Clinical Chemistry 59:1
296–305 (2013)

Copy Number and Expression Alterations of miRNAs in the Ovarian Cancer Cell Line OVCAR-3: Impact on Kallikrein 6 Protein Expression

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BACKGROUND: Kallikrein-related peptidase 6 (*KLK6*), a member of the serine protease family of kallikrein (KLK) genes, is dysregulated in ovarian carcinomas (OCa) and its overexpression is associated with poor prognosis. Regulation of its expression is poorly understood and is likely to be influenced by multiple mechanisms. The KLK locus is subject to copy number changes and heterogeneity in serous OCas. These copy number imbalances generally correlate with KLK6 protein expression; however, this is not always the case. In this study we explored the role of miRNAs in the posttranscriptional control of KLK6 expression and the contributions of copy numbers, not only of the KLK locus, but also of the miRNAs predicted to regulate it.

METHODS AND RESULTS: By miRNA profiling of the *KLK6*-overexpressing OCa cell line, OVCAR-3, we identified overexpressed and underexpressed miRNAs. Publically available miRNA databases identified the human miRNA lethal 7 (hsa-let-7) family members as putative regulating miRNAs, from which hsa-let-7a was chosen for functional analysis. The transient transfection of hsa-let-7a to OVCAR-3 resulted in a decrease of KLK6 secreted protein. Moreover, such transfection was also able to weakly affect the expression of another member of the KLK gene family, *KLK10* (kallikrein-related peptidase 10). Cytogenomic analysis, including array comparative genomic hybridization, fluorescence in situ hybridization, and spectral karyotyping revealed the overall net copy number losses of hsa-let-7a and other miRNAs predicted to target *KLK6*.

CONCLUSIONS: The hsa-let-7 family member hsa-let-7a is a modulator of KLK6 protein expression that is independent of the *KLK6* copy number status.

Cancer arises through the dysregulated interplay and regulatory networks between DNA and the expression of proteins and pathways encoded within it. Elucidating these regulatory mechanisms provides insight into the observed changes seen at the DNA, RNA, and protein levels, which are frequently used as cancer biomarkers. The kallikrein (KLK)[4] family of serine proteases is a putative source of such cancer biomarkers *(1, 2)*. KLK6, the protein encoded by the Kallikrein-related peptidase 6 (*KLK6*)[5] gene, is a promising biomarker that is frequently overexpressed at the mRNA and protein levels in tissues and fluids derived from patients with ovarian cancer (OCa) and implicated in a variety of normal physiological processes *(3)*. This observed overexpression been associated with poor prognosis and enhanced malignancy. Several prognostic studies have shown that the combination of KLK6 with CA-125 enhances their diagnostic power *(4, 5)*. Increases in the *KLK6* gene copy number have been previously reported in OCa *(6)*, and the KLK locus (19q13.3/4) is subject to copy number changes in OCa *(7, 8)*. Early cytogenetic studies *(8)* revealed that the entire locus was involved in the copy number change, rather than individual members, a result that was recently confirmed by array studies *(9)*. The highly aneuploid and structurally abnormal OCa karyotypes also showed that the locus was subject to copy number het-

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Received July 20, 2012; accepted October 19, 2012.

Previously published online at DOI: 10.1373/clinchem.2012.193060

[4] Nonstandard abbreviations: KLK, kallikrein; OCa, ovarian carcinoma; UTR, untranslated region; FISH, fluorescence in situ hybridization; SKY, spectral karyotyping; aCGH, array comparative genomic hybridization; NCBI, National Center for Biotechnology Information; RT-PCR, reverse-transcription PCR; ABI, Applied Biosystems; TLDA, TaqMan low-density array; CT, threshold cycle.

[5] Human genes: *KLK6*, kallikrein-related peptidase 6; *KLK10*, Kallikrein-related peptidase 10; *MYC*, v-myc myelocytomatosis viral oncogene homolog (avian); *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; *HMGA2*, high mobility group AT-hook.

erogeneity *(10, 11)*, reflecting chromosomal instability often not revealed by the averaging algorithms of genome-wide methods *(12)*. Although copy number gains of *KLK6* were associated with increased KLK6 protein expression, a number of cancers with either 2 copies and/or a subpopulation containing 1 copy of the locus exhibited protein levels comparable to cancers with extra copies or amplification of the locus (approximately 28.0%). Conversely, some cancers with populations of copy number gains or amplifications exhibited KLK6 protein expression deemed as moderate/low (approximately 15.0%). Therefore, these data suggest that in addition to copy number, other mechanisms influence the observed overexpression, particularly posttranscriptionally *(13–15)*. Although a number of mechanisms have been investigated, more study is needed *(3)*. A growing body of evidence shows that miRNAs are key players in regulating and fine-tuning protein expression *(16)*. miRNAs comprise a class of noncoding RNAs, in which perfect complementarity between the miRNA and the target gene mRNA 3′ untranslated region (UTR) results in the cleavage and degradation of the target mRNA, whereas less than perfect pairing represses the translation process. In this fashion, miRNAs can target different mRNAs, increasing the diversity of gene regulation. Alterations in miRNAs have been analyzed in many cancers, including OCa *(17, 18)*, and are believed to be affected by copy number *(17, 19, 20)*. Recent bioinfomatic and experimental findings *(21–23)* suggest that the KLK genes are also subject to regulation by miRNAs. In this study, we examined the contribution of copy number, of both the KLK locus and of miRNAs predicted to target *KLK6*, to the observed protein expression of KLK6 in a representative KLK6-overexpressing OCa cell line, OVCAR-3.

