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Three-dimensional imaging of intramural perineural invasion in colorectal cancer: three-dimensional reconstruction approach with multiple immunohistochemically stained sections

Running Title: Intramural perineural invasion in CRC

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Abbreviations & acronyms

List of abbreviations

PNI: perineural invasion, CRC: colorectal cancer, 2D: two-dimensional, 3D: three-dimensional, IHC: immunohistochemistry, AE1/AE3: cytokeratin AE1/AE3, S-100: anti-S-100 antibody

Conflict of Interest: The authors declare that there is no conflict of interest.

Abstract

Perineural invasion (PNI) at Auerbach's plexus in colorectal cancer (CRC), known as intramural PNI, is associated with adverse prognostic outcomes. This study aimed to characterize the three-dimensional (3D) architecture of CRC with intramural PNI and to evaluate the morphological features of tumor invasion around nerve tissue. Serial tissue sections from two cases of CRC were stained with cytokeratin AE1/AE3 and an anti-S-100 protein antibody. 3D models were reconstructed by scanning the virtual slides. In one case, intramural PNI was observed at the horizontal invasive front. The 3D reconstruction model showed tumor cells that appeared to infiltrate along the nervous meshwork, the structure of which was preserved. In the other case, intramural PNI was observed both at and behind the horizontal invasive front, and the 3D reconstruction model showed that the tumor cells appeared to be involved with nerve cells at the focal part of the horizontal invasive front. The nervous meshwork structure, however, was not well identified in cancer-involved areas. This is the first study to characterize the 3D structure of tumor invasion around nerve tissue in CRC, demonstrating the morphological features of intramural PNI in CRC.

Keywords: 3D reconstruction, intramural perineural invasion, colorectal cancer

Introduction

Perineural invasion (PNI) is a pathologic process characterized by tumor invasion of nervous structures and spread along nerve sheaths.[1] PNI has been recognized as an important pathologic feature of various malignant tumors and is associated with poor prognostic outcomes.[2] In the literature, PNI is observed in 10% to 35% of cases of colorectal cancer (CRC).[3, 4] The Japanese Society for Cancer of the Colon and Rectum has proposed a PNI grading system based on the location within the bowel and classifications of extramural and intramural PNI,[4] which are associated with adverse prognostic outcomes.[5] The pattern of horizontal cancer spread along the Auerbach's plexus area is defined as myenteric spread regardless of the presence of intramural PNI.[6] In regions of myenteric spread, cancer seems to develop by replacing nerve tissue, with a complete disappearance of nerve structures behind the invasive front. The typical finding of PNI, where cancer cells directly involve the nerve plexus, is rarely observed except at the invasive frontal region.[6, 7] Structural features of intramural PNI cannot be well evaluated with only two-dimensional (2D) examinations using traditional pathological techniques.

Techniques of three-dimensional (3D) reconstruction of tissue at microscopic resolution have been developed to investigate the microarchitecture of tumors.[8-10] Serial sectioning and scanning of tissue sections combined with immunohistochemistry (IHC) have given some insight into the 3D tumor architecture, and we have demonstrated the feasibility of this technique.[11]

In this study, our objective was to characterize the 3D structure of CRC with intramural PNI and evaluate the morphological features of tumor invasion around nerve tissue.

Materials and Methods

Ethical considerations

This study was approved by the institutional review board of Kyushu University, Fukuoka, and National Defense Medical College, Saitama, Japan (permission number: 30-109, 2545, respectively).

Case selection

The tissue samples were obtained from two patients undergoing surgery at National Defense Medical College Hospital between 2014 and 2016. Myenteric spread was defined as horizontal cancer spread along Auerbach's plexus zone. PNI was defined as the histological finding of tumor cells located at and invading nerve fascicles. Myenteric spread and PNI were assessed on Hematoxylin and eosin (H&E) slides by a gastrointestinal pathologist (H.S). Two cases were selected according to the following criteria: colorectal adenocarcinoma, tumor size ≥5 cm, depth of wall invasion pT2 or more, intramural PNI observed at the invasive front, and no extramural PNI confirmed. These two cases were arbitrarily named Case A and Case B. The clinicopathological characteristics are presented in Table 1.

