Chromosomal rearrangements of the kallikrein locus in human cancer cell lines

Jane Bayani, Chris Planque, Miltiadis Paliouras, Shannon J.C. Shan, Cassandra Graham ,Jeremy A. Squire, Eleftherios P. Diamandis.

[1]Department of Laboratory Medicine and Pathobiology, University of Toronto

[2]Samuel Lunenfeld Research Institute, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada

Mount Sinai Hospital — The Samuel Lunenfeld Research Institute

University Health Network — Ontario Cancer Institute

Advanced Centre for Detection of Cancer — www.acdclab.org

ABSTRACT

The association of kallikreins (KLK) with cancer has been well documented. Their expression correlates with diagnosis, prognosis, monitoring, and disease progression. Cancer cell line work has shown that the kallikreins are either tightly regulated via hormone dependent pathways or show dysregulated expression with no sensitivity to hormone stimulation. The kallikrein serine protease family consists of 15 genes organized in a single locus of 300 kb, at chromosome band 19q13.4. This region is non-randomly rearranged in many solid tumors, but to date there has been no systematic analysis of the extent of genomic rearrangements affecting the kallikrein gene cluster. Recurrent chromosomal translocations may be specifically associated with the activation of a novel fusion oncoprotein. Alternatively, translocations may lead to perturbations in gene expression in the vicinity of the translocation breakpoint, by position effect or other types of *cis*-acting epigenetic disruption. Thus, we investigated the molecular cytogenetics of the kallikrein locus to determine whether chromosomal rearrangements could account for the differential expression patterns of kallikreins in cancer cell lines. Fluorescence *in-situ* hybridization (FISH) was carried out to analyze large-scale genomic changes and abnormalities of the locus in the following cancer cell lines, Breast (MCF-10A, MCF-7, T47D, BT474, and MDA-MB-468), Ovarian (OVCAR-3 and HOSE) and Prostate (LNCaP and 22RV1). We found that the kallikrein locus is disrupted in a number of cell lines, as a result of unbalanced translocations that typically lead to gains of large segments of cytoband 19q13.4. The breast cancer cell lines MDA-MB-468, BT474 and MCF-7 all showed net gains of the 19q13.4 region, resulting from unbalanced translocations affecting KLKs: KLK2, KLK4, KLK6 and KLK13. FISH analysis of Patient Ascites also revealed the copy-number change and rearrangement of the locus. We conclude that the kallikrein locus is frequently translocated in many cancer cell lines.

METHODS

Patient Specimens and Cell Lines

The ovarian cancer cell line OVCAR-3; breast cancer cell lines T47D, MCF10A, MDA-MB-468, MCF7, BT474; and prostate cancer cell lines LNCAP and 22RV1.were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained according to the product specifications. Ascites fluid were collected by routine methods. All patient specimens were handled according to the guidelines set for by the Research Ethics Boards for the University Health Network and Mount Sinai Hospital The ascites cultures were maintained in alpha-MEM, supplemented with 15%(v/v) fetal calf serum (Invitrogen)/1% (v/v) penicillin-streptomycin (Invitrogen)/1%(v/v) L-glutamine (Invitrogen)

Fluorescence in situ Hybridization (FISH)

Cell line, patient and control lymphocyte cultures were prepared for cytogenetic analysis with 0.1mg/mL Colcemid (Gibco/BRL) for 2-3 h, followed by osmotic swelling in KCl (0.075 M), and fixed in methanol: acetic acid (3:1).

For translocation studies, DNA from the BAC clones RP11-76F7 and RP11-10I11 were extracted by standard methods. These clones have been previously shown to include the following kallikrein genes: KLK3, KLK2, KLK4, KLK5, KLK6, KLK7,KLK8, KLK9, KLK10, KLK11, KLK12, KLK13 and KLK14 as described by Yousef et al (Yousef, 2000) and Figure 1. The extracted BAC DNA was directly labeled with Spectrum Orange(Vysis/Abbott Laboratories) by nick translation using the Vysis Nick Translation Kit (Vysis/Abbott Laboratories). FISH to normal human lymphocytes confirmed the genomic location to the 19q13.3/19q13.3 region. For each cell line and patient specimen, metaphase preparations were hybridized with both a Spectrum Green labeled, whole chromosome 19 paint(Vysis/Abbott Laboratories) and the Spectrum Orange-labeled BAC probes and processed as described above. In each case, at least 10 metaphase spreads were analysed.