Materials and Methods

MOLECULAR CYTOGENETICS

The OVCAR-3 cell line was obtained from the ATCC and maintained according the product insert. For fluorescence in situ hybridization (FISH) analysis, differentially labeled bacterial artificial chromosome clones mapping to 3 regions of chromosome 19q (19q12, 19q13.2, the KLK locus at 19q13.3/4) were used as described previously *(7)* and hybridized to OVCAR-3 metaphase chromosomes *(24)*. For spectral karyotyping (SKY) analysis, metaphase preparations were pretreated and hybridized with SKY Paints (Applied Spectral Imaging) and processed as described *(25)*. The images were collected using a Zeiss Axioplan microscope (Carl Zeiss) and processed using the ASI image capturing and analysis system (ASI). For array comparative genomic hybridization (aCGH) analysis, the Agilent human genome 244K microarray platform was used (Agilent Technologies). The array design included a total of 5045 features and internal controls based on the UCSChg17 Build [National Center for Biotechnology Information (NCBI) build 35, May 2004]. The aCGH assay was performed as previously described *(8)*. The normalized ratios for all features on the array are provided in Table 1 of the Data Supplement accompanying the online version of this article at http://www.clinchem.org/content/vol59/issue1.

miRNA PROFILING

We extracted miRNAs using the mirVana™ miRNA isolation kit (Ambion Life Technologies) and stored at −80 °C until ready for use. Quantification of miRNA expression was performed by reverse-transcription (RT)-PCR using the TaqMan® MicroRNA v1.0 system [Applied Biosystems Inc. (ABI), Life Technologies] and compared to normal miRNAs derived from total ovarian tissues (Ambion). The TaqMan low-density array (TLDA) human miRNA panel and multiplex RT pools of 8 48-miRNA sets (ABI) were used for global miRNA expression. Briefly, 10 ng of miRNAs were converted into specific cDNAs and subsequently quantified using the TaqMan MicroRNA TLDA card, containing 365 lyophilized human TaqMan miRNA sequences plus 3 small nucleolar RNAs (RU6B, RNU4, and RNU44) as endogenous controls. Data were quantified and analyzed using Sequence Detection System (version 2.3) (ABI). miRNA relative expression was normalized against endogenous controls and normal ovarian miRNAs according to the following threshold cycle (CT) calculation: $2^{-\Delta CT}$, where $\Delta CT = (CT_{miRNA} - CT_{snoRNAs})$ *(26)*. The final log10-transformed and normalized expression values for the miRNAs on the array can be found in online Supplemental Table 2.

IDENTIFICATION OF PREDICTED miRNAS TARGETING *KLK6*

To identify putative miRNAs predicted to target the 3′UTR of KLK6, 3 public databases were accessed: the Sanger Institute miRNA Registry *(27)*, Memorial Sloan-Kettering Cancer Center's miRNA database *(28)*, and TargetScan *(29)*. Predicted miRNAs common to all 3 databases were considered candidates for further study.

TRANSIENT TRANSFECTION OF miRNAS TO OVCAR-3

For transient transfections, the PremiR™ miRNA Precursor Kit (Ambion) was used. The hsa-let-7a-5p precursor miRNA (PM10050, Ambion, Life Technologies), corresponding to identical mature miRNAs of hsa-let-7a-1, hsa-let-7a-2, and hsa-let-7a-3 loci, plus a scrambled miRNA negative control (Life Technologies) were prepared to a final concentration of 6.25 μmol/L. OVCAR-3 cells were trypsinized and adjusted

to a final concentration of 1×10^5 cells/mL. In 6-well culture plates, 2.4 mL of OVCAR-3 cells (performed in duplicate) were combined with the transfection mixture comprised of the Ambion siPORT *NeoFX* transfection agent and Opti-Mem I medium, as instructed. The cultures were incubated at 37 °C in a CO_2 incubator for 24 h, after which time the medium was replaced with fresh normal growth medium. Biological replicates for each condition were prepared. After 72 h, the supernatant was collected and stored at −80 °C, the cells were counted by trypan blue staining, and the cell pellet was stored for later protein and RNA extraction.

QUANTITATIVE RT-PCR

Quantification of mature hsa-let-7a, using the TaqMan miRNA Assays (ABI, ID RT000377, and TM000377), including a control small nuclear RNA, RNU44 (ABI, ID RT001094, and TM001094), were performed according to the manufacturer's instructions and analyzed as described above. The assays were performed in triplicate for each sample.

MEASUREMENT OF KLK6 AND KLK10 BY ELISA AND WESTERN BLOTTING

The assays were based on sandwich-type ELISA principles, with one antibody used for capture and one for detection. We used a monoclonal–monoclonal ELISA configuration for the immunodetection of KLKs 6 and 10 *(8)*, and for standard Western analysis we used the same antibody clones for *KLK6* (26 kDa) and 10 (30 kDa) with β-actin (41 kDa) as a control (Abcam).

