Impact of cytogenetic and genomic aberrations of the kallikrein locus in ovarian cancer

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ABSTRACT

The tissue kallikrein (KLK) genes are a new source for biomarkers in ovarian cancer. However, there has been no systematic analysis of copy number and structural rearrangements related to their protein expression. Chromosomal rearrangements and copy number changes of the KLK region were studied by FISH with protein levels measured by ELISA. Ovarian cancer and cell lines revealed the KLK region was subject to copy number imbalances or involved in unbalanced translocations and were associated with increased protein expression of KLKs 5, 6, 7, 8, 9, 10 and 11. In this initial study, we introduce the potential for long-range chromosomal effects and copy number as a mechanism for the previously reported aberrant expression of many KLK genes in ovarian cancers.

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1. Introduction

Chromosomal changes, including copy number and structural rearrangements, are a hallmark of many cancers, since they may significantly alter gene function. Classical cytogenetic and more contemporary high-resolution integrative genomic strategies have identified genomic signatures that are common and specific to many tumor types. These findings facilitated identification of candidate tumor suppressor genes and oncogenes, as well as specific translocations as genomic biomarkers. Serum biomarkers, such as prostate-specific antigen (PSA), also known as kallikrein 3 (KLK3), have already been shown to be important for screening and monitoring of prostate cancer (Borgono et al., 2004a). The overexpression of other members of the kallikrein family in ovarian, breast and other cancers (Borgono et al., 2004b) makes this protease family an attractive source of new biomarkers. However, little is known about the relationship between chromosomal and genomic aberrations of this gene family and protein expression.

The human tissue kallikreins (KLKs) are a family of secreted serine proteases. All 15 genes are located in tandem

on chromosome 19q13.4 and share, on average, 30–50% sequence homology (Borgono et al., 2004a,b; Yousef and Diamandis, 2001). The KLKs are aberrantly expressed in several cancer types; most strikingly, 12 KLKs are concurrently over-expressed in ovarian cancer (Borgono et al., 2004b). Accumulating evidence suggests that these kallikreins are potential prognostic and diagnostic cancer biomarkers (Borgono et al., 2004a,b; Yousef and Diamandis, 2001; Diamandis and Yousef, 2002; Paliouras et al., 2007) and that several may also be involved in cancer progression (Haese et al., 2003; Cohen et al., 1992; Anisowicz et al., 1996; Magklara et al., 2003; Ghosh et al., 2004). In order to more thoroughly define the roles of KLKs in cancer pathophysiology and clinical management, it is important to investigate the mechanisms that influence their dysregulation in cancer.

Somatically acquired chromosomal aberrations are a major mechanism for gene activation. This is well-known in hematologic malignancies, where specific and recurrent chromosomal translocations result in oncogenic fusion transcripts. Recent data indicate that common epithelial tumors, such as prostate cancer, may also harbor recurrent translocations (Tomlins et al., 2005, 2006). Changes in gene expression may be regulated by epigenetic modifications, as well as by gene copy number and chromosomal translocations (Mitelman et al., 2007). The karyotypes of many carcinomas including ovarian (Taetle et al., 1999a,b; Bayani et al., 2002; Rao et al., 2002), breast (Pandis et al., 1995; Adeyinka et al., 2000; Rummukainen et al., 2001) and prostate cancers (Beheshti et al., 2000; Wang et al., 2005) are complex and are characterized by numerical and structural rearrangements. However, it is clear that more advanced molecular cytogenetic and genomic mapping methods may be required to delineate the precise chromosomal rearrangements that may influence the expression of specific loci.

Chromosome 19 and the 19q13 region, which harbors the KLK family of genes, are a site of frequent rearrangements in neoplasms (Mitelman et al., 2006) and a site for copy number imbalances in breast and ovarian cancer, as determined by metaphase (mCGH) and array comparative genomic hybridization (aCGH) (Baudis, 2007). Due to the known overexpression of the KLK genes in ovarian, breast and prostate cancers, the objective of this study was to investigate the copy number and structural rearrangement status of the KLK locus by fluorescence in situ hybridization (FISH) and/or high-resolution oligonucleotide array comparative genomic hybridization (aCGH), in ovarian, breast and prostate cancer cell lines and ovarian cancer tumors.

2.   Results

2.1.   Fine structural analysis of chromosomal rearrangements affecting 19q13 in cancer cell lines

Following the confirmatory mapping of the RP11-76F7 and RP11-10I11 BAC clones to normal human lymphocyte metaphase spreads (data not shown), a dual-color FISH strategy was used to identify the presence of translocations of the 19q13 region in various cancer cell lines. For each case, the combined Spectrum Orange-labeled 19q13 BACs (RP11-76F7 and RP11-10I11) were hybridized with a Spectrum Green-labeled whole chromosome 19 painting probe (WCP19). Rearrangements were identified when the red KLK FISH signals (and the corresponding WCP19 signal), were associated with chromosomes that did not contiguously hybridize with the whole chromosome 19 paint. Using this strategy, numerical and structural rearrangements involving the KLK locus were identified in the ovarian cancer cell line CAOV-3 and the breast cancer cell lines, MDA-MB-468, MCF-7 and BT474 (Figure 1B). No structural changes involving 19q were identified in the prostate cancer cell lines 22RV1 and LNCaP, consistent with previous SKY analysis (Beheshti et al., 2000; van Bokhoven et al., 2003) or the breast cancer cell lines MCF10A and T47D. In addition, for these cell lines, there was no change in copy number relative to the ploidy of the genome. Thus, 22RV1 and MCF10A maintained two copies of the KLK locus within the diploid genome and LNCaP possessed four copies of the KLK locus against a tetraploid genome. However, T47D, with a pseudo-triploid karyotype, was shown to possess a population of cells with an additional chromosome 19. Approximately 70% of cells enumerated possessed two copies of the KLK locus within chromosome 19, with the remaining 30% of cells possessing three copies of the KLK locus, due to a gain of chromosome 19.

Sequential SKY and FISH analysis was performed on CAOV-3, MDA-MB-468 and MCF7 (Figure 2) to determine the position of rearrangements relative to the KLK genes and to assign copy numbers for the gene cluster in each cell line. CAOV-3 was shown to be a hypertriploid line showing many complex structural rearrangements (Figure 2A). Two KLK signals were located at their usual 19q location, whilst another two KLK loci were involved in a duplicated complex, unbalanced translocation involving chromosomes 19, 2 and 13. These paired rearrangements suggest that this structural abnormality occurred prior to tetraploidization. Similarly, in MDA-MB-468, two KLK signals were present at their usual chromosome 19q location, along with an aberrant KLK signal, due to an unbalanced translocation with chromosome 20 (Figure 2B). MCF-7, a hypotriploid line, possessed three unbalanced translocations and only one apparently normal chromosome 19. One of the rearrangements, the der(19)t(17::11::19), appears to be a derivative of the der(19)t(11;19) and the other rearrangement is an unbalanced translocation with portions of 12q translocated adjacent to the KLK locus (Figure 2C). The hypertetraploid BT-474 cell line showed two chromosomes 19 containing the KLK locus, and three additional unbalanced translocations, each containing the KLK locus, consistent with previously published SKY findings (Kytölä et al., 2000). The unbalanced rearrangements included a der(7)t (7:19), der(10)t(10;19), and der(8)t(8::20::19) (data not shown).

