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REVIEW

# Detecting circulating tumor material and digital pathology imaging during pancreatic cancer progression

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## Abstract

Pancreatic cancer (PC) is a leading cause of cancer-related death worldwide. Clinical symptoms typically present late when treatment options are limited and survival expectancy is very short. Metastatic mutations are heterogeneous and can accumulate up to twenty years before PC diagnosis. Given such genetic diversity, detecting and managing the complex states of disease progression may be limited to imaging modalities and markers present in circulation. Recent developments in digital pathology imaging show potential for early PC detection, making a differential diagnosis, and predicting treatment sensitivity leading to long-term survival in advanced stage patients. Despite large research efforts, the only serum marker currently approved for clinical use is CA 19-9. Utility of CA 19-9 has been shown to improve when it is used in combination with PC-specific markers. Efforts are being made to develop early-screening assays that can detect tumor-derived material, present in circulation, before metastasis takes a significant course. Detection of markers that identify circulating tumor cells and tumor-derived extracellular vesicles (EVs) in biofluid samples offers a promising non-invasive method for this purpose. Circulating tumor cells exhibit varying expression of epithelial and mesenchymal markers depending on the state of tumor differentiation. This offers a possibility for monitoring disease progression using minimally invasive procedures. EVs also offer the benefit of detecting molecular cargo of tumor origin and add the potential to detect circulating vesicle markers from tumors that lack invasive properties. This review integrates recent genetic insights of PC progression with developments in digital

pathology and early detection of tumor-derived circulating material.

**Key words:** Circulating tumor cells; Digital pathology; Early detection; Exosomes; Pancreatic cancer



**Core tip:** Pancreatic cancer (PC) is a leading cause of cancer-related death. PC mutations accumulate 20 years before patient death with metastatic mutations occurring late in the process. Metastatic risk increases dramatically when tumor diameter is greater than 1 cm. Most PC cases are diagnosed at late metastatic stages when survival is short. Outcomes could be improved if non-invasive methods could detect early stages of the disease and guide treatment decisions. Recent studies indicate this may be possible with application of digital pathology imaging, screening of CA 19-9 with additional markers, and detecting circulating tumor material in early-stage PC patients.

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## INTRODUCTION

Pancreatic cancer (PC) is the third leading cause of cancer-related death in men and women in the United States surpassing breast cancer[1,2]. Projections indicate PC will outpace colorectal cancer and become the second leading cause of cancer-related death in the United States by 2020[2]. The majority of pancreatic tumors (90%) are classified as adenocarcinomas arising from the ductal epithelium with an annual incidence of 45220 patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) in the United States[1,3]. Estimates suggest that only 1.3%-10% of patients diagnosed with PC have familial basis for the disease where a genetic component is inherited from a relative[4]. The remaining majority of PDAC cases display large genomic heterogeneity[5]. Five-year survival is about 25% for localized stages but only 2% for advanced disease[1]. The best curative treatment is surgical resection, if performed early it presents a 5-year survival in 25%-30% lymph node negative patients but only 10% for those with positive lymph nodes[6-8]. Less than 20% of PC cases are diagnosed early enough for surgical intervention[2]. Relapse rate after surgery is typically high (80%) for this type of cancer and surgery is often followed by adjuvant chemotherapy or chemoradiation[2,9]. Approximately 80% of PDAC patients are diagnosed late when the disease becomes locally advanced or metastatic, where palliative chemotherapy is the only treatment option[10]. Since 1997, Gemcitabine has been commonly used over 5-fluorouracil (5-FU) albeit with only a modest median overall survival (OS) advantage of 5.6 mo (Gemcitabine) *vs* 4.4 mo (5-FU) in patients presented with advanced stage[11]. Extensive efforts have been made over the past decade, including numerous randomized phase Ⅲ clinical trials, to evaluate combinatorial drug treatments for patients with advanced disease[12]. To date erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, plus gemcitabine is the only course with a targeted therapy agent approved by the United States Food and Drug Administration (FDA) for first-line use in advanced PC[13-15]. FOLinic acid, Fluorouracil, IRINotecan, and OXaliplatin (FOLFIRINOX) and nab-paclitaxel/gemcitabine have emerged as combinatorial treatments with results that may reach the one-year survival barrier[16,17]. Adjuvant combination chemotherapy comprising gemcitabine with capecitabine has also shown statistically improved survival over gemcitabine monotherapy in PDAC subjects (ESPAC-4, Phase 3)[18]. Great focus has been extended into developing methods for improving early detection of the disease and exploring alternate treatment options that can extend survival in patients with late stage presentation[14]. This review provides description of the genetic fingerprints that drive disease progression and discusses selected features relevant to detection and treatment in this biological context. We further highlight recent advances in digital pathology, improvements in CA 19-9 testing, and detection of circulating tumor cells and tumor-derived extracellular vesicles (EVs) in biofluids of PC subjects (Table 1). Particular attention is made to literature that provides examples of material isolated from human PC subjects along with cell culture or animal model systems that explore mechanistic underpinnings.

## PC PROGRESSION AND GENETICS

Computational modeling of primary pancreatic tumors supports the observations that metastatic probability increases exponentially with tumor size[19,20]. A patient with a primary tumor size of 1 cm in diameter is predicted to have a 28% probability of harboring metastasis at the time of diagnosis. This dramatically increases to 73% probability with a tumor size of 2 cm and elevates to 94% chance for a tumor size of 3 cm[20]. This clearly suggests that systemic treatments that target rapidly growing cells need to be administered early before log-phase growth is reached. For conventional therapies to improve survival, it will become paramount to detect early lesions before significant invasion takes course. The term pancreatic intraepithelial neoplasia (PanIN) was first coined in 1999 to describe ductal lesions which form as precursors to invasive cancer[21]. A progression model was soon after proposed where *HER-2/neu* overexpression and *KRAS* mutations are observed early, *p16 (CDKN2A/INK4a)* gene inactivation occurs at intermediate stages, with inactivation of *p53*, *DPC4*, and *BRCA2* occurring late[21].

**Table 1 Summary of demonstrated clinical uses for digital pathology, circulating tumor cells and extracellular vesicles for pancreatic cancer**

| | Digital pathology | CTCs | EVs |
|---|---|---|---|
| Screening in population | Relies on invasive biopsies | Detection of KRAS mutations[92] | Early detection possibility (GPC1+ EVs)[117] |
| Diagnosis | Differential diagnosis of mucinous cancers[62] | Pancreatic CTC detected by ISET[82] and CellSearch[81] | GPC1+ EVs detected in IPMNs[117]<br>EVs express mutated KRAS and p53 in PDAC serum[123]<br>EVs detected in pancreaticobiliary cancers[124] |
| Staging | Early stage detection in mice[60]<br>Distinguish Grade Ⅰ / Ⅱ in humans[61] | (C-MET, CK20, CEA) + CTCs elevated in late stages[96] | miR-17-5p in serum exosomes correlates with stage[128] |
| Prognosis | Potential | CTC positivity has prognostic value in locally advanced pancreatic cancer[81]<br>CK20 expression in CTC indicates shorter overall survival[94] | Potential |
| Monitor treatment | Potential | CTC levels decrease during 5-FU therapy[91] | Potential |
| Drug sensitivity/ pharmacokinetics | CT scans can predict drug transport[35] | CTC apoptosis can be detected after 5-FU therapy[91] | Demonstrated for breast cancer[111] |
| Monitor recurrence | Potential | CTC positivity correlates with postoperative staging[94-97] | potential |

EVs: Extracellular vesicles; CTCs: Circulating tumor cells; 5-FU: 5-Fluorouracil; PDAC: Pancreatic ductal adenocarcinoma; CT: Computed tomography; PDAC: Pancreatic ductal adenocarcinoma; CEA: Carcino-embryonic antigen.

This model predicts that PC evolves slowly with defined mutational characteristics and presents clinically at late stage. This progression paradigm of gradual pace has recently been challenged by Notta *et al*[22] who propose a punctuated equilibrium hypothesis where tumorigenic mutations arise from a cataclysmic event that rapidly leads to invasive cancer and metastasis. Data from this model suggests PC development is neither gradual nor follows the accepted mutation order which may be supported by observations showing that not all clonally expanded precursor lesions lead to a tumor lineage[5,22,23].

Recent evidence suggests that the development of metastatic cancers from primary tumors can take up to two decades, based on genomic sequence comparisons and mathematical analysis. The development of parental clones from an initiated tumor cell is estimated to take an average of 11.7 years, with an additional 6.8 years for expansion of metastatic subclones, and another 2-3 years before tumors disseminate to distant organs leading to patient death[24]. The founder mutations present only in the parental clones accumulate in a large number of driver genes involved in tumorigenesis such as *KRAS, TP53* and *SMAD4*. The resulting subclones, giving rise to metastatic lesions, contain additional progressor mutations which vary highly among subclones[24]. This suggests that distant metastasis occurs late during the genetic evolution of PC also supporting the punctuated equilibrium model of progression. These observations are consistent with findings that show more than 50% of the genomic rearrangements occur early during tumor progression being present in both primary and metastatic clones in the patient[25]. If these rearrangements could be narrowed to distinct genes or protein signaling pathways, they could serve as powerful targets for therapeutics made highly effective by reaching both primary and metastatic sites. In addition to identifying mutation hot-spots in metastatic clones, it will be important to compare founder mutations in primary tumors between patients with different survival outcomes to discover early factors that commit patients to a high risk course[26].

PCs were shown to have gene expression alterations in 69 gene sets, half of which cover at least twelve core signaling pathways with functional relevance in 67%-100% of observed neoplasias[27]. Even though these 12 overlapping cascades appear to be genetically altered in majority of the tumors, alterations of the pathway components themselves vary greatly between individual tumors[27,28]. This implies that therapies directed against these actionable targets may need to implement multi-targeted approaches based on selected patient subgroups, or consist of cocktails that effectively abolish entire signaling cascades[14,29].

