Hormonal regulation of human tissue kallikrein 9 in human cancer cell lines

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ABSTRACT:

Human tissue kallikreins (*KLKs*) are a group of 15 genes, tandemly located on chromosome 19, with considerable similarity at the gene and amino acid structure. Classical kallikreins such as *KLK2* and *KLK3* (also known as prostate specific antigen, PSA) are currently used as serological biomarkers for diagnosis and monitoring of prostate cancer. Numerous reports indicate that many of the newly identified kallikreins are differentially expressed in ovarian, breast and prostate cancer. Kallikrein 9 (*KLK9*), identified in our laboratory, is one of the newly characterized members of the kallikrein gene family. Our earlier clinical studies indicate that *KLK9* mRNA is differentially expressed in cancer. Ovarian and breast cancer patients with *KLK9*-positive tumors have longer progression-free and overall survival compared to those who are *KLK9*-negative.

In this study the hormonal regulation of *KLK9* in LNCaP, PC-3, and PC-3 (AR6) prostate cancer cell lines, the CAOV-3 ovarian cancer cell line and the BT-474, MCF-7, MDA-MB-468, and BT-20 breast cancer cell lines was investigated. Cancer cells stimulated with estradiol, dihydrotestosterone (DHT), norgestrel, aldosterone, dexamethasone or ethanol alone (as a control) were examined. Cells treated with 10-8 M of either of these steroids dissolved in ethanol were grown until 80% confluent. Total RNA was extracted using Trizol reagent. 1.6 µg of RNA was reverse-transcribed using oligo(dT) primers and Superscript II reverse transcriptase in a final volume of 20 µl. One µl of cDNA was amplified using Taq DNA polymerase. The PCR products were separated on a 1% agarose gel, and photographed under UV light. β-actin was used as a control gene. PSA expression was simultaneously measured and was used as a positive control.

Actin was uniformly expressed in all cell lines regardless of stimulant used. PSA was strongly expressed in LNCaP cells under all growth conditions. BT-474 cells expressed significant amounts of PSA only when stimulated with DHT and norgestrel, as expected. In contrast to PSA, *KLK9* mRNA expression in LNCaP cells was very weak and was only detected after 40 PCR cycles. In this cell line *KLK9* expression was stimulated with dexamethasone and norgestrel. In PC-3 and PC-3 (AR6) cells, *KLK9* was detected as a strong band under all growth conditions. In the MCF-7 breast cancer cell line, estradiol, DHT, norgestrel and dexamethasone and in BT-474 cells estradiol, aldostrone and norgestrel, resulted in significant up-regulation of *KLK9*. No differential expression of *KLK9* was apparent in BT-20 or any other cell lines tested. In summary, our results indicate that similar to other kallikreins *KLK9* mRNA expression is regulated by steroid hormones.

INTRODUCTION:

- Human tissue kallikreins (*KLK*) are a subgroup of serine proteases with diverse physiological function present in a variety of tissues and biological fluids.
- For many years the human tissue kallikrein gene family was considered to be composed of only three genes: the pancreatic/renal kallikrein (*KLK1*), the human glandular kallikrein 2 (*KLK2*), and prostate specific antigen (*KLK3*).
- These three genes are now considered as the classical human tissue kallikrein. In the recent years twelve additional genes with significant homology with the previously known kallikreins have been identified by different investigators.
- The term 'tissue kallikrein' is currently used to describe a group of fifteen enzymes with highly conserved gene and protein structure, with considerable similarities.
- All 15 human tissue kallikrein genes are tandemly located in a 300 kb region on chromosome 19q13.3-19q13.4 and are composed of five coding exons of similar or identical size.
- Kallikreins are predicted to encode for serine proteases. They are synthesized as preproenzymes and then processed to the mature active forms. Predicted to be secreted proteins, kallikreins are expected to circulate in the peripheral blood.
- The most well known member of this group of enzymes *KLK3*, also known as Prostate-Specific Antigen (PSA), is currently used in the diagnosis and monitoring of prostate cancer.
- In 1987, Stamey et al. showed that serum concentrations of PSA increase in patients with prostate cancer, and the elevated levels correlate with tumor volume and clinical stage of disease.
- Since then numerous studies have confirmed the value of serum PSA determinations in the diagnostic and monitoring of prostate cancer patients. Recent reports indicate that *KLK2* may soon find applicability as an additional prostatic and breast cancer biomarker.
- In the recent years, the value of the newly identified members of the human kallikrein gene family as potential biomarkers of cancer is being intensely investigated.
- Numerous reports indicate that messenger RNA of many of the kallikreins is differentially expressed in ovarian, breast and prostate cancer.
- Kallikrein 9 (*KLK9*), identified in our laboratory, is one of the newly characterized members of the kallikrein gene family.
- Similar to other kallikreins, *KLK9* is located on chromosome 19, has 5 coding exons, and is predicted to encode for a serine protease.
- Our earlier clinical studies indicate that *KLK9* mRNA is differentially expressed in cancer. Ovarian and breast cancer patients with *KLK9*-positive tumors have longer progression-free and overall survival compared to those who are *KLK9*-negative.

RATIONALE:

- Clinical studies indicate that mRNA of many of the newly identified kallikreins is differentially expressed in ovarian, breast and prostate cancer.
- *KLK9* mRNA expression is down-regulated in ovarian and breast cancer patients.
- *KLK2* and *KLK3* are currently used in the diagnosis and monitoring of prostate cancer. Gene expression of these two kallikreins is reported to be hormonally regulated by androgens and progestins in human cancer cell lines.
- *KLK9* gene expression in human prostate, ovarian, and breast cancer cell lines may also be under hormonal regulation.

