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Untouched tumor cell isolation allows for subsequent enrichment of rare cancer stem cells and improves NGS analysis

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Background

Solid tumors are vascularized and infiltrated by stromal cells. The amount and composition of these infiltrating non-tumor cells varies, for example, depending on tumor entity and stage or treatment history and may complicate sample analysis. Contaminating cells, for instance, lead to a significant reduction of sensitivity in next-generation sequencing (NGS) due to the measurement of irrelevant signals. To overcome these limitations, we combined semi-automated tissue dissociation and automated immunomagnetic cell isolation. A negative selection strategy enables the isolation of heterogeneous tumor cells independent of specific surface markers by depletion of non-tumor cells. Here, we have applied the method to isolate human tumor cells from primary and metastatic ovarian carcinoma specimens, as well as from a thymoma sample. The purified tumor cell fraction of ovarian carcinoma was further used for the positive isolation of CD133⁺ cancer stem cells. Bulk tumor and isolated tumor cells were subjected to whole exome sequencing (WES) to assess the impact of non-tumor cell depletion on the sensitivity of downstream NGS analyses. This application note is based on reference 1.

Materials and methods

Tumor Dissociation

Specimens of human primary and metastatic ovarian carcinomas and a thymoma sample were dissociated into single-cell suspensions using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, human according to the datasheet.

Isolation of untouched heterogeneous tumor cells followed by CD133⁺ enrichment of cancer stem cells

Non-tumor cells were magnetically depleted from dissociated tumor samples with the Tumor Cell Isolation Kit, human on the automated autoMACS® Pro Separator. Untouched isolated ovarian carcinoma cells were further processed to enrich CD133⁺ cancer stem cells with the CD133 MicroBead Kit – Tumor Tissue, human.

Flow cytometry

One aliquot of each sample was stained with CD326 (EpCAM) Antibody, anti-human, VioBlue®, REAfinity™ and CD45 Antibody, anti-human, PE-Vio® 770, REAfinity before and after isolation of tumor cells and analyzed using the MACSQuant® Analyzer 16. For the detection of CD133⁺ cancer stem cells, isolated CD133⁺ cells were stained using Labeling Check Reagent, FITC.

Whole exome sequencing

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. Read mapping and variant calling were conducted following standard bioinformatics procedures.

Results

Untouched isolation results in pure heterogeneous tumor cells and enables subsequent magnetic enrichment of rare CD133⁺ cancer stem cells

Using the Tumor Cell Isolation Kit, human, contaminating non-tumor cells were eliminated from cell suspensions by automated cell separation in less than 20 min. The results show successful tumor cell isolation regardless of the tumor entity, even from low initial tumor cell frequencies, such as thymoma (tumor cells <2%; fig. 1). As the tumor cells are not magnetically labeled after separation, this approach allows for the subsequent immunomagnetic isolation of subpopulations (e.g. isolation of human CD133⁺ cancer stem cells), which would otherwise be lost in the background.