A recent study performing whole-genome sequencing and copy number variation (CNV) analysis found a total of 857971 point mutations, insertions and deletions in 100 samples of PDAC[30]. The four most commonly mutated genes observed in PDAC patients are the oncogene *KRAS* (75%-90%), tumor suppressor genes *TP53* (74%), *CDKN2A/p16* (35%), and *SMAD4* (31%), along with inactivating mutations in the Rac exchange factor *PREX2*, the tumor suppressor *RNF43*, and the histone demethylase *KDM6A* observed in 10%-18% of subjects[30]. Focal amplification of druggable oncogenes such as *ERBB2, MET, FGFR1, CDK6*, *PIK3R3* and *PIK3CA* is observed at very low prevalence among only 1%-2% of patients[30]. Levels of protein expression or activity were not determined in these studies, however, to understand the functional significance of the focal amplifications. Integrated genomic analysis of PDAC identified 32 mutated genes that comprise 10 signaling

pathways: KRAS, transforming growth factor (TGF)-beta, WNT, NOTCH, ROBO/SLIT signaling, G1/S transition, SWI-SNF, chromatin modification, DNA repair and RNA processing[31]. Four tumor subtypes were identified based on differential expression of transcription factors and downstream targets: Squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX) tumors. These tumor subtypes were sorted by gene programs to identify genetic factors that impact OS in PDAC subjects[31].

PDAC primary tumors can also be sorted into three distinct subtypes based on gene expression patterns and drug sensitivity: Classic, quasimesenchymal and exocrine-like[32]. The classic subtype, more sensitive to the EGFR inhibitor erlotinib, expresses high levels of adhesion-associated epithelial genes such as *AGR2, S100PBP* and *GATA6*. The quasi-mesenchymal subtype is more sensitive to gemcitabine and expresses high levels of mesenchymal genes such as *TWIST1* and *S100A2*. The exocrine-like subtype has high expression of tumor cell derived digestive enzyme genes such as *REG3A* and *PRSS1*[32]. These findings open the possibility for stratifying patients based on tumor gene expression patterns as a means for predicting drug sensitivity.

Taken together, these observations demonstrate that primary and metastatic tumors of the pancreas are highly heterogeneous and contain several distinct clonal populations with unique molecular signatures which develop over a long period of time. This makes targeted therapy difficult, unless common pathways are found that can be effectively blocked by personalized drug regimens[5].

## PANCREATIC STROMA

Another source of genetic diversity can be found within the pancreatic stroma. PDAC cells are surrounded by a rich stroma that is typically far more abundant in cell types other than the tumor. Pancreatic stroma contains a variety of cells including stellate cells, immune cells, fibroblasts, vascular endothelial cells and the extracellular matrix which make up the tumor micro-environment (TME)[33]. TME plays a pivotal role in tumor behavior including proliferation, drug resistance, invasion and localized immune response[33,34]. A clinical study investigating intraoperative gemcitabine infusions during PDAC resection showed that high stromal density inhibits hENT1-mediated drug incorporation into the tumor[35,36]. Investigators in this study derived mass transport parameter (MTP) cutoff values based on expression of the nucleoside transporter hENT1 in the tumor, and pancreatic stromal density scores calculated from CT scans[35]. Applying MTP cutoffs to a cohort of 110 patients, who received gemcitabine therapy, revealed a 5-year survival rate of 40% in subjects with favorable transport parameters compared to a 15% survival rate in subjects who did not reach the parameter cutoff point[35]. This study demonstrates that stromal density and drug transport properties can be measured during surgery, using routine contrast-enhanced CT scans and immunohistochemistry, as a highly effective means for predicting significant response to cancer therapy. hENT1 expression in tumor cells permits bidirectional transport of pyrimidine nucleosides such as gemcitabine, capecitabine and 5-FU[37]. High expression of hENT1 in PC patients treated with gemcitabine is predictive of improved survival[36,38,39]. These studies open the possibility for determining drug sensitivity in resected patients through screening morphological features of the stroma combined with assessment of pharmacogenomic profiles[40].

The pancreatic stroma is enriched with large diversity of constituents, making it difficult to score clinically. A recent study applied a blind source separation technique called non-negative matrix factorization (NMF) to analyze gene expression from a microarray dataset that included 145 primary and 61 metastatic PDAC tumors in comparison to 134 normal tissue samples[41]. This technique effectively generated gene expression signatures sorted by tumor, stromal and normal cellularity. Patients that were identified with a "classical" tumor subtype had a median survival of 19 mo compared to patients with a "basal-like" tumor subtype that demonstrated a significantly worse survival of 11 mo. Additionally, two stromal subtypes were identified in patients: A "normal" subtype with 24 mo-median survival and an "activated" stromal subtype with significantly worse median survival of 15 mo. These techniques lead the way for identifying genetic markers that may otherwise be obscured by confounding material from normal and stromal tissue[41].

Mounting evidence supports the hypothesis that pancreatic TME s play a significant role in pathological outcome and treatment response and should therefore be clinically evaluated as a standard practice. The use of digital imaging combined with pharmacogenomic analysis could extend the application of existing treatments for personalized medicine. Best clinical outcomes come from early diagnosis of the disease. Leveraging the biological properties of pancreatic adenocarcinomas and their surrounding micro-environment for early detection and diagnosis would provide maximum benefit for patient survival.

## CURRENT DIAGNOSTIC METHODS USING SERUM

Presently, there are no suitable PC screening strategies effective for early detection of PC in the general population. Diagnosis of PDAC is made by pathological assessment of a tissue biopsy. The current gold standard is *via* an endoscopic ultrasound technique coupled with fine needle aspirations (EUS-FNA) which has a sensitivity of 75%-94% and specificity of 78%-95%[8,42]. For patients who have non-diagnostic FNAs or cannot undergo endoscopy, treatment decisions are based on imaging or determining CA 19-9 serum levels[8]. The only serum biomarker approved by the FDA for PC is the sialylated Lewis (a) blood group antigen CA 19-9 which is not tumor specific and is frequently elevated during many malignancies,

**Table 2 Clinical uses for biomarker panels that increase predictive value of CA 19-9 for pancreatic cancer**

| | CA 19-9 | Sensitivity | Specificity | Ref. |
|---|---|---|---|---|
| Screening in population | EUS-FNA | 75%-94% | 78%-95% | [42] |
| | CA 19-9[1] | 60%-70% | 70%-85% | [45,46] |
| Differential diagnosis | CA 19-9 | 60% | 83% | [44] |
| | CA 19-9 + CA 125 | 87% | 77% | [44] |
| | CA 19-9 + ICAM-1 + OPG | 78% | 94% | [49] |
| | CA 19-9 + CEA + TIMP-1 | 71% | 89% | [49] |
| Staging | PAM4-reactive mucins | 76% | 85% | [51] |
| | CA 19-9 + PAM4-reactive mucins | 84% | 82% | [51] |
| Monitor treatment | Response to chemotherapy | | | [47] |
| Monitor recurrence | Low levels post-surgery correlate with survival | | | [45] |

[1]Values reflect subjects presented with pancreatobilliary disease. EUS-FNA: Endoscopic ultrasound and fine needle aspiration; OPG: Osteoprotegerin; ICAM-1: Intercellular adhesion molecule 1; CEA: Carcinoembryonic antigen; TIMP-1: Tissue inhibitor of metalloproteinases 1; clivatuzumab monoclonal antibody (PAM4) to MUC5AC.

pancreatitis, cholangitis, obstructive jaundice, hepatobiliary cancer, and benign biliary obstruction[43,44]. CA 19-9 alone has not been shown to be an effective screening marker for PDAC among the general population based on most studies[45]. However, sensitivity (60%-70%) and specificity (70%-85%) of CA 19-9 improve significantly in patient cohorts presented with pancreatobiliary disease[45,46]. Low serum CA 19-9 levels following surgery correlate with improved survival[45]. Oncologists occasionally use CA 19-9 to track response to chemotherapy but the predictive significance of CA 19-9 for this purpose has reported some variability[43,45,47].

Measuring CA 19-9 in combination with other markers such as CEA, CA242, and TIMP1, however, was shown to improve its predictive value (Table 2)[45,48,49]. Barnett *et al*[49] could identify PDAC patients using two independent panels: CA 19-9, CEA, and TIMP-1; and a second panel containing CA 19-9, ICAM-1, and OPG. Both panels demonstrated increased sensitivity and specificity over CA 19-9 alone (Table 2)[49]. Recently, O'Brien *et al*[44] discovered CA 19-9 (> 37 U/mL) and CA 125 (> 30 U/mL) serum levels can be elevated up to two years before PDAC diagnosis based on a nested case control study. CA 125 has been reported to distinguish malignant from benign PC tumors with 60.8% sensitivity and 83.3% specificity which improved to 87.8% and 77.8% respectively when combined with CA 19-9[50]. PAM4, an antibody which binds mucin MUC1 and MUC5AC epitopes expressed in PC, was capable of identifying 64% of stage Ⅰ PDAC patients with high discriminatory power compared to those with benign pancreatic disease[51]. PAM4 is capable of distinguishing normal pancreas from PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 lesions, intraductal papillary mucinous neoplasia (IPMN) lesions, as well pancreatic adenocarcinomas of various grades[52]. Combining CA 19-9 with a PAM4-reactive marker improved sensitivity (84%) without a loss in specificity (82%) in a serum-based enzyme immunoassay (EIS)[51]. Despite some propensities for false positivity, CA 19-9 continues to be a benchmark serum marker for evaluating PC in the clinical setting. It will be important to test combinations of other markers in addition to CA 19-9 to improve its diagnostic utility in larger populations.

## TUMOR IMAGING AND DIGITAL PATHOLOGY

In addition to biopsies and serum marker tests, lesions and primary tumors can be characterized by clinical imaging. Computed tomography (CT) and magnetic resonance imaging (MRI) are the most frequently used imaging method for diagnosis and clinical staging[33,43]. Additional screening approaches using imaging multi-modalities include endoscopic ultrasonography (EUS), magnetic resonance cholangiopancreatography (MRCP), and endoscopic retrograde cholangiopancreatography (ERCP)[4]. However, these approaches are limited to surveillance centers with robust PC programs and are typically only performed on high-risk patients[53,54]. MRI and EUS have been proposed for use as first line modalities but often fail to distinguish benign from malignant lesions[55]. Emerging imaging modalities and molecularly targeted imaging agents are of great interest as early detection strategies but may be cost prohibitive and inaccessible to many patients[56].

Upon diagnosis, patients are staged based on the AJCC 7$^{th}$ Edition Staging Manual criteria before proceeding to surgery[8,57]. This is typically accomplished through cross-sectional imaging (CT or MRI) along with tissue biopsy[8]. Among those staged with resectable disease by biopsy, only 70%-85% actually present with resectable tumors, intraoperatively[8]. This indicates a need for improvements in staging methodology which may be enhanced by digital pathology[8]. The field of digital pathology has recently grown to complement histological diagnosis performed by pathologists[58]. These methods extract and quantify histological features from whole slide images thus improving on the subjective nature of the work[59].