METHODOLOGY:

- Hormonal regulation of *KLK9* in LNCaP, PC-3, and PC-3 (AR6) prostate cancer cell lines, Caov-3 ovarian cancer cell line and BT-474, MCF-7, MDA-MB-468, and BT-20 breast cancer cell lines was investigated.
- Cancer cells stimulated with estradiol, dihydrotestosterone (DHT), norgestrel, aldosterone, dexamethasone or ethanol were examined.
- Cells treated with 10^{-8} M of either of these steroids dissolved in ethanol were grown until 80% confluent. Cells stimulated with 100% ethanol were included as controls.
- Culture cells were then harvested and total RNA was extracted from the cell lines using Trizol reagent (Gibco BRL) following the manufacturer's instructions.
- RNA concentration was determined spectrophotometrically. 1.6 µg of total RNA was reverse-transcribed into first-strand cDNA using oligo(dT) primers and Superscript II reverse transcriptase in a final volume of 20 µl.
- One µl of cDNA was amplified using primers specific to *KLK9*. Primers were chosen from different exons to avoid contamination by genomic DNA.
- A plasmid containing *KLK9* mRNA as an insert was used as a template to provide positive control for PCR reactions.
- PCR was carried out in a 25 µl reaction mixture, containing 10 mM Tris-Cl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 100 ng of the forward and reverse primers, and 2.5 units of HotStar Taq DNA polymerase.
- The PCR conditions were 95ºC for 10 minutes for initial heat activation of the enzyme, followed by 39 cycles of denaturation at 94ºC for 40 seconds, annealing at 62ºC for 40 seconds, and extension at 72ºC for 50 seconds and a final extension at 72ºC for 10 minutes.
- Equal amounts of PCR products were separated on 1% agarose gels. The gels were stained with ethidium bromide, visualized and photographed under UV light.
- As a control gene, the expression of β-actin (a housekeeping gene that is not regulated by steroid hormones) was measured in all cell lines.
- PSA expression (up-regulated by androgens and progestins) was simultaneously measured and was used as a positive control.

RESULTS:

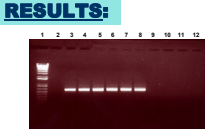

Fig. 1 LNCap cell line- PSA

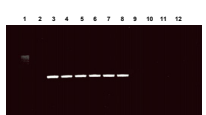

Fig. 3 LNCap cell line- Actin

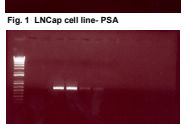

Fig. 2 BT-474 cell line -PSA

Fig. 4 BT-474 cell line- Actin

LEGEND:

Lane 1 - Marker
Lane 2 – Negative control
Lane 3 - Estradiol
Lane 4 - DHT
Lane 5 - Norgestrel
Lane 6 - Aldosterone
Lane 7 - Dexamethasone
Lane 8 - Ethanol
Lane 9 - Blank
Lane 10 – PCR negative control
Lane 11 - Blank
Lane 12 - Blank

RESULTS:

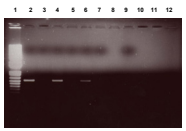

Fig. 5 LNCap cell line-*KLK9*

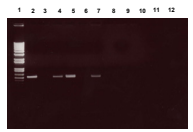

Fig. 7 BT-474 cell line-*KLK9*

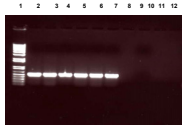

Fig. 6 PC-3 (AR6) cell line-*KLK9*

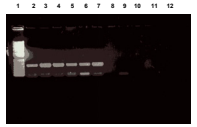

Fig. 8 BT-20 cell line-*KLK9*

LEGEND:

Lane 1 - Marker
Lane 2 - Estradiol
Lane 3 - DHT
Lane 4 - Norgestrel
Lane 5 - Aldosterone
Lane 6 - Dexamethasone
Lane 7 - Ethanol
Lane 8 - Blank
Lane 9 – PCR negative control
Lane 10 - Blank
Lane 11 – Blank
Lane 12 – Blank

DISCUSSION:

- β-actin, was uniformly expressed in all cell lines regardless of stimulant used, this was as expected since expression of actin is not under hormonal regulation.
- PSA was strongly expressed in LNCaP cells under all growth conditions. BT-474 cells expressed significant amounts of PSA only when stimulated with DHT and norgestrel, as expected.
- *KLK9* mRNA expression in LNCaP cells was very weak and was only detected after 40 PCR cycles. In this cell line KLK9 expression was stimulated with estradiol, dexamethasone, and norgestrel. In PC-3 and PC-3 (AR6) cells, KLK9 was detected as a strong band under all growth conditions.
- In the MCF-7 breast cancer cell line, estradiol, DHT, norgestrel and dexamethasone and in BT-474 cells estradiol, aldostrone and norgestrel, resulted in significant up-regulation of *KLK9*.
- No differential expression of *KLK9* was apparent in BT-20 or any other cell lines tested.

CONCLUSION:

- Our results indicate that similar to other kallikreins *KLK9* mRNA expression is regulated by steroid hormones. Hormonal regulation of *KLK9* in a variety of additional prostate, ovarian, and breast cancer cell lines is currently under investigation.
- We have recently developed anti-*KLK9* specific antibodies. *KLK9* protein in the supernatant of the cultured cancer cell lines will be probed using these antibodies.

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