Isolation of primary human tumor cells significantly reduces bias in molecular analysis and improves culture of target cells

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Introduction

Solid tumors are infiltrated by cells of non-tumor origin, including heterogeneous lymphocyte subpopulations, fibroblasts, and endothelial cells[1]. The amount and composition of infiltrating cells is highly variable and patient dependent, which makes analyses of primary tumor samples difficult. The contaminating cells lead to hybridization of non-tumor cell–derived mRNA molecules to probes on microarrays, and a significant reduction of sensitivity caused by measurement of irrelevant signals during next-generation sequencing or proteome analysis can be expected. In addition, the culture of human tumor cells is frequently hampered by fibroblasts overgrowing the target cells, which bias assays such as drug sensitivity tests.
To overcome these limitations, 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). A negative selection strategy enables the isolation of the tumor cell population without specific knowledge of surface marker expression on these cells. Even from tissues that initially contained low numbers of tumor cells (<20%), the cells could be isolated to purities of higher than 95% in less than 20 minutes. 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). Whole genome expression profiling was carried out to define the specific signatures among those samples. Finally, we performed whole exome sequencing (WES) of bulk human ovarian carcinoma and thymoma samples and compared the results to isolated tumor cells of the respective samples.

Results

1 Rapid isolation of human tumor cells

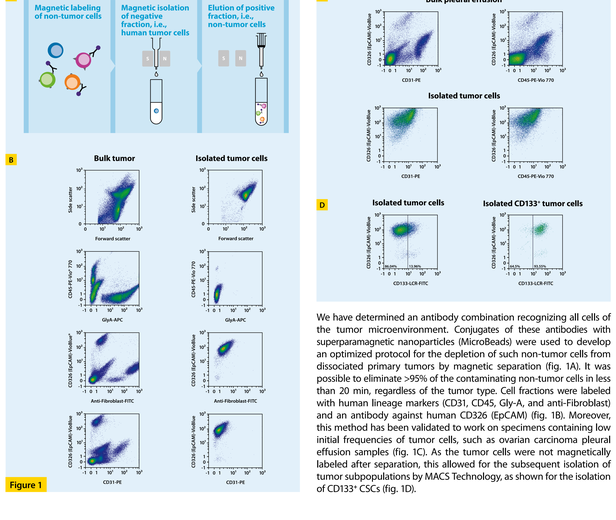


Figure 1

We have determined an antibody combination recognizing all 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 (CD31, CD45, Gly-A, and anti-Fibroblast) and an antibody against human CD326 (EpCAM) (fig. 1B). Moreover, this method has been validated to work on specimens containing low initial frequencies of tumor cells, such as ovarian carcinoma pleural effusion samples (fig. 1C). As the tumor cells were not magnetically labeled after separation, this allowed for the subsequent isolation of tumor subpopulations by MACS Technology, as shown for the isolation of CD133+ CSCs (fig. 1D).

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 impair efficient plating of tumor cells, fibroblasts attach and expand more efficiently, thereby overgrowing the target cells. Even when the target cells attach and grow well, *in vitro* cell culture assays (e.g. drug cytotoxicity testing) are problematic since mathematical correction for effects originating from contaminating cells is impossible in most cases. Upon magnetic separation, the original and negative fractions were cultured for seven days, fixed, and stained for a fibroblast marker (vimentin) and the human-specific tumor marker CD326 (EpCAM) (fig. 2A). Even after seven days of culture, a nearly pure population of human tumor cells was observed in the negative fraction. Moreover, cultivation of an ovarian cancer pleural effusion sample for three days with and without tumor cell isolation clearly indicated the benefit of removal of debris and non-tumor cells, reflected in homogeneous cultures (fig. 2B).