Results

IDENTIFICATION OF PREDICTED miRNAS REGULATING KLK6 AND miRNA PROFILING

The most commonly predicted miRNAs for the 3′ UTR of KLK6 were from the members of the hsa-let-7 family of miRNAs, namely *Hsa-let-7a*, *Hsa-let-7b*, *Hsa-let-7c*, *Hsa-let-7d*, and *Hsa-let-7e* (Fig. 1A). With the use of the TLDA arrays, miRNA profiling of the *KLK6*-overexpressing OVCAR-3 showed the dysregulation of these miRNAs compared to normal ovarian-derived miRNA, with the decrease in expression of hsa-let-7 family members. Table 1 summarizes the expression of miRNAs that were present on the TLDA array and predicted to target *KLK6*. Moreover, Table 1 indicates only those miRNAs that were identified in 2 or more databases. A comprehensive list detailing the log10 ratios of these and other miRNAs predicted to target *KLK6* is provided in online Supplemental Table 3. On the basis of the miRNAs present on the TLDA array, and those previously predicted *(23)*, 10 of the 13 miRNAs predicted to target *KLK6* showed decreased expression (see online Supplemental Table 3). Expression profiling of the other predicted miRNAs showed a similar trend, with 13 of 24 miRNAs showing decreased expression (see online Supplemental Table 3). Because hsa-let-7a has been identified as an important member of the OCa miRNA signature *(17)*, it was chosen as a candidate modulator of KLK6 protein expression. Validation of the miRNA findings by mature hsa-let-7a–specific qPCR confirmed the relative low-level expression of hsa-let-7a in OVCAR-3 compared to the normal ovarian miRNA control (Fig. 1B).

To test whether these candidate miRNAs could affect protein expression, hsa-let-7a-5p was transiently transfected into OVCAR-3. After a 72-h transfection with either hsa-let-7a-5p or a scrambled miRNA control, the amount of secreted *KLK6* in the supernatants was determined by ELISA. After adjustment for the final cell number, a mean decrease of 25%–35% in the concentration of KLK6 protein in the hsa-let-7a-5p–treated cells was detected compared to the controls. Western blotting confirmed the results observed with ELISA, with a modest decrease in KLK6 protein from the cell lysate (Fig. 1C). On the basis of previous studies showing the modulation of *KLK6* and 10-protein expression by hsa-let-7f *(21, 23)*, we surveyed the databases from miRNAs predicted to target *KLK10* and their expressions. Table 2 summarizes the relative miRNA expression of those miRNAs predicted to target *KLK10* in 2 or more databases. Similarly, a detailed list of the relative log10 expression ratios of these miRNAs and others is provided in online Supplemental Table 4. Of the miRNAs identified in all 3 databases, 3 of 4 miRNAs showed decreases in expression, including hsa-let-7b, hsa-miR-214, and hsa-miR-485–5p (Table 2; also see online Supplemental Table 4). Of the 20 miRNAs identified in 2 databases, 11 miRNAs also showed a relative decrease in expression (see online Supplemental Table 4). On the basis of these database algorithms, among the hsa-let-7 family members hsa-let-7b is more strongly predicted to target *KLK10* than hsa-let-7a, hsa-let-7c, hsa-let-7d, or hsa-let-7e, with hsa-let-7a identified in 2 databases as a putative regulating miRNA. Nevertheless, we tested whether hsa-let-7a could also affect KLK10 protein expression. ELISA and Western blotting for KLK10 in the hsa-let-7a-5p transfected line showed an 8%–15% decrease in secreted KLK10 protein compared to the controls (Fig. 1C).

OVCAR-3 CYTOGENOMICS

SKY analysis of OVCAR-3 showed a hypertriploid cell line (59–70 chromosomes) with numerous simple and complex structural rearrangements consistent with previous reports from the NCBI SKY/M-FISH and CGH Database. Structural rearrangements involving chromosome 19 were identified in addition to 2 appar-