Langer *et al*[60] developed a method that can accurately predict early pancreatic lesions with a 93% success rate

in an independent test set using tissue obtained from mouse models of early-stage PDAC. The program uses a top-down object learning paradigm similar to the methodology used by human pathologists. Initially, ducts, nuclei and tumor stroma are identified and segmented. From those, secondary morphological features such as duct deformation and nuclei malformations are measured. These data sets are then used to train a predictive model that distinguishes normal tissue from premalignant cancer lesions[60]. Similar techniques can be extended to accomplish classification of PDAC by grade using human tissue samples[61]. Diagnosis of PDAC was made based on three parts: Segmentation and feature extraction; model learning and validation; and diagnosis. Training data measuring ducts, consisting of the lumen and epithelial nuclei, can distinguish normal human subjects and those with grade Ⅰ and grade Ⅱ PDAC with an accuracy of 94%[61]. Automated systems have been developed for making a differential diagnosis of rare lesions such as cystic neoplasms of the pancreas using human biopsy tissue[62]. Song *et al*[62] were able to distinguish benign serous from malignant mucinous cystadenomas using a computer-aided design technique. Cystic regions were identified and epithelial cells surrounding the lumen were discerned. Three classes of features were analyzed by the program to achieve a differential diagnosis: The number and size of cysts, characteristics of the surrounding epithelium, and indication of mucus production[62].

Current applications of digital pathology for PC do not offer much more beyond histological diagnosis performed by a pathologist but indicate potential for detecting early lesions. Improvements could be made, for example, by developing digital pathology methods for images annotated with clinical data from population-based repositories. This could potentially aid the discovery of morphological features associated with treatment and survival outcomes.

The intended goal beyond research is to incorporate digital tools into clinical practice as a way to standardize histological diagnosis in patients at high risk of developing the disease. This could improve staging and determination of resectability. Some concerns raised include public health consequences if misdiagnosis is caused by improper use or analysis of poor quality images[63]. The Food and Drug Administration recently released a guidance for technical performance assessment of digital pathology whole slide imaging (WSI) devices[64]. Currently, WSI devices are classified as Class Ⅱ for methods that provide adjunct analysis after a primary diagnosis is made using glass slides. WSI devices which make a primary diagnosis alone are classified as high-risk Class Ⅲ devices if their intended use is new and lacks a Class Ⅱ predicate. A *de novo* process provides a less resource-intensive approval path to Class Ⅰ/Ⅱ classification if special controls are presented that provide reasonable assurance of safety and effectiveness. A more clearly-defined approval process for manufactures would enhance innovation and commercialization potential of digital pathology instruments and software[65].

## DETECTING TUMOR CELLS IN CIRCULATION

Performing invasive biopsies for routine screening of the general population is not reasonably a feasible option. Detecting tumor material in the blood or other biofluids would be ideal for many reasons. A test assessing a panel of markers in biofluids could be ordered by physicians in most clinical centers, and collected by non-invasive or minimally invasive procedures. Performing additional tests using the same starting material could easily lead to diagnostic refinement. Diagnostic tests can be expanded to cover non-tumor biomaterial such as components of the immune system, blood/serum, pancreatic juice, stool, oral and gut microbiota, and markers of metabolic activity. Given patient variability, measuring systemic profiles of markers not directly derived from tumors may not yield the specificity and sensitivity necessary to accurately determine risk for developing advanced PC. A search for "pancreatic cancer" in the published literature can easily generate over 50000 returns which documented more than 2500 individual genes as potential PC biomarkers due to their overexpression patterns[66]. A compendium of PC biomarkers identified at least 1000 molecules with evidence of upregulation in precursor lesions[66]. Early detection of these precursor lesions particularly before invasive cells establish colonization would be ideal.

A critical study, using genetically engineered mouse models of PanIN, showed that cells from preneoplastic lesions can breach the basement membrane and spread into the stroma[67]. Contrary to conventional wisdom, these cells undergo epithelial-to-mesenchymal transition (EMT) and enter the blood into circulation with no evidence of carcinoma. These findings suggest that EMT transition can occur as an early phenomenon even before histologic emergence of cancer. These cells acquire a mesenchymal phenotype, exhibit stem cell properties, have tumor-initiating capacity, and are most abundantly observed at inflammatory foci[67]. Induction of pancreatitis and immunosuppressive treatment with dexamethasone have strong effects on dissemination supporting a link between early precursor cell invasion and localized inflammation[67]. Typical circulating tumor cell (CTC) markers such as EpCAM are expressed in less than 20% of the PanINs in this model system[67]. This has implications for commercially available methods which may overlook these circulating precursors, because they rely on such epithelial markers for CTC detection.

EMT in primary cells was shown to be associated with acquisition of stem cell-like characteristics[68]. Both normal and cancer stem cells possess the ability to self-renew and produce differentiated progeny[29]. Cancer stem cells are further functionally defined by having enhanced tumor initiating capacity when transplanted to a permissive host[67]. *In vivo*, CTCs detach from the primary tumor and enter the blood where they can be

transported to distant sites with only 0.01% surviving to form metastases[69]. CTC detection has been extensively used for prognosis (progression free survival and OS) and predicting response to treatment in breast, prostate, colorectal and lung cancers[70-74]. CTCs have also been detected in PC patient samples but their prognostic potential remains to be optimized outside the limitations of a small sample of subjects[69,75-77].

## CTC DETECTION METHODS

Circulating tumor cells are present at very low concentrations in the blood, typically one CTC per billion blood cells. For this reason, CTCs need to be enriched to differentiate them from the vast hematopoietic cell background, and characterized to verify their tumor origin[69]. Several enrichment media for density-gradient centrifugation are commercially available including LymphoPrep™ (Axis-Shield), Ficoll-HyPaque™ (Sigma-Aldrich), Oncoquick® (Greiner Bio-One), and RosetteSep ™ Human Circulating Epithelial Tumor Cell Cocktail with SepMate™ (StemCell Technologies). Enrichment is typically followed by targeted isolation. Four strategies are available to isolate and capture CTCs: Positive selection using antibodies attached to solid-support, negative selection, cell size-based methods such as filtration, and physical property-based methods[77]. Most CTC detection methods rely on either positive immunoselection of cells expressing the epithelial cell adhesion molecule (EpCAM) or negative selection by depleting leukocytes from the blood using CD45-binding antibodies[78]. Commercial immunomagnetic bead separation systems are available including EasySep cell separation (StemCell Technologies), Dynabeads (Invitrogen), CellSearch CTC system (Janssen Diagnostics) and MACS (Miltenyi). CellSearch CTC is the only system approved by the FDA for capturing and enumerating CTCs of epithelial origin by $CD45^-$, $EpCAM^+$ and $cytokeratin^+$ selection[79]. CellSearch has been cleared by the FDA for management of breast, colorectal and prostate cancers and has also been tested in PDAC patients with detection rates varying from 11%-45%[44,78,80-85].

Once isolated, circulating tumor cells are typically characterized by immunocytochemical (ICC) staining or nested real-time polymerase chain reaction (RT-PCR). Detection strategies typically assess epithelial mRNA profiles which include *EpCAM*, epithelial carcinoembryonic antigen (*CEA*), *CEACAM5*, *CK19*, *BIRC5* and *MUC1*[76]. There are currently more than 40 assay platforms for CTC detection and enrichment that have been widely publicized[86]. Among these, the utilization of microfluidics and microarray technology in CTC detection is expanding.

CTC detection was investigated as a prognostic tool in a LAP07 international multicenter randomized study to assess if patients with locally advanced pancreatic carcinoma (LAPC) would benefit from chemoradiotherapy over continuation of chemotherapy[81]. Bidard *et al*[81] were able to achieve a CTC detection rate of 11% using a low cut-off of one or more CTCs/7.5 mL of blood using the CellSearch system. This is lower than the 50% detection rate typically reported for metastatic PC patients[84]. CTC positivity nonetheless was a prognostic factor for OS which was lower in CTC positive LAPC patients[81]. More CTCs can be detected in the blood of PC patients using ISET (Isolation by Size of Tumor Cell) based on a comparative study which found detection of 26 CTCs/7.5 mL blood using ISET compared to 2 CTCs/7.5 mL blood by CellSearch[82]. ISET also detected CTCs in a much higher proportion of patients (93%) *vs* CellSearch (40%)[82]. ISET is a filtration-based, marker-independent method that sorts by cell size and morphology using filter modules offered by a company started by the inventor of the technology (Rarecells Diagnostics)[87,88]. Thus ISET may offer a significant advantage over CellSearch which relies on expression of EpCAM for CTC identification. PDAC cells, among carcinomas, are more prone to epithelial-mesenchymal transition (EMT) which reduces the expression of EpCAM[78,89,90]. This presents a problem for PC detection using CTCs as most of the current CTC detection methods rely on EpCAM or other epithelial molecules for CTC detection[69,86].

## CTC CHARACTERIZATION

There exists a critical need for the development of assays that can additionally identify CTCs which undergo EMT and lose expression of typical epithelial surface antigens. Ren *et al*[91] detected CTCs in peripheral blood of advanced stage PC patients before (in 80% of patients) and after treatment (in 29% of patients) with 5-FU by immunostaining for CA19-9 and CK8/18 expression. The mean concentration of blood CTC decreased from 16.8 cells/7.5 mL of blood before chemotherapy to 3.8 cells/7.5 mL blood after a seven-day cycle of 5-FU chemotherapy[91]. Evidence of apoptosis induced by 5-FU was observed in CTCs obtained from patients and in pancreatic cell line models (PL45 and PANC-1 cells)[91]. These studies open the possibility for using CTC assays to monitor chemotherapy efficacy and extent of remission although they fail to selectively identify mesenchymal antigens expressed by CTCs. Other potential mesenchymal protein marker candidates include Cadherin 2, Vimentin, Snail/Slug, zinc finger E-box binding homeobox1 (ZEB1), and Twist family basic helix-loop-helix transcription factor 1 (TWIST1)[79]. These mesenchymal markers could be combined with PC-specific markers to increase specificity.

