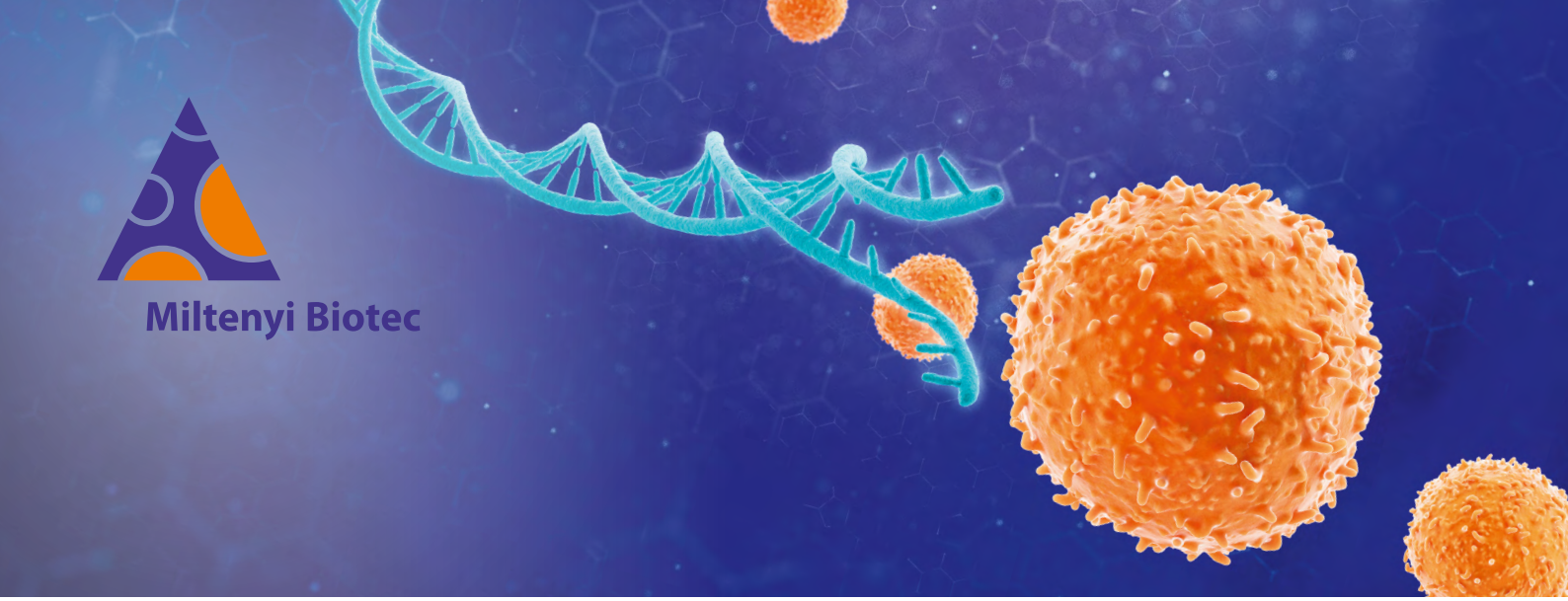


Tumor cell isolation allows accurate mutation analysis of FFPE gastric cancers with low and diffuse tumor content

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Background

Targeted sequencing using next generation sequencing (NGS) offers new prospects for drug selection for cancer patients based on alterations in their genome.[1–3] The accuracy in the mutation analysis using NGS relies on the tumor content of the tissue samples, which are commonly formalin-fixed paraffin-embedded (FFPE) tissues. FFPE tissue samples with low tumor cell content are typically subjected to tumor cell enrichment using macrodissection. This technique consists of isolating the tumor-specific areas within the sample. However, this is often unsuitable for samples containing diffused-tumor cells, such as diffuse-type gastric cancer or lobular breast cancer. In those cases alternative methods for tumor cell isolation are necessary to enable the accurate detection of mutations in the sequencing of target gene panels.

This application note summarizes some of the findings observed in the study performed by Hatakeyama *et al.*[4] to investigate the effect of tumor cell isolation from FFPE tissue sections of diffuse-type and intestinal gastric cancers on the detection of mutations by targeted sequencing.

Materials and methods

FFPE tissue dissociation

FFPE tissue samples collected between 2014 and 2019 from diffuse-type and intestinal gastric cancer patients were used in the study.

FFPE tissue blocks were cut into 10, 20, and 50 µm thick sections, and for each thickness the number of sections cut was adjusted to process a total amount of 100 µm. The sections were dewaxed and rehydrated prior to heat-induced antigen retrieval. Afterwards, the samples were dissociated using the FFPE Tissue Dissociation Kit in combination with a gentleMACS™ Octo Dissociator with Heaters. All steps were performed according to the protocol provided in the data sheet of the FFPE Tissue Dissociation Kit.

