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Identification of Prostate Cancer Prognostic Markers

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The objective of this project is to develop prognostic markers for prostate cancer (PCa) and identify potential therapeutic targets. The specific aims are: 1) To profile bone metastasis samples to identify genomic alterations of prostate cancer metastases that can be retrieved in their corresponding primary tumors, 2) To evaluate the prognostic value of specific genomic alterations in localized primary tumors with clinical follow-up database, 3) To ascertain the relevance to disease progression of genes residing in genomic alterations of prostate cancer metastases. Progress has been made in the three aims. Ethics approval has been obtained for the samples collection of AIM1. The chromosome 16p13.3 gain was found to be associated with high Gleason score, advanced tumor stage, and early biochemical recurrence suggesting a prognostic value for this genomic alteration (AIM2). The analyses of 16q23 and 10q23 deletions are under way. Toward AIM3, a specific antibody for GABARPL2 detected the endogenous expression of GABARAPL2 in prostate cell lines. siRNA effectively knocked down GABARAPL2 expression in PCa cells which was rescued with a GABARAPL2 expressing vector. These tools will serve for the functional studies of AIM3. Overall, the work accomplished shall lead to the realisation of the objective of this project.

Prostate cancer, Genomic alteration, Fluorescence in situ hybridization (FISH), Prognostic markers, ectopic expression, gene silencing, cDNA cloning, Prostate cancer cell lines

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Introduction

We hypothesize that specific genomic alterations, determinant of tumor behaviour, could be detected in primary tumor and predict clinical outcome. The objective is to develop prognostic markers for prostate cancer (PCa) and identify potential therapeutic targets. To that goal we proposed the following specific aims: 1) To profile bone metastasis samples to identify genomic alterations of prostate cancer metastases that can be retrieved in their corresponding primary tumors, 2) To evaluate the prognostic value of specific genomic alterations in localized primary tumors with clinical follow-up database, 3) To ascertain the relevance to disease progression of genes residing in genomic alterations of prostate cancer metastases. We have made progress in the three aims which are detailed below.

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In regard to AIM1 and AIM2, we have recently obtained the final approval from the Ethics committee of our hospital after a lengthy process. We have made the arrangement with the orthopaedic surgeons to collect the bone metastasis samples for profiling (AIM1). We have set up the conditions to extract and profile samples with low nucleic acid inputs and we are now ready to process samples as the will become available to us.

In regard to AIM2, we have surveyed a tissue microarray (TMA) of 300 of formalin fixed paraffin embedded PCa samples from radical prostatectomies using FISH probes for chromosome 16p13.3 (Figure 1A). The 16p13.3 gain was detected in 86 out 267 scorable cases (32%, Figure 1B). Most of the 16p13.3 gains consisted of single copy gain as shown in Figure 1C. The 16p13.3 gain was associated with high Gleason grade (P<0.0001, Figure 2A) and advanced stage (T3 vs T2, P=0.0004, Figure 2B). Although not statistically significant, there was a trend toward an association between the 16p13.3 gain and high levels of preoperative PSA (P=0.07, Figure 2C). These results suggested that the 16p13.3 gain may be associated with an aggressive phenotype of PCa. The biochemical recurrence status of PCa was available for 245 of the cases analyzed. Using Kaplan-Meier analysis, we found that the 16p13.3 gain was associated with early biochemical recurrence (P=0.0007, Figure 3A). Moreover, this association was still significant when we considered only the cases with a Gleason score of 7 or less (P=0.02, Figure 3B). This is a very relevant finding because the Gleason score of 7 or less cases represent the most challenging subset for prognostication. We are now carrying out more advanced statistical analyses to assess whether the prognostic value of 16p13.3 gain is independent of the other known prognostic tools such as Gleason score, tumor stage, and preoperative PSA.

We have just completed the FISH scoring for the 16q23 deletion on the same sample set and we are looking forward to evaluate its prognostic value. Based on our previous analysis of a published prostate cancer genomic profiling data set with clinical follow-up [1], we expect that the 16q23 deletion to be associated with early biochemical recurrence. It will be particularly interesting to see if a combination of the two markers may provide a better patient stratification. We have also started the FISH hybridization to assess the 10q23 deletion status on these samples.