To determine the copy number and mapping status of specific KLKs, PCR or genomic fragments of individual kallikreins were cloned into bacterial vectors. The DNAs were extracted, labeled directly by nick translation and hybridized as described above using either Spectrum Green (Vysis/Abbott Laboratories), Spectrum Red (Vysis/Abbott Laboratories) or DEAC (Aqua).

Agilent Oligonucleotide Array 244k - Array Comparative Genomic Hybridization (aCGH)

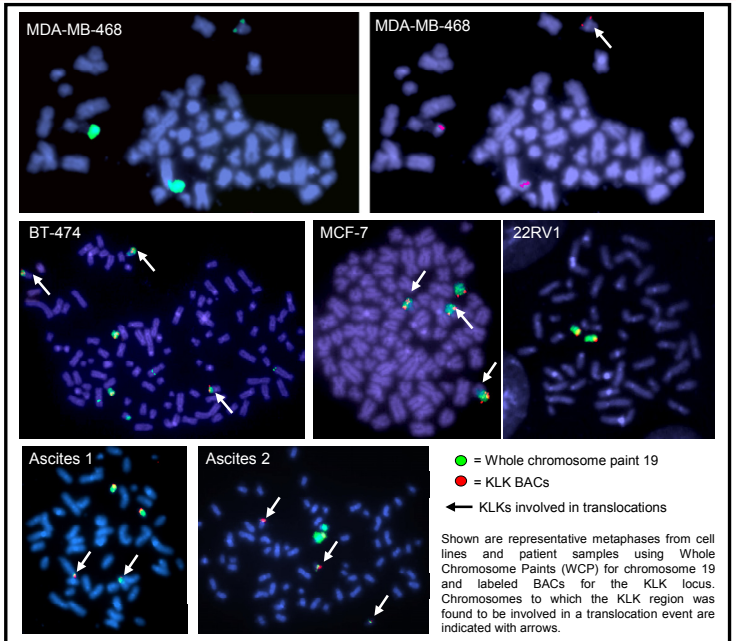
DNAs from the cell lines were extracted by standard phenol:chloroform extraction methods. The Agilent Human Genome microarray containing unique 60-mer oligonucleotide probes (Agilent Technologies, Inc., Palo Alto, CA, USA). 3 µg of DNA of male genomic DNA reference (Promega) and 3 µg of cell line genomic DNA samples were prepared according to the manufacturer's protocol. Labelling reactions were performed with 2 µg of purified digested DNA using the Invitrogen Bioprime labeling kit (Invitrogen) according to the manufacturer's directions either Cy5-dUTP for the experimental sample or Cy3-dUTP for the 46,XY male reference (PerkinElmer Life and Analytical Sciences). "Dye-swaps" were carried out for all experiments. Prior to hybridization, the 500 ul hybridization mixtures were denatured at 100ºC for 1.5 minutes and incubated at 37ºC for 30 minutes. The sample was applied to the array using an Agilent microarray hybridization chamber and hybridization was carried out for 40 hrs at 65ºC in a rotating oven (Robbins Scientific) at 20 rpm. The arrays were washed for 5 minutes at room temperature in 0.5X SSPE/0.005% NLS, followed by 3 minutes at 37ºC in 0.1X SSPE/0.005% NLS. Slides were dried using the Agilent drying solution and scanned using an Agilent 2565AA DNA microarray scanner (Agilent Technologies).

aCGH Data Analysis

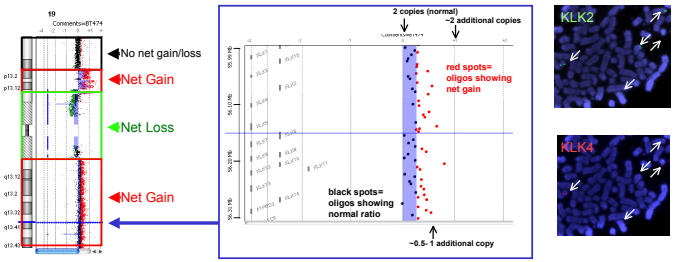
The CGH Analytics software version 3.4 (Agilent Technologies) was used to analyze the aCGH data. Copy number aberrations were objectively detected in replicate (dye swap) experiments using an aberration calling method based on computing significance scores for all genomic intervals. All CGH data represent the average of dye-flip experiments for each of the tumor samples.