Tumor cell isolation and flow cytometric analysis

Fully automated isolation of tumor cells was performed using Anti-Cytokeratin MicroBeads, human and an autoMACS® Pro Separator according to the protocol included in the data sheet of the Anti-Cytokeratin MicroBeads, human. Unseparated FFPE cell suspensions, separated tumor cell fractions, and the residual fractions after separation were stained using Cytokeratin Antibody, anti-human, FITC REAfinity™ (clone REA831); Vimentin Antibody, anti-human, APC REAfinity (clone REA409); and CD235a (Glycophorin A) Antibody, anti-human, PE REAfinity (clone REA175) and were analyzed using a MACSQuant® Analyzer 10. The nuclei were stained with DAPI Staining Solution.

DNA isolation

DNA was extracted from FFPE tissue using a GeneRead™ DNA FFPE Kit (Qiagen). Purified DNA was quantified using a NanoDrop™ and Qubit™ 2.0 Fluorometer (ThermoFisher Scientific). To check the quality of DNA, the DNA integrity number (DIN) was determined using TapeStation (Agilent Technologies).

Targeted sequencing of the gene panel

For targeted sequencing of genes in the DNA isolated from the FFPE tissue, a library of 225 genes was constructed using a hybridization-based enrichment protocol (SureSelect Custom panel, Agilent). Details on the panel of genes, library construction, and data analysis are described in Hatakeyama *et al.*[4]

Results

Efficient tumor cell isolation from FFPE tissue sections of diffuse and intestinal gastric cancers of varying thickness

FFPE tissue sections of 10, 20, and 50 µm in thickness were obtained from two diffuse-type (D1 and D2) and two intestinal (S1 and S2) gastric cancers. The tumor cellularity estimated by a pathologist was of 20% tumor content for both diffuse-type samples, and 60% and 50% in the intestinal-type samples. After dissociation of the FFPE sections, tumor cells were efficiently isolated using automated magnetic cell separation from both gastric cancer types based on the expression of cytokeratin. The population considered to be of tumor cells (cytokeratin-positive, vimentin-negative) were increased in the tumor fractions compared to the unseparated samples, whereas in the residual fraction, these populations were decreased (fig. 1). No differences in tumor cell isolation due to the thickness of the FFPE tissue sections were observed.

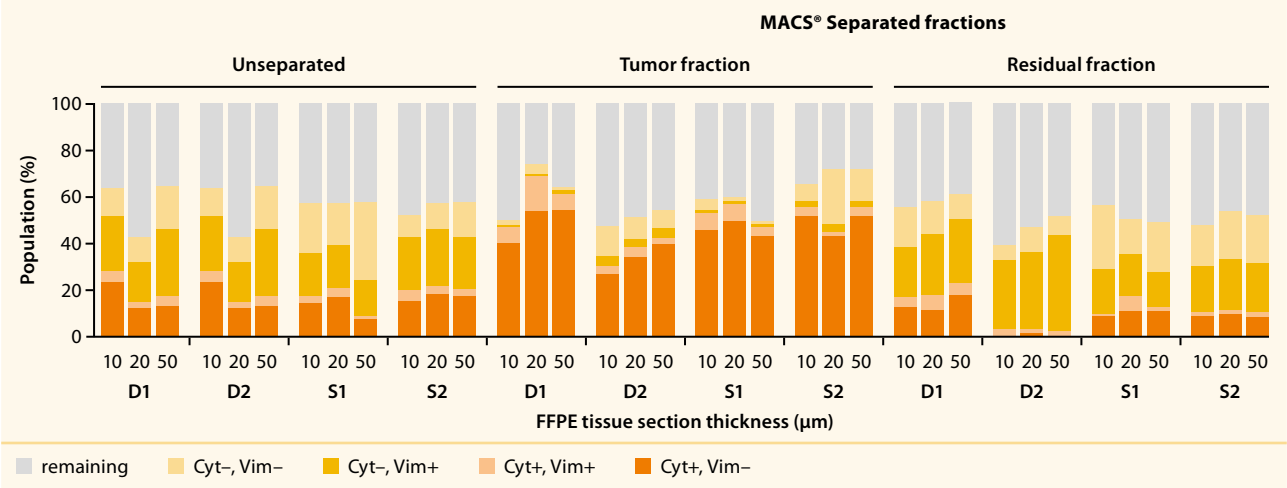


Figure 1: Analysis of the tumor cell content before and after tumor cell isolation from FFPE diffuse and intestinal gastric cancers. FFPE tissue sections of varying thickness (10, 20, and 50 µm) were dissociated into cell suspensions using the FFPE Tissue Dissociation Kit and the gentleMACS Octo Dissociator with Heaters. Next, tumor cells were magnetically isolated from the cell suspensions using Anti-Cytokeratin MicroBeads, human and an autoMACS Pro Separator. The amount of tumor cells in the unseparated and separated fractions was determined by flow cytometry based on the expression of cytokeratin (Cyt) and vimentin (Vim) from nucleated cells (DAPI-positive). Remaining events represent DAPI-negative and CD235-expressing cells (erythrocytes).