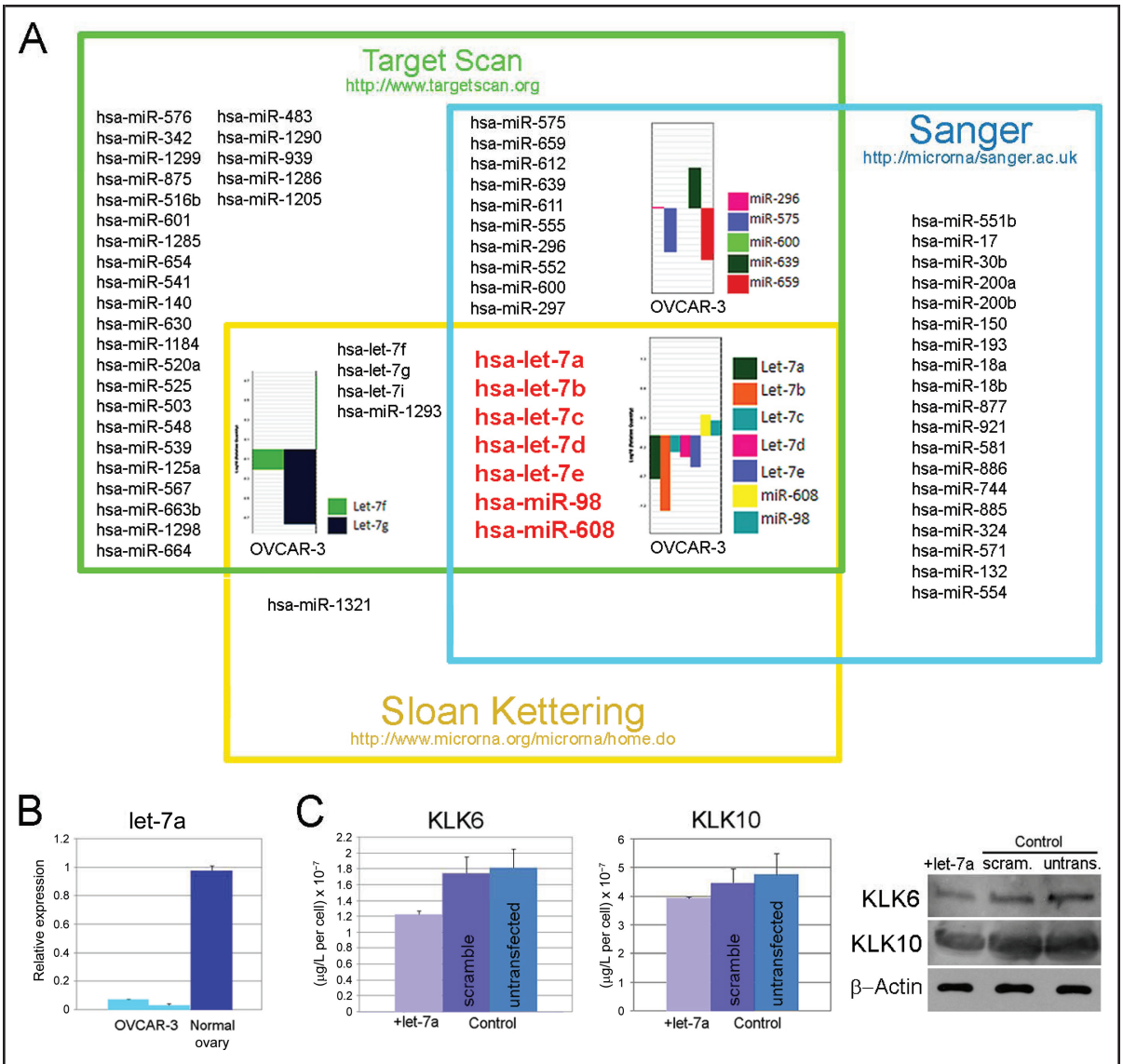

Fig. 1. miRNA Profiling of OVCAR-3 and miRNAs predicted to target KLK6.

(A), Venn diagram depicting the results of a search performed by using the publically accessible databases, including the miRNA Registry at the Sanger Institute, the Memorial Sloan-Kettering Cancer Center's miRNA database, and TargetScan [Grimson et al. *(29)*]. miRNAs common to all databases are shown in red. The expression of those miRNAs present on the TLDA microarray is also depicted and reflects the relative expression of normal ovarian miRNAs (performed in duplicate). (B), hsa-let-7a specific RT-PCR confirming the relative decrease in expression of hsa-let-7a in the same OVCAR-3 miRNAs used for TLDA analyses, and compared to normal ovarian miRNAs in technical triplicates (SDs of 0.006 and 0.03 were seen for OVCAR-3 samples and normal ovarian samples, respectively). (C), KLK6 and KLK10 ELISA and Western blotting of supernatants and cell lysates, respectively, from hsa-let-7a–transfected OVCAR-3 compared to the OVCAR-3 controls, consisting of both transfected-scrambled (scram.) miRNA and untransfected (untrans.) OVCAR-3. Both assays showed the decrease in KLK6 and KLK10 protein upon transient transfection. SDs for hsa-let-7a–transfected OVCAR-3 were 0.06 (error, 0.04) and 0.05 (error, 0.04), respectively, for KLK6 and KLK10. SDs for KLK6 for both controls were 0.2 (error, 0.2) and for KLK10 of scramble-transfected and untransfected OVCAR-3 were 0.7 (error, 0.5) and 0.8 (error = 0.8), respectively.

Table 1. Summary of miRNA expression and copy number changes of miRNAs predicted to target *KLK6*.[a]

Database	miRNA	aCGH locus	Net copy number	miRNA expression mature miRNA
Target Scan	**hsa-let-7a-1[b]**	**9q22.32**	**Balanced[c]**	**Decreased**
Sloan Kettering	**hsa-let-7a-2[b]**	**11q24.1**	**Loss**	
Sanger	**hsa-let-7a-3[b]**	**22q13.3**	**Loss**	
	hsa-let-7b[b]	**22q13.3**	**Loss**	**Decreased**
	hsa-let-7c	**21q21.21**	**Gain**	**Decreased**
	hsa-let-7d[b]	**9q22.32**	**Balanced**	**Decreased**
	hsa-let-7e[b]	**19q13.3**	**Loss**	**Decreased**
	hsa-miR-98[b]	**Xp11.22**	**Gain**	**Increased**
Target Scan	hsa-let-7f-1[b]	9q22.32	Balanced	Decreased
Sloan Kettering	hsa-let-7f-2[b]	Xp11.22	Gain	
	hsa-let-7g	3p21.1	Balanced	Decreased
Target Scan	hsa-miR-296	20q13.32	Gain	Increased
Sanger	hsa-miR-575	4q21.22	Balanced	Decreased
	hsa-miR-639	19p13.2	Loss	Increased
	hsa-miR-659	22q13.1	Loss	Decreased
	hsa-miR-140[b]	16q22.1	Loss	Decreased

[a] Shown are the combined expression and cytogenomic analyses of miRNAs predicted to target KLK6, which were identified in 2 or more databases. miRNAs in bold are those miRNAs identified in all 3 publically accessible databases as described in the Materials and Methods.
[b] miRNAs previously reported by White et al. *(23)* to be predicted to target KLK6.
[c] Balanced, net copy number of 2/no change in copy number; loss, net decrease in copy number; gain, net increase in copy number.

ently normal copies of chromosome 19. A complex chromosome described as a der(22)(16;19;22) was shown to contain a large homogeneously staining region indicative of amplification (Fig. 2A). aCGH analysis of the cell line revealed a high-level amplification spanning 19q11 to 19q13.2 and focal amplification at 19q13.4 (Fig. 2B). These copy number changes were consistent with other aCGH findings for this cell line *(9)*. Using a multicolor 19q probe set *(7)*, we confirmed the copy number findings identified by aCGH. Amplification of 19q11 (green) and 19q13.2 (blue) were identified on the der(22), as evidenced by the colocalization of FISH signals. Single hybridization signals, representing the KLK locus, were observed only on the 2 normal versions of chromosome 19 (red) (Fig. 2C). aCGH ratios for the KLK locus suggested its net loss, reflecting the limitations of CGH to account for the ploidy status of the cell line and the possibility of nonclonal losses and gains of chromosome 19. Thus, at most, there are 2 copies of the KLK locus in this triploid cell line.

