

Introduction

Solid tumors are infiltrated by cells of non-tumor origin, including heterogeneous lymphocyte subpopulations, fibroblasts, and endothelial cells¹. The amount and composition of infiltrating cells is highly variable and patient dependent, which makes analyses of primary tumor samples difficult. We have developed a fast and easy method to isolate untouched human tumor cells from primary tissue. This procedure is based on the comprehensive depletion of cells of non-tumor origin by combining automated tissue dissociation and magnetic cell sorting (MACS® Technology). Here, we have applied the method

to isolate human tumor cells from primary and metastatic ovarian carcinoma, as well as thymoma specimens. The purified human ovarian carcinoma tumor cell fraction was further used for the isolation of CD133⁺ cancer stem cells (CSCs). We performed Whole Exome Sequencing (WES) and gene expression profiling to i) compare genomic characteristics of isolated tumor cells and unpurified samples, ii) identify tumor cell- and CSC-specific expression signatures, and iii) compare the latter expression data with that of ovarian cancer cells (GSE29450)², which had been collected by laser capture microdissection (LCM).

Results

1 Rapid isolation of human tumor cells

We have determined an antibody combination recognizing cells of the tumor microenvironment. Conjugates of these antibodies with superparamagnetic nanoparticles (MicroBeads) were used to develop an optimized protocol for the depletion of such non-tumor cells from dissociated primary tumors by magnetic separation (fig. 1A). It was possible to eliminate >95% of the contaminating non-tumor cells in less than 20 min, regardless of the tumor type. Cell fractions were labeled with human lineage markers, i.e., CD31 (shown as example), CD45, Gly-A, and anti-Fibroblast as well as an antibody against human CD326 (EpCAM) (fig. 1B). Subsequently, tumor subpopulations were isolated by MACS Technology, as shown for the isolation of CD133⁺ CSCs (fig. 1C).

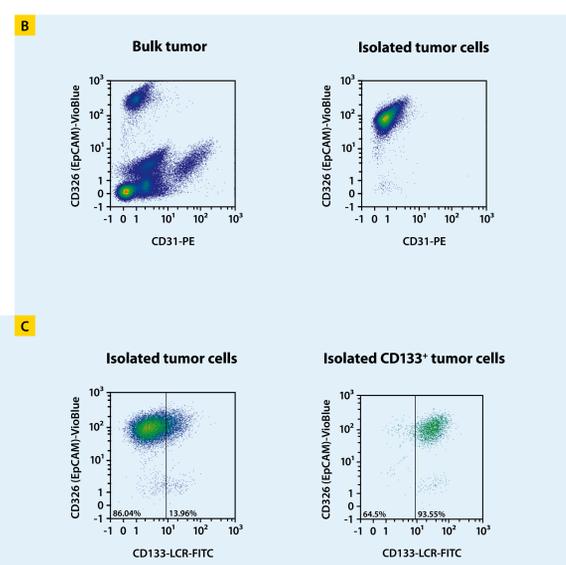


Figure 1

2 Depletion of non-tumor cells improves downstream culture of target cells

The culture of human tumor cells from primary specimens is frequently hampered by the presence of fibroblasts, red blood cells, and debris. While debris and red blood cells impede efficient plating of tumor cells, fibroblasts attach and expand more efficiently, thereby overgrowing the target cells. Upon magnetic separation, the original

and isolated tumor cell fractions were cultured for seven days, fixed, and stained for a fibroblast marker (Vimentin) and the human-specific tumor marker CD326 (EpCAM) (fig. 2). Even after seven days of culture, a nearly pure population of human tumor cells was observed in the isolated fraction.



