-let-7f	3	1
KLK7	hsa-mir-199a	2	1
KLK7	hsa-mir-199b	2	1
KLK7	hsa-mir-30a	2	1
KLK7	hsa-mir-30e	2	1
KLK7	hsa-mir-33a	2	1
KLK7	hsa-mir-338	2	1
KLK7	hsa-mir-33b	2	1
KLK7	hsa-mir-369	2	1
KLK7	hsa-mir-509	2	1
KLK7	hsa-mir-519a	2	1
KLK7	hsa-mir-519c	2	1
KLK7	hsa-mir-591	2	1
KLK7	hsa-mir-628	2	1
KLK9	hsa-mir-18a	2	1
KLK9	hsa-mir-18b	2	1
KLK9	hsa-mir-198	2	1
KLK9	hsa-mir-33a	2	1
KLK9	hsa-mir-33b	2	1
KLK9	hsa-mir-376a	2	1
KLK9	hsa-mir-376b	2	1
KLK9	hsa-mir-431	2	1
KLK9	hsa-mir-548c	2	1
KLK9	hsa-mir-598	2	1
KLK9	hsa-mir-612	2	1
KLK9	hsa-mir-663	2	1
KLK10	hsa-let-7a	3	1
KLK10	hsa-let-7b	2	1
KLK10	hsa-let-7c	3	1
KLK10	hsa-let-7d	3	1
KLK10	hsa-let-7e	3	1
KLK10	hsa-let-7f	3	1
KLK10	hsa-let-7g	2	1
KLK10	hsa-mir-148a	2	1
KLK10	hsa-mir-148b	2	1
KLK10	hsa-mir-197	2	2
KLK10	hsa-mir-214	2	2
KLK10	hsa-mir-224	2	1
KLK10	hsa-mir-326	2	2
KLK10	hsa-mir-377	2	1
KLK10	hsa-mir-496	2	2
KLK10	hsa-mir-516a	2	3
KLK10	hsa-mir-516b	2	3
KLK10	hsa-mir-598	2	1
KLK10	hsa-mir-98	3	1
KLK11	hsa-mir-186	2	1
KLK11	hsa-mir-409	2	1
KLK11	hsa-mir-495	2	1
KLK11	hsa-mir-511	2	1
KLK11	hsa-mir-542	2	1
KLK13	hsa-mir-1	2	1
KLK13	hsa-mir-141	2	1
KLK13	hsa-mir-181b	2	1
KLK13	hsa-mir-181c	2	1
KLK13	hsa-mir-181d	2	1
KLK13	hsa-mir-206	2	1
KLK13	hsa-mir-34a	3	1
KLK13	hsa-mir-34c	2	1
KLK13	hsa-mir-409	2	1
KLK13	hsa-mir-449	3	1
KLK13	hsa-mir-453	2	1
KLK13	hsa-mir-455	2	1
KLK13	hsa-mir-494	2	1
KLK13	hsa-mir-515	2	1
KLK13	hsa-mir-542	2	1
KLK14	hsa-mir-612	2	1
KLK14	hsa-mir-661	2	1
KLK15	hsa-mir-224	3	1
KLK15	hsa-mir-498	2	1
KLK15	hsa-mir-552	2	1
KLK15	hsa-mir-608	2	1
KLK15	hsa-mir-638	2	1
KLK15	hsa-mir-663	2	1

[a]A list of the different programs used for target prediction is given in the materials and methods section.
[b]The number of predicted target sites on the 3′ untranslated region of the corresponding KLK mRNA.

zawa et al., 2004). The let-7f miRNA was selected for experimental verification.

Cell line transfection

The breast cancer cell-line MDA-M8-468 was transfected with let-7f and appropriate controls, as outlined in the materials and methods section. Day 3 after transfection, when KLK protein levels were detectable for ELISA measurements, was taken as a baseline measurement. As shown in Figure 3, let-7f transfection led to a significant decrease in KLK6 and KLK10 protein levels ($p<0.05$), and this was most apparent between days 3 and 7 post-transfection. Control transfection did not cause a significant decrease in KLK levels. Co-transfection of let-7f and anti-let-7f inhibitor was able to partially restore KLK6 and KLK10 levels. Quantitative RT-PCR analysis confirmed that the levels of let-7f showed a three- to four-fold increase in transfected compared to non-transfected cells (Figure 4).

