Towards prevention of metastatic prostate cancer: recent molecular insights from the direct analysis of metastatic precursor cells

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Metastasis is the major cause of cancer-related deaths in patients with solid cancers (1). Prostate cancer (PC) is the second most frequently diagnosed cancer in males, detected in about every fourth man, and the fifth leading cause of death in men worldwide (2). Somehow surprisingly, the 5-year survival rate of M0-stage patients (i.e., patients without evidence of distant metastasis) is close to 100% (1), because PC is very slowly progressing. The growth of metastasis often takes more than 10 years and death due to metastasis frequently occurs more than 15 years after diagnosis (3,4). Consequently, most men die with PC, not due to PC.

On the other hand, most patients who eventually develop metastasis will succumb to the disease; here, the 5-year survival of M1-stage patients (i.e., patients that have been diagnosed with distant metastasis) is less than 30% (1). This fatal condition arises in about one third of patients treated for localized disease (5). Therefore, the most critical problem is to identify patients that will relapse early on (6). The extent of this problem is illustrated by the fact that it is necessary to screen more than 1,000 patients to detect and treat 40 patients in order to eventually prevent the death of a single patient (7,8). Treatment itself is associated with reduced quality of life (9), and therefore active surveillance is offered to patients at low risk to die of metastatic PC; again, the problem arises that active surveillance may miss the optimal time-window for treatment (10).

It is therefore due time to directly analyze the metastatic seed residing in distant organs and the hidden disease that is smoldering undetectably for so long periods of time.

We need to understand the conditions that affect disease progression towards manifest metastasis. The groups of Robert Vessella and Colm Morrisey belong to the few that have engaged in this research for long time and together with Min Fang have now started to address the molecular characteristics of putative metastasis founder cells in PC at high resolution (11). We will therefore discuss their study together with other work in the field.

Identifying the hidden malignant seed

Increasing evidence suggests that cancer cells often disseminate early during the development of primary tumors (12). Cancer cells entering the vasculature (commonly termed circulating tumor cells, CTCs) spread to distant sites, and eventually lodge within the tissues of distant organs, thereby becoming disseminated cancer cells (DCCs). Therefore, the presence of DCCs indicates successful seeding from the primary tumor and successful invasion of distant organs. Cancer cells remaining in the body after surgical removal of the primary tumor must comprise the founder cells of later arising lethal metastases. Therefore, DCCs are the targets of adjuvant therapies aiming to prevent or delay the outgrowth of metastasis. Although CTCs in the blood are easier and less invasive to obtain than DCCs, which for example can be detected in bone marrow (BM) or lymph nodes, their biological and clinical relevance in early stage PC remains unclear, mainly because they can hardly be detected. For example, two

teams showed that in early PC the detection rate of cells thought to be CTCs (which are identified by cytokeratin expression and absence of CD45 expression) was the same for cancer patients and healthy controls (13,14). On one hand, this illustrates the rareness of the CTCs, possibly due to the rapid clearance of CTCs from bloodstream upon surgery (15), but also that histogenetic profiling does not suffice to pinpoint a cancer cell. In contrast, CTC are more frequently detected in M1 patients (16). These CTCs are most likely shed from secondary growths, and therefore serve as surrogate biopsy of these secondary growths, which are often not easily accessible.

DCCs can be detected in BM of PC patients using staining against epithelial markers [e.g., cytokeratins (CK) or epithelial cell adhesion molecule (EpCAM)] (17,18). Their clinical relevance was demonstrated repeatedly; finding DCCs in the BM of M0-stage PC patients was associated with poor prognosis (17,19,20). Equally important, bone is the preferred site of metastatic outgrowth in PC (21) and therefore DCCs from BM offer the opportunity to track progression towards metastasis: they mark cancer cell dissemination and comprise *bona fide* founder cells of metastasis. Thereby, DCC research may offer new opportunities to improve adjuvant therapies. Since many targeted therapies are of little help in the adjuvant setting (22) and therapy decision is based on molecular characteristics of the primary tumors (1), it has been suggested that the underlying reason for the failure of systemic therapies is the molecular disparity between primary tumors and disseminated metastasis founder cells (23). Therefore, the design of successful targeted therapies requires detailed molecular characterization of metastasis founder cells, a sub-population of DCCs. However, similar to blood-derived CTCs, DCCs are rare and found in BM samples at a frequency of 10^{-6}. Usually, only one or few cells can be detected in up to 60% of the samples of M0-stage patients (17-19) and therefore progress of the field critically requires technologies suitable for single cell analysis.