2.2.   Chromosomal rearrangements of 19q13 in ovarian cancer patient samples

To determine whether the genomic and chromosomal alterations affecting KLK and the 19q13 region in cancer cell lines were also present in ovarian cancer patient tumors, FISH analysis was performed on cytogenetic preparations
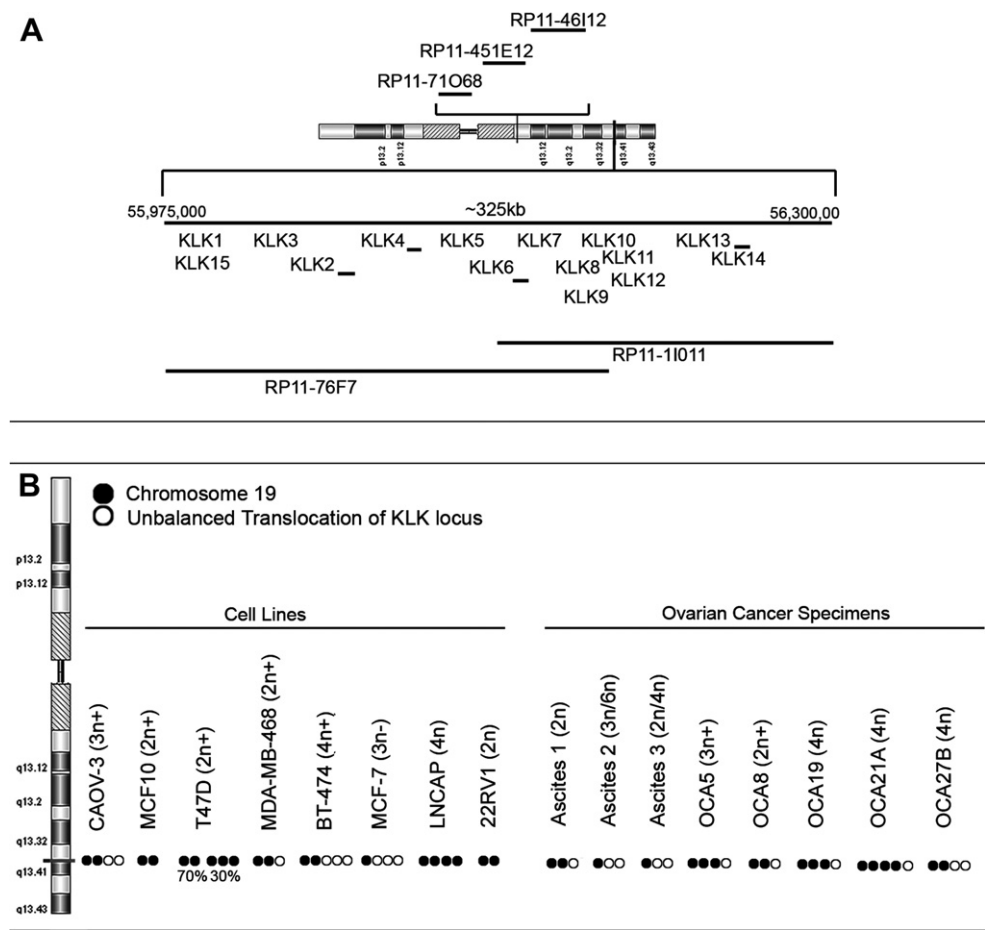
Figure 1 – Summary of FISH findings of the KLK locus in cancer cell lines and ovarian cancer patients. (A) Schematic illustrating the BAC clones used in this study. (B) Summary of FISH studies in cell line and tumors. Chromosome 19 was analyzed using the paint probe WCP19 and BACs that cover most of the 325-kb KLK genomic region using dual color FISH. Closed circles indicate the presence of the KLK locus located at the resident chromosome 19q site. Open circles indicate the presence of the KLK locus associated with either an intra-chromosomally or inter-chromosomal structural rearrangement. Also indicated for each analysis is the ploidy established by chromosomal counts. MCF10A and 22RV1 did not show copy number changes or involvement of translocation. In 70% of cells, T47D also showed a normal diploid pattern, but 30% showed a whole chromosomal gain of 19. LNCAP showed four copies of chromosome 19 with four copies of the KLK locus, showing no net gain over ploidy, but two copies over a normal diploid cell. The remaining cell lines and tumors displayed net gains of the KLK locus by unbalanced translocations.

from three representative ascites fluid samples from ovarian cancer patients. In all three samples, copy number imbalances of the KLK region were detected (Figure 1B). In Patient 1, two copies of the KLK locus were identified at the resident chromosome 19 site. An additional signal was detected in an unbalanced rearrangement. For Patient 2, the presence of different cell populations based on chromosome number (ploidy) were identified, however FISH identified the presence of at least one chromosome 19 containing the KLK locus at its usual location, and the presence of up to two additional KLK signals involved in translocations (Figure 3A,B). In addition, inserted portions of chromosome 19 were detected throughout the karyotype. Spectral karyotyping (SKY) of this patient sample revealed a karyotype possessing complex structural changes, as illustrated in Figure 3C. Aberrations involving chromosome 19 were identified in at least six chromosomes with one reflecting an intact 19, consistent with initial FISH results.

Patient 3 also displayed genomic heterogeneity, possessing at least two populations of cells, primarily an abnormal diploid population (~30%) and a tetraploid population (~60%). The tetraploid population was found to be similar to the diploid clone, suggesting that it arose from duplication of a diploid progenitor following failed cytokinesis. FISH identified the presence of five KLK signals within the tetraploid population, and three signals within the diploid population, yielding a net gain of the KLK locus per cell. Four of the five signals were shown to result from two isochromosomes 19(q) (Figure 3D,E arrow) with the remaining signal localized at the resident chromosome 19q (Figure 3F). Other additions and insertions of chromosome 19 material were also seen within the genome (Figure 3F) and confirmed by SKY analysis (Figure 3G).