Tissue sample and 3D reconstruction

From each of these two cases, a 5-mm-thick tissue block was made from the horizontal edge of the tumor. PNI was confirmed by IHC for cytokeratin AE1/AE3 (AE1/AE3) and anti-S-100 antibody (S-100).[12, 13] The procedure for creating the 3D model was carried out as reported previously. [11] Alternate sections were stained with AE1/AE3 and S-100, and every eleventh section was stained with H&E. A total of 70 slides were made for each immunohistochemical stain. IHC was performed with the AE1/AE3 (clone MAB3412, Chemicon, 1:1000 dilution) and S-100 (Z0311, Dako, 1:2000 dilution) antibodies. All sections were captured with a slide scanner (Axio Scan Z1®), and the scanned virtual slides were loaded into Amira 6®. The color balance was adjusted to distinguish each stain. After

visualization of only the labelled region and conversion to each color tone, tumor cells stained for AE1/AE3 were labelled in orange, and nerve cells stained for S-100 were labelled in green.

Results

Macroscopic and microscopic features of Case A are shown in Figure 1. Panels C-H are high-power magnifications of the horizontal invasive front, as indicated by the *triangles* in Panel B, which indicate the existence of isolated PNI in the Auerbach's plexus area. The 3D reconstruction models are shown in Figure 2 and Supplementary Video 1. In this case, tumor cells appeared to infiltrate along the nervous meshwork, the structure of which was preserved. The closest point between the tumor and nerve cells was detected and the 3D reconstruction model is shown in the second half of Supplementary Video 1. A cancer cell nest was coming into contact with nerve cells and pushing the nervous meshwork structure, but the nervous meshwork structures were preserved.

In Case B, macroscopic and microscopic features are shown in Figure 3. Myenteric cancer spread and intramural PNI were observed both in and behind the horizontal invasive front (Panel C). The microscopic appearance of the horizontal invasive front is shown in Panels D-G. The 3D reconstruction models in this region are shown in Figure 4 and Supplementary Video 2. Tumor cells appeared to invade along the nerve cells in some parts, but nervous meshwork structures were not well identified in cancer-involved areas. We focused on one point in this area at a higher magnification, where PNI was prominent with 2D evaluation (Figure 3E-G). The 3D reconstruction model in this region is shown in the second half of Supplementary Video 2. Tumor cells were directly involved with the nerve structure, and nervous structures had been completely destroyed in the tumor-sided portion. In contrast, nervous meshwork structures were preserved in the intact side in this region.

Discussion

This is the first study to show the 3D structure of tumor invasion around Auerbach's plexus in CRC. There are no standardized criteria for PNI in cancer. Seefeld et al. regarded PNI as tumor involvement of the perineural and endoneural spaces.[14] Some authors defined PNI as the presence of cancer cell nests inside the perineurium.[15, 16] One possible mechanism for PNI is that tumor cells that spread along nerves preferentially develop within a low-resistance plane (path of low resistance).[2] Another possible mechanism is that PNI may involve mutual signaling interactions between tumor and nerve cells (neurotrophic factors).[17]

Meanwhile, several studies have assessed 3D imaging of colorectal tissue.[18, 19] However, the morphological features of CRC spreading at Auerbach's plexus have not been clarified.

Our 3D models demonstrated structures of tumor invasion around Auerbach's plexus. In Case A, tumor cells were observed to infiltrate along the nervous meshwork. Furthermore, the closest point between the tumor and the nerve cells was identified, showing that a cancer cell nest was directly pushing on the nerves at that point. It is conceivable that the tumor may have developed preferentially within the sparse connective tissue at Auerbach's plexus. In Case B, 3D models showed tumor cells involving the nerve cells at one point, which was thought to be the invasive front of intramural PNI. At this invasive front, tumor and nerve cells were overlapping, suggesting that cancer invasion may be mediated by adhesion or growth factors contained in neurotrophic factors. Collectively, a region where the tumor and the nerve cells were close to each other was detected in both cases. Patterns of PNI at Auerbach's plexus in these two cases may reflect a variety of the mechanisms. Case A possibly reflects the 'path of low resistance' mechanism, and Case B mainly reflects the 'influence of neurotrophic factors' mechanism. Another possibility is that the differential PNI patterns may also reflect the differences in time phase aspect. Conceivably, these two

distinctive findings (A and B) might represent early and late phases, respectively, in the general time course of neural invasion, where cancer cells firstly come and touch neural plexus; subsequently, reactive cytokines start affecting the interactions between cancer cells and nerve cells.