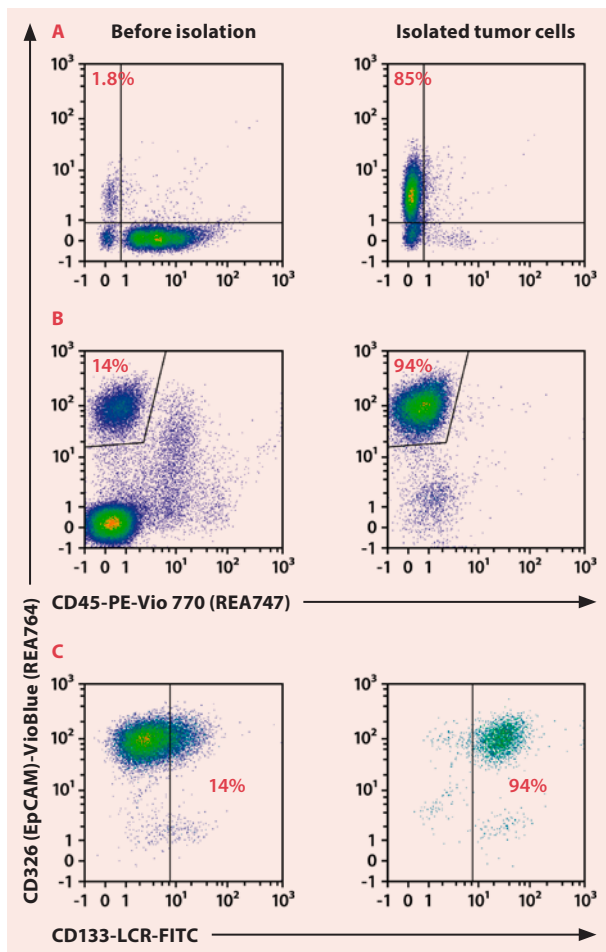


Figure 1: Exemplary untouched isolation of low frequent tumor cells and subsequent enrichment of CD133⁺ cancer stem cells. Non-tumor cells were magnetically depleted from dissociated human thymoma (A) and ovarian carcinoma (B) specimens with the Tumor Cell Isolation Kit, human on the autoMACS Pro Separator. One aliquot of each sample was stained before and after tumor cell isolation to verify the depletion efficiencies on the MACSQuant Analyzer 16. Isolated ovarian carcinoma cells were further processed to enrich CD133⁺ cancer stem cells with the CD133 MicroBead Kit – Tumor Tissue, human and stained with Labeling Check Reagent (LCR) for flow cytometry analysis (C).

Isolated tumor cells allow for sensitive and robust detection of somatic mutations via NGS

An important aspect in the context of tumor analysis is the detection of loss of heterozygosity (LOH). To examine the influence of prior tumor cell isolation on LOH detectability, the number of single-nucleotide polymorphisms (SNPs) with variant frequencies ≥ 0.95 or ≤ 0.05 were compared between bulk and isolated tumor cells. In the two ovarian carcinoma specimens, a much higher number of SNPs fulfilling these criteria was detected in the isolated tumor cells compared to bulk tumor indicating an improved detectability of LOH after tumor cell isolation (fig. 2). Frequencies of LOH events detected in the bulk versus isolated thymoma sample varied less, which can be explained by the low mutational burden in these samples.

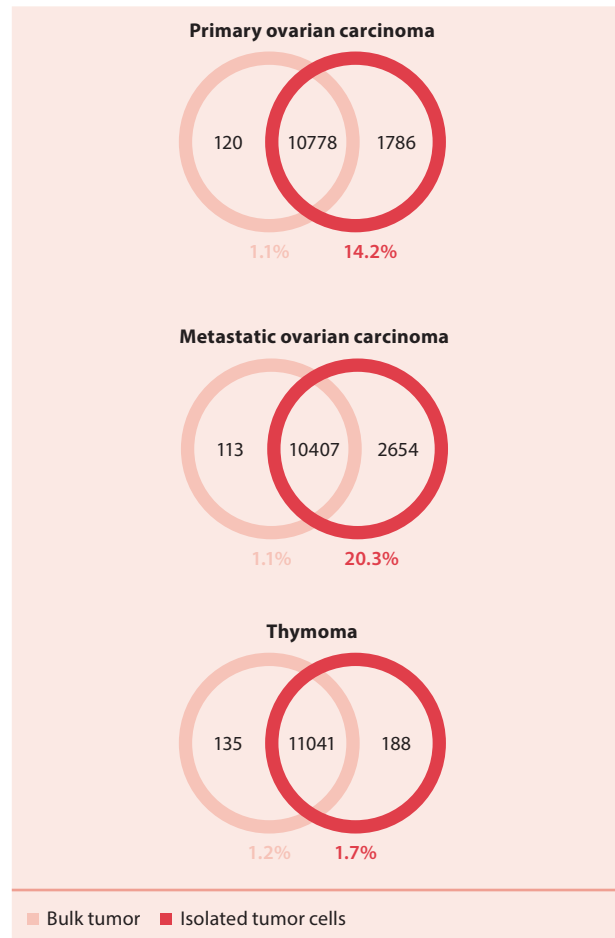


Figure 2: Successful detection of somatic mutations via NGS after tumor cell isolation. DNA from bulk tumor or isolated tumor cells was subjected to WES. The number of SNPs with variant frequencies ≥ 0.95 or ≤ 0.05 were compared between bulk tumor and isolated tumor cells.