Discussion

Identification of miRNA targets usually involves two steps: target prediction using bioinformatic approaches, followed by target validation. At the moment, there is no 'gold standard' program for miRNA target prediction analysis. In a recent review, TargetScan, PicTar and miRanda were chosen as the most reliable target predic-

Table 2 Chromosomal locations of KLK-targeting miRNAs.

miRNA[a]	Band[a]	Targets
hsa-let-7a-1, -2, -3	9q22, 11q24, 22q13	KLK10
hsa-let-7b	22q13	KLK10
hsa-let-7c	21q21	KLK10
hsa-let-7d	9q22	KLK10
hsa-let-7e	19q13	KLK10
hsa-let-7f-1, -2	9q22, Xp11	KLK6, KLK10
hsa-let-7g	3p21	KLK10
hsa-mir-106a	Xq26	KLK13
hsa-mir-106b	7q22	KLK5
hsa-mir-1-1, -2	20q13, 18q11	KLK5
hsa-mir-122	18q21	KLK5
hsa-mir-125a	19q13	KLK5
hsa-mir-125b-1, -2	11q24, 21q21	KLK5
hsa-mir-141	12p13	KLK13
hsa-mir-143	5q33	KLK5
hsa-mir-148a	7p15	KLK10
hsa-mir-148b	12q13	KLK10
hsa-mir-17	13q31	KLK5
hsa-mir-181b-1, -2	1q31, 9q33	KLK13
hsa-mir-181c	19p13	KLK13
hsa-mir-181d	19p13	KLK13
hsa-mir-185	22q11	KLK5
hsa-mir-186	1p31	KLK11
hsa-mir-18a	13q31	KLK9
hsa-mir-18b	Xq26	KLK9
hsa-mir-197	1p13	KLK10
hsa-mir-198	3q13	KLK9
hsa-mir-199a-1, -2	19p13, 1q24	KLK7
hsa-mir-199b	9q34	KLK7
hsa-mir-206	6p12	KLK13
hsa-mir-20b	Xq26	KLK5
hsa-mir-211	15q13	KLK2
hsa-mir-214	1q24	KLK10
hsa-mir-224	Xq28	KLK10, KLK15
hsa-mir-299	14q32	KLK5
hsa-mir-30a	6q13	KLK7
hsa-mir-30e	1p34	KLK7
hsa-mir-324	17p13	KLK2
hsa-mir-326	11q13	KLK5, KLK10
hsa-mir-337	14q32	KLK2
hsa-mir-338	17q25	KLK7
hsa-mir-33a	22q13	KLK7, KLK9
hsa-mir-33b	17p11	KLK7, KLK9
hsa-mir-34a	1p36	KLK13
hsa-mir-34c	11q23	KLK13
hsa-mir-369	14q32	KLK7
hsa-mir-376a-1, -2	14q32	KLK9
hsa-mir-376b	14q32	KLK9
hsa-mir-377	14q32	KLK10
hsa-mir-409	14q32	KLK11, KLK13
hsa-mir-422a	15q22	KLK4
hsa-mir-431	14q32	KLK9
hsa-mir-449a	5q11	KLK13
hsa-mir-453	14q32	KLK13
hsa-mir-455	9q32	KLK13
hsa-mir-491	9p21	KLK5
hsa-mir-494	14q32	KLK13
hsa-mir-495	14q32	KLK11
hsa-mir-496	14q32	KLK10
hsa-mir-498	19q13	KLK15
hsa-mir-502	Xp11	KLK2
hsa-mir-509-1, -2, -3	Xq27	KLK7
hsa-mir-511-1, -2	10p12	KLK11
hsa-mir-515-1, -2	19q13	KLK13
hsa-mir-516a-1, -2	19q14	KLK10
hsa-mir-516b-1, -2	19q15	KLK10
hsa-mir-519a-1, -2	19q16	KLK7
hsa-mir-519c	19q13	KLK7
hsa-mir-519d	19q13	KLK5
hsa-mir-519e	19q13	KLK5
hsa-mir-542	Xq26	KLK11, KLK13
hsa-mir-548c	12q14	KLK9
hsa-mir-548d-1, -2	8q24, 17q24	KLK4
hsa-mir-552	1p34	KLK15
hsa-mir-591	7q21	KLK7
hsa-mir-598	8p23	KLK9, KLK10
hsa-mir-608	10q24	KLK15
hsa-mir-612	11q13	KLK9, KLK14
hsa-mir-625	14q23	KLK5
hsa-mir-628	15q21	KLK7
hsa-mir-637	19p13	KLK4
hsa-mir-638	19p13	KLK15
hsa-mir-661	8q24	KLK14
hsa-mir-663	20p	KLK9, KLK15
hsa-mir-765	1q23	KLK4
hsa-mir-98	Xp11	KLK10