Because copy number has been shown to be a contributing mechanism to changes in miRNA expression *(17, 19, 20)*, we assessed the copy number status of the 3 genes encoding for an identical mature hsa-let-7a miRNA *(30)*, *Hsa-let-7a-1* (9q22.32), *Hsa-let-7a-2* (11q24.1), and *Hsa-let-7a-3* (22q.13.3), using the aCGH data (Fig. 3). Both *Hsa-let-7a-2* and *Hsa-let-7a-3* mapped to loci generating ratios indicative of an overall net loss, whereas *Hsa-let-7a-1* indicated ratios indicative of 2 copies. Because OVCAR-3 possesses a triploid karyotype, with both clonal and nonclonal changes of these chromosomes, there is the presence of at least 2 copies of *Hsa-let-7a-1*, and at least 1, but not more than 2 copies each of *Hsa-let-7a-2* and *Hsa-let-7a-3*, consistent with the SKY findings. When we analyzed the structural rearrangements of those chromosomes to which these miRNAs mapped, we found that although the majority of metaphases possessed these structural rearrangements, which affect these loci, for some only 50% (5/10) of metaphases possessed these derivative chromosomes (Fig. 3). For *Hsa-let-7a-1* (and *Hsa-let-7f-1*) mapping to 9q22.3, 2 normal versions of chromosome 9 were detected in all cells (10/10); however, the der(9)t(8;9) was detected in only 60% of metaphases, with the loss of material telomeric to 9q12. For *Hsa-let-7a-2* mapping at 11q24.1, no normal versions of chromosome 11 were identified, however, 8/10 of metaphases possessed the der(11)t(11;14) rearrangement, accounting for copies of *Hsa-let-7a-2* at the telomeric end of the chromosome. However, the der(11)t(11;16;18) rearrangement, present in only

Table 2. Summary of miRNA expression and copy number changes of miRNAs predicted to target *KLK10*.[a]

Database	aCGH			miRNA expression mature miRNA
	miRNA	Locus	Net copy number	
Target Scan	**hsa-let-7b**[b]	**22q13.3**	**Balanced**	**Decreased**
Sloan Kettering	hsa-miR-214[b]	1q24.3	Gain	Decreased
Sanger	hsa-miR-224[b]	Xq28	Balanced	Increased
	hsa-miR-485-5p	14q32.3	Balanced	Decreased
Sanger	**hsa-let-7a-1**[b]	**9q22.32**	**Balanced**	**Decreased**
Target Scan	**hsa-let-7a-2**[b]	**11q2.1**	**Loss**	**Decreased**
	hsa-let-7a-3[b]	**22q13.3**	**Loss**	**Decreased**
	hsa-let-7c	**21q21.21**	**Gain**	**Decreased**
	hsa-let-7d	**9q22.32**	**Balanced**	**Decreased**
	hsa-let-7e	**19q13.3**	**Loss**	**Decreased**
	hsa-miR-148b	12q13.13	Gain	Increased
	hsa-miR-152	7q21.32	Gain	Decreased
	hsa-miR-197	1p13.3	Loss	Decreased
	hsa-miR-326	11q13.4	Amplified	Increased
	hsa-miR-98[b]	**Xp11.22**	**Gain**	**Increased**
Target Scan	hsa-miR-1-1	20q13.33	Gain	Decreased
Sloan Kettering	hsa-miR-1-2	18q11.2	Loss	Decreased
	hsa-miR-143[b]	5q32	Loss	Decreased
	hsa-miR-18a	13q31.1	Loss	Increased
	hsa-miR-192	11q13.1	Gain	Decreased
	hsa-miR-193b	16p13.2	Loss	Decreased
	hsa-miR-206[b]	6p12.2	Balanced	Increased
	hsa-miR-215	1q41	Gain	Increased
	hsa-miR-510	Xq27.3	Loss	Decreased
	hsa-miR-515-3p	19q13.42	Amplified	Decreased
	hsa-miR-613	12p13.1	Gain	Increased
	hsa-miR-646	20q13.3	Gain	Increased
White et al. *(23)*	**hsa-miR-125b-1**[b]	**11q24.1**	**Loss**	**Decreased**
	hsa-miR-125b-2[b]	**21q21.1**	**Gain**	**Decreased**
	hsa-miR-140[b]	**16q22.1**	**Loss**	**Decreased**
	hsa-miR-149[b]	2q37.3	Gain	Increased
	hsa-miR-432[b]	14q32.2	Balanced	Decreased

[a] Shown are the combined expression and cytogenomic analyses of miRNAs predicted to target KLK10 in two or more databases. miRNAs in bold represent those miRNAs also predicted to target KLK6.
[b] miRNAs previously reported by White *et al. (23)* to be predicted to target *KLK10*.
[c] Balanced, net copy number of 2/no change in copy number; gain, net increase in copy number; loss, net decrease in copy number.