Tumor cell isolation from 10 µm thick FFPE tissue sections from diffuse and intestinal gastric cancers leads to sufficient cell amounts and high quality DNA for NGS analysis

The amount and quality of the DNA extracted from the unseparated and separated fractions were suitable for NGS (figs. 2A, 2B), and the read depth of the unseparated and separated fractions was comparable (fig. 2C).

These results were independent of the thickness of the FFPE tissue sections used, indicating that the use of 10 µm thick FFPE sections was suitable to be used as starting material for targeted sequencing. The subsequent NGS-mutation analysis was carried out with the extracted DNA of the cell suspensions obtained from the 10 µm thick FFPE sections.

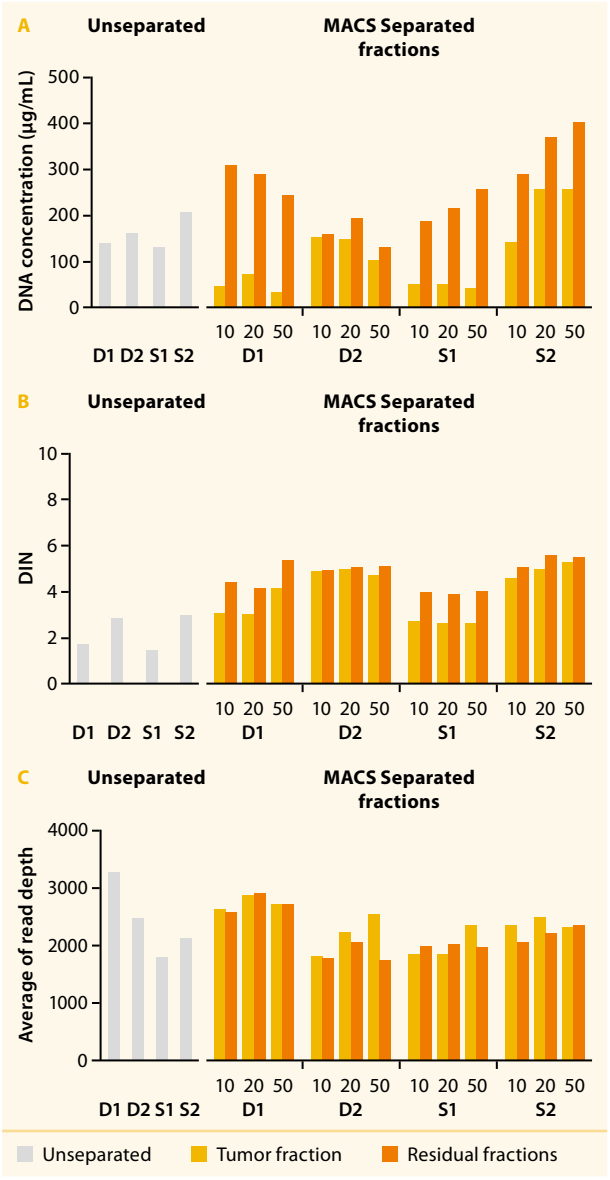


Figure 2: Evaluation of the quality of the DNA extracted from the unseparated and separated fractions after dissociation of FFPE tissue sections of 10, 20, or 50 µm thickness. DNA concentration (A), DNA integrity number (DIN) (B), and read depth (C), were determined from the extracted DNA from the different cell suspensions. The unseparated cell suspensions were obtained from 10 µm thick FFPE tissue sections only. The separated fractions correspond to the tumor fractions isolated using the Anti-Cytokeratin MicroBeads, human together with an autoMACS Pro Separator, and the residual fractions consist of the flow through of cells not captured by the MicroBeads.

