Two different, mutually exclusively distributed, TP53 mutations in ovarian and peritoneal
tumor tissues of a serous ovarian cancer patient: indicative for tumor origin?

**SUPPLEMENTARY INFORMATION**

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**Characterization of proliferating CD45⁺/EpCAM⁺ double positive cells from ascites**

In addition, in ascites of the HGSOC patient with two different functional TP53 mutations, substantial numbers of an unusual cell population were identified, which co-express the pan-leucocyte cell marker CD45 and the epithelial cell surface marker EpCAM. Interestingly, quantitative IF analyses of embedded cells from ascites revealed the presence of a free floating CD45 and EpCAM double positive cell population in a much higher frequency (6.2% to 9.2%) compared to other HGSOC patients (0.0% to 4.0%, N=17).

Accurate visual inspection of the images of other patients corresponding to the 4% CD45⁺/EpCAM⁺ double positive cells proved that these signals were false positively identified cells from the automated cell image analysis software, which resulted from infiltrating CD45⁺ immune cells into aggregated EpCAM⁺ tumor cells (Suppl. Figure S1).
CD45+/EpCAM+ double positive cells occurred mostly either as single cells or as small aggregates consisting of two to five cells. Their cell nuclei were larger than typical nuclei of non-cancer cells visible with DAPI staining. CD45 and EpCAM fluorescence intensity was weaker compared to single positive CD45+ leukocytes or single positive EpCAM+ tumor cells (Suppl. Figure S1C-F). We used two different fluorescence antibody clones against human EpCAM (rabbit anti-EpCAM IgG, clone E144 (ab32392), Abcam, Cambridge, UK and mouse anti-EpCAM IgG1 VU1D9, clone VU1D9, Cell Signaling, Cambridge, UK) to rule out unspecific binding of the antibody and CD45 (rat anti-CD45 IgG2b, clone orb96558, Biorbyt, Cambridge, UK and rabbit anti-CD45 IgG, clone E19-G, DB Biotech, Kosice, Slovak Republic). Further bead-staining with human anti-EpCAM (Dynabeads® Epithelial Enrich magnetic beads, Ber-EP4, Invitrogen, CA, USA) and rabbit anti-CD45 (clone E19-G, BD Biotech, NJ, US) antibodies coupled to micro beads confirmed the existence of the CD45+/EpCAM+ cells (Suppl. Figure S1B).

Phenotypic characterization of these cells with IF staining with additional markers including CD14, CD16, and CD44 showed weak co-expressions of these immune cell and stem cell markers. In contrast, they were negative for Pan-cytokeratin (Ck8, Ck18, and Ck19). IF staining for p53 showed mostly negative free floating CD45+/EpCAM+ cells in ascites (Suppl. Figure S1C-F). According to the TP53 mutational analysis, picked free floating CD45 and EpCAM double positive single cells, labelled with two different sized Dynabeads, did not contain any of the two described TP53 mutations (Figures 2C and 3).

Presence of this unique cell population has been reported previously (Ramakrishnan, Mathur et al. 2013). Here, as possible genesis a fusion of a macrophage with a tumor cell was discussed (Ramakrishnan, Mathur et al. 2013). Thus, a cell-fusion of immune cells and tumor cells with subsequent consolidation of chromosomes can be one of the explanations for this unusual type of cells. Moreover, epithelial Langerhans cells were also known to express both, epithelial and immune cell markers (Gaiser, Lammermann et al. 2012). The existence of immune cells expressing epithelial cell marker EpCAM is also thinkable.
Ki-67 staining (together with CD45 and EpCAM), a nuclear proliferation marker, revealed that some double positive cells as well as tumor and immune cells proliferate (Suppl. Table S1). A tumorigenic origin of these free floating double-positive cells cannot be proven, since picked CD45+/ EpCAM+ double positive single cells did not contain any of the two described TP53 mutations (Figure 2C and 3).

**Materials and Methods**

**Enrichment of EpCAM positive tumor and CD45 positive immune cells from ovarian and peritoneal tissues and ascites preparations.** For enrichment of CD45 and EpCAM positive cells from ascites and processed tumor tissues Magnestat technology (prototype, Clinical Genomics Pty Ltd, Sydney, Australia) was used. At least 5 x 10^6 cells were re-suspended in 7.5 ml DMEM and 2.5 ml 4 x Miltenyi buffer was added. The well mixed CD45 or EpCAM beads were washed in 1ml Dynabeads wash buffer containing 1 x PBS, 0.1% BSA, 0.6% sodium citrate on a magnetic stand. Beads were re-suspended in 50 µl PBS-T and added to the prepared sample. The mixture was incubated for 20 min while shaking at 4°C and bead bound cells were subsequently separated using Macs multi and Vario Macs columns according to the manufacturer’s instructions.