To verify tumor origin, isolated CTCs can also be screened for genes expressed or mutated predominantly in PC such as *KRAS*. Court *et al*[92] detected *KRAS* mutations in 92% of PC patients using a NanoVelcro/laser capture microdissection (LCM) platform. This technique captures CTCs on a microfluidic chip using biotinylated anti-EpCAM antibodies and is followed by identification through ICC staining of CD45, CEA, and staining of pancytokeratin for nuclear morphology. Mutations in *KRAS* were not observed in white blood cells and overall reliability of the assay required isolation of only 10-100 circulating tumor cells[92]. Chausovsky *et al*[93] detected the

expression of Cytokeratin 20 (*CK20*) in 22/28 PC patients using RT-PCR analysis of peripheral blood CTCs. Soeth *et al*[94] found that *CK20* was expressed in CTCs of 33% of patients in a larger cohort ($n$ = 154) who had significantly shorter OS. Cytokeratin 7 (*CK7*) and cytokeratin 20 (*CK20*) are expressed in a variety of epithelial neoplasms including majority of pancreatic carcinomas (62%)[95]. A variety of commercial platforms are now available for detection of amplified CTC DNA such as TruSeq Amplicon (Illumina) and Ion Torrent AmpliSeq™ (Life Technologies).

Levels of RNA expression can also be measured by RT-PCR or directly imaged by *in situ* RNA hybridization using platforms such as ViewRNA™ CTC Platform (Affymetrix). Zhou *et al*[96] measured mRNA expression of *h-TERT, C-MET, CK20*, and *CEA* by RT-PCR in CTCs isolated by immuno-magnetic enrichment using EpCAM. This method can distinguish PC patients from benign control subjects with high degree of specificity. Further, when pancreatic patients were in later stages, the expression rate for C-MET (67%), CK20 (75%) and CEA (75%) were statistically higher than during earlier stages[96]. These findings open the utility of CTC detection for monitoring disease progression. Two independent studies have also found that preoperative CTC positivity correlated with postoperative staging[94,97]. This indicates that in addition to diagnostic value, CTC detection has prospects for PC staging[8].

The genetic content of CTCs can also be sequenced for molecular discovery[92]. Yu *et al*[98] adapted a microfluidic device to capture CTCs which were subjected to single-molecule RNA sequencing. Using a mouse PC model, *Wnt2a* gene was identified to be enriched in CTCs isolated from mice and in 5/11 human PC cases[98]. Ting *et al*[99] used focusing-enhanced microfluidic capture of CTCs (CTC-iChip) from primary PC tumors followed by deep-RNA sequencing. RNA-seq profiles identified enrichment of stem-cell-associated genes such as *Aldh1a2* and the extracellular growth factor binding protein *Igfbp5* which localized focally at the tumor epithelial-stromal interface[99]. CTCs of mouse and human origin also expressed elevated levels of gene expression of the stromal-derived extracellular matrix protein (SPARC), which increases invasive and migratory potential of PDAC cell lines[99]. Whole exome sequencing of CTCs has been successfully accomplished in metastatic prostate cancer cells, PDACs, and pancreatic carcinoma neoplasms with acinar differentiation[100-102].

To improve prognostic value, CTC analysis can be combined with other methods such as direct detection of circulating free DNA (cfDNA) in the blood. Mutated *KRAS* cfDNA, isolated from plasma, was observed in 26% of patients with resectable and advanced stage disease and correlated strongly with decreased OS compared to mutant *KRAS* free subjects (60 d *vs* 772 d)[85]. Patients with panreatobiliary carcinomas were accurately diagnosed using cfDNA sequencing with a 92% sensitivity and 100% specificity[103]. CTCs were detected in peripheral blood of 20% of metastatic disease patients using the CellSearch system by CD45 positive cell depletion. CTC positive PDAC patients had decreased OS of 88 d (95%CI: 27-206) compared to 393 d (95%CI: 284-501) in CTC negative subjects[85]. Circulating tumor DNA (ctDNA) can also serve as a detection strategy on its own or in combination and can be found in other articles that focus on this topic[78,104,105]. For example, Berger *et al*[104] were able to distinguish patients with Intraductal Papillary Mucinous Neoplasm (IPMN) lesions from controls by detecting mutation hot-spots in circulating cell-free DNA from patient blood samples.

Collectively, the utility of circulating tumor cells as a diagnostic marker in PC is gaining more ground. CTC detection offers the benefit of a low-risk safety profile which may be a cheaper alternative to FNA biopsies[8]. The cost of obtaining a diagnosis by EUS-FNA in the United States can be approximately $16000 compared to $370 Medicare reimbursement for the CellSearch CTC-based Assay[106,107]. A broader range of epithelial and mesenchymal markers are needed to create techniques that adequately capture a wide range of PC-specific circulating tumor cells. Finally, selected CTC techniques need to be tested in larger patient cohorts to pass the same FDA clinical guidelines that made the CellSearch CTC-based assay a successful clinical tool for breast, prostate and colon cancer.

## EVS

The study of EVs has gained significant momentum in recent years, because their cargo represents material of tumor which can shed light on the state of disease progression. EVs are membrane-bound organelles secreted by a variety of cells including cancer cells. The cytosol-derived lumen of EVs is enclosed by a lipid-bilayer forming a delivery vehicle for a variety of nucleic acids, proteins and lipids which can be horizontally transferred into recipient cells altering their biological properties. This allows cancer cells to continually modify their local microenvironment as well as distant sites when EVs enter circulation[108]. Because the molecular composition and function of these organelles represents their tumor origin, insight into EV biology provides great potential for tumor screening, diagnosis and prognosis. However, not all EVs are alike. The subcellular origin determines the type of cargo and mechanism of release from the cell. Large microvesicles (100-1000 nm) that bud outward from the plasma membrane are called ectosomes or ARRDC1-mediated microvesicles (ARMMs)[109]. Small EVs (30-150 nm) are called exosomes, which originate as intraluminal vesicles found in endosomal membranes and are secreted through fusion of multivesicular bodies (MVB) with the plasma membrane[110]. Several studies have identified exosomal subtypes based on molecular content that may hold diagnostic and prognostic value for diseases such as PDAC[110,111].

Exosomes play an active role in disease progression by promoting tumorigenesis, metastasis, tissue remodeling, immune evasion, and chemoresistance[111,112]. This is reported to be achieved by the delivery of microRNA,

mutated genomic DNA fragments, proteins and lipids which alter the biology of tissues that take up cancer-derived exosomes[112]. Exosomes offer several detection advantages over other biomarkers. Because exosomes travel across the endothelium into circulation they can be detected in serum and/or urine which can be collected over time when monitoring a patient[112]. Exosomal content can be dispersed within the lipid membrane bi-layer but can also be found in the lumen where it is protected from degradation by external nucleases and proteases[113]. Once exosomes are isolated, their content can be much easier to detect by sensitive techniques such as RT-PCR, next generation sequencing, gene expression microarrays, and mass spectrometry[111,114]. The first challenge in establishing exosome biomarkers as clinical tools depends on the ability to isolate them in sufficient quantity at high purity.

Initial isolation depends on crude physicochemical properties such as particle size, density and solubility. Isolation by differential centrifugation is the most classical method used by the biomedical research community. However, differential centrifugation typically results in low yield and always presents with some degree of contamination[108]. Recent developments have improved yield and purity through precipitation, affinity-based sorting by magnetic beads, and particle size-based isolation such as ultrafiltration and size exclusion chromatography[108,113]. Exosome isolation kits are now readily commercially available[108,115]. The identity and enrichment of exosomes in a biochemical fraction can be further defined by detection of endosome-specific tetraspanins (CD9, CD63, CD81), membrane transport and fusion proteins (flotilin, GTPases), MVB biogenesis-related proteins (Syntenin, Alix, ESCRT, TSG101), and heat-shock proteins (Hsp60, Hsp70, Hsp90)[110,113,116].

## EXOSOMAL CARGO

Given that most cells secrete exosomes, it can be a difficult task to distinguish cancer-specific material to that of healthy cells. When evaluating pathological relevance of exosome studies, purification methodology, exosome identification and presence of cancer-specific markers are essential components that should be taken into consideration. One of the most widely acclaimed PDAC exosome studies was recently presented by Melo *et al*[117]. The authors identified the presence of heparin proteoglycan GPC1, in exosomes isolated from breast and PC patients by ultracentrifugation and sucrose density gradient separation (followed by CD9, CD81 and flotillin 1 detection). Baseline GPC1 positivity was found in only 2.3% of healthy donors while elevated GPC1 expression was found in 75% of breast cancer subjects and among 100% of pancreas cancer patients ($n$ = 190). Relative concentrations of exosomes were much higher in the sera of cancer patients compared to healthy subjects. $GPC1^+$ exosomes were also detected prior to formation of PanIN lesions in 16-d-old mouse models of PDAC ($Ptf1a^{cre/+}$; $LSL\text{-}Kras^{G12D/+}$; $Tgfbr2^{L/L}$) with increased proportionality over time. Serum $GPC1^+$ exosomes in these models were present in circulation early before the onset of histological signs or MRI-detectable lesions. Further, the authors were able to use GPC1 positivity to distinguish healthy donors and those with benign pancreatic disease (BPD) from patients with histologically validated PC precursor legions (intraductal papillary mucinous neoplasm-IPMN) with a high degree of specificity (75%) and sensitivity (82%). Taken together, these findings suggest that PC cells secrete elevated levels of GPC1 positive exosomes which may be useful for early detection of tumors even prior to histologic manifestation[117].

EGFR is a receptor tyrosine kinase (RTK) activated in a subset of PC cells[118]. Adamczyk *et al*[119] found pancreatic cell lines (BxPC3, MiaPaca2, Panc1, Paca44 and A818-4 cells) secrete a 110 kDa soluble form of the EGFR ligand-binding extracellular domain (sEGFR) directly into conditioned media. A 170 kDa intact receptor and a 65 kDa processed form, including the intracellular kinase domain, are secreted as constituents of exosomes. Exosomes were separated from the secretome by ultracentrifugation and confirmed by exosome markers Alix, CD9, CD63 and Syntenin. The full-length EGFR was enriched 20-fold in exosomes along with 1600 other proteins found in the fraction by mass spectrometry[119]. The reason for compartmentalized release of these processed EGFR forms is currently not known. Soluble EGFR may provide a method for distant receptor transactivation or may confer EGFR positivity in cancer cells lacking EGFR expression. $EGFR^+$ exosomes may also enhance drug resistance by serving as a decoy for therapeutic antibodies. This has been observed in $HER2^+$ exosomes secreted by breast cancer cells that were shown to inhibit cell proliferation effects of Trastuzumab but not Lapatinib[120]. The next important step will be detecting $EGFR^+$ exosome in healthy and PC patients. Whether these isoforms possess any oncogenic mutations also needs to be explored before clinical use of these exosomes as cancer-specific biomarkers is considered.