Tumor cell isolation helps to identify somatic mutations with low variant allele frequency in diffuse and intestinal gastric cancers

The number of mutations detected in the tumor fractions was greater than in the unseparated fractions, except for one diffuse-type sample that had a similar number of mutations. There were fewer mutations detected in the residual fractions (fig. 3A). Moreover, 19% of the mutations detected in the tumor fractions were tumor fraction-specific and could not be detected in the unseparated or residual fractions (fig. 3B). The tumor fraction-specific mutations had a significantly lower variant allele frequency (VAF) compared to the rest of the mutations detected (fig. 3C). Interestingly, these mutations were increased in the tumor fractions from the diffuse gastric cancer types[4]. These findings show that the isolation of tumor cells from 10 µm FFPE tissue sections by the described method helped in the identification of somatic mutations that otherwise would remain undetected. Furthermore, it enabled a more accurate mutation analysis in diffuse-gastric cancer samples with low and diffuse-tumor content.

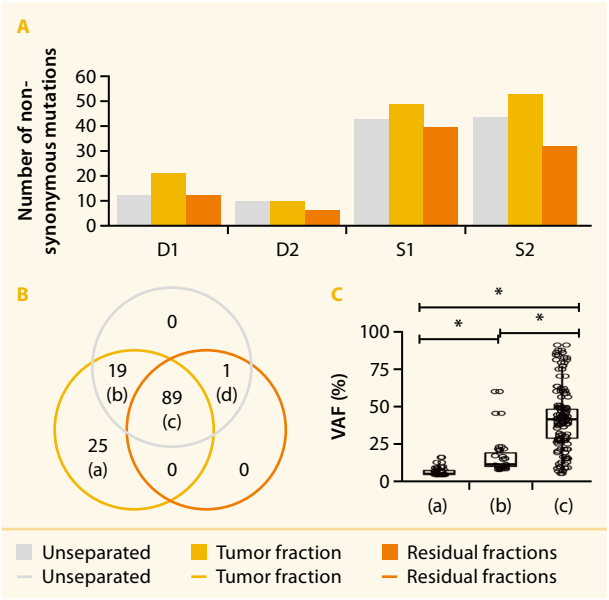


Figure 3: Influence of tumor cell isolation on mutation analysis of gastric cancers. Comparison of non-synonymous mutations between the unseparated and separated samples (A). Venn diagram of nonsynonymous mutations detected in each separated fraction and in the unseparated samples (B). Variant allele frequency (VAF) of the tumor fraction-specific mutations (a), the mutations detected in both the tumor and unseparated fraction (b), and detected in all fractions (c) (C).

*p<0.003 (Welch's t-test with Bonferroni correction).

Conclusions

- Tumor cells from gastric cancers with low and diffuse-tumor content can be efficiently isolated using automated magnetic cell isolation after dissociation of FFPE tissue sections of 10, 20, and 50 µm in thickness.
- The dissociation and tumor cell isolation from 10 µm FFPE tissue sections of both gastric cancers allows the isolation of sufficient amounts of high quality DNA for downstream NGS-based targeted sequencing.
- Tumor cell isolation helps to increase the success rate of target panel sequencing of gastric cancers, particularly those with diffuse-tumor cell content.

References

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Product	Order no.
Tumor dissociation	
FFPE Tissue Dissociation Kit	130-118-052
gentleMACS Octo Dissociator with Heaters	130-096-427
gentleMACS C Tubes	130-093-237
Pre-Separation Filters (70 µm)	130-095-823
Tumor cell isolation and analysis	
Anti-Cytokeratin MicroBeads, human	130-123-094
autoMACS Pro Separator Starter Kit	130-092-545
Flow cytometric analysis	
Cytokeratin Antibody, anti-human, FITC REAfinity (clone REA831)	130-112-931
Vimentin Antibody, anti-human, APC REAfinity (clone REA409)	130-118-497
CD235a (Glycophorin A) Antibody, anti-human, PE REAfinity (clone REA175)	130-120-613
DAPI Staining Solution	130-111-570
MACSQuant Analyzer 10	130-096-343

Additional information

The figures shown in this application note correspond to modified figures published in the article cited in reference 4. The modification and use of the modified figures in this application note have been approved by the author of the mentioned article, Keiichi Hatakeyama, and is permitted by a Creative Commons Attribution 4.0 International License *https://creativecommons.org/licenses/by/4.0/.*

PROJECT HOPE

Project HOPE (High-tech Omics-based Patient Evaluation)
The methods described in this application note are part of the results of Project HOPE. In January, 2014, the Shizuoka Cancer Center launched the Project HOPE, a clinical study aiming at the ideal cancer medicine. The features of this project are: No restrictions for cancer types, integrated method of analysis called multiomics is employed for genetic analyses, and it's a research within one single site, Shizuoka Cancer Center, which is equipped with a research institute in addition to its function as a hospital.
For more information about the project, please access the following URL or QR code.

▸ **https://www.scchr.jp/en/institute/projecthope.html**

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