In AIM3, we are ascertaining the relevance to disease progression of genes residing in genomic alterations of prostate cancer metastases. Our focus is on GABARAPL2, the only gene which expression was significantly and negatively associated with the 16q23 deletion status. GABARAPL2 protein is involved in the critical cellular process of autophagy, but there is a lack of knowledge about GABARAPL2 in general and its role in PCa in particular. To that end, we have been successful at the isolation the full length cDNA of GABARAPL2 by RT-PCR in PC-3 prostate cancer cell line. We have generated an expression vector for the ectopic expression of a V5 tagged GABARAPL2, which can be detected using an anti V5 antibody. A challenge that we encountered for this part of the project was the lack of specific antibody to detect the endogenous
expression of GABARAPL2. We have finally found one that is specific and sensitive enough to detect the endogenous expression of GABARAPL2 in cell lines by Western blot. Using this antibody, we were able to detect the expression of GABARAPL2 in the most common prostate cell lines. As shown in Figure 4, its expression varied across the cell line with a maximum expression in 22RV1 PCa cells and a minimum in LAPC4 PCa cell line (Figure 4). Using siRNA, we were able to knockdown by 80% GABARAPL2 expression in the most expressing cell line 22RV1 and rescued its expression by ectopic expression with our vector (Figure 5). This experiment also showed that the new antibody is specific and can be used for the project. This laboratory work carried out so far will serve for the functional studies which are part of AIM3.

**Key research accomplishments**
1. Chromosome 16p13.3 gain and 16q23 deletion status were assessed by FISH in a set of 267 PCa samples.
2. Chromosome 16p13.3 gain was detected in 32% (86/267) of the samples and associated with high Gleason grade and advanced tumor stage.
3. The 16p13.3 gain was associated with early biochemical recurrence of prostate cancer.
4. GABARAPL2 expression was surveyed in most of the laboratory prostate cell lines using a specific antibody.
5. Endogenous expression of GABARAPL2 was knocked down by siRNA and rescued by ectopic expression in prostate cancer cell lines 22RV1.

**Reportable outcomes**

Poster and oral presentations:


Conclusions
1. The FISH probes for 16p13.3 gain and 16q23 deletion are specific and suitable for prostate tissues assessment.
2. Our results suggest that chromosome 16p13.3 gain is associated with aggressive prostate cancers and may serve as biomarker to predict biochemical recurrence.
3. GABARAPL2 is expressed at various levels in all prostate cell lines surveyed. GABARPL2 expression could be effectively knocked down by siRNA in the most expressing PCa cell line and rescued by ectopic expression.

References
Figure 1. Dual-color FISH analysis of 16p13.3 gain in formalin-fixed paraffin-embedded PCa samples. A) BAC DNA mapping to chromosome 16p13.3 was fluorescently labelled and co-hybridized with fluorescent centromere 16 control probe to detect the gain in tumor samples. B) 16p13.3 gain status of 267 primary PCa samples determined by interphase FISH. C) On the left panel, representative FISH images of PCa without gain show two red signals (16p13.3 locus) and two green signals (centromere 16) in most of the nuclei. On the right panel, FISH images show greater than or equal to three red signals (16p13.3 locus) and two green signals per nucleus, indicating a 16p13.3 gain.
Figure 2. 16p13.3 gain and Gleason grade, tumor stage, and preoperative PSA levels. Distribution of 16p13.3 gain as detected by FISH in relation to Gleason grade (A), stage (B), and preoperative PSA (C) in 267 cases of PCa treated by radical prostatectomy. Chi-squared test, $P$ value indicated.
Figure 3. Prognostic value of 16p13.3 gain in PCa. A) Kaplan-Meier recurrence-free survival analysis based on 16p13.3 gain status determined by FISH on the entire samples set with clinical follow-up (A, n=245) and on Gleason score 6 and 7 only samples subset (B, n=220). $P$-value (log-rank test) indicated.
Figure 4. GABARAPL2 expression in RWPE-1 (non tumor cell line), 22RV1, LAPC4, LNCaP, PC-3, and DU145 PCa cell lines. Cells were grown in maintenance medium and assessed for basal levels of expression of GABARAPL2 by Western blot analysis (anti-GABARAPL2 antibody, Abcam). Actin was used as control (anti-actin, CHEMICON).

Figure 5. siRNA knockdown of GABARAPL2 in 22RV1 prostate cancer cell line and ectopic expression. 22RV1 cells were transfected with mock or V5-GABARAPL2 expressing vector in combination with siCtrl or siGABARPL2. Western blot analysis was done with anti-GABARAPL2 antibody (Abcam) and anti-actin (CHEMICON) as loading control.