Figure 2

3 Accurate detection of loss of heterozygosity events in isolated tumor cells and enhanced detectability of somatic mutations

DNA from bulk tumor and isolated tumor cells was analyzed by Whole Exome Sequencing (75 bp paired-end reads) on a MiSeq® instrument (Illumina®). After adapter clipping (trimmomatic) and read mapping (bwa), SNP calling restricted to the targeted regions was conducted using VarScan. Finally, snpEff/snpSift was applied for SNP effect prediction and SNP annotation. A SNP was defined as a difference between the sequenced sample and the reference genome (hg19). An important task in the context of tumor analysis is the detection of LOH (loss of heterozygosity). For that purpose, the numbers of SNPs with variant frequencies ≥ 0.95 or ≤ 0.05 were determined. In three out of four tumor specimens (OvCa_1, OvCa_3, OvCa_3_Met), a much higher number of SNPs was detected in the isolated tumor cells compared to bulk tumor (fig. 3A) indicating an improved detectability

of LOH after tumor cell isolation. Figure 3B exemplifies this finding in tumor sample OvCa_3 for a deleterious SNP (ENST00000413465: c.112C > T p.Q38*, COSM236889, not in dbSNP) causing a stop gain within a subset of transcript variants of the TP53 gene. Some bulk tumor specimens contain very low amounts of tumor cells, e.g., 2% in the thymoma sample analyzed here. The analysis of those samples, in which the frequency of somatic mutations is often below the detection limit, particularly benefits from prior enrichment of tumor cells. Figure 3B illustrates a somatic mutation in the CTNNB1 gene (NM_001904.3:c.133T > C p.S45P, COSM5663, rs121913407, ClinVar: (likely) pathogenic, associated with hepatocellular carcinoma) that could only be detected in isolated tumor cells but not in bulk tumor.

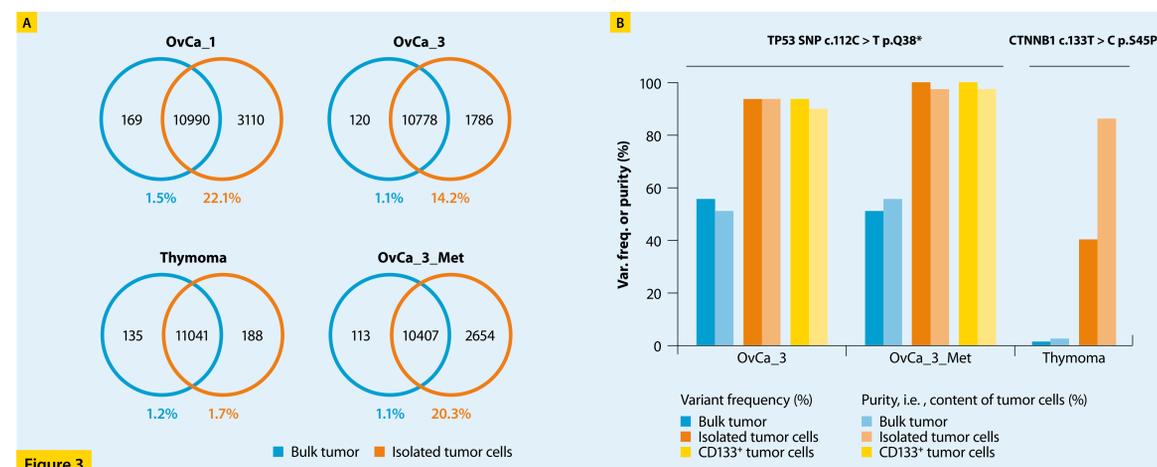


Figure 3

4 Risk of misinterpretation when tumor subpopulations are compared to bulk tumor instead of purified tumor cells

Typically, gene expression profiles are compared between isolated tumor cell subpopulations, such as CSCs, and heterogeneous samples of bulk tumor. Here, in contrast, whole genome microarray analyses of isolated tumor cells and subsequently sorted CD133⁺ CSCs were compared. The median log₂ intensity values (three tumors) of CD133⁺ cells and bulk tumor or isolated tumor cells were compared (fig. 4A). The color represents the fold change (log₂) between CD133⁺ CSCs and isolated tumor cells or bulk tumor, respectively. Many immune response-related genes showed high expression only in bulk tumor. Figure 4B shows enrichment scores for associations of the top 200 regulated genes with selected pathways. The expression signatures of tumor microenvironment (i.e. cell adhesion and immune responses) were depleted upon isolation of tumor cells, and the identification of tumor-specific pathways such as Wnt signaling was enhanced. Subpopulation-specific functions could