*KRAS* is an oncogene that is mutated in 90% of PC cases[121,122]. Kahlert *et al*[123] isolated large (> 10 kb) double stranded genomic DNA fragments from EVs originating from PC cell lines and from serum of PDAC patients. Exosomes were purified from cell lines (Panc-1, T3M-4) and serum isolated from patients prior to surgical resection using ultracentrifugation after filtration. Exosomes were further verified by expression of CD9, TSG101, and CD63 by FACS analysis. By using whole genome sequencing, the authors demonstrated that PDAC serum exosomes contain not just mutated *KRAS* and *p53* oncogenes but also genomic DNA fragments spanning all chromosomes[123]. This suggests that genomic fragments can be isolated from purified PC exosomes and sequenced for analysis. Exosomes isolated from peripheral blood and pleural effusions can be sequenced to profile the genomes and transcriptomes of patients with pancreaticobiliary cancers[124]. Traditional tissue biopsies for these deeply located visceral cancers are difficult to safely acquire in

**Table 3 Challenges and potential solutions for pancreatic cancer diagnosis and treatment**

| Challenges | Potential solutions |
|---|---|
| Metastatic probability increases dramatically with larger tumor size | Promote development of early detection methods (circulating tumor cells, extracellular vesicles, molecular cargo in CTCs and EVs, cfDNA, ctDNA) |
| Tumor mutations develop up to two decades with metastatic mutations occurring late in the process | Identify founder mutations that correlate with unusual survival outcomes |
| Pancreatic stroma influences treatment sensitivity | Promote research on stromal characterization |
| Transporter expression in the tumor impacts drug delivery | Identify expression features that correlate with treatment sensitivity to a variety of drugs |
| CA 19-9 is not pancreatic cancer specific | Promote development of assays for biomarker panels that increase CA 19-9 utility that will be eligible for FDA approval |
| Prediction of resectability is only 70%-85% accurate | Improve staging based on biopsies by implementing clinical use of digital pathology methods |
| No FDA-approved digital pathology methods exist for pancreatic cancer | Combine digital pathology with accepted primary diagnostic methods and test special controls for digital imaging that will permit FDA application through a more streamlined *de novo* pathway |

CTC: Circulating tumor cells; EVs: Extracellular vesicles; cfDNA: Circulating free DNA; ctDNA: Circulating tumor DNA; FDA: Food and Drug Administration.

less specialized clinical centers. These studies create the possibility of performing genomic panel tests to identify oncogenic material from exosomes isolated from patients suspected of having elevated risk of PC or those where traditional biopsies are not feasible to obtain.

In addition to carrying genomic DNA, exosomes can also directly inhibit translation or target mRNA for degradation through the delivery of microRNA[125]. Exosomal miR-21, miR-212-3p and miR-203 and have been shown to enhance chronic pancreatitis, modulate immune response, and induce drug resistance[125-127]. Que *et al*[128] found elevated levels of miR-17-5p in serum exosomes of PC patients which correlate with metastatic stage, compared to healthy controls. Levels of exosomal miR-21 were also higher in PC patients *vs* healthy and chronic pancreatitis subjects but did not correlate with PC differentiation or stage[128]. The concentration of EVs in serum or plasma is almost one thousand times higher than in urine, a less invasive biofluid where exosomes remain stable at room temperatures for up to a week[115]. Ymir Genomics has developed a novel precipitation reagent, Ymirite, which isolates extracellular nucleic acids and vesicles from urine samples. Exiqon offers two exosome enrichment kits (miRCURY) for serum/plasma and urine isolation and a qPCR detection system (LNA™) for miRNA detection which enables profiling of biofluids where microRNA levels are extremely low. Further improvements in exosome and oncosome cargo characterization will significantly improve the clinical prospects of EVs.

## EXOSOMAL MARKERS THAT INDICATE PATHOGENIC EFFECTS OF PDAC

Once released into circulation, the destination of tumor-secreted exosomes can be directed through expression of membrane proteins that guide cellular targeting such as integrins, tetraspanins, phosphatidylserine receptors and heparin sulfate proteoglycans[117,129-131]. These features enable exosomes to reach distant sites where they can exert pathogenic effects secondary to the primary cancer. For example, PDAC derived exosomes have been shown to promote liver metastasis through expression of macrophage migration inhibitory factor (MIF) which induces a fibrotic microenvironment when taken up by liver resident Kupffer cells[112,132]. PDAC derived exosomes can also secrete TGF-beta which activates hepatic stellate cells to secrete fibronectin which in turn arrests bone-marrow derived macrophages and neutrophils to produce pro-tumorigenic cytokines in the liver[127,132].

Diabetes is a risk factor for PC but the association is complex[133]. Studies by Javeed *et al*[134] suggest that adrenomedullin (AM), secreted into circulation by pancreatic exosomes, reaches remote pancreatic beta cells to induce beta-cell dysfunction by inhibiting insulin secretion. The authors showed $AM^+$ exosomes, isolated by differential centrifugation, are secreted into cultured media by PC patient-derived primary cell lines as well as into portal/peripheral venous blood of PC patients. Additionally, these $AM^+$ exosomes also contain CA 19-9 making them an attractive PC biomarker[134]. Another PDAC-exosomal protein Bip, also impairs insulin secretion through interactions with pro-insulin[127]. These studies hold promise for potential diagnostic methods which may predict secondary complications to PC.

Detection of exosomes and their cargo presents some attractive qualities as a liquid biopsy technique. Advantages include the ability to capture tumor-derived material circulating before and during metastatic colonization, enable monitoring of treatment effectiveness and recurrence, enhance prognostic capability based on classifying molecular signatures, and serving to indicate secondary complications. Vesicle enrichment methods have been streamlined and standardized through the availability of commercial kits. The discovery of highly cancer-specific exosomal markers such as GPC1 will provide a foundation that could serve as the basis for

accurate and non-invasive diagnostic tests.

## CONCLUSION

The genetic evolution of PC is complex and may take up to two decades with metastatic mutations occurring relatively late in the process. Diagnosis is made at late stage where large genetic heterogeneity is observed within the tumor. With genotyping costs decreasing, it may be possible to predict drug sensitivity following resection through a combination of genomic profiling of the tumor, stromal density image processing and transporter expression determination. Digital analysis of the stroma from CT images has demonstrated the ability to predict a significant survival benefit for patients who undergo gemcitabine treatment. These methods pave the way for future applications in digital pathology as a means to increase prognostic potential and augment treatment decisions for personalized medicine.

While there is some room for refining existing treatment options to extend survival of late-stage PC patients, overcoming challenges for early detection of the disease will be paramount to significantly decrease the burden on the population (Table 3). Detecting physiologically relevant markers in exosomes and circulating tumor cells offers an advantage of testing with little or no discomfort to the patient. This creates the possibility to obtain serial samples of body fluids over time to allow monitoring of disease progression while eliminating risks associated with invasive biopsies. Combining information gained from these two types of tests could potentially increase diagnostic potential during early stages of PC development.

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This article is dedicated to Sgt. Mark Diehl.

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ORIGINAL ARTICLE

Retrospective Cohort Study

# Value of macrobiopsies and transanal endoscopic microsurgery in the histological work-up of rectal neoplasms: A retrospective study

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Author contributions: Bökkerink GMJ designed the study, collected the data, performed analysis and wrote the manuscript; de Jong D provided the endoscopy-reports and contributed to the discussion; van der Wilt GJ assisted in the statistical analysis and calculations to compare various work-up schemes; van Krieken HHJM provided the histopathological reports and contributed to the discussion; de Wilt JHW supervised the first author; Bleichrodt RPB and Bremers AJA designed the study, performed the definitive surgery and supervised the first author.

Institutional review board statement: This study was performed in accordance to the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments; this study was exempted from informed consent according to Dutch regulations.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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## Abstract

### *AIM*

To evaluate a step up approach: Taking macrobiopsies and performing excision biopsies in patients with suspected rectal cancer in which biopsies taken though the flexible endoscope showed benign histology.

### *METHODS*

Patients with a rectal neoplasm who underwent flexible endoscopy and biopsies were included. In case of benign biopsies rigid rectoscopy and macrobiopsies were employed. If this failed to prove malignancy, transanal

endoscopic microsurgery (TEM) was used in a final effort to establish a certain preoperative diagnosis. The pre-operative results were compared with the findings after surgical excision and follow up to calculate the reliability of this algorithm.

***RESULTS***

One hundred and thirty-two patients were included. One hundred and ten patients with a carcinoma and 22 with an adenoma. Seventy-five of 110 carcinomas were proven malignant after flexible endoscopy. With the addition of rigid endoscopy and taking of macrobiopsies, this number increased to 89. Performing TEM excision biopsies further enlarged the number of proven malignancies to 100.

***CONCLUSION***

The step-up approach includes taking macrobiopsies through the rigid rectoscope and performing excision biopsies using transanal endoscopic microsurgery in addition to flexible endoscopy. This approach, reduced the number of missed preoperative malignant diagnoses from 32% to 9%.

**Key words:** Rectal cancer; Histology; Biopsy; Macrobiopsy; Transanal endoscopic microsurgery; Sampling error



**Core tip:** Increasing the number of biopsies taken through a flexible endoscope, taking macrobiopsies and performing excision biopsies with transanal endoscopic microsurgery can reduce the number of missed preoperative malignant diagnoses in patients with rectal cancer.

Bökkerink GMJ, van der Wilt GJ, de Jong D, van Krieken HHJM, Bleichrodt RP, de Wilt JHW, Bremers AJA. Value of macrobiopsies and transanal endoscopic microsurgery in the histological work-up of rectal neoplasms: A retrospective study. *World J Gastrointest Oncol* 2017; 9(6): 251-256 Available from: URL: http://www.wjgnet.com/1948-5204/full/v9/i6/251.htm DOI: http://dx.doi.org/10.4251/wjgo.v9.i6.251

## INTRODUCTION

Adequate pre-treatment histological sampling is of paramount importance for the optimal treatment of rectal neoplasms. A wide spectrum of surgical and neoadjuvant treatments is available. In case of benign disease, surgical excision alone, will suffice. For a majority of the malignant tumors however, a combination of neoadjuvant therapy and total mesorectal excision is indicated to optimize local control[1-5]. High complete response rates after chemoradiation therapy have led to the development of organpreserving strategies[6-8].

Although the oncological benefits of neoadjuvant treatments are evident, the acute toxicity and long term side effects of chemoradiation therapy are considerable.