Figure 3 exemplifies how tumor cell isolation enables an accurate detection of SNP zygosity. The variants TP53 c.112C>T p.Q38* for ovarian carcinoma as well as CHECK2 c.349A>G p.R117G for thymoma are depicted. While the frequencies of both variants in the bulk tumor suggest heterozygous conditions, LOH events could be detected in the isolated tumor cell fraction. Furthermore, the somatic CTNNB1 mutation c.133T>C p.S45P in the thymoma sample, which is (likely) pathogenic and associated with hepatocellular carcinoma according to ClinVar2, could only be detected in isolated tumor cells.

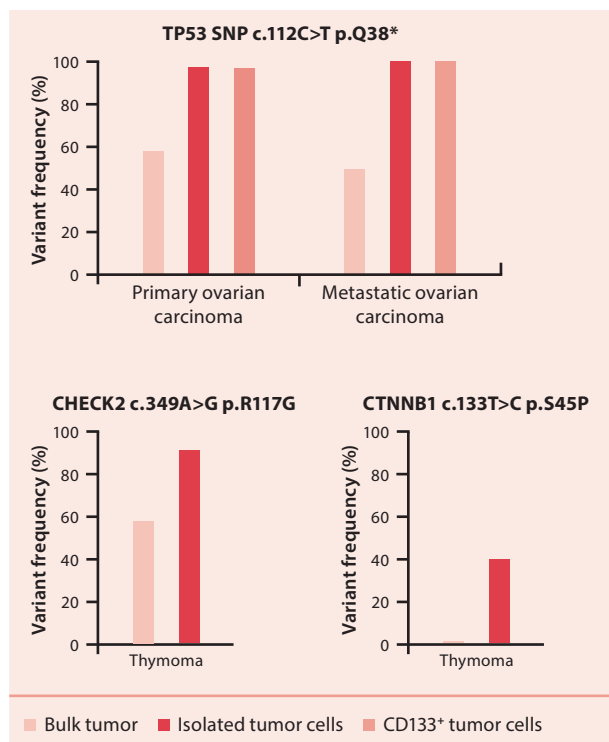


Figure 3: Successful detection of SNP zygosity after tumor cell isolation. SNP detection and evaluation of zygosity is shown for TP53 c.112C>T p.Q38* (in primary and metastatic ovarian carcinoma samples) and for CHECK2 c.349A>G p.R117G and CTNNB1 c.133T>C p.S45P (in a thymoma sample).

Conclusions

- The Tumor Cell Isolation Kit, human allows untouched isolation of a wide population of tumor cells from dissociated specimens without the need for a highly expressed cancer marker.
- Cell isolation can be easily automated and performed in <20 min with the autoMACS Pro Separator and allows for subsequent magnetic isolation of rare tumor cell subpopulations (e.g. cancer stem cells).
- Cell isolation enables accurate downstream NGS analysis of tumor cells avoiding bias caused by contaminating cells from the tumor microenvironment.
- Some bulk tumor specimens contain very low amounts of tumor cells. Molecular analysis of those samples, in which the frequency of somatic mutations is often below the detection limit, particularly benefits from prior tumor cell enrichment.

References

1. Willnow, L. *et al.* Isolation of primary human tumor cells significantly reduces bias in molecular analysis and improves culture of target cells. [abstract]. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16–20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016; 76 (14 Suppl): Abstract nr 174.
2. Landrum, M. J. *et al.* (2016) *Nucleic Acids Res.* 44: D862–868.

Product	Order no.
Tumor dissociation	
gentleMACS Octo Dissociator with Heaters	130-096-427
gentleMACS C Tubes	130-093-237
Tumor Dissociation Kit, human	130-095-929
Tumor cell isolation	
Tumor Cell Isolation Kit, human	130-108-339
CD133 MicroBead Kit - Tumor Tissue, human	130-100-857
autoMACS Pro Separator	130-092-545
Flow cytometry analysis	
MACSQuant Analyzer 16	130-109-803
CD326 (EpcAM) Antibody, anti-human, VioBlue, REAfinity	130-111-121
CD45 Antibody, anti-human, PE-Vio 770, REAfinity	130-110-772
Labeling Check Reagent, FITC	130-124-701



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