be identified based on the direct comparison of CD133⁺ cells and isolated tumor cells. The expression profiles of isolated tumor cells obtained by magnetic separation with our tumor cell isolation method showed striking similarities with data obtained from tumor cells isolated by LCM (GSE29450)². The heatmaps (fig. 4C) show the overlap of differentially expressed genes (fold change >2 or <-2; p < 0.05; displayed as median log₂ intensities, centered per gene for each dataset) between the LCM samples (ovarian epithelial cells from healthy donors and clear cell ovarian carcinoma,) and our tumor cell isolation data set. The majority of genes showed similar expression trends, suggesting that bulk tumor contained many cells with similarities to normal healthy ovarian epithelium. The tumor cell isolation method using magnetic beads therefore provides a fast and comparable alternative to other methods for the isolation of tumor cells.

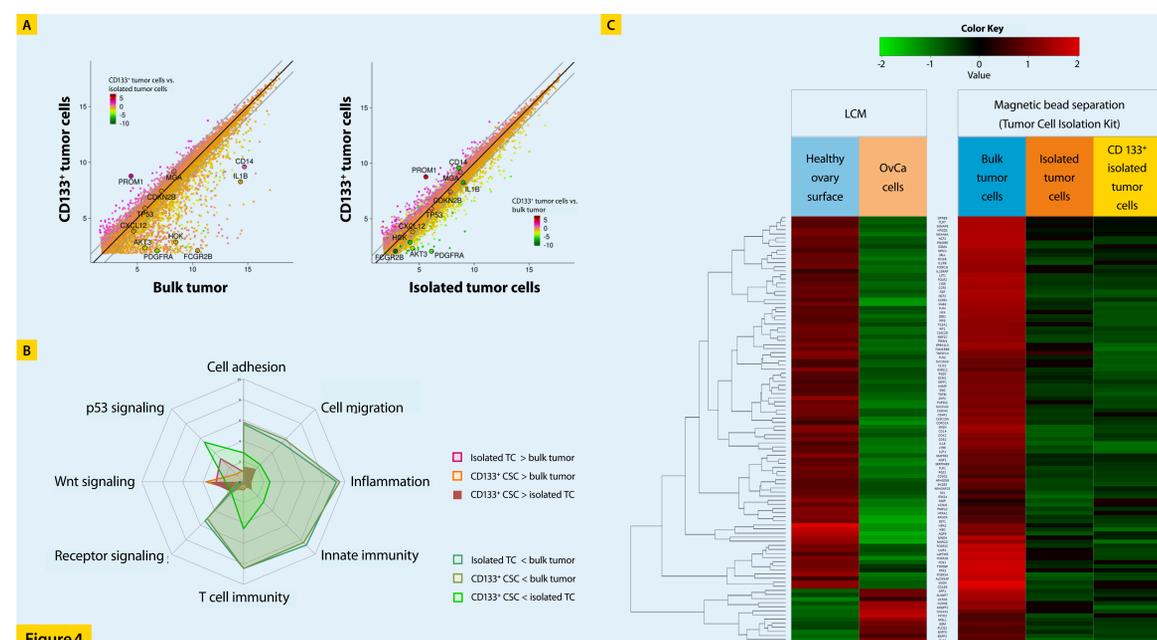


Figure 4

Conclusion

- Removal of non-tumor cells from bulk tumor tissue improves:
 - tumor cell culture,
 - analysis by NGS,
 - gene expression microarray analysis.
- Isolation of tumor cells lowers the complexity/heterogeneity of the sample.
- Characteristics of tumor cell subpopulations (i.e. CSCs) can be more reliably identified by direct comparison with purified tumor cells than in the presence of cells from tumor microenvironment.

References

- Hanahan, D. and Weinberg, R. A. (2011) Cell 144: 646–674.
- Stany, M.P. et al. (2011) PLoS One 6: e21121.