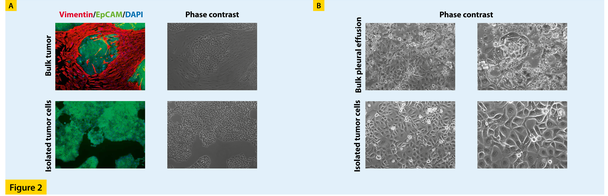


Figure 2

3 Accurate detection of loss of heterozygosity events in isolated tumor cells

DNA from bulk tumor or isolated tumor cells was used to produce exome-captured sequencing libraries applying the Nextera® Rapid Capture Exome Kit (Illumina®). For sequencing on a MiSeq® instrument (Illumina) the MiSeq Reagent Kit v3 (150 cycle, Illumina) was utilized to generate 75-bp paired-end reads. After adapter clipping (trimmomatic v0.33[2]), the reads were mapped against the human genome (bwa v0.7.12[3]). Duplicate reads were removed (MarkDuplicates, Picard Tools v1.134[4]), and SNP calling was conducted using VarScan v2.4.0[5] restricted to the regions targeted by the Nextera Rapid Capture Exome Kit. Finally, snpEff/snpSift v4.2[6] was applied for SNP effect prediction and SNP annotation. As no healthy control tissue was available for comparison, 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 number of SNPs with variant frequencies ≥0.95 or ≤0.05 were compared between bulk tumor and isolated tumor cells. In three out of four tumor specimens (OvCa_1, OvCa_3, OvCa_3_Met), a much higher number of SNPs fulfilling this selection criterion 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[7], not in dbSNP[8]) causing a stop gain within a subset of transcript variants of the TP53 gene. While a variant frequency in the bulk tumor of 57% suggested a heterozygous SNP, an LOH event was indicated in the isolated tumor cells (97%) and the CD133+ cells (96%).

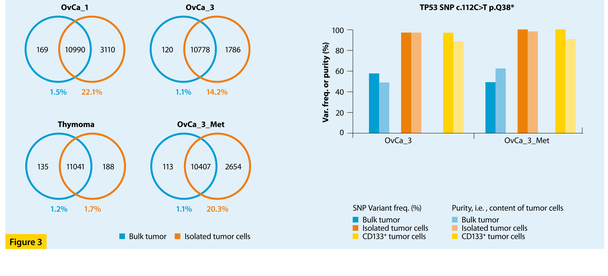


Figure 3

4 Enhanced detectability of somatic mutations

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. This is shown for two examples derived from the thymoma samples in figure 4. Figure 4A illustrates a somatic mutation in the CTNNB1 gene (NM_001904.3:c.133T>C p.S45P, COSM5663, rs121913407, ClinVar[9]: (likely) pathogenic, associated with hepatocellular carcinoma) that could only be detected in isolated tumor cells but not in bulk tumor (right-hand part generated using the Integrative Genome Viewer (IGV[10]). Figure 4B depicts a SNP in the CHECK2 gene (NM_007194.3:c.349A>G p.R117G, rs28909982) that is associated with hereditary cancer-predisposing syndrome and familial breast cancer according to ClinVar. Here, with the help of tumor cell isolation, it could be demonstrated that the donor was initially heterozygous for this mutation (58% var. freq. in bulk tumor at a purity of 2%) but switched to homozygosity in the tumor cells (91% var. freq. at a purity of 86%).