In addition, five primary, untreated ovarian cancers, previously described (Bayani et al., 2002), by SKY and mCGH were analyzed to determine whether specific alterations leading
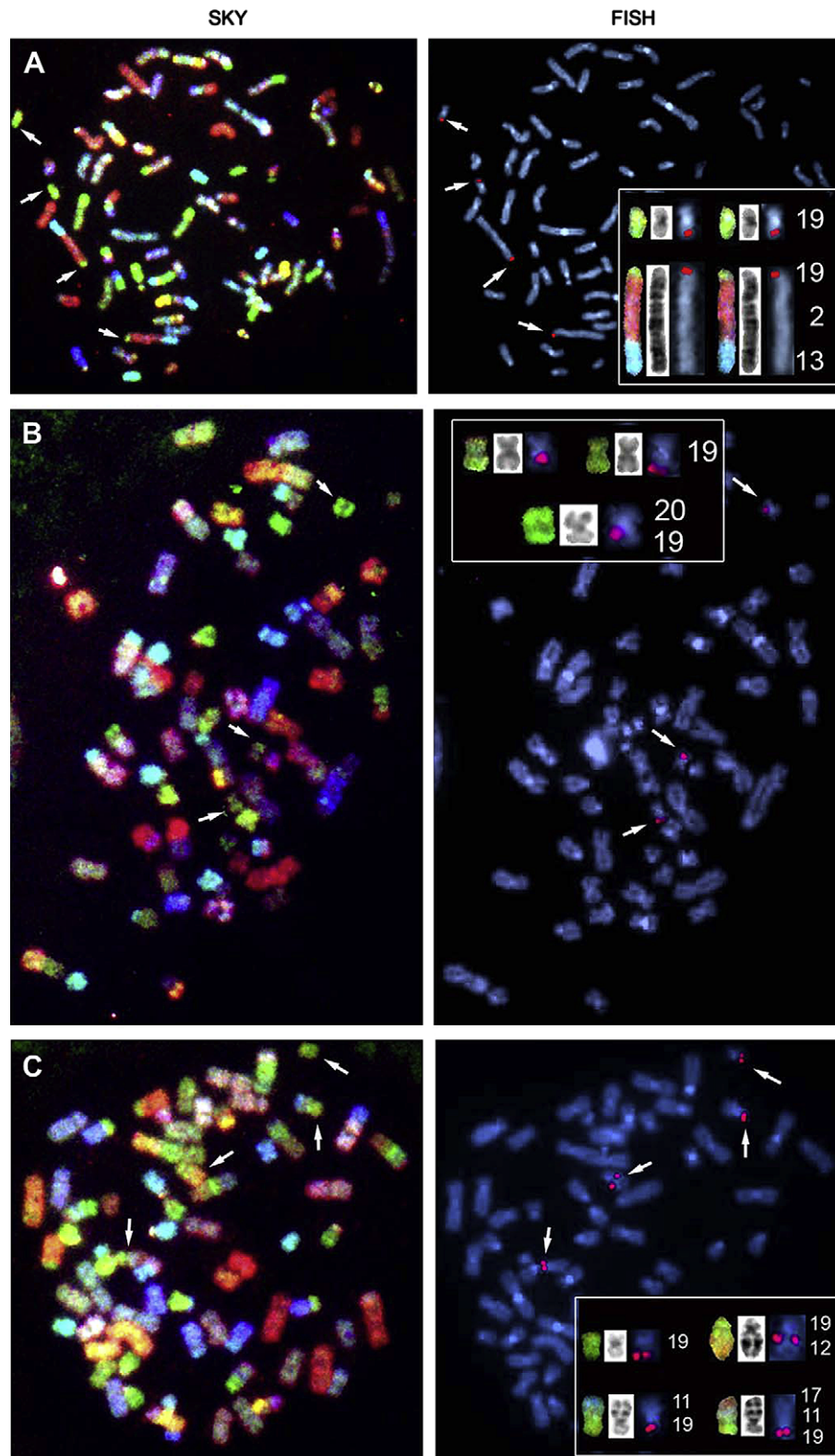
Figure 2 – KLK status in cancer cell lines by sequential SKY and FISH. Shown are representative metaphases of CAOV-3 (A), MDA-MB-468 (B) and MCF-7 (C) by SKY and FISH using the KLK BACs (red). The affected chromosomes are indicated by arrows and shown in the inset.

to KLK imbalances and structural rearrangements were also present (Figure 1B). The BAC RP11-716O8, mapping to 19q11/12, was used to identify affected chromosomes. In all five tumors, copy number imbalances, either due to whole copy number gains of chromosome 19 or unbalanced translocations were identified. Moreover, complex structural rearrangements, including a ring chromosome in OCA5 (Figure 3H,I) were shown to include the KLK locus, as well as the detection

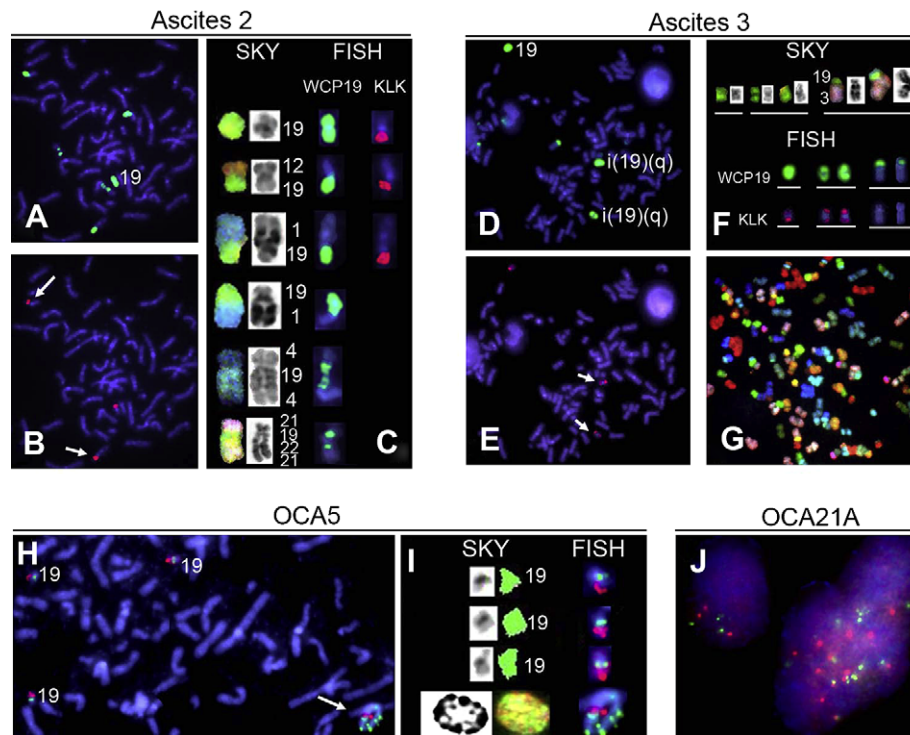Figure 3 – Status of the KLK locus in patient ascites. In each case, WCP19 (green) was hybridized with BACs for the KLK locus (red) to metaphase preparations. (A–C) Patient ascites 2. (A and (B) show the presence of chromosome 19 material (green) dispersed throughout the genome (A), with one KLK signal mapping to the resident chromosome 19, with the remaining two copies involved in translocations (B) (arrows). (C) Integrative FISH and SKY analysis of affected chromosomes. Inverted DAPI banding in shows the banding pattern of the metaphase chromosomes hybridized with the SKY paints (left) with FISH of corresponding chromosomes showing chromosome 19 paint (green) and the KLK locus (red) from A/B (left). By SKY complex rearrangements involving two or more chromosomal partners can be seen through the change of color along the length of a contiguous chromosome. The RGB (red–green–blue) display color for chromosome 19 is bright green, with a normal chromosome 19 (top–bottom), followed by five abnormal chromosomes involved in structural rearrangements with other chromosomal partners. The corresponding FISH using the KLK and whole chromosome 19 painting probes confirmed that structurally rearranged chromosomes by SKY also included the KLK locus. (D–G) Patient ascites 3 shows the presence of a KLK signal at the resident chromosome 19 and the presence two isochromosomes 19q (i(19q)) (D,E). The net result is the gain of KLK copy number due to these isochromosomes. (F) Integrative SKY and FISH analysis revealed the involvement of chromosome 19 in translocations with chromosome 3, but without involvement of the KLK locus. (G) Representative SKY metaphase of patient ascites 3. (H,I) Sporadic and untreated primary cancer OCA5. Shown in red is the KLK locus and a BAC clone for the 19q12 region, shown in green revealing the net gain of the KLK locus through whole gains of chromosome 19 and the presence of a multi-centric (green) ring chromosome containing the KLK locus (red). (I) Integrative SKY and FISH of OCA5. SKY and FISH confirms that no other chromosomes are involved in structural rearrangements on the three chromosomes 19, but the possibility exists for other chromosomal fragments in the ring chromosome. (J) Interphase nuclei of OCA21A shows variability in copy numbers of the KLK locus from cell to cell.