60% (6/10) of metaphases, possesses a deletion telomeric to 11q23. Together, these account for the net deletion of the locus. Finally, *Hsa-let-7a-3*, which maps to 22q13.2, showed a net loss and contributed primarily by the combination of losses of the locus in the following rearrangements: i(22)(p10) (8/10 metaphases) and der(22)t(11;8;16) (5/10 metaphases), and the presence of the locus in the der(22)t(16;22) (7/10 metaphases) and der(22)t(16;19;22) (9/10 metaphases). Table 1 summarizes the net copy number changes for the miRNAs predicted to target *KLK6* in at least 2 of the databases and for those previously identified *(23)* by combined aCGH and cytogenetic analysis, and accounting for the ploidy. For each miRNA, there is a

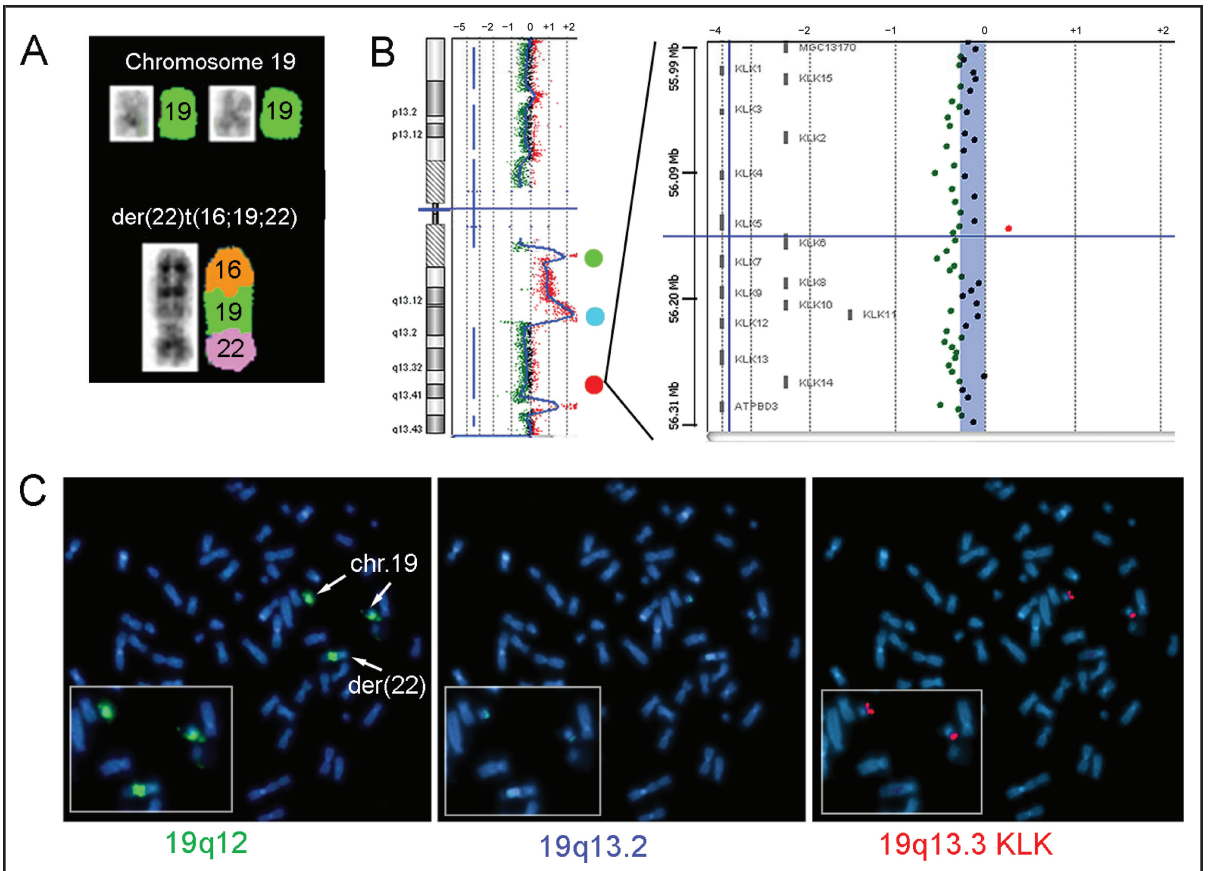

Fig. 2. Cytogenomic Analysis of OVCAR-3.

(A), SKY showing the presence of 2 normal versions of chromosome 19 (chr.19) (green) and a structurally abnormal der(22) comprised of chromosomes 16 (orange), 19 (green), and 22 (pink). (B) aCGH results of chromosome 19 in OVCAR-3 showing the copy number amplifications, gains, and losses along the chromosome. An enlargement of the KLK locus at 19q13.3/4 shows the net copy number loss of the entire KLK locus. Green, blue, and red circles beside the ideogram depict the location of the multicolor FISH probes used to verify the copy -number status of KLK6 as described in the Methods. (C), Locus-specific FISH to OVCAR-3 metaphases confirming the amplification of 19q12 (green signals) on the der(22) and single copies at the normal chromosome 19 position (left panel); amplification of 19q13.3 (blue signals) on the der *(22)* and single copies at the normal mapping position (middle panel); single copy of the KLK locus (red signals) only on the normal chromosomes 19.

general concordance in the association between overexpression of the mature miRNAs and net copy number gains of their genomic mapping locations, or decreased expression associated with net copy number losses. The exceptions to these findings are hsa-let-7c, hsa-let-7f-2, and hsa-miR-639. Table 2 summarizes the net copy number changes by combined aCGH and cytogenetic analysis for the miRNAs predicted to target *KLK10* in at least 2 of the databases and for those previously identified *(23)*. These results show a similar trend of positive association between the copy number status of the miRNAs and their expression, with the exception of 7 that showed an inverse association.