Immunophenotyping of single DCCs

Survival data already give a first insight into the functional heterogeneity of DCCs. First, not all patients with DCCs developed metastasis during follow-up and secondly, patients without epithelial marker-positive DCCs may also develop metastasis, although at lower rate, suggesting the existence of DCC subpopulations with and without

epithelial detection markers. Immunophenotyping was used as first approach to address the functional heterogeneity of DCCs, for example by analyzing cell cycle status or apoptosis. Using PCNA as proliferation marker, Muller *et al.* could not detect $CK^+/PCNA^+$ cells (24). In contrast, in BM samples selected for positivity of PSA mRNA, which was used as screening assay for DCC positive BM samples, Cher *et al.* found an association between Gleason score and proliferation status. Patients with a Gleason's score <7 harbored 29% $CK^+/Ki-67^+$ (another proliferation marker) cells in BM as opposed to 53% double positive cells for higher Gleason scores (25). The discrepancy between the two studies has not been addressed so far. More recently, BM of PC patients was analyzed for the presence of apoptotic cells using staining against M30 (caspase-cleaved CK18), and it was found that 36% of patients contain $M30^+$ cells (26), however, the authors could not detect associations with clinical follow-up or risk factors, in contrast to other studies (17,19). In summary, immunophenotyping raised several questions, which motivated the development of additional, more comprehensive approaches.

Genomic profiling of single DCCs

Metaphase-based comparative genomic hybridization (mCGH) was the first technology developed to comprehensively analyze the genome of single DCCs. Early on, it was observed that in many types of carcinomas, including PC, genomes of CK^+ M0-DCC were quite heterogeneous, as opposed to the genomes of M1-DCCs (27). Further work demonstrated that CK^+ M0-DCCs showed genetic heterogeneity both within and between patients, and lacked similarity to the genomes of primary tumors (17). On the other hand, CK^+ M1-DCC genomes showed more similarities to the genomes of primary tumors (17) as well as harbored genomic changes characteristic of PC (28). It should be mentioned that PT samples or CK^+ M1-DCCs contained on average significantly more aberrations per cell than CK^+ M0-DCCs (17). Further advance of the technologies enabled analysis of the genomes of DCCs using array-based CGH (aCGH). While it is considered that mCGH has a resolution of about 10 Mbp, the resolution of aCGH is in the kbp range. Holcomb *et al.* used BAC-based aCGH to interrogate the genomes of cell pools containing 10–20 $EpCAM^+$ DCCs (29). They validated the method using LNCaP cell line and achieved 0.4 Mbp resolution and then analyzed the pools of DCCs isolated from PC patients with localized or advanced disease, as well

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as cells of primary tumors. Overall, they found concordance of genomic aberrations present in DCC pools isolated from PC patients with localized and advanced disease, but also between DCC pools isolated from patients with localized disease and primary tumors. However, DCC pools from patients with localized disease overall had less genomic aberrations than DCC pools from advanced disease.