of i(19)(q) in OCA19. In many cases, the heterogeneity of these short-term cultures was revealed, with some tumors showing low-level copy number variation due to clonal and non-clonal changes, as shown by the analysis of interphase nuclei (OCA21A; Figure 3J).

2.3.   High-resolution aCGH and KLK-specific FISH analysis confirms copy number imbalances

Since the RP11-76F7 and RP11-10I11 BACs span the entire KLK family locus, aCGH using the Agilent oligonucleotide platform was performed on the BT474 cell line to determine the copy number status of each KLK. Array CGH of BT-474 showed a net gain of 19q12-19qter, with a ratio of approximately +0.5 (Figure 4), suggesting an average copy number

of five per cell. These findings are consistent with initial FISH findings using the combined BAC probes (Figure 1, Table 1).

To confirm the aCGH findings from this study and others (Neve et al., 2006), a multi-color KLK-specific FISH strategy was followed in a subset of lines (Figure 4, Table 1). Figure 4 illustrates the KLK-specific FISH findings in BT474, which confirm the copy number gain and the movement of the entire KLK family locus in unbalanced translocations.

Based on these aCGH findings and to those published by others (Shinoda et al., 2007), it was also determined that among the cell lines analyzed, no specific breakpoint locus within 4 Mb centromeric to the KLK locus was identified, with most imbalances arising from whole copy number alterations of 19q. FISH using BAC clones within the 19q13.3 and
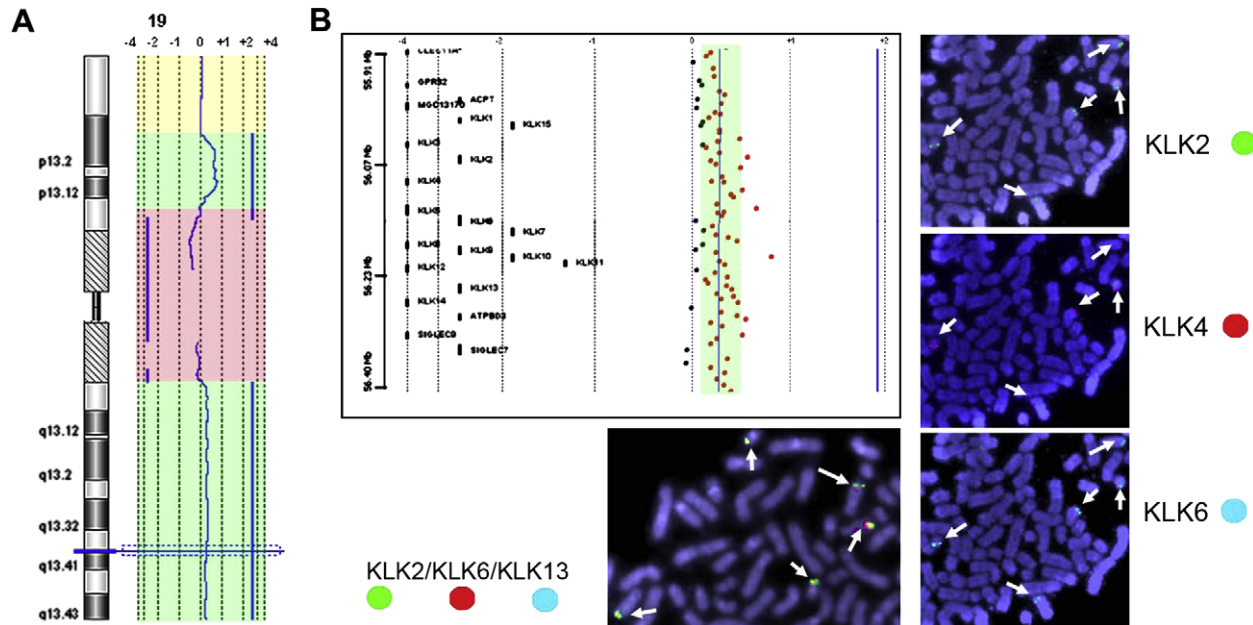
Figure 4 – aCGH and FISH validation of copy number imbalances of the KLK locus in BT-474. (A,B) aCGH for chromosome 19. Regions of gain are highlighted in green with regions of loss indicated by red, and no net change in yellow. (B) Magnification of the KLK locus shows an average ratio approaching +0.5 indicating a net gain of the region. Due to the ploidy of the genome, the net gain suggests one copy over ploidy, thus 1 + 4n = 5 copies of the KLK locus. Side panels: FISH Validation of individual KLKs in BT474. A three-color FISH approach was used for KLK2 (green), KLK4 (red) and KLK6 (aqua) to BT474 metaphase spreads. Bottom panel: KLK13 validation in BT474. In this three-color experiment, KLK2 (green), KLK6 (blue) and KLK13 (red) were co-hybridized and also shown to co-localize and be present in copy numbers consistent with aCGH findings.

19q11/19q12 regions confirmed these observations (data not shown).

2.4. Protein expression and relationship to copy number and structural rearrangements in cancer cell lines and ovarian specimens

KLK protein levels were measured prospectively in all cell line tissue culture supernatants and ascites fluid; and retrospectively, in the primary ovarian cancer specimens, either in cultured cell supernatants and/or cell lysates, or in tumor extracts (Table 2). Of the breast cancer lines, MCF-10A, a near normal breast epithelial cell line produced relatively very low levels of all KLKs, with highest expression detected for KLK10. Similar findings were found for T47D, with low protein levels for all KLKs tested, with the exception of KLK9 and KLK10. Neither line possessed structural rearrangements involving the KLK locus, nor were there any copy number changes detected in MCF-10A. T47D, as discussed earlier, did possess a population of cells (30%) containing the whole gain of chromosome 19. The remaining breast and ovarian lines showed variable KLK protein expression (higher levels of KLK proteins are shown in brackets). CAOV-3 (KLKs 5, 6, 7, 8, 9, 10); MDA-MB-468 (KLK 5, 6, 7, 8, 9, 10); BT-474 (KLK9); MCF-7 (KLKs 6, 7, 9, 11); of which all were associated with copy number changes of the KLK locus due to unbalanced translocations. ELISA results of the prostate cancer cell lines LNCaP and 22RV1 revealed significant amounts of only KLKs

2, 3 and 9, consistent with previous findings (Borgono and Diamandis, 2004a; Diamandis and Yousef, 2002). The cytogenetic results of these lines revealed normal copy number for 22RV1 and no net change of copy number for LNCaP, based on the ploidy status of the cell. Moreover, no structural change of the KLK locus was identified among these two lines.