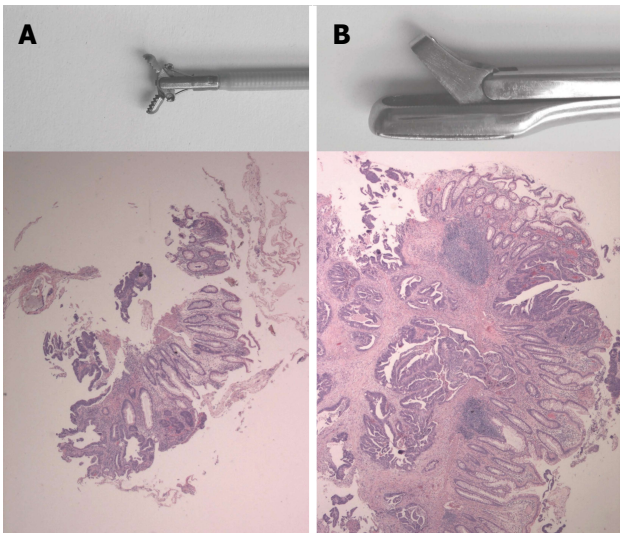


**Figure 1 Biopsy focepses and histological slides.** A: Biopsy forceps used with the flexible endoscope: Representative slide of a malignant tumor, HE × 20; B: Biopsy forceps used with the rigid endoscope, representative slide of the same tumor, HE × 20.

Therefore, administration of neoadjuvant chemoradiation therapy requires definite proof of malignancy. As the diagnosis of malignancy based on imaging alone may be erroneous because of the risk of overstaging MRI based imaging, these neoadjuvant treatments require histological evidence of malignancy before treatment can commence.

A preoperative histological diagnosis is usually obtained by taking biopsies through a flexible endoscope. Flexible endoscopy offers a high tumor detection rate[9] and the possibility to take biopsies. However, from limited evidence available, sensitivity for malignancy on these biopsies is suboptimal at best[10-12]. The most important reason for this is that biopsies taken through flexible endoscopes are small and sometimes too superficial to demonstrate high grade neoplasia[13]. In case of superficial biopsies, the diagnosis of malignancy relies solely on tissue structure and atypical appearance of cells (Figure 1). One way to overcome this problem is to take more biopsies. Indeed, several authors demonstrated a correlation between sensitivity and the number of biopsies taken from a suspected lesion. When 3 or 4 biopsies were taken, the sensitivity for invasive growth varied between 50% and 86%[10-12]. By taking up to 10 biopsies, the sensitivity increased to 78% to 100% (Table 1).

Another way to increase the sensitivity of pre-treatment histological sampling for the detection of malignancy is to increase the volume and depth of the biopsy. Although considered old-fashioned by many clinicians, rigid rectoscopy is an easy, cost effective, fast and well-tolerated tool for examination of the rectum[14], that enables the endoscopist to take so-called "macrobiopsies". Macrobiopsies are 2-10 times larger in three dimensions and approximately 50 times larger in volume than those obtained with flexible rectoscopy. The

**Table 1 Flexible endoscopy: Sensitivity for invasive growth; correlation with the number of biopsies**

| Number of biopsies | | ⩽ 2 | 3 | 4 | 5 | 6 | 7 | 8 | ⩾ 9 |
|---|---|---|---|---|---|---|---|---|---|
| Marshall (1993) | Sens | | | 68.3 | | 78.3 | | 78.3 | 78.3 |
| | *n* = 70[1] | | | 70 | | 70 | | 70 | 70 |
| Colleypriest (2009) | Sens | 80% | 86% | 86% | 88% | 98% | 100% | 98% | 100% |
| | *n* = 217 | Not specified | | | | | | | |
| Dabos (2011) | Sens | | 50% | 72% | 70% | 76% | 88% | 91% | 100% |
| | *n* = 149 | Not specified | | | | | | | |
| Current study | Sens | 40% | 30% | 76% | 75% | 83% | 50% | 91% | 72% |
| | *n* = 113 | 7 | 12 | 21 | 17 | 14 | 16 | 13 | 13 |

[1]Authors studied the value of reviewing 2, 4 and 6 additional biopsies, taken in every patient.

rigidity of the biopsy forceps also enables the endoscopist to push the forceps against the tumor so that deeper layers of the rectal wall can be included in the biopsy, and to "palpate" the lesion and take the biopsies from the firmer parts of the lesion selectively. For these reasons, rigid rectoscopy may perform better with respect to sampling error than flexible endoscopy.

Sometimes, even macrobiopsies may fail to demonstrate invasive growth. In an ultimate effort to obtain sufficient histological confirmation of malignancy without interfering with the optimal treatment strategy, transanal endoscopic microsurgery (TEM) may be used in these cases to perform an excision biopsy. TEM is an invasive way to obtain a histological diagnosis. However, it does have the advantage that it can sometimes be used as a definitive treatment for low risk T1 carcinomas.

Although there are sound theoretical grounds to expect that rigid rectoscopy and TEM can boost the sensitivity of the pre-treatment histological work-up for suspected rectal cancer, this has never been empirically investigated. The aim of this article, therefore, is to assess the accuracy, therapeutic value and tolerability of taking additional macrobiopsies and performing excision biopsies with TEM in patients with suspected rectal cancer: a step-up approach.

## MATERIALS AND METHODS

### Patients

All patients who underwent biopsy through a flexible endoscope, as part of the work-up for surgery of a rectal neoplasm, between January 2005 and January 2011 in the Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands were analyzed. Patient selection was based on the database of surgical procedure in our hospital. All patients who underwent surgical excision of a rectal neoplasm [local excision; transanal endoscopic microsurgery or total or partial mesorectal excision: Abdomino perineal resection or (low) anterior resetion] where selected. The medical records of all patients were reviewed for demographic characteristics and for endoscopy, pathology and surgical reports.

### Diagnostic and therapeutic algorithm

This is a retrospective analysis of the diagnostic and therapeutic step-up algorithm, which was followed during the study period. This algorithm is shown in Figure 2. Macrobiopsies were taken through the rigid sigmoidoscope in case of benign histology after flexible endoscopy and persisting clinical or radiological suspicion for malignancy, macrobiopsies were taken through rigid rectoscopy. TEM was performed in case of a benign or cT1 tumor on endorectal ultrasound (ERUS).

### Equipment

Flexible endoscopes were the CF140S 70 cm sigmoidoscope and CF 140 I colonoscope (Olympus, Tokyo, Japan). For flexible endoscopy, a 2.2 mm radial jaw biopsy forceps was used (Boston Scientific, Natick, United States) (Figure 1). For colonoscopy complete bowel preparation was used. Sedation and analgesia given upon request. During colonoscopy multiple biopsies were taken from any suspicious lesions. A 250 mm × 18 mm disposable rectoscopy tube, Heine, Herrsching, Germany was used for rigid rectoscopy. Biopsies were taken with a Franital biopsy forceps with a 5 mm × 10 mm bite (Figure 1). Bowel preparation before rigid and flexible sigmoidoscopy consisted of a single soap enema. All procedures were performed by, or under direct supervision of, consultant level surgeons or gastroenterologists.

TEM-surgery was performed by one of the authors (AB) as first described by Buess[15] using the stereo-optic Wolf rectoscope (Wolf, Knittlingen, Germany).

### Statistical analysis

The additional yield of taking macrobiopsies and performing excision biopsies was analyzed by comparing all biopsies with the definitive excision specimen. The differences in sensitivity between the number of samples taken through the flexible endoscope was tested with the chi square test for trends.

## RESULTS

### Patients

One hundred and thirty-two patients (82 males and 50 females) underwent flexible endoscopy with biopsies as part of the work-up for a rectal neoplasm (tumor located below 15 cm from the anal verge). Median age was 63 years (range: 27-92).

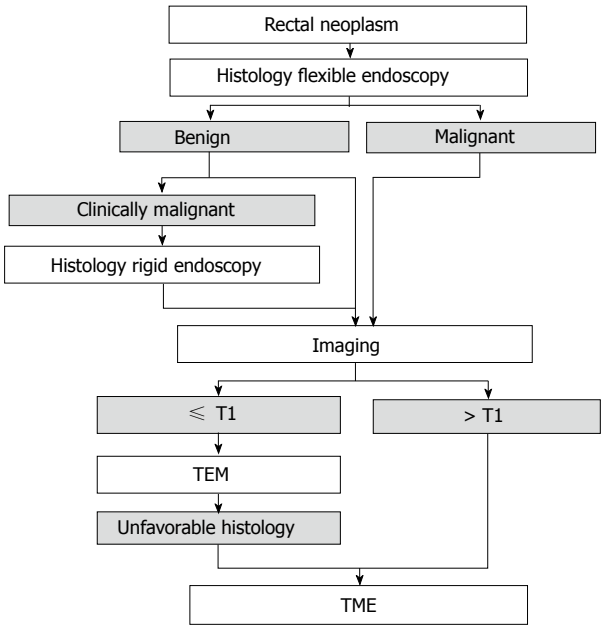


**Figure 2 Diagnostic and therapeutic algorithm.** TEM: Transanal endoscopic microsurgery.

### *Flexible endoscopy*

The histological work-up of all 132 patients is shown in Figure 3. At final pathology 110 patients had an adenocarcinoma, of which 75 (68%) were detected with flexible endoscopy only. The other 22 patients had a villous adenoma. One of the tumors, classified as malignant based on biopsies taken through the flexible endoscope (snare polypectomy), showed benign histology after (transanal) resection.

The number of biopsies was documented for 113 patients and varies from 1 to 14, with a median of 4 biopsies (Table 1). There was a significant correlation between the number of biopsies and a correct histological diagnosis ($P$ = 0.020; 2-sided $\chi^2$ test for trends). Taking 4 or more biopsies resulted in a significant higher sensitivity than taking 3 or less ($P$ = 0.004; 2-sided $\chi^2$ test for trends). Prior probability of malignancy was 83.3% in this group. Sensitivity and specificity were 68% and 95% respectively. A malignant result is useful with a posterior probability of malignancy of 99% (95%CI: 92%-100%). Benign histology after flexible endoscopy is clearly inconclusive, leaving a posterior probability of malignancy of 62% (95%CI: 55%-69%).