[a] miRNA names and locations are given according to release 10.0 of the miRBase database of the Sanger Institute (http://microrna.sanger.ac.uk/targets/v4/).

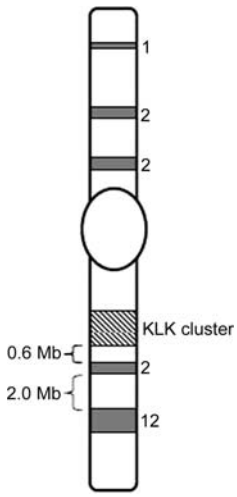

Figure 2 Relative locations of miRNAs and their targeted kallikreins on chromosome 19.
The centromere location is shown by a circle, with the short arm on top of it and the long arm below. The solid gray bars represent the locations of KLK-targeting miRNAs that are located on the chromosome, and the number adjacent to each bar indicates the number of miRNAs within that location. The patterned bar represents the KLK gene cluster. This distance between the KLK cluster and the closest miRNAs is shown (Figure not drawn to scale).

tion programs available, with a sensitivity rate of 65–68%. Programs use a variety of factors to generate miRNA target predictions, including RNA folding and thermodynamics, complementarity of the miRNA to its target sequence, and conservation of the target site across species (Sethupathy et al., 2006; Maziere and Enright, 2007), and this makes it difficult to interchange results between them.

Validating predictions of miRNA targets is challenging, and so far there is no simple, high-throughput method for biologically validating miRNA targets. The method most commonly used implements tissue culture assays

Table 3 KLK-targeting miRNAs that are documented to be dysregulated in other malignancies.

miRNA	Target	Cancer type[a]
hsa-let-7a	KLK10	Breast, colorectal, head and neck, liver, lung Prostate, stomach, thyroid, uterus
hsa-let-7b	KLK10	Head and neck, liver, prostate
hsa-let-7c	KLK10	Cervix, head and neck, liver, lung, prostate, thyroid
hsa-let-7d	KLK10	Breast, head and neck, liver, pancreas, prostate, uterus
hsa-let-7e	KLK10	Liver, uterus
hsa-let-7f	KLK6, KLK10	Breast, colorectal, head and neck, liver, lung, prostate, thyroid, uterus
hsa-let-7g	KLK10	Liver, lung, prostate, thyroid
hsa-mir-106a	KLK13	Colorectal, lung, pancreas, prostate
hsa-mir-106b	KLK5	Lung
hsa-mir-122	KLK5	Breast, liver
hsa-mir-125a	KLK5	Breast, liver, lung, pancreas, prostate, thyroid, uterus
hsa-mir-125b	KLK5	Brain, breast, head and neck, liver, pancreas, prostate, stomach, uterus
hsa-mir-141	KLK13	Liver, lung, prostate, thyroid
hsa-mir-143	KLK5	Cervix, colorectal, liver, lung, prostate, breast
hsa-mir-148a	KLK10	Lung, prostate, thyroid
hsa-mir-148b	KLK10	Pancreas
hsa-mir-17	KLK5	Breast, colorectal, lung, pancreas, prostate
hsa-mir-181b	KLK13	Brain, breast, pancreas, prostate, thyroid
hsa-mir-181c	KLK13	Brain, liver, lung, pancreas, thyroid
hsa-mir-18	KLK9	Head and neck, liver
hsa-mir-18	KLK9	Head and neck, liver
hsa-mir-197	KLK10	Lung, pancreas, prostate, thyroid, uterus
hsa-mir-198	KLK9	Lung, prostate, thyroid
hsa-mir-199a	KLK7	Liver, lung, pancreas, prostate, thyroid, uterus
hsa-mir-199b	KLK7	Breast, liver, lung, thyroid
hsa-mir-206	KLK13	Breast, pancreas, prostate
hsa-mir-214	KLK10	Colorectal, liver, lung, pancreas, prostate, stomach, thyroid
hsa-mir-224	KLK10, KLK15	Breast, liver, lung, pancreas, thyroid
hsa-mir-299	KLK5	Thyroid, uterus
hsa-mir-30a	KLK7	Lung, pancreas, prostate, thyroid, uterus
hsa-mir-324	KLK2	Pancreas, uterus
hsa-mir-326	KLK5, KLK10	Pancreas
hsa-mir-33a	KLK7, KLK9	Lung, pancreas, thyroid
hsa-mir-33b	KLK7, KLK9	Stomach
hsa-mir-34a	KLK13	Breast, liver, lung, prostate, thyroid
hsa-mir-34c	KLK13	Pancreas
hsa-mir-369	KLK7	Pancreas, uterus
hsa-mir-376a	KLK9	Pancreas
hsa-mir-376b	KLK9	Uterus
hsa-mir-449a	KLK13	Head and neck
hsa-mir-491	KLK5	Prostate
hsa-mir-498	KLK15	Prostate
hsa-mir-98	KLK10	Head and neck