Analysis of prospective and retrospective ovarian specimens showed that patient ascites 1 contained high levels of KLKs 1, 5, 6, 7, 8, 9, 10, 11; patient ascites 2 contained high levels of KLKs 5, 6, 7, 8, 10, 11; patient ascites 3 contained high levels of KLKs 5, 6, 7, 8, 10, 11 (italic values indicate levels > 10 μg/L). ELISA results of retrospective ovarian cancer samples were associated with high levels of the KLKs 5, 6, 7, 8, 9, 10 and 11. In all cases, copy number changes were

Table 1 – KLK-specific FISH[a]				
	KLK2[b]	KLK4	KLK6	KLK13
NHL (2n)	2	2	2	2
MCF-10A (2n)	2	2	2	2
T47D (2n and pseudo 3n)	2	2	2	2
	3	3	3	3
BT474 (4n+)	5	5	5	5
MDA-MB-468 (2n+)	3	3	3	3

a See also Table 1 for abbreviations and associated information.
b Number of copies.

Table 2 – ELISA findings for cancer cell lines and patient specimens[a]

Specimen	KLK1	KLK2	KLK3	KLK4	KLK5	KLK6	KLK7	KLK8	KLK9	KLK10	KLK11
CaOV3 (3n+)[b]	<0.2	<0.005	<0.005	<0.2	227 ± 17	14.5 ± 0.75	3.9 ± 0.27	12.4 ± 0.47	3.2 ± 0.3	50 ± 16	<0.2
MCF-10A (2n)	<0.2	<0.005	<0.005	<0.2	0.25 ± 0.04	<0.2	0.4 ± 0.06	<0.2	<0.5	2.8 ± 1.3	<0.2
T47D (2n+, pseudo 3n)	<0.2	<0.005	<0.005	<0.2	<0.05	<0.2	<0.2	0.2 ± 0.02	13.8 ± 2.6	0.7 ± 0.04	0.08 ± 0.02
MDA-MB-468 (2n+)	<0.2	<0.005	<0.005	<0.2	640 ± 29	103 ± 14	1.3 ± 0.2	2.1 ± 0.06	39 ± 2.4	21 ± 3.5	0.21 ± 0.15
BT-474 (4n+)	<0.2	<0.005	<0.005	<0.2	<0.05	<0.2	<0.2	<0.2	13 ± 5.0	<0.05	0.11 ± 0.02
MCF-7 (3n−)	<0.2	<0.005	<0.005	<0.2	<0.05	0.3 ± 0.07	0.44 ± 0.19	<0.2	13 ± 2.5	<0.05	2.3 ± 0.6
LNCaP (4n)	<0.2	28 ± 7.0	410 ± 23	0.13 ± 0.09	<0.05	<0.2	<0.2	<0.2	16 ± 5.0	<0.05	<0.2
22RV1 (2n)	<0.2	5 ± 2.5	68 ± 50	<0.2	<0.05	<0.2	<0.2	<0.2	9 ± 4.8	<0.05	<0.2
Ascites											
Patient 1 (2n)	6.0	<0.005	<0.005	<0.2	3.8	84	0.62	5.0	1.2	17	97
Patient 2 (3n/6n)	0.75	<0.005	<0.005	<0.2	41	388	2.5	81	0.65	65	94
Patient 3 (2n+/4n+)	1.3	<0.005	<0.005	<0.2	11.9	80	9.4	14	0.2	35	90
OCA5 (3n+): 1 week culture sup	<0.2	<0.005	<0.005	<0.005	<0.05	0.4	0.3	0.2	5.8	0.5	<0.2
OCA8 (2n+): 1 week culture sup	<0.2	<0.005	<0.005	<0.005	2.4	1.3	2.4	0.8	7.2	7.2	2.1
OCA19 (4n): tumor tissue extract	<0.2	<0.005	<0.005	<0.005	0.6	0.1	<0.2	0.1	0.8	0.2	0.8
OCA21 (4n): pelleted cultured cancer cells	<0.2	<0.005	<0.005	<0.005	17	16	34	11	2.2	30	64
Patient OCA27 (4n): 1 week culture sup	<0.2	<0.005	<0.005	<0.005	14	18	4.7	34	2.6	20	40

KLK12 was not measured; no ELISA assay available; sup, supernatant.
a Data for KLKs 13, 14 and 15 are not shown, since the values were low in all cases. All values are in μg/L ± standard deviation.
b Indicated in brackets is the ploidy of the cell as determined by cytogenetic analysis.

associated with whole chromosomal gains of chromosome 19 and/or unbalanced translocations of the KLK locus, suggesting that in breast and ovarian cancers, copy number gains of the KLK locus, particularly through unbalanced translocations, may be a mechanism for the high expression of KLKs 5, 6, 7, 8, 9, 10 and 11.

3.   Discussion

The human tissue KLKs are a family of secreted serine proteases which are often co-expressed within the same tissues and thought to participate in enzymatic cascades (Borgono et al., 2004b). The KLKs are aberrantly expressed in several cancer types and they are potential biomarkers for diagnosis, prognosis and prediction of therapy response (Borgono and Diamandis, 2004a; Borgono et al., 2004b; Yousef and Diamandis, 2001; Diamandis and Yousef, 2002; Paliouras et al., 2007). The regulation of gene expression appears to be influenced by steroid hormones (Paliouras and Diamandis, 2007) and epigenetic changes (Sidiropoulos et al., 2005; Pampalakis et al., 2006; Pampalakis and Sotiropoulou, 2006); however, the mechanisms that mediate aberrant KLK expression in cancer have not been entirely delineated. For many neoplasms, altered gene expression is linked to gene copy number, as in the case of *PTEN* in prostate cancer (Yoshimoto et al., 2006) and *EGFR* in lung cancer (Sasaki et al., 2007). To date, there has been no cytogenetic study that has examined the copy number status or the possible translocation of KLK gene members, in association with their relative protein expression in ovarian, breast or prostate cancer cell lines or

tissues. Recently, Shinoda et al. reported the overexpression of KLK5 in urinary bladder carcinoma cell lines, which were associated with changes in gene copy number (Shinoda et al., 2007), and Ni et al. demonstrated by Southern blot and RT-PCR analysis that the overexpression of KLK6 in a subset of ovarian cancers was associated with copy number gains (Ni et al., 2004).

In this study, we analyzed the copy number, positional mapping status and protein level of the KLK genes in breast, ovarian and prostate cancer cell lines. Additionally, since many KLKs are overexpressed in ovarian cancers (Borgono and Diamandis, 2004a; Borgono et al., 2004b; Yousef and Diamandis, 2001), a cohort of primary tumors and ascites samples was subjected to detailed cytogenetic and genomic analyses.