Higher resolution, even down to the single cell level, was made available recently by researchers from Seattle (11). They used SNP-arrays to interrogate the presence of copy number variations (CNVs) in samples of 1–40 cells. The performance of the method was demonstrated on the LNCaP cells, where all previously reported CNVs were detected. Overall, the achieved resolution was in <1 MBp range and up to 5 kbp. Next, they analyzed pools of EpCAM+ DCCs containing between 5 and 20 cells isolated from eight PC patients with the advanced disease. As in previous similar studies, aberrations in the regions containing genes known to be altered during PC progression were observed. Interestingly, the analyzed samples showed great heterogeneity in the fraction of the genome containing CNVs, as well as the sizes of CNVs (the sizes of observed aberrations were between 21.59 kbp to 154.91 Mbp for gains and and 2.99 kbp to 135.37 Mbp for losses). Of note, for two patients matched samples of metastasis tissues from multiple locations were available for genomic analysis. Interestingly, the majority of aberrations were shared between DCCs and metastases; however, metastases isolated from different organs showed some heterogeneity both in the fraction of the genome containing CNVs as well as the type of CNVs within the patient. Furthermore, two patients differed in the percentage of aberrations unique to DCCs. These data support the hypothesis that cancer cells present at different sites in advanced disease arise from independently disseminated and evolved clones (12). The high resolution, if applied systematically to early DCCs of patients before and at the M1-stage may eventually help to resolve the evolutionary paths of systemic PC.

Transcriptomic profiling of single DCCs

Transcriptome analysis of DCC in PC was the last technology to come of age. In 2014 two studies explored different aspects of the biology of PC-DCCs. The team of Colm Morrissey used microarrays (30) to investigate transcriptomes of EpCAM+ DCCs isolated from patients with no evidence of disease for at least 7 years and patients

with the advanced disease (31). They focused on cells expressing low levels of erythroid-lineage genes and high levels of prostate- and PC-specific genes. Not unexpectedly, hierarchical clustering revealed that DCCs from patients without recurrence and with advanced disease differed in their expression profiles. In addition, cells from advanced disease clustered in two distinct groups, one more characterized by activation of genes involved in cytokine and chemokine signaling, while the other characterized by activation of genes involved epithelial to mesenchymal transition. However, the major difference between DCCs from non-progressed and advanced disease patients was the activation of a so-called "dormancy" signature (32) in the former group. This work is complementary to our work (18), where we characterized PC-DCCs by combined analysis of genome and transcriptome of single cells. Transcription profiling of DCCs isolated from M0- and M1-stage patients revealed a surprising and unexpected plasticity of DCC transcriptomes. Specifically, PC-DCCs were shown to express both transcripts characteristic for epithelial/prostate cells and transcripts characteristic for hematopoietic/ erythroid cells. The data suggested that more recently disseminated and not yet fully malignant DCCs display combined epithelial/hematopoietic transcriptome, while genomically more advanced DCCs contain less non-epithelial transcripts. While at the moment these patient-derived data cannot be modeled *in vitro* or *in vivo*, it is tempting to speculate that this unexpected transcriptome plasticity may help DCCs to adapt to and survive in ectopic environments, before they acquire the ability to progress and display epithelial/prostate characteristics.

Conclusions

While the work on PC-DCCs had seen the significant advances in the last decade, many questions remain unanswered, among them the crucial question about the identity and molecular characteristics of metastasis founder cells. The main obstacle is the rarity of DCCs, as well as inability to examine different regulatory layers of these cells (genome, transcriptome, methylome, miRNAome, proteome) at the same time in the same cell. This is further complicated by their ongoing evolution and the dynamics of phenotype changes. Inevitably, any analyzed cell represents only a snapshot. Therefore, one of the major challenges is to track DCCs within individual patients for the prolonged periods of time. This could be achieved by analyzing DCCs from different stages of disease [M0 at surgery, M0 post-

surgery, biochemical relapse (BR), M1 pre- and post-treatment] in large cohorts of patients. Furthermore, we should strive to integrate as many "-omics" layers as possible into analysis, in order to obtain a better understanding of the often decade-lasting dark stage of cancer progression. Combined "omics" analyses are slowly becoming a routine (18,33), and should be used to identify novel modes of therapeutic intervention.

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Footnote

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