Supplementary information for

Combined genome and transcriptome analysis of single disseminated cancer cells from bone marrow of prostate cancer patients reveals unexpected transcriptomes

Supplementary Methods

Processing of BM samples

Upon arrival, the BM sample was washed with Hank's salt solution (HSS; Biochrom AG) to remove fat and thrombocytes. The sample was centrifuged at 170 x g, for 10 min at 4°C and supernatant was discarded. The washing was done twice. Next, the cell pellet was resuspended in 9 ml of HSS and overlaid on 6 ml of 65% Percoll solution (ρ = 1.083 g/cm³; GE Healthcare). The sample was centrifuged at 1000 x g, for 20 min at 4°C, to remove erythrocytes and granulocytes. After centrifugation, the interphase containing mononuclear cells (MNCs) was carefully collected and washed with phosphate buffered saline (PBS). Cell suspension was centrifuged at 500 x g, for 10 min at 4°C. The number of MNCs and erythrocytes was determined using a haemocytometer. To enrich the DCC-containing fraction the sample was depleted of the majority of hematopoietic cells using negative immunomagnetic selection. To achieve this, the cell pellet was resuspended in MACS buffer (0.5% BSA, 2 mM EDTA in PBS; 90 µl/10⁷ MNC). Then, the cell suspension was incubated with APC-conjugated antibodies against CD11b, expressed by monocytes, granulocytes, macrophages, and natural killer cells (10 µl/10⁷ MNC; antibody concentration was not supplied by manufacturer, Miltenyi Biotec), CD33, expressed by myeloid cells (5 µl/10⁷ MNC; Invitrogen), and CD45, common antigen of leukocytes (5 µl/10⁷ MNC; Invitrogen). After 15 min incubation at 4°C, the suspension was washed in MACS buffer. The cell pellet was again re-suspended in MACS buffer (60 µl/10⁷ erythrocytes). Then,
anti-APC beads (20 µl/10⁷ MNC; Miltenyi Biotec) and anti-235a beads (glycophorin A, expressed on mature erythroid cells; 20 µl/10⁷ erythrocytes; Miltenyi Biotec) were added. After 15 min incubation at 4°C, the suspension was washed in MACS buffer. The cell pellet was re-suspended in 2 ml of MACS buffer, run through the 40 µm cell sieve, and then run on LS MACS column (Miltenyi Biotec), previously equilibrated with MACS buffer. The column was washed with 9 ml of MACS buffer. Eleven ml of the eluate containing the marker-negative cell fraction were collected on ice and cell number determined on haemocytometer.

**Staining of BM samples and cell lines**

On average, two million BM cells were re-suspended in 100 µl of blocking solution (10% human AB serum (Biotest) in PBS) and incubated for 15 min under constant rotation at 4°C. Next, 2 µl of anti-EpCAM-PE (HEA125; antibody concentration was not supplied by manufacturer, Miltenyi Biotec) antibody were added and incubated for 15 min on the roller at 4°C. After incubation, the antibody solution was removed and the cell pellet washed with PBS. Staining with isotype antibodies (Dako) was performed in 12 cases (10% of all BM samples) and in no case unspecific staining was observed. Remaining samples were not stained with the isotype antibody due to the lack of sufficient number of cells (the same BM samples were used for other *in vivo* and *in vitro* studies). All experiments with cell lines involved included controls using isotype antibodies (Supplementary Figure S2).

Cell lines (WPE-Stem, DU145, PC3, WPE-Int, LNCaP and VCaP) were grown according to instructions from ATCC. At 80% confluence, the medium was discarded, cell monolayer washed with PBS, and cells fixed using 4% formaldehyde. After fixation, cells were washed with PBS. Further immunostaining steps were performed as described above. Final washing step was done in presence of 0,5 ng/µl dilution of DAPI (Roche) in
PBS. The DAPI solution was incubated for 5 min. This solution was replaced with fresh PBS and the cells observed under microscope.