FISH and SKY analysis of the eight ovarian cancers studied revealed that the KLK region was frequently involved in copy number gains in the form of unbalanced translocations. Similarly, FISH analysis of all three patient ascites samples and five ovarian carcinomas identified the presence of copy number changes of the KLK locus due to unbalanced rearrangements. Analysis of the KLK cluster by aCGH for the breast cancer cell line BT474 showed that imbalance of a large region of chromosome 19 was associated with KLK copy number increases. To determine whether rearrangements took place within the KLK gene locus, cluster-specific FISH for KLK2, KLK4, KLK6 and KLK13 was used to confirm the contiguous presence of five copies of each of these genes. These findings suggested that copy number changes usually affect the entire KLK locus (approx. 300 kb) and that elevated expression is not associated with genomic disruption of the cluster. Similar

findings were revealed for the other cell lines tested (Table 1). A major advantage of aCGH is the ability to ascertain the loci that are involved in breakage, resulting in copy number change, revealing translocation breakpoints (Selvarajah et al., 2006). Our aCGH data, as well as those of others (Neve et al., 2006), have shown that the majority of the copy number transition sites in these cell lines occur centromeric to the KLK locus at 19q12, a region closely associated with the centromere. This suggests that in a subset of tumors the copy number and unbalanced translocation events likely occur at the level of the whole chromosome arm. FISH analysis of this region confirmed these observations, however, also implicate the presence of more subtle genomic aberrations between 19q12 and a 4-Mb region centromeric to the KLK locus, in BT474, MDA-MB-468 and CAOV-3 cells. These data draw attention to the possibility that chromosomal alterations, or gains in the genomic vicinity of the KLK locus, may indirectly contribute towards elevating the transcript and protein levels of kallikreins. However, we consider it less likely that a classical oncogenic fusion event involving the KLK cluster itself is taking place, since no common translocation partner or recurrent rearrangement was observed in the cell lines and ovarian tumors with 19q13 alterations.

KLK protein analysis revealed a relationship between copy number gains of the KLK locus and KLK gene family members. The most consistent data were seen in the prospective analysis of ascites fluid, whereby high levels of KLKs 5, 6, 7, 8, 10 and 11 were observed (Table 2). Similar elevations were also detected in CAOV-3 and MDA-MB-468 (Table 2), suggesting that copy number changes may influence the expression of genes in this region. The retrospective analysis of cryopreserved primary ovarian cancers and primary cultures also revealed elevations in KLK protein, although at lower levels, in comparison to freshly obtained specimens.

Human chromosome 19, although being one of the smallest chromosomes, is the most gene-rich (Venter et al., 2001) and possesses the highest number of CpG islands (Craig and Bickmore, 1994). The lack of constitutional rearrangements involving chromosome 19 appears to correlate to the finding of selection against translocations involving gene-dense chromosomes (Bickmore and Teague, 2002). Since chromosome 19 possesses a high CpG content, transcriptional regulation by methylation-dependent mechanisms may be compromised as a result of the translocation event. Indeed, studies have demonstrated the transcriptional silencing of genes due to the introduction of heterochromatin. Changes in the spatial orientation of chromosomal domains within the nucleus may also influence the extent of gene transcription (Sadoni et al., 1999; Cremer et al., 2000, 2004). Furthermore, the extent of chromatin compaction at the translocation site/region could enhance or hinder the access of transcription factors or other regulatory binding sites, offering an attractive mechanism for the aberrant expression of KLKs. The translocation event can also alter the proximity or sensitivity to hormone responsive elements, which may have important implications for breast, ovarian and prostate carcinogenesis.

In conclusion, in this preliminary analysis of cell lines and patient specimens, we have demonstrated that there is an association between copy number changes and changes in protein expression of certain KLK family members, and that these copy number imbalances primarily result from unbalanced translocations. Moreover, we have shown in this initial survey that these events are extremely common and may be characteristic of late stage and progressive ovarian cancer, however additional studies are necessary. The careful examination of early stage disease and other malignancies will also be required, to determine whether these genomic changes have prognostic or predictive value.

4. Materials and methods

4.1. Patient specimens and cell lines

The ovarian cancer cell line CAOV-3; the breast cancer cell lines T47D, MCF-10A, MDA-MB-468, MCF-7, BT474; and the 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. Excess ascites fluid from ovarian cancer patients, collected for routine diagnostic testing, was used for short-term cultures. All patient specimens were collected and handled with protocols approved by the Research Ethics Boards of the University Health Network and Mount Sinai Hospital (Toronto). The ascites cultures were maintained in alpha-MEM, supplemented with 15% (v/v) fetal calf serum (Invitrogen Canada, Burlington, ON, Canada), 1% (v/v) penicillin–streptomycin (Invitrogen) and 1% (v/v) L-glutamine (Invitrogen). In addition, heparinized whole blood from a normal female donor was cultured with phytohemagglutinin for 72 h in a CO_2 incubator in RPMI 1640 medium, supplemented with 20% (v/v) fetal calf serum, 1% (v/v) L-glutamine and 1% (v/v) penicillin–streptomycin.

4.2. Fluorescence in- situ hybridization (FISH)

Cell line, patient and control lymphocyte cultures were prepared for cytogenetic analysis with 0.1 μg/mL Colcemid (Invitrogen) for 2–3 h, followed by osmotic swelling in KCl (0.075 M) and fixed in methanol:acetic acid (3:1), as previously described (Bayani and Squire, 2000).

For translocation studies, DNA from the BAC clones RP11-76F7 and RP11-10I11 was 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 (Yousef et al., 2000), (Figure 1A). Other BAC clones used in this study include RP11-716O8, RP11-451E12 and RP11-46I12. The extracted BAC DNA was directly labeled with Spectrum Orange (Vysis/Abbott Laboratories, Des Plaines, IL) by nick translation using the Vysis Nick Translation Kit, according to the manufacturer's instructions. FISH to normal human lymphocytes confirmed the genomic location to the 19q13.3/19q13.4 region.

Approximately 300 ng of labeled probes were precipitated in excess human Cot-1 DNA (Invitrogen) and sonicated salmon sperm DNA (Roche) and resuspended in a 50% formamide, 10% dextran sulfate and 2× SSC hybridization buffer (DAKO, Mississauga, ON, Canada). The probes were heat-denatured and hybridized to pepsin-treated and denatured

normal human metaphase chromosomes, as described previously (Bayani and Squire, 2000). Following a rapid-wash technique consisting of one wash in 0.4× SSC and 0.3% NP-40 at 72 °C for 3 min, followed by a 5-min wash at room temperature in 2× SSC and 0.1% NP-40, slides were mounted in DAPI/Antifade (Vector Laboratories, Burlington, ON, Canada) and visualized with a Zeiss Axioskop fluorescence microscope (Carl Zeiss Canada). For each cell line and patient specimen, metaphase preparations were hybridized with both a Spectrum Green-labeled, whole chromosome 19 paint (Vysis) and the Spectrum Orange-labeled BAC probes and processed as described above. In each case, at least 10 metaphase spreads were analyzed.