### *Rigid rectoscopy and macrobiopsies*

In 29 of the 56 patients who were diagnosed with a benign tumor after flexible endoscopy, additional rigid endoscopy was performed. With this addition, 14 previously undetected carcinomas were diagnosed. In this selected group of 29 patients who underwent rigid endoscopy, prior probability of malignancy was 75.9%. Sensitivity and specificity were 64% and 100% respectively, which makes a malignant histology after rigid endoscopy useful with a posterior probability of malignancy of 100% (95%CI: 68%-100%). Benign histology after rigid endoscopy is leaves a posterior probability of malignancy of 53%

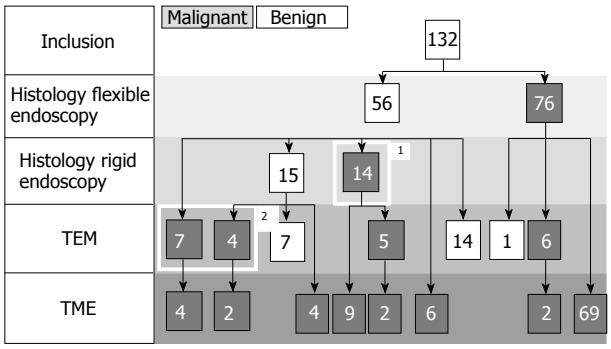


**Figure 3 Yield of macrobiopsies en excision biopsies.** [1]Fourteen not earlier detected carcinomas; [2]Eleven not earlier detected carcinomas.

(95%CI: 41%-68%). The remaining 27 patients did not undergo additional macrobiopsies taken through a rigid endoscope because there was no clinical suspicion of malignancy and endorectal ultrasound did not show invasion deeper than the submucosa (clinical benign or clinical T1). Further management was not dependent on histology analysis, since these lesions were regarded as indication for TEM for complete removal.

### *TEM*

A total of 44 patients underwent TEM (Figure 3), 32 patients after benign biopsies (combined flexible and rigid), 12 after malignant biopsies (clinical and radiological T1). With this addition, another 11 invasive carcinomas were detected. The number of detected carcinomas increased from 89 out of 110 (81%) to 100 out of 110 (91%).

Histology after TEM showed 18 adenomas, 4 *in situ* carcinomas, and 22 carcinomas. After TEM, 10 patients underwent a completion TME because of unfavorable histological findings. The excision specimen of one of these 10 patients was perforated at the former local excision site. One patient with an ypT3 tumor was unfit to undergo a total mesorectal excision and was treated with short course radiotherapy and TEM after a 6 wk interval. No major complications were observed nor preoperative perforations or conversions to laparotomy after TEM in this group. One patient with postoperative rectal blood loss needed transfusion.

### *Neoadjuvant treatment*

A total of 79 patients received neoadjuvant treatment in 4 different schemes according to tumor stage and general condition. Thirty-eight patients received 5 Gy × 5 Gy in the week prior to surgery according to protocol for T2 and T3 tumors. Thirty Patients with a radiologically involved circumferential resection margin received neoadjuvant chemoradiation therapy (25 Gy × 2 Gy with concomitant capecitabine) and delayed surgery after 8 wk. Eleven patients whose general condition did not allow chemoradiation therapy (CRT) and who required tumor regression received 5 Gy × 5 Gy ($n$ = 9) or long course radiotherapy (24 Gy × 2 Gy) ($n$ = 2) and delayed surgery as decided by a multidisciplinary team.

### *Surgery*

Forty-four patients underwent TEM, 53 underwent a LAR and a further 34 underwent APR, 1 patient with MSH6 mutation underwent a subtotal colectomy with LAR. After TEM 10 patients underwent a completion TME. Definitive histology after resection showed 18 adenomas, 4 *in situ* carcinomas, 101 carcinomas and 9 complete responses after neoadjuvant treatment.

## DISCUSSION

In the present study we demonstrated that macrobiopsies obtained through a rigid endoscope and excision biopsies by TEM are valuable additional tools to obtain a correct preoperative histological diagnosis in a significant number of patients with suspected rectal cancer.

Over time, flexible endoscopy has replaced rigid rectoscopy because of its superior (videoscopic) visualization of the entire colon, better mobility and deeper intubation[16-20] and subsequently a good tumor detection rate[9]. However, when it comes to the diagnostic sensitivity to detect malignancy in rectal tumors, our results are in accordance with the literature and confirm the disappointing overall performance of flexible endoscopy. The proportion of false negative biopsies after flexible endoscopy alone was 32%. This can be explained by the number of biopsies taken in our study. With a median number of biopsies of 4, a sensitivity of 70% can be expected.

Increasing the number of biopsies with flexible endoscopy can increase the number of detected malignancies in the group of suspicious rectal neoplasms (Table 1). However, increasing the number of biopsies through flexible endoscopy, as suggested by some authors[10-12], was not our main strategy to increase diagnostic sensitivity, because these biopsies are often too superficial to show high grade neoplasia[13]. Our algorithm included rigid endoscopy and TEM as additional steps.

In terms of accuracy, the selected group of patients with false negative biopsies after flexible endoscopy, 14 additional patients with a malignancy were identified with rigid endoscopy, and with TEM, another 11 patients. In total, 100 of 110 malignancies could be diagnosed preoperatively. This means that the proportion of carcinomas of which the malignant nature would have been proven in time was 32% with flexible endoscopy alone and was reduced to 9% in the evaluated algorithm. This is a significant reduction with high therapeutic value.

Regarding procedure-related morbidity, both rigid endoscopy and TEM were well-tolerated. In our experience, TEM did not cause an increase in positive circumferential resection margins (CRM) in TME as determined by standardized pathological evaluation according to Quircke[21].

### *Conclusion*

With the current treatment options for patients with rectal cancer, optimal preoperative histological diagnosis is essential. Besides the combinations with radical surgery, multimodality organ sparing treatments are becoming more and more accepted. Short-term results show high percentages of pathologic complete response[6,22] and acceptable oncological outcome[6,7], adequate histological sampling seems of paramount importance for these new treatment strategies, not only before but also after (chemo)radiation therapy.

In the present study we demonstrated that macrobiopsies obtained through a rigid endoscope and excision biopsies by TEM are valuable additional tools in obtaining a correct preoperative histological diagnosis in a significant number of patients with suspected rectal cancer. Prospective trials are needed to compare the yield of these strategies to increasing the amount of biopsies through flexible endoscopy. Evidence-based recommendations for guidelines regarding the histological work-up of rectal neoplasms can be based on those trials.

## COMMENTS

### *Background*

Histological sampling is one of the key components of the work-up for rectal neoplasms. For neoadjuvant and radical surgical treatments histological proof of invasive growth is mandatory. It can be difficult to obtain this proof with flexible endoscopy only. There are only a few publications available in which the sensitivity for malignancy of biopsies taken through a flexible endoscope is discussed. The aim of this study was to evaluate a step up approach: Taking macrobiopsies and performing excision biopsies in patients with suspected rectal cancer in which biopsies taken though the flexible endoscope showed benign histology.

### *Research frontiers*

An important subject in current rectal cancer research is the evaluation of organ sparing treatment techniques. Adequate pre-treatment histological sampling is of paramount importance for this treatment technique.

### *Innovations and breakthroughs*

Other studies evaluating the value of macrobiopsies and excision biopsies are not available in literature. More studies with larger populations need to be done to confirm the results from this study.

### *Applications*

This study can motivate the reader to take macrobiopsies and perform excision biopsies in daily practice.

### *Terminology*

Excision biopsy: Transanal local excision of (a part of) a rectal malignancy with the intention to assess its histology; Macrobiopsy: Large biopsy taken through a rigid recto- or sigmoidoscope.

### *Peer-review*

Bökkerink *et al* describe the use of macrobiopsies in the diagnosis of rectal cancer.

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ORIGINAL ARTICLE

Retrospective Study

# Effects of age on survival and morbidity in gastric cancer patients undergoing gastrectomy

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Author contributions: Fujiwara Y, Fukuda S and Tsujie M planned this study, and performed the data collection and statistical analysis; Ishikawa H, Kitani K, Inoue K, Yukawa M and Inoue M supported this study; all authors have read and approved the final version.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Nara Hospital, Kindai University.

Informed consent statement: Comprehensive agreement of clinical study was obtained in all patients at admission of our hospital. The analysis used anonymous and the detail of this study are published on the homepage of Nara Hospital, Kindai University.

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## Abstract

### *AIM*

To evaluate clinicopathological features and surgical outcomes of gastric cancer in elderly and non-elderly patients after inverse probability of treatment weighting (IPTW) method using propensity score.

### *METHODS*

We enrolled a total of 448 patients with histologically confirmed primary gastric carcinoma who received gastrectomies. Of these, 115 patients were aged > 80 years old (Group A), and 333 patients were aged < 79 years old (Group B). We compared the surgical outcomes and survival of the two groups after IPTW.

### *RESULTS*

Postoperative complications, especially respiratory complications and hospital deaths, were significantly more common in Group A than in Group B ($P < 0.05$). Overall survival (OS) was significantly lower in Group A patients than in Group B patients. Among the subset of patients who had pathological Stage I disease, OS was significantly lower in Group A ($P < 0.05$) than Group B, whereas cause-specific survival was almost equal in the two groups. In multivariate analysis, pathological stage, histology, and extent of lymph node dissection were

independent prognostic values for OS.

***CONCLUSION***

When the gastrectomy was performed in gastric cancer patients, we should recognized high mortality and co-morbidities in that of elderly. More extensive lymph node dissection might improve prognoses of elderly gastric cancer patients.

**Key words:** Gastric cancer; Mortality; Morbidity; Elderly; Lymphadenectomy; Propensity score matching; Prognosis; Survival



**Core tip:** Inverse probability of treatment weighting (IPTW) attempts to reduce the bias due to confounding variables in estimates of treatment effects. In the present study, we compared the surgical outcomes and survival of elderly gastric cancer patients with that of general population after IPTW. The overall survival of pStage Ⅰ gastric cancer patients in elderly was lower survival due to death of other diseases. We found that extent of lymph nodes dissection were independent prognostic factors. When the gastrectomy was performed in gastric cancer patients, we should recognized high mortality and comorbidities in that of elderly. This study was reviewed and approved by Nara Hospital, Kindai University review board on human research.

Fujiwara Y, Fukuda S, Tsujie M, Ishikawa H, Kitani K, Inoue K, Yukawa M, Inoue M. Effects of age on survival and morbidity in gastric cancer patients undergoing gastrectomy. *World J Gastrointest Oncol* 2017; 9(6): 257-262 Available from: URL: http://www.wjgnet.com/1948-5204/full/v9/i6/257.htm DOI: http://dx.doi.org/10.4251/wjgo.v9.i6.257

## INTRODUCTION

Gastric cancer is the fifth most common malignancy after cancers of the lung, breast, colorectal area and prostate; patients in Eastern Asia account for about half of the world's incidence[1]. In the past decade, incidence of gastric cancer in elderly patients has increased in Japan because of longer life spans of the general population[2]; decisions regarding gastric cancer surgeries in elderly patients have therefore also increased. Many surgeons are reluctant to have elderly patients undergo gastrectomies because of the considerably higher risk of complications from gastrectomies. There were some retrospective studies compared the outcomes of elderly gastric cancer patients to that of general populations, but the effects of age on morbidity, mortality from gastrectomy and/or prognosis are controversial, as no randomized studies have been conducted to our knowledge[3-18]. Also, no standard definition of "elderly" exists; thresholds vary from 65 to 80 years. Therefore, no standard guidelines for the treatment of elderly gastric cancer patients are available.