Discussion

In this study, we investigated the role of miRNAs in the regulation of KLK6 protein expression in a representative OCa cell line, OVCAR-3, and whether copy number may contribute to this mode of regulation. Mining of the publically available miRNA databases identified the hsa-let-7 family of miRNAs as likely regulators of *KLK6*. Together with aCGH and molecular cytogenetic validation, the copy number status of these and other miRNAs was shown to be subject to copy number losses, in keeping with the observed decrease in their expression, as determined by miRNA profiling. In-

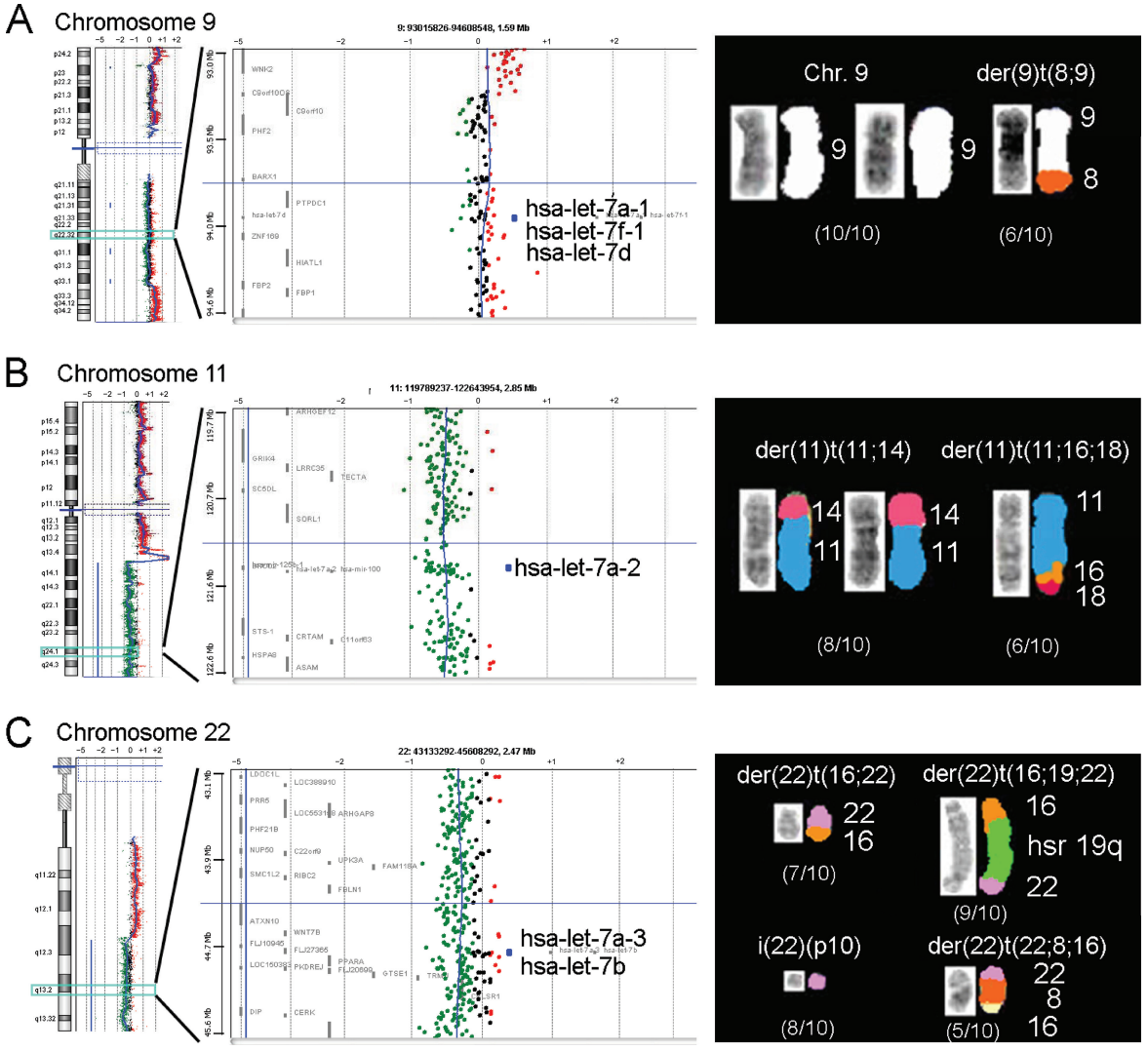

Fig. 3. Cytogenomic analysis of hsa-let-7a family members in OVCAR-3.

(A), Mapping of hsa-let-7a-1 to chromosome 9q22.32 together in a cluster with hsa-let-7f-1 and hsa-let-7d showing a 1:1 ratio in copy numbers, indicating 2 copies. Cytogenetic analysis of chromosome 9 confirms the presence of 2 normal versions of chromosomes 9 and 1 der(9)t(9;8) with a breakpoint centromeric to the mapping location of the miRNA cluster. (B), hsa-let-7a-2 mapping to chromosome 11q24.1 showing the net loss in copy number, suggesting that no more than 2 copies for the locus exist. Cytogenetic analysis for chromosome 11 shows the presence of 2 abnormal versions of chromosome 11 as a der(11:14) in 8/10 cells, accounting for the copies of the miRNA; and the presence of a der(11)t(11;16;18) present in 6/10 cells with a deletion of the terminal region of chromosome 11 containing hsa-let-7a-2. (C), hsa-let-7a-3 mapping to chromosome 22q13.3, also showing the net loss of the locus, with no more than 2 copies present. Cytogenetic analyses for chromosome 22 show the presence of several structural aberrations involving chromosome 22, with such structural aberrations resulting in the loss of the locus.

deed, the transient transfection of hsa-let-7a into OVCAR-3 resulted in the decrease of KLK6 protein expression.