[a]Information regarding dysregulation of miRNAs in different cancers is based on our compiled database of differential miRNA expression in cancer extracted from the literature (the complete list of published studies that were used is available from the authors upon request).

using luciferase reporter gene constructs fused to target sequences (Lewis et al., 2003; Burgler and Macdonald, 2005). These constructs are used to transfect cells expressing the relevant miRNA, or sometimes miRNA is experimentally overexpressed. If such a construct is actively regulated by miRNAs already present in the transfected cells, this should lead to production of lower levels of the reporter than the mutant construct. Future development of high-throughput target validation techniques will be necessary to increase the specificity and sensitivity of miRNA target prediction algorithms.

The findings that multiple miRNAs can target the same kallikrein and the presence of multiple binding sites on the same kallikrein transcript are not unprecedented. miRNAs have been shown to be capable of functioning in a collaborative, combinatorial manner (Bentwich, 2005). The finding that structurally related miRNAs or miRNAs of the same family can target multiple KLKs (Table 1) is not surprising and may be of biological significance. It was recently reported that miRNAs might work in combination to accomplish their function (Yu et al., 2006). Since the kallikrein gene cluster is very close to the largest miRNA cluster of the genome, they could both be simultaneous targets for a control mechanism that affects this region, e.g., a locus control region or chromosomal insertion or deletions.

Since there are many identified splice variants for kallikrein genes, some of which differ in their 3′UTR (Kurlender et al., 2005), it is thus important to determine if these splice variations can affect protein expression by altering miRNA binding sites.

In conclusion, our combined bioinformatics and experimental approach provides the first preliminary analysis of the miRNA-KLK axis. More detailed analyses are needed

Table 4 Partial list of KLK-targeting miRNAs that are located in fragile sites of different malignancies.

miRNA	Targets	Cancer type
hsa-let-7a-1	KLK10	Breast, ovarian
hsa-let-7a-3	KLK10	Breast, ovarian
hsa-let-7b	KLK10	Ovarian
hsa-let-7d	KLK10	Breast, ovarian
hsa-let-7e	KLK10	Ovarian
hsa-let-7f-1	KLK6, KLK10	Breast, ovarian
hsa-let-7f-2	KLK6, KLK10	Breast
hsa-let-7g	KLK10	Ovarian
hsa-mir-106b	KLK5	Ovarian, prostate
hsa-mir-1-1	KLK5	Ovarian
hsa-mir-122	KLK5	Breast
hsa-mir-125a	KLK5	Breast, ovarian
hsa-mir-141	KLK13	Ovarian
hsa-mir-143	KLK5	Breast
hsa-mir-148a	KLK10	Breast, ovarian
hsa-mir-148b	KLK10	Breast, ovarian
hsa-mir-181b-1	KLK13	Breast, ovarian
hsa-mir-181c	KLK13	Ovarian, prostate
hsa-mir-181d	KLK13	Ovarian, prostate
hsa-mir-186	KLK11	Ovarian
hsa-mir-197	KLK10	Ovarian
hsa-mir-199a-1	KLK7	Ovarian, prostate
hsa-mir-199b	KLK7	Breast, ovarian
hsa-mir-211	KLK2	Ovarian
hsa-mir-224	KLK10, KLK15	Breast
hsa-mir-338	KLK7	Ovarian
hsa-mir-34a	KLK13	Breast
hsa-mir-498	KLK15	Ovarian
hsa-mir-511-1	KLK11	Ovarian
hsa-mir-511-2	KLK11	Ovarian
hsa-mir-519c	KLK7	Ovarian
hsa-mir-519d	KLK5	Ovarian
hsa-mir-519e	KLK5	Ovarian
hsa-mir-548d-1	KLK4	Prostate
hsa-mir-591	KLK7	Breast
hsa-mir-598	KLK9, KLK10	Breast
hsa-mir-608	KLK15	Prostate
hsa-mir-637	KLK4	Ovarian, prostate
hsa-mir-638	KLK15	Ovarian, prostate
hsa-mir-661	KLK14	Ovarian, prostate
hsa-mir-765	KLK4	Ovarian
hsa-mir-98	KLK10	Breast