To determine the copy number and mapping status of specific KLKs in a subset of tumors, PCR products of genomic fragments of KLK2, KLK4, KLK6 and KLK13 were cloned into TOPO TA cloning vectors (Invitrogen). The DNAs were extracted, labeled directly by nick translation and hybridized, as described above using either Spectrum Green, Spectrum Orange or the aqua-fluorescing DEAC (Applied Biosystems).

4.3.   Spectral karyotyping (SKY)

Metaphase preparations (described above) were pre-treated and hybridized with SKY Paints (Applied Spectral Imaging, Carlsbad, CA) and processed as described elsewhere (Bayani and Squire, 2000). The images were collected using a Zeiss Axioplan fluorescence microscope (Carl Zeiss Canada) and processed using the spectral karyotyping image capturing and analysis system (ASI). Karyotypic descriptions were assigned according to the guidelines of the International System for Human Cytogenetic Nomenclature (2005). For sequential SKY to FISH experiments, the coverslip was removed and the previously SKYed slide washed in 0.3% NP-40/0.1× SSC for 5 min and dehydrated in an ethanol series. The slide was then denatured in 70% formamide/2× SSC at 72 °C for 3 min and dehydrated. Heat-denatured probe, as described above, was applied to the slide and allowed to hybridize overnight at 37 °C. The slides were washed as described above.

4.4.   Array comparative genomic hybridization (aCGH)

The Agilent Human Genome 244K microarray platform was used (Agilent Technologies, Inc., Palo Alto, CA, USA cat. G4411B) containing 236,381 unique 60-mer oligonucleotide features. The array design included a total of 5045 features used as internal controls. Features were based on the UCSC hg17 (NCBI build 35, May 2004) Build.

DNA was extracted by standard phenol:chloroform methods, RNase-treated and resuspended in sterile RNase/DNase-free water (Sigma Canada). The direct labeling of DNAs, probe evaluation, clean-up, hybridization to the array and post-hybridization washes were carried out using reagents and equipment, according to Agilent protocols (http://www.chem.agilent.com). Briefly, at least 3 μg of male genomic DNA reference (Promega) and 3 μg of test genomic DNA samples were digested with AluI (12.5 units) and RsaI (12.5 units) (Promega), then verified using the DNA 1000 LabChip Kit and Agilent 2100 Bioanalyzer, according to the

manufacturer's instructions (Agilent Technologies). Individual reference and experimental samples were then filtered using the Qiaquick PCR Cleanup Kit (Qiagen Inc., Hilden, Germany). Labeling reactions were performed with 2 μg of purified digested DNA using the Invitrogen Bioprime labeling kit, according to the manufacturer's directions in a 50-μL volume, consisting of a modified dNTP pool of 120 mM each of dATP, dGTP, dCTP, 60 mM dTTP, and 60 mM of either Cy5-dUTP for the experimental sample or Cy3-dUTP for the 46, XY male reference (Perkin Elmer Life and Analytical Sciences, Woodbridge, ON, Canada). "Dye-swaps" were carried out for all experiments. Labeled targets were subsequently filtered using a Centricon YM-30 filter (Millipore, Bedford, MA, USA). Experimental and reference targets for each hybridization were pooled, mixed with 50 μg of human Cot-1 DNA (Invitrogen), 50 μL of Agilent 10× blocking agent and 250 μL of Agilent 2× hybridization buffer. Prior to hybridization, the 500-μL hybridization mixtures were denatured at 100 °C for 1.5 min and incubated at 37 °C for 30 min. The sample was applied to the array using an Agilent microarray hybridization chamber and hybridization was carried out for 40 h at 65 °C in a rotating oven (Robbins Scientific, Sunnyvale, CA) at 20 rpm. The arrays were washed for 5 min at room temperature in 0.5× SSPE/0.005% NLS, followed by 3 min at 37 °C in 0.1× SSPE/0.005% NLS. Slides were dried using the Agilent drying solution and scanned using an Agilent 2565AA DNA microarray scanner (Agilent Technologies).

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. According to the Agilent CGH Analytics software, a ratio of 0 represents the presence of two copies within the tumor/test genome, and a ratio of +1 represents the net gain of two additional copies (i.e. net gain of four copies in the tumor/test at 2n), hence the ratio of 0.5 suggests the net gain of one copy, based on a normal diploid genome (i.e. three copies at 2n or five copies at 4n).

4.5.   Measurement of KLKs by ELISA

We have previously described our ELISA methodologies for all KLKs. Briefly, the assays are based on "sandwich"-type ELISA principles with one antibody used for capture and one for detection. More details on procedures and performance can be found elsewhere (Shaw and Diamandis, 2007). All assays are highly specific, with no cross-reactivity from other KLKs.

For KLK analysis of cell lines, we used supernatants collected after 7 days of culture. No steroid hormonal stimulation was used. Ascites fluid was analyzed after centrifugation, to remove cells. Primary cultures of ascites cells were maintained for 1–7 days and the supernatants were collected for analysis. Also, cell pellets were lysed and analyzed in a similar fashion. When solid tumor was available (one case, OCA19), tumor tissue was homogenized and KLKs extracted in a lysis buffer containing NP-40 sulfactant, as described elsewhere. All KLK values were expressed as μg/L.

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REFERENCES

Adeyinka, A., Kytola, S., Mertens, F., Pandis, N., Larsson, C., 2000. Spectral karyotyping and chromosome banding studies of primary breast carcinomas and their lymph node metastases. Int. J. Mol. Med. 5, 235–240.

Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S.C., Sager, R., 1996. A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med. 2, 624–636.

An International System for Human Cytogenetic Nomenclature, 2005. In: Shaffer, L.G., Tommerup, N. (Eds.), Recommendations of the International Standing Committee on Human Cytogenetic Nomenclature. S Karger AG, Basel.

Baudis M. Progenetix cytogenetics online database. 2007.

Bayani, J., Squire, J.A., 2000. Chapter 22: Cell biology of chromosomes and nuclei. In: Morgan, K. (Ed.), Current Protocols in Cell Biology. John Wiley & Sons, Inc., Bethesda, MD.

Bayani, J., Brenton, J.D., Macgregor, P.F., et al., 2002. Parallel analysis of sporadic primary ovarian carcinomas by spectral karyotyping, comparative genomic hybridization, and expression microarrays. Cancer Res. 62, 3466–3476.

Beheshti, B., Karaskova, J., Park, P.C., Squire, J.A., Beatty, B.G., 2000. Identification of a high frequency of chromosomal rearrangements in the centromeric regions of prostate cancer cell lines by sequential giemsa banding and spectral karyotyping. Mol. Diagn. 5, 23–32.

Bickmore, W.A., Teague, P., 2002. Influences of chromosome size, gene density and nuclear position on the frequency of constitutional translocations in the human population. Chromosome Res. 10 (8), 707–715.

Borgono, C.A., Diamandis, E.P., 2004a. The emerging roles of human tissue kallikrein in cancer. Nat. Rev. Cancer 4, 876–890.