ELISA

The concentration of each KLK was measured with specific and quantitative immunofluorometric ELISA assays developed in our laboratory. In brief, 96 well polystyrene plates were first coated with 500 ng/well of an hK-specific capture antibody. After overnight incubation, the plates were washed, 50 µL of culture supernatant or standards and equal volume of assay buffer were added and incubated at room temperature for 2 hours. Plates were washed and biotinylated antibodies were subsequently added. Following incubation with biotinylated antibodies, alkaline phosphatase-conjugated streptavidin was added. Finally, diflunisal phosphate (DFP) and terbium-based detection solutions were added and fluorescence was measured with the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada). The calibration and data reduction were performed automatically.



Shown are representative metaphases from cell lines and patient samples using Whole Chromosome Paints (WCP) for chromosome 19 and labeled BACs for the KLK locus. Chromosomes to which the KLK region was found to be involved in a translocation event are indicated with arrows.



Shown above are the aCGH findings for BT-474 revealing the net gain of the 19q. The fluorescence ratios for the KLK family members reveal a net gain corresponding at a ratio midway to the ratios corresponding to 2 additional copy numbers. Since BT-474 possesses a tetraploid (4n) genome, the ratios reflect the 3 copies above the normal genome ploidy of 2n. Thus, these findings suggest the average copy number of 5 copies per cell. Multi-colour FISH validation (right panel) confirms the copy number status of KLK2 (green), KLK4 (red) and KLK6 (blue).

Specimen	Tumour Type	Translocations Involving KLK Region	Copy Number Change
NHL (2n)	Normal	no	no 2 signals = 2x chr19
HOSE (2n)	normal	no	no 2 signals = 2x chr19
T47D (3n-)	Breast	no	no 2 signals/3 signals
MCF10A (2n)	Breast	no	no 2 signals = 2x chr 19
MDA-MB-468 (2n+)	Breast	yes	yes – gain 3 signals= 2x chr19 + 1xt
MCF7 (3n-)	Breast	yes	yes – gain 4 signals=1x chr19 + 3x t
BT474 (4n+)	Breast	yes	yes – gain 5 signals=2x chr19 + 3x t
LNCAP (4n)	Prostate	no	yes 4 signals = 4x chr19
22RV1 (2n)	Prostate	no	no 2 signals = 2x chr19
Patient Ascites 1 (2n)	Ovarian	yes	Yes 3 signals= 2x chr19 +1x t
Patient Ascites 2 (3n)	Ovarian	yes	Yes 3 signals= 1x chr19 +2x t

Summary of FISH findings for Ovarian, Breast, Prostate Cell Lines and Patient Specimens: For each case, the ploidy has been noted. Translocations involving the KLK region refer to the identification of KLK signals on chromosomes involved in rearrangements with other chromosomal partners as determined using whole chromosomal paints for chromosome 19. The total number of signals are also described with the number identified as involved in a chromosomal rearrangement.

Summary of KLK ELISA results in breast cancer cells lines. (Paliouras and Diamandis, 2007)

	KLK concentrations [µg/L]								
	KLK2	PSA	KLK5	KLK6	KLK7	KLK8	KLK10	KLK11	KLK13
MCF10A	0	0	0	0	0	0	30	0	0
T47D	0	0	0	0.01	0	0.1	3	0.2	0
BT474	0	0	0.02	0.05	0	0	0.1	1	0
MCF7	0	0	1	2.5	0	0.1	1.5	25	0
MDA-MB-468	0	0	420	27	6	9	9	0.2	0

DISCUSSION and CONCLUSIONS

- We observed a number of translocations with amplifications of the entire kallikrein locus in BT474, MCF7 and MDA-MB-468 breast cancer cell lines. The whole locus appears to be amplified, as FISH with *KLK* specific probes all show the same hybridization pattern. Also confirmed by aCGH analysis of BT474.
- It's clear that the genomic location of the *KLK* locus amplification event is correlated to dysregulated *KLK* gene expression (see KLK ELISA summary table), and/or other rearrangements within the genome may also impact *KLK* regulation.
- Rearrangements of the *KLK* locus observed in ascites samples indicate that 19q13.4 is a region of the genome that is sensitive to translocation/amplification events.
- Will continue analyzing for changes in *KLK* gene copy number and gene structure by genomic Southern blotting.

References

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Yousef, G. M., Chang, A., Scorilas, A., and Diamandis, E. P. (2000). Genomic organization of the human kallikrein gene family on chromosome 19q13.3-q13.4. *Biochem Biophys Res Commun.* 276:125-133.

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