In silico analyses of chromosomal hotspots of different cancers were performed using the Mitleman databases of chromosomal aberration in cancer, through the Cancer Genome Anatomy Project.

for target validation and exploration of the possible involvement of miRNAs in controlling kallikrein gene expression in cancer and other diseases.

Materials and methods

Bioinformatics analysis

We used four programs to perform bioinformatics-based target prediction analysis. These are miRBase Targets V4 (http://microrna.sanger.ac.uk/targets/v4/), miRanda (http://www.microrna.org/), TargetScan 4.0 (http://www.targetscan.org/), and PicTar predictions (http://pictar.bio.nyu.edu/). A positive prediction was only included if it was detected by at least two programs (except in Figure 1, in which all predictions are included). The TargetScan program was used to identify the location and number of miRNA target site(s) on the 3′UTR of the *KLK* transcripts for each miRNA. miRNAs with the same mature sequences are treated as one miRNA.

Experimental validation

Cell line preparation and transfection The breast cancer cell line MDA-M8-468 was selected for transfection as it expresses high levels of KLK6 and KLK10. Cells were propagated in RPMI (Gibco, Burlington, Canada) with 10% fetal bovine serum (Gibco) in T175 flasks in a humidified incubator containing 5% CO_2 at 37°C. The siPORT™ NeoFX™ transfection system (Ambion, Streetsville, Canada) was used. Cells were transfected with Pre-miR hsa-let-7f Precursor molecules (Ambion), and Pre-miR negative control precursor molecules (random sequence), Anti-miR hsa-let-7f inhibitors (Ambion), and Anti-miR negative control (Ambion) were all added at a concentration of 2 μM (after reconstitution with RNase-free water) (Ambion). All transfections were performed on day 0 (at the same time point) using siPORT Neo-FX transfection reagent (Ambion) according to the manufacturer's recommended protocol. Cells from the stock flasks were trypsinized and counted using a hemocytometer; and subsequently diluted to 3.0×10^5 cells/ml. Transfection agent/RNA complex formation was carried out in Opti-MEM Reduced-Serum Media (1×) (Gibco). Transfection efficiency was assessed by RT-PCR. Experiments were carried out in duplicate and the average was calculated. The conditioned medium was not changed throughout the experiment. A 100-μl aliquot of the medium was collected from each transfection on days 0, 3, 5, and 7.

RNA extraction and quantitative RT-PCR Cells were collected on day 7 after transfection according to a standard trypsinization protocol. Total RNA was extracted using a mirVana extraction kit and protocol (Ambion). Quantitative miRNA RT-PCR was performed using a TaqMan microRNA assay® kit using the supplier's protocol (Applied Biosystems, Streetsville, Canada). The let-7f transcript was first reverse-transcribed into cDNA using specific let-7f primers. let-7f cDNA was quantified in the subsequent PCR step using an ABI7500 standard system and let-7f-specific probes (Applied Biosystems). Experiments were carried out twice and the average was calculated. Expression values were normalized to a housekeeping gene (*GAPDH*).