Borgono, C.A., Michael, I.P., Diamandis, E.P., 2004b. Human tissue kallikreins: physiologic roles and applications in cancer. Mol. Cancer Res. 2, 257–280.

Cremer, T., Kreth, G., Koester, H., Dietzel, S., Fakan, S., 2000. Chromosome territories, interchromatin domain compartment, and nuclear matrix: an integrated view of the functional nuclear architecture. Crit. Rev. Eukaryot. Gene. Expr. 10, 179–212.

Cremer, T., Kupper, K., et al., 2004. Higher order chromatin architecture in the cell nucleus: on the way from structure to function. Biol. Cell 96, 555–567.

Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., Rosenfeld, R.G., 1992. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. 75, 1046–1053.

Craig, J.M., Bickmore, W.A., 1994. The distribution of CpG islands in mammalian chromosomes. Nat. Genet. 7, 376–382.

Diamandis, E.P., Yousef, G.M., 2002. Human tissue kallikreins: a family of new cancer biomarkers. Clin. Chem. 48, 1198–1205.

Ghosh, M.C., Grass, L., Soosaipillai, A., Sotiropoulou, G., Diamandis, E.P., 2004. Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. 25, 193–199.

Haese, A., Graefen, M., Becker, C., et al., 2003. The role of human glandular kallikrein 2 for prediction of pathologically organ confined prostate cancer. Prostate 54, 181–186.

Kytölä, S., Rummukainen, J., Nordgren, A., Karhu, R., Farnebo, F., Isola, J., Larsson, C., 2000. Chromosomal alterations in 15 breast cancer cell lines by comparative genomic hybridization and spectral karyotyping. Genes Chromosomes Cancer 28, 308–317.

Magklara, A., Mellati, A.A., Wasney, G.A., et al., 2003. Characterization of the enzymatic activity of human kallikrein 6: autoactivation, substrate specificity, and regulation by inhibitors. Biochem. Biophys. Res. Commun. 307, 948–955.

Mitelman F, Johansson B, et al. Mitelman Database of Chromosome Aberrations in Cancer. 2006.

Mitelman, F., Johansson, B., Mertens, F., 2007. The impact of translocations and gene fusions on cancer causation. Nat. Rev. Cancer 7, 233–245.

Neve, R.M., Chin, K., Fridlyand, J., et al., 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10, 515–527.

Ni, X., Zhang, W., Huang, K.C., Wang, Y., Ng, S.K., Mok, S.C., Berkowitz, R.S., Ng, S.W., 2004. Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br. J. Cancer 91, 725–731.

Paliouras, M., Diamandis, E.P., 2007. Coordinated steroid hormone-dependent and independent expression of multiple kallikreins in breast cancer cell lines. Breast Cancer Res. Treat. 102, 7–18.

Paliouras, M., Borgono, C.A., Diamandis, E.P., 2007. Human tissue kallikreins: The cancer biomarker family. Cancer Lett. 249, 61–79.

Pampalakis, G., Sotiropoulou, G., 2006. Multiple mechanisms underlie the aberrant expression of the human kallikrein 6 gene in breast cancer. Biol. Chem. 387, 773–782.

Pampalakis, G., Diamandis, E.P., Sotiropoulou, G., 2006. The epigenetic basis for the aberrant expression of kallikreins in human cancers. Biol. Chem. 387, 795–799.

Pandis, N., Jin, Y., Gorunova, L., et al., 1995. Chromosome analysis of 97 primary breast carcinomas: identification of eight karyotypic subgroups. Genes Chromosomes Cancer 12, 173–185.

Rao, P.H., Harris, C.P., Yan Lu, X., et al., 2002. Multicolor spectral karyotyping of serous ovarian adenocarcinoma. Genes Chromosomes Cancer 33, 123–132.

Rummukainen, J., Kytola, S., Karhu, R., Farnebo, F., Larsson, C., Isola, J.J., 2001. Aberrations of chromosome 8 in 16 breast cancer cell lines by comparative genomic hybridization, fluorescence in situ hybridization, and spectral karyotyping. Cancer Genet. Cytogenet 126, 1–7.

Sadoni, N., Langer, S., Fauth, C., et al., 1999. Nuclear organization of mammalian genomes. Polar chromosome territories build up functionally distinct higher order compartments. J. Cell Biol. 146, 1211–1226.

Sasaki, H., Kawano, O., Endo, K., et al., 2007. EGFRvIII mutation in lung cancer correlates with increased EGFR copy number. Oncol. Rep. 17, 319–323.

Selvarajah, S., Yoshimoto, M., Park, P.C., et al., 2006. The breakage–fusion–bridge (BFB) cycle as a mechanism for generating genetic heterogeneity in osteosarcoma. Chromosoma 115, 459–467.

Shaw, J.L.V., Diamandis, E.P., 2007. Distribution of 15 human kallikreins in tissues and biological fluids. Clin. Chem. 53, 423–432.

Shinoda, Y., Kozaki, K.I., Imoto, I., et al., 2007. Association of KLK5 overexpression with invasiveness of urinary bladder carcinoma cells. Cancer Sci. 98, 1078–1086.

Sidiropoulos, M., Pampalakis, G., Sotiropoulou, G., et al., 2005. Downregulation of human kallikrein 10 (KLK10/NES1) by CpG island hypermethylation in breast, ovarian and prostate cancers. Tumour Biol. 26, 324–336.

Taetle, R., Aickin, M., Panda, L., et al., 1999a. Chromosome abnormalities in ovarian adenocarcinoma: II. Prognostic impact of nonrandom chromosome abnormalities in 244 cases. Genes Chromosomes Cancer 25, 46–52.

Taetle, R., Aickin, M., Yang, J.M., et al., 1999b. Chromosome abnormalities in ovarian adenocarcinoma: I. Nonrandom chromosome abnormalities from 244 cases. Genes Chromosomes Cancer 25, 290–300.

Tomlins, S.A., Rhodes, D.R., Perner, S., et al., 2005. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310, 644–648.

Tomlins, S.A., Mehra, R., Rhodes, D.R., et al., 2006. TMPRSS2:ETVA4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res. 66, 3396–3400.

van Bokhoven, A., Caires, A., Maria, M.D., Schulte, A.P., Lucia, M.S., Nordeen, S.K., Miller, G.J., Varella-Garcia, M., 2003. Spectral karyotype (SKY) analysis of human prostate carcinoma cell lines. Prostate 57, 226–244.

Venter, J.C., Adams, M.D., Myers, E.W., et al., 2001. The sequence of the human genome. Science 291, 1304–1351.

Wang, Y., Xue, H., Cutz, J.C., et al., 2005. An orthotopic metastatic prostate cancer model in SCID mice via grafting of a transplantable human prostate tumor line. Lab. Invest. 85, 1392–1404.

Yoshimoto, M., Cutz, J.C., Nuin, P.A., et al., 2006. Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias. Cancer Genet. Cytogenet. 169, 128–137.

Yousef, G.M., Chang, A., Scorilas, A., 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.

Yousef, G.M., Diamandis, E.P., 2001. The new human tissue kallikrein gene family: structure, function and association to disease. Endocr. Rev. 22, 184–204.