**Screening of BM and isolation of single cells and cell pools**

Each BM sample was manually screened for the presence of EpCAM\(^+\) cells on an inverted fluorescent microscope (Olympus or Zeiss), equipped with micromanipulator (Patchman NP2, Eppendorf) and pump (CellTram, Eppendorf). Cell suspension containing \(~0.3 \times 10^6\) of stained BM cells in 30 \(\mu l\) was transferred into the chamber of 8-chamber microscope slide (Nunc) containing 170 \(\mu l\) of PBS. Single cells with integer morphology and positively stained for EpCAM were isolated using a glass capillary attached to the micromanipulator. After extraction of the single cell, it was transferred into the chamber containing 200 \(\mu l\) of PBS, to ensure that only one cell was isolated. Then, selected single cells were isolated manually using a micropipette, by transferring the single cell in 1 \(\mu l\) of PBS into microtubes 0.2 ml tubes containing 10 ng tRNA (Roche), dissolved in 4 \(\mu l\) of lysis buffer (mTRAP kit, Active Motif). The microtubes were immediately stored at -80°C. tRNA was added to prevent unspecific binding of lysed single cell RNA to the tube wall. In addition, after screening and isolation of single cells, a pool of \(~2000\)-3000 cells was isolated by taking 1 \(\mu l\) of cell suspension and transferring it to the microtubes with tRNA and lysis buffer. After isolation of single cells, 1 \(\mu l\) of cell-free PBS, from which individual cells were isolated, was also isolated for subsequent WTA as control.

**Whole transcriptome amplification (WTA) of single cells**

Single cells in 1 \(\mu l\) of PBS were isolated using micromanipulation and deposited in tubes containing \(6.4 \mu l\) of lysis buffer (Active Motif) containing 10 ng of tRNA (Roche), 1 \(\mu g\) of protease (Active Motif), and 1 \(\mu l\) of 37.5 \(\mu M\) solution of biotinylated oligo-dT peptide nucleic acids (PNAs; Active Motif). Proteolytic digestion was performed by incubating the
samples for 10 min at 45°C, followed by inactivation of protease at 75°C for 1 min, and annealing of PNA to poly-A tails of mRNAs, at 22°C for 15 min. PNA-mRNA complexes were precipitated in magnetic force field using streptavidin-conjugated metal beads. While precipitated in magnetic racks, bead pellets were washed with 10 μl of wash buffer 1 (50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 0.25% Igepal), 20 μl of wash buffer 2 (50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 0.5% Tween-20), and again with 20 μl of wash buffer 1. These DNA-containing supernatant were transferred to a tube, containing 0.8 μl of polymerized 0.25% polyacrylamide as a carrier, for subsequent precipitation and WGA. Reverse transcription on solid phase was performed for 45 min under rotation at 44°C, in 20 μl reaction mixture containing 0.5 mM of each dNTP (GE Healthcare), 200 U of SuperScript II reverse transcriptase (Invitrogen), 0.25% Igepal, 5 mM DTT, 30 μM of C15GTCTAGAN8 primer, 15 μM of C15GTCTAGACTTGAGT24VN primer (Metabion), and 1x first strand buffer (Invitrogen). Primers were annealed at room temperature for 10 min, prior to addition of the enzyme. Following reverse transcription, beads were precipitated in magnetic racks and washed in 20 μl of wash buffer 3 (50 mM KH2PO4, 1 mM DTT, 0.25% Igepal), and resuspended in 10 μl of buffer for tailing (4 mM MgCl2, 0.1 mM DTT, 0.2 mM dGTP, 10 mM KH2PO4). Reaction mixture was overlaid with 40 μl of mineral oil, and the cDNA single strands released from beads by incubating the mixture at 95°C for 5 min, followed by incubation on ice for 3 min. Addition of dGTPs on 5’ termini of single stranded cDNAs was performed by adding 10 U of terminal dNTP transferase (TdT; USB-Affymetrix) and incubating the mixture for 60 min at 37°C. After inactivation of TdT at 70°C for 5 min, we added 35 μl of WTA reaction mix 1 (4 μl of buffer I (Expand Long Template, Roche), 3% deionized formamide). Hotstart PCR was performed by incubating the sample to 78°C and adding 5.5 μl of WTA reaction mix 2 (3.2 mM each dNTP, 12 mM TCAGAATTTCATGC15 primer, and 7.5 U of PolMix (Expand Long Template, Roche)). WTA consisted of 40 cycles in MJ Research PCR cycler – 20 cycles of 15 s at 94°C, 30 s at
65°C, and 2 min at 68°C, and 20 cycles with an increase of elongation step for 10 s/cycle, followed by final cycle with 7 min of elongation.