**Picking of single cells, labeled with magnetic beads of different sizes and DNA extraction**

Previously frozen ascites samples (around 10^6 ascites cells) were thawed, washed and resuspended in 500 µl 1 x PBS. The cell suspension was first incubated with 1 µl/ml rabbit anti-CD45 (clone E19-G, BD Biotech, NJ, US) (4°C, at least 20 min, rolling) and washed by centrifugation. Subsequently, the cell pellet was resuspended in a mix of 20 µl Dynabeads® M280 sheep anti-rabbit IgG with a size of 2.8 µm to label CD45+ antibody coupled cells and 32.5 µl/ml, 4.5 µm small human anti-EpCAM Dynabeads (Dynabeads® Epithelial Enrich magnetic beads, Ber-EP4, Invitrogen, CA, USA) to label EpCAM+ cells (4°C, at least 20 min, rolling). Single CD45+ or EpCAM+ cells (labeled with 2.8 µm or 4.5 µm Dynabeads, respectively) were picked in 1 x PBS using mmi CellEctor Plus system with mmi CellTools v.4.3.2 software (mmi, Glattbrugg, Switzerland), (Figure 2B).
In order to extract DNA from single or few picked cells, cells were lysed with 5 µl lysis buffer containing 200 mM KOH and 50 mM dithiothreitol (DTT), mixed 1:1 according to the previously described protocol (Kim, Yoon et al. 2009). The suspension was mixed gently, spun down and incubated at 65°C for 10 min. The reaction was stopped in 10 µl stop solution with neutralization buffer (900 mM Tris-HCl pH 8.3, 300 mM KCl, 200 mM HCl) and 3 µl 3 M NaOAc pH 5.3 and 1 µl Glycogen were added together with abs. EtOH for precipitating of the DNA by centrifugation at 16,000 x g, 4°C for 1 h. After two washing steps of the DNA pellet using -20°C 70% EtOH, the pellet was re-suspended in nuclease free water. Whole genome amplification was carried out according to manufacturer’s instructions using the Repli-g Single Cell Kit (QIAGEN, Venlo, Nederland). 16.8 µl purified DNA was digested with a mix of 1 µl restriction endonuclease MseI (U/ml) and 0.2 µl BSA in 2 µl 10 x NE Buffer and incubated at 37°C for 2 h and at 65°C for 20 min. DNA was analyzed further with ddPCR as described in the manuscript.

Supplementary Table S1. Quantification of EpCAM+ tumor cells, CD45+ immune cells, and CD45+/EpCAM+ double positive (DP) cells in ascites from IF stainings of embedded ascites cells of the HGSOC patient.

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*Quantification of proliferating tumor cells (or immune cells) relate to total EpCAM+ tumor cells (CD45+ immune cells) in ascites. **Quantification of DP cells relates to EpCAM+/CD45+ DP cells (not to total quantified cells).
Supplementary Figure S1. (A) IF staining of infiltrating immune cells shows embedded ascites cells containing aggregated EpCAM+ tumor cells and CD45+ immune cell, which are infiltrated into the aggregates (indicated with white arrows on merged image). These immune cells were shown to be quantified as EpCAM+/CD45+ double positive cells in the control patient groups with the CellProfiler. Images are visualized with laser scanning microscopy at 200 x magnifications. Double positive cells are marked on merged image by white arrows. (B) Microbead staining shows CD45 positive immune cells (white arrows) covered with small beads and EpCAM positive tumor cells circled with larger beads (black arrows). Double positive cells bound to both small and large beads are tagged with blue arrows (scale bar 30 µm). (C-F) IF staining of CD45+/EpCAM+ double positive (DP) cells. For staining of ascites cell populations CK (C), CD44 (D), CD16 (E), p53 (F), Ki67 (F), CD45 (C-F), and EpCAM (C-F) markers are used and imaged on laser scanning microscopy at 200x magnifications. The cell nuclei were counterstained with DAPI. Double positive cells are indicated by white arrows on merged image.