Limitations of this study are that it contains only two cases and that we examined the focal parts of the tumor regions. Although the findings revealed a relationship between invasive carcinoma and nerve tissue, the relationship indicated above may not be generalizable to all such tumors. Accumulation and analysis of more cases is needed in the future. In conclusion, the present study demonstrated the 3D structures of intramural PNI. With our 3D reconstruction models, it was possible to evaluate the morphological features of tumor invasion around nerve cells. This method has the potential to provide key insights into the biology of PNI in CRC.

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Disclosure statement

None Declared.

Author Contributions

Y.M. and S.K. performed the experiments and captured the images with a slide scanner, and Y.M. wrote the paper. T.I. designed the experiments, performed the 3D reconstruction process, and co-wrote the paper. E.S. designed the experiments, selected the samples for 3D reconstruction, analysed the staining data and co-wrote the paper. S.O. designed the experiments and analysed the staining data. H.S. diagnosed the myenteric spread and intramural PNI and selected the samples for 3D reconstruction. E.O., H.U., Y.O., and M.M. supervised the study. All authors contributed to the discussion and revision of the manuscript.

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Tables

Table 1. Clinicopathological characteristics of the two cases.

Figure Legends

Figure 1. Photograph of tumor tissue and 2D images of H&E and immunohistochemically stained sections of Case A.

A: Photograph of the tumor tissue of Case A.

B: H&E slide of Case A, the area marked by the red square in A. Intramural PNI was observed (arrowhead). Scale bar: 1 mm.

C: High-power magnification of the horizontal invasive front stained by H&E. Scale bar: 200 μm.

D: High-power magnification of the horizontal invasive front stained for cytokeratin AE1/AE3. Scale bar: 200 μm.

E: High-power magnification of the horizontal invasive front stained for S-100 protein. Scale bar: 200 μm.

F: The closest point between the tumor and nerve cells stained by H&E. Scale bar: 50 μm.

G: The closest point between the tumor and nerve cells stained for cytokeratin AE1/AE3. Scale bar: 50 μm.

H: The closest point between the tumor and nerve cells stained for S-100 protein. Scale bar: 50 μm.

Figure 2. Selected 3D reconstruction models in Case A.

Tumor cells are labelled in orange, and nerve cells are labelled in green. The closest point between the tumor cells and the nerve cells in Case A is shown in the upper right corner.

Figure 3. Photographs of tumor tissue and 2D images of H&E and immunohistochemically stained sections of Case B

A: Photograph of the tumor tissue of Case B.

B: Cross section of Case B.

C: H&E slide of Case B, the area marked by the red square in A, B. Myenteric spread (arrowhead) was observed both at and behind the horizontal invasive front. Scale bar: 2m m.

D: H&E slide of the horizontal invasive front in Case B. Intramural perineural invasion (PNI) was observed (arrowhead). Scale bar: 1mm.

E: High-power magnification of intramural PNI stained by H&E. Scale bar: 100 μm.

F: High-power magnification of intramural PNI stained for cytokeratin AE1/AE3. Scale bar: 100 μm.

G: High-power magnification of intramural PNI stained for S-100 protein. Scale bar: 100 μm.

Figure 4. Selected 3D reconstruction models in Case B.

Tumor cells are labelled in orange, and nerve cells are labelled in green. The closest point between the tumor cells and the nerve cells in case B is shown in the upper left corner.

Supporting Information

List of supplementary videos.

Video 1,2: 3D reconstruction models. Tumor cells are labelled in orange and nerve cells are labelled in green.

Video 1: 3D reconstruction model at the horizontal invasive front of Case A. The 3D reconstruction model was rotated around x-axis. In the second half of the video, the closest point between the tumor and nerve cells of Case A is shown. The picture at the end of this

video was reconstructed from two microscopic images of serial sections stained for AE1/AE3 and S-100, respectively.

Video 2: 3D reconstruction model at the horizontal invasive front of Case B. The 3D reconstruction model was rotated around the x-axis. In the second half of the video, one point of the horizontal invasive front of Case B is shown, where PNI was prominent with 2D evaluation. The picture at the end of this video was reconstructed from two microscopic images of serial sections stained for AE1/AE3 and S-100, respectively.