**Whole genome amplification (WGA)**

Sixty microliters of DNA- and carrier PAA-containing supernatant were mixed with 120 μl of ice-cold absolute ethanol and left at -20°C to precipitate. Subsequently, the tubes were centrifuged at 4°C for 45 min at 20800 x g. The supernatant was removed, and the pellet was washed with 180 μl of 70% ice-cold ethanol, for 10 min at 18°C in theromixer (Eppendorf) set on 350 rpm. Next, the tubes were centrifuged at room temperature for 10 min at 20800 x g. These washing steps were repeated twice. After final centrifugation, the pellet was air-dried, and resuspended in 3.5 μl of water, and incubated for 18 h at 18°C in thermomixer (Eppendorf) set on 350 rpm. Subsequent WGA procedure was performed using the Ampli1™ WGA Kit (Silicon Biosystems), which is based on the method described previously (10, 13).

**Establishing conditions for specific end-point PCR**

Primers were selected based on the cDNA sequence of each gene of interest (48, 49), using Primer3 online tool (50). Specificity of each primer was checked using BLAST (51). This analysis showed that each primer pair detects all isoforms of the corresponding transcript. The only exception was the transcript of MAGEA2, were primers detect 6 out of 7 isoforms. Amplification of MAGEA genes proved to be very difficult, so we used already published primers (27, 52).

WTA products of different single cell line cells (VCaP, PC3, A431 and BT20) and BM cells were used to establish conditions for PCR amplification of selected transcripts (Supplementary Figure S11). These WTA products were from our collection of cell line WTA products. All annealing temperatures were selected based on results of gradient
PCR. PCR was performed in 10 μl of water solution containing 0.1 μl of WTA cDNA, 10 mM Tris-HCl, 50 mM KCl, 1 mM MgCl₂, 0.1 mM equimolar mixture of dNTPs (GE Healthcare), 0.4 pM of each primer (Supplementary Table S1), 0.5 μg/μl of BSA (Roche), and 0.5 U of Taq DNA polymerase (Pan Biotech). Thermal cycling was performed as follows: 1st phase: denaturation at 94°C for 2 min, annealing (transcript-specific, Supplementary Table S1) for 30 sec, and extension at 72°C for 2 min for the first cycle; 2nd phase: denaturation at 94°C for 15 sec, annealing for 30 sec, and extension at 72°C for 20 sec for 14 cycles; 3rd phase: denaturation at 94°C for 15 sec, annealing for 30 sec, and extension at 72°C for 20 sec for 25 cycles; 4th phase: extension at 72°C for 2 min. All PCR products were electrophoresed through a 3% agarose gel and stained with ethidium bromide for visualization under ultraviolet light. To confirm the identity of amplified fragments, we digested all PCR products with adequate restriction endonuclease (NEB; Supplementary Figure S11 and Supplementary Table S1).

Restriction digestion was performed in a 30 µl water solution containing 15 µl of PCR reaction product, 3 µl of corresponding restriction endonuclease (Supplementary Table S1) and 3 µl of adequate reaction buffer (NEB; NEBuffer 1 for Tsp509I, NEBuffer 3 for BsrI and NEBuffer 4 for Hpy188I, Hpy188III, HpyCH4IV, HpyCH4V, HaeIII and NlaIII). For enzymes Hpy188III and NlaIII reaction was supplemented with 10 ng/μl of BSA (Roche). Incubation for Hpy188I, Hpy188III, HpyCH4IV, HpyCH4V, and NlaIII was at 37°C for 3 h followed by inactivation at 65°C for 20 min, for enzyme HaeIII at 37°C for 3 h and inactivation at 80°C for 20 min, for Tsp509I at 65°C for 3 h without inactivation and for BsrI at 65°C for 3 h and inactivation at 80°C for 20 min.

Additionally, all primers were tested on human genomic DNA (Invitrogen) and failed to produce the correct fragment, demonstrating their specificity for cDNA.
Supplementary References