Our TLDA profiling of this *KLK6*-overexpressing OCa cell line showed that the majority of miRNAs predicted to target *KLK6* had decreased expression (Table 1; also see online Supplemental Table 3). All hsa-let-7 family members present on the array showed decreased expression of their mature miRNAs, compared to miRNAs derived from normal ovary, consistent with previous findings of decreased hsa-let-7 expression in OCas *(17)*.

The KLK locus is subject to copy number heterogeneity, and cancers deemed to have net losses of the locus often possess minor subpopulations of cells with 2 or more copies *(7, 8)*. Moreover, cytogenetic studies support the observation that chromosome 19 is typically involved in both clonal and nonclonal numerical and structural aberrations *(8, 31)*, and that the vast majority of OCas are polyploid *(8, 10, 11, 32)*. Therefore, the averaging algorithms of aCGH used in studies resulting in the frequent loss of the locus *(33, 34)* failed to take into account ploidy and copy number heterogeneity *(12)*. FISH showed 2 copies of the KLK locus at the normal mapping location (19q13.3/4). Moreover, SKY analysis showed OVCAR-3 to be structurally and numerically abnormal, consistent with the reported karyotypic changes. Complementing the karyotypic analyses, aCGH confirmed the shift in ratio indicating the net loss of the KLK locus, including *KLK6* (Fig. 2B), in keeping with identification of 2 copies within a triploid background. Therefore, OVCAR-3 represents the *KLK6*-overexpressing and diploid/net loss copy number scenario, observed in our primary tumor study *(7)*.

Because copy number has been suggested to play a role in miRNA expression *(17, 19, 20)*, we assessed the copy number status of the *Hsa-let-7* family members predicted to target *KLK6* (Table 1). There was concordance between the copy number status and the expression of the miRNA (Table 1, Table 2). The *Hsa-let-7* members map to regions susceptible to frequent copy number changes *(9, 33)*, and the net copy number changes exhibited in this cell line are representative of the genomic profiles seen in most OCas. With the exception of hsa-let-7f-2 and hsa-let-7c, we found that expression of miRNAs was associated with their copy number status. The combined aCGH and karyotypic analyses of *Hsa-let-7a*, which is encoded by 3 distinct genes and was processed to result in an identical mature hsa-let-7a, showed the overall net loss of genomic material (Fig. 3). Because hsa-let-7a has been identified in many OCa miRNA profiling and functional studies to be an important member of the OCa miRNA signature *(17)*, and because the mature form results from 3 distinct copies within the normal human genome, hsa-let-7a was chosen as a candidate modulator of KLK6 protein expression. When hsa-let-7a miRNA was transiently transfected into OVCAR-3, there was a decrease in KLK6 protein in the hsa-let-7a–transfected cell line supernatant and cell lysate, compared to the scrambled and untransfected controls (Fig. 1C). These findings were consistent with those of other investigators *(21–23)*, who showed the decrease in *KLK6* expression by hsa-let-7f, which possesses the identical seed sequence to hsa-let-7a *(30)* and binding site on the *KLK6* 3′ UTR *(21–23)*. Additionally, previous bioinfomatic analyses *(23)*, as well as our own (Table 2; also see online Supplemental Table 4), led to the identification of hsa-let-7a as a possible modulator of *KLK10*. ELISA for KLK10 showed that hsa-let-7a was able to decrease KLK10 protein expression but to a lower extent than *KLK6*. Like *KLK6*, *KLK10* overexpression has also been reported in OCa and associated with unfavorable prognosis and late-stage disease *(35)*.

These findings add to the growing number of mRNA targets for the hsa-let-7 members, including *MYC* [v-myc myelocytomatosis viral oncogene homolog (avian)] *(36)*, *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) *(37)*, and *HMGA2* (high mobility group AT-hook) *(38)*, whose increased expression contributes to cancer progression. Indeed, although the increase of *KLK6* mRNA and protein expression has been demonstrated to enhance malignant potential *(3)*, the increased *KLK6* transcript may also act to sequester such miRNAs with tumor-suppressive qualities from their endogenous targets. Interestingly, the bioinfomatic predictions performed to date suggest that members of the hsa-let-7 family may preferentially target *KLK6 (21–23)*, and to a lesser extent, *KLK10* (Table 1; also see online Supplemental Table 3). Although this theory is speculative and requires further investigation, we envision that the preferential targeting of *KLK6* by the members of the let-7 family acts to simultaneously decrease *KLK6*-associated aggressive phenotypes while permitting the expression of the recently elucidate tumor suppressive properties of *KLK10 (39)*. The downregulation of hsa-let-7a has been consistently reported among OCas *(17)*, and its relative expression has been shown to have an impact on survival outcomes of patients treated with chemotherapy *(40)*. The dysregulation of hsa-let-7a expression could have profound biological consequences linked to changes in the concentrations of target proteins, suggesting that such proteins could be putative biomarkers. Identifying and verifying the putative targets of this and other miRNAs provides the opportunity to discover relevant biomarkers and protein-based signatures that could enhance genomically and transcriptomically derived signatures and define important clinical subgroups.

In summary, we demonstrated that the hsa-let-7 family member, hsa-let-7a, is a modulator of *KLK6* protein expression that is independent of the *KLK6* copy number status. Additionally, we demonstrated that hsa-let-7a can also weakly affect the protein expression of *KLK10*. Our cytogenomic analyses showed the strong contribution of copy number and miRNA expression in this representative OCa cell line. With the continued elucidation of other hsa-let-7a targets, it is possible that a clinically significant proteomic signature (including *KLK6*) can be developed to improve the diagnostic and predictive needs in OCa.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: E.P. Diamandis, *Clinical Chemistry*, AACC.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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