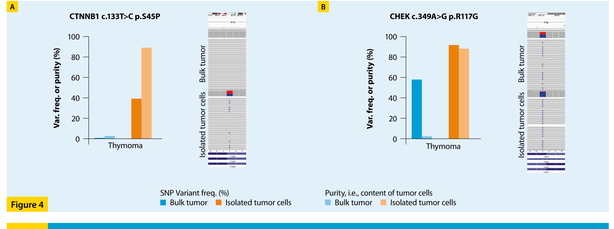


Figure 4

5 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+ tumor cells were compared and the cells' expression differences related to bulk tumor. Differential expression was determined by filtering quantile-normalized log2 data by Anova and Tukey post-hoc tests with p ≤ 0.05, ±2-fold change, and detectability in at least two out of three samples with higher expression in the group comparisons.
Volcano plots (-log10 P-value versus log2 ratio) of all detectable genes (fig. 5A) demonstrate predominantly lower expression in both CD133+ and isolated tumor cells compared to bulk tumor. Only few and less significant expression differences were observed between CD133+ tumor cells and isolated tumor cells. This is further confirmed in a heat map (fig. 5B) of differentially expressed genes by coclustering of CD133+ and isolated tumor cell samples separated from bulk tumor samples. Shown are the median centered log2 expression values of candidate genes after hierarchical clustering (Euclidean distance, complete linkage) of genes and samples.
The scatter plots (fig. 5C) compare the median log2 intensity values of CD133+ cells and bulk tumor or isolated tumor cells, and are colored by the fold change (log2) between the CD133+ and isolated tumor cells or bulk tumor, respectively. Many immune response–related genes showed high expression only in bulk tumor. These genes had similar expression in CD133+ and isolated tumor cells. Thus, purification of tumor cells facilitates the identification of characteristic gene signatures of CD133+ tumor subpopulations independently of signatures typical for the tumor microenvironment.

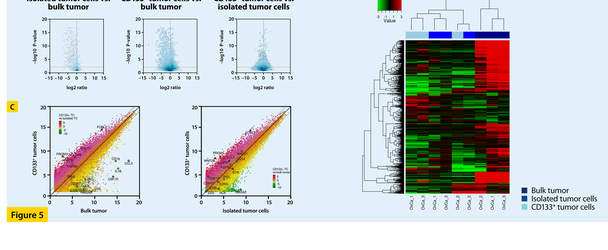


Figure 5

6 Isolation of tumor cells lowers the complexity/heterogeneity of the sample

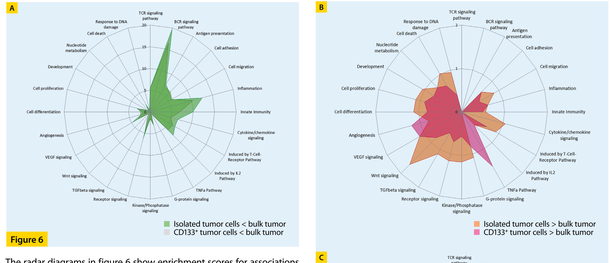


Figure 6 (cont.)

The radar diagrams in figure 6 show enrichment scores for associations of the top 200 regulated genes with selected pathways. The isolation of tumor cells significantly depleted gene expression signatures of the tumor micro-environment (fig. 6A). Genes that were down-regulated in isolated and CD133+ tumor cells compared to bulk tumor were strongly enriched for pathways and functions associated with immune responses. Figure 6B shows associations of the top 200 genes that were up-regulated in isolated and CD133+ tumor cells compared to bulk tumor. Characteristic tumor-specific functions or pathways, such as Wnt signaling and angiogenesis, were enriched. Isolation of tumor cells lowered complexity/heterogeneity of the sample (fig. 6C). Comparison of isolated tumor cells and CD133+ cells based on the top 200 differentially regulated genes allowed a clearer identification of subpopulation-specific functions than the comparison to bulk tumor.

Conclusion

- We have developed an easy and fast (<20 min) cell separation method, which allows for accurate downstream analysis of human tumor specimens, avoiding bias caused by contaminating cells.
- The contaminating non-tumor cells are specifically labeled prior to their depletion from the dissociated tissue. Labeling of the target cells is not required. Therefore, the procedure can be used for the isolation of tumor cells from all kinds of solid human cancer entities without the need for a positive marker expressed on the target cells.
- Removal of non-tumor cells from bulk tumor tissue significantly improves the analysis of human tumor material by next-generation sequencing and gene expression analysis.
- Based on our data, subpopulations isolated from tumor cells, such as CSCs, appeared to be very similar to isolated tumor cells.
- The tumor-specific gene expression signature is masked by genes expressed in the tumor microenvironment.
- Characteristics of tumor cell subpopulations can be more reliably identified by the direct comparison of purified tumor cells and CSCs in the absence of cells from the tumor microenvironment.

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