ELISA KLK6 and KLK10 ELISAs were performed to measure the amount of protein secreted in conditioned medium, using specific antibodies as described elsewhere (Luo et al., 2006; Shaw and Diamandis, 2007). In brief, white polystyrene microtiter plates were coated with 500 ng/well of monoclonal antibody (specific for KLK6 or KLK10) in 100 μl of coating buffer (50 mM Tris-HCl, pH 7.8) overnight. Aliquots of 50 μl of calibrator or sample diluted in 6% BSA solution were added to the wells along with 50 μl of assay buffer. Plates were incubated for 2 h with shaking. An aliquot of 100 μl of biotinylated monoclonal detection antibody (50 ng) diluted in assay buffer was added to each well and incubated for 1 h. After washing, 100 μl (5 ng) of alkaline phosphatase-conjugated streptavidin diluted in 6% BSA was then added to each well and incubated for 15 min. After washing, 100 μl of diflunisal phosphate solution was added to each well and incubated for 10 min. Finally, 100 μl of developing solution was added to each well and mixed for 1 min. Fluorescence was measured using an Envision time-resolved fluorometer (Perkin-Elmer, Waltham, USA). The assay covered a linear range of 0.05–10 ng/ml. Protein levels were standardized to the number of cells initially seeded on day 0.

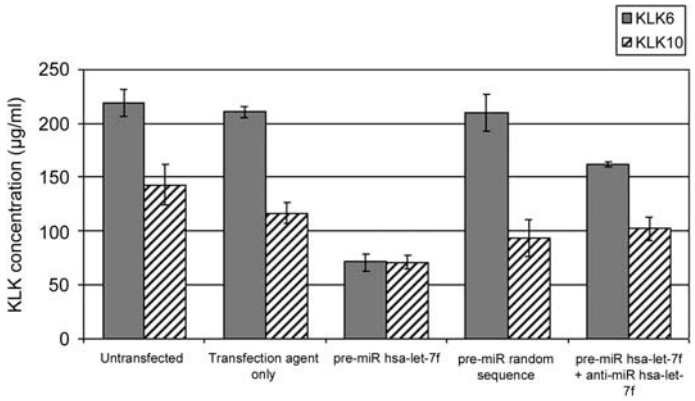

Figure 3 Effect of let-7f transfection on KLK6 and KLK10 protein expression levels.
The breast cancer cell line MDA-M8-468 was selected for transfection as it expresses high levels of KLK6 and KLK10. Transfection experiments were carried out in RPMI medium with 10% fetal bovine serum. Bars represent the percentage increase in protein levels between day 3 and day 7 post-transfection. Compared to controls, pre-miR hsa-let-7f transfection led to a significant decrease in secreted protein levels that was partially rescued by co-transfection of pre-miR hsa-let-7f and anti-miR hsa-let-7f inhibitor.

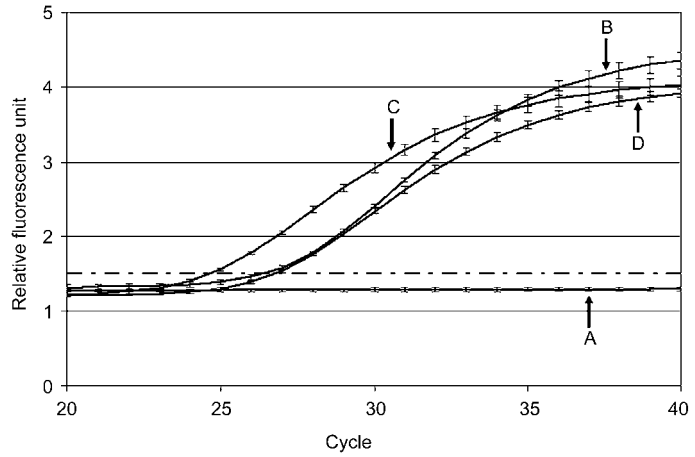

Figure 4 Representative quantitative RT-PCR graph of pre-miR hsa-let-7f analysis in transfected and non-transfected cells.
Relative fluorescence is shown on the longitudinal axis, and the horizontal axis represents the cycle number. Transfected cells showed a three-fold increase in the total amount of let-7f (after adjusting to the control level). Cells were collected and measured on day 7 after transfection. (A) Negative PCR control reaction, (B) untransfected cells, (C) cells transfected with pre-miR has-let-7f, and (D) cells transfected with pre-miR random sequence. The dotted line is the user-defined threshold value for the RT-PCR and was set to 1.5.

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