Recently, the concept of propensity score matching (PSM) and inverse probability of treatment weighting (IPTW) has garnered some attention. PSM and IPTW attempts to reduce bias due to confounding variables in estimates of treatment effects[19]. In the present study, we first evaluated the clinicopathological features and surgical outcomes of gastric cancer treated in our department among patients aged 80 years and older, and compared them with those of patients aged 79 years and younger, after IPTW. We then analyzed these data to find optimal cut-off ages for elderly patients with gastric cancer.

## MATERIALS AND METHODS

A total of 448 patients with histologically confirmed primary gastric carcinoma had gastrectomies in our department between 2005 and 2013. Of these, 115 patients were aged ⩾ 80 years old (Group A), and 333 patients were aged ⩽ 79 years old (Group B). All patients were American Society of Anesthesiologists risk less than three and there was no selection bias in each groups. Clinicopathological data for these patients were obtained from hospital records. Characteristics of two groups are shown and compared in Table 1. Postoperative complications were evaluated according to CTCAE Version 3.0; complications of grade ⩾ 2 were regarded as significant[20]. Tumor location, clinical or pathological stage, degree of lymph node dissection (D0, D1 or D2), and curability (R0, R1 or R2) were assessed according to the Japanese Classification of Gastric Carcinoma, 13$^{th}$, and then 14$^{th}$ editions[21,22]. Surgical mortality, morbidity, and hospital mortality were compared between two groups. Mean follow-up time for all patients was 34.57 mo (range: 0.16-113.13 mo). Recurrences were confirmed by computed tomography, tumor markers, and endoscopic examinations. Overall survival (OS) was defined as the time from the date of surgery to patient death (including surgery-associated death or hospital death), or the date of last available information concerning vital status. Cause-specific survival (CSS) is cancer survival in the absence of other cause of death or death from other cancers. CSS and OS were evaluated after IPTW method. This study was approved by our institute's committee on human research (Approval No.399): Comprehensive informed consent was obtained from all patients when they admitted our hospital prior to surgery.

### *Statistical analysis*

Clinicopathological variables between two groups were compared using the Mann-Whitney test or $\chi^2$ test. Survival analysis was carried out using Kaplan-Meier methods, and log-rank test was used to assess survival differences. $P < 0.05$ was considered significant. The propensity score (PS) was calculated using a multivariable logistic regression model with the two age groups as the dependent variables, and sex, cancer site, cT (14$^{th}$

**Table 1 Characteristics of patients in this study**

| | Group A | Group B | *P* value |
|---|---|---|---|
| Patients number | 115 | 333 | |
| Sex (Male: female) | 73/42 | 135/198 | |
| Mean age (yr) | 83.44 | 65.87 | < 0.05 |
| Occupied lesion | | | 0.693 |
| U | 24 | 81 | |
| M | 39 | 114 | |
| L | 52 | 138 | |
| Clinical stage (13th edition) | | | 0.446 |
| Ⅰ A | 40 | 137 | |
| Ⅰ B | 30 | 65 | |
| Ⅱ | 20 | 48 | |
| Ⅲ A | 9 | 39 | |
| Ⅲ B | 8 | 26 | |
| Ⅳ | 8 | 18 | |
| Lymph nodes metastasis | | | 0.639 |
| Negative | 76 | 212 | |
| Positive | 39 | 121 | |
| Histological type | | | 0.1224 |
| Intestinal | 70 | 175 | |
| Diffuse | 45 | 158 | |
| Operative procedures | | | 0.074 |
| Distal gastrectomy | 68 | 218 | |
| Total gastrectomy | 34 | 95 | |
| Proximal gastrectomy | 5 | 4 | |
| PPG | 3 | 11 | |
| Partial gastrectomy | 5 | 4 | |
| PD | | 1 | |
| Lymph nodes dissection | | | < 0.05 |
| D0 | 18 | 8 | |
| D1 | 60 | 61 | |
| D2 | 36 | 264 | |
| Curability | | | < 0.05 |
| Curative | 97 | 310 | |
| Non-curative | 18 | 23 | |

PPG: Pylorus preserving gastrectomy; PD: Pancreaticoduodenectomy.

edition), cN, clinical stage, operative procedures, and histological type (Lauren classification) as independent variables. Inverse probability of treatment weight (IPTW) was then calculated using PS. To evaluate the sensitivity and specificity of age in predicting 3-year OS, a time-dependent receiver operating characteristic (ROC) curve was calculated, and Youden's index was estimated to determine the optimal cutoff age. Univariate and multivariate analyses used the Cox proportional hazard model for OS after IPTW method. A stepwise method was used to estimate predictive variables for OS in multivariate analysis. Statistical analysis was performed using STATA version 14 (Stata Corp LP, College Station, TX, United States), R version 3.1.0 (R Project for Statistical Computing, Vienna, Austria), and SPSS Statistics version 22 (IBM, Tokyo, Japan).

## RESULTS

Patients' characteristics are shown in Table 1. Degree of lymph node dissection was significantly more extensive in Group B ($P < 0.05$), and non-curative dissection was more frequency in Group A ($P < 0.05$). Optimal cutoff age for gastrectomy in terms of OS was 79.2 years old (AUC = 0.642, TP = 0.536, FP = 0.248, Figure 1). Therefore, we set the cut-off age at 80 years old.

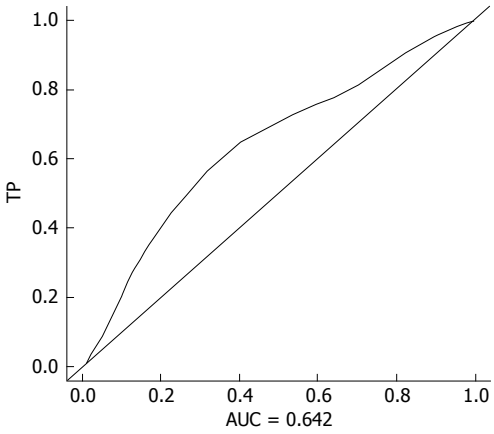


**Figure 1 Receiver operating characteristic curve for three years survival (AUC = 0.642, TP = 0.536, FP = 0.248).**

Postoperative complications are shown in Table 2. Respiratory complications and hospital death (including surgery-associated death) were more common in Group A ($P < 0.05$). After IPTW method, we found OS was significantly lower in Group A patients ($P < 0.05$; Figure 2A). The OS rates for Group A were 3-year: 46.6%, 5-year: 36.8%; those for Group B were 3-year: 74.8%, 5-year: 68.8%. Also, estimated CSS rates were significantly lower in Group A patients at 3-year, 5-year: 59.7% for Group A; and 3-year: 74.9%, 5-year: 69.1% in Group B ($P < 0.05$, Figure 2B). Among patients with pStage Ⅰ disease, OS was significantly lower in Group A ($P < 0.05$, Figure 3A), whereas CSS was almost equal in both groups (Figure 3B); their estimated 5-year CSS and OS rates were CSS: 92.07%, OS: 62.18% in Group A and CSS, OS: 94.7% in Group B. OS was lower in Group A because of death by other cancers and other diseases, included pneumonia.

Among patients with pStage Ⅱ-Ⅲ disease, CSS and OS rates were almost equal in the two groups. The 5-year estimated CSS/OS rates (same rates) for patients with pStage Ⅱ disease were 67.5% in Group A and 67.96% in Group B. Estimated 5-year CSS and OS rates for patients with pStage Ⅲ disease were CSS: 42.4%, OS: 22.16% in Group A and CSS, OS: 23.23% in Group B. However, among patients with pStage Ⅳ disease, estimated OS/CSS (same rates) were significantly lower in Group A than in Group B; estimated 5-year CSS/OS were 27, 1% in Group B and 0% in Group A, respectively (Figure 4).

Univariate analysis of prognostic factors for OS in Group A is shown in Table 3. We found pStage, radicality, lymph node metastasis and extent of LN dissection significantly affected prognoses ($P < 0.05$). In multivariate analysis, pStage, histology, and extent of lymph node dissection were independent prognostic values for OS (Table 4).

## DISCUSSION

In the present study, we evaluated clinicopathological

**Table 2 Postoperative complications compared between two aged group**

| | Group A ($n$ = 115) | Group B ($n$ = 333) | $P$ value |
|---|---|---|---|
| Anastomotic leakage | 5 (4.3) | 8 (2.4) | NS |
| Respiratory complications | 7 (6.0) | 7 (2.1) | < 0.05 |
| Other complications | | | |
| Pancreatitis | 3 (2.6) | 7 (2.1) | NS |
| Intraabdominal abscess | 0 (0) | 5 (1.5) | NS |
| Ileus | 1 (0.87) | 1 (0.3) | NS |
| Duodenal stump perforation | 1 (0.87) | 1 (0.3) | NS |
| Hepatic failure | 1 (0.87) | | |
| Cholecystitis | 0 (0) | 1 (0.3) | NS |
| Hospital death | 5 (4.3) | 3 (0.9) | < 0.05 |

**Table 3 Univariate analysis of overall survival in Group A patients after IPTW method**

| Variants | HR | 95%CI | $P$ value |
|---|---|---|---|
| Sex (male:female) | 0.941 | 0.515-1.720 | 0.845 |
| Tumor location (U:M:L) | 0.967 | 0.779-1.202 | 0.768 |
| Operative procedures (total:others) | 1.005 | 0.813-1.242 | 0.961 |
| Extent of LN dissection (D0:D1:D2) | 0.661 | 0.4233-1.032 | 0.009 |
| pStage (13th edition) (Ⅰ:Ⅱ:Ⅲ:Ⅳ) | 2.12 | 1.616-2.782 | 0.001 |
| Radicality (curative:non-curative) | 1.529 | 0.083-0.280 | 0.001 |
| pLN metastasis (negative:positive) | 2.332 | 1.274-4.272 | 0.006 |
| Postoperative complications (negative: positive) | 1.432 | 0.642-3.195 | 0.379 |
| Histology (Lowren) (intestinal:diffuse) | 2.637 | 1.470-4.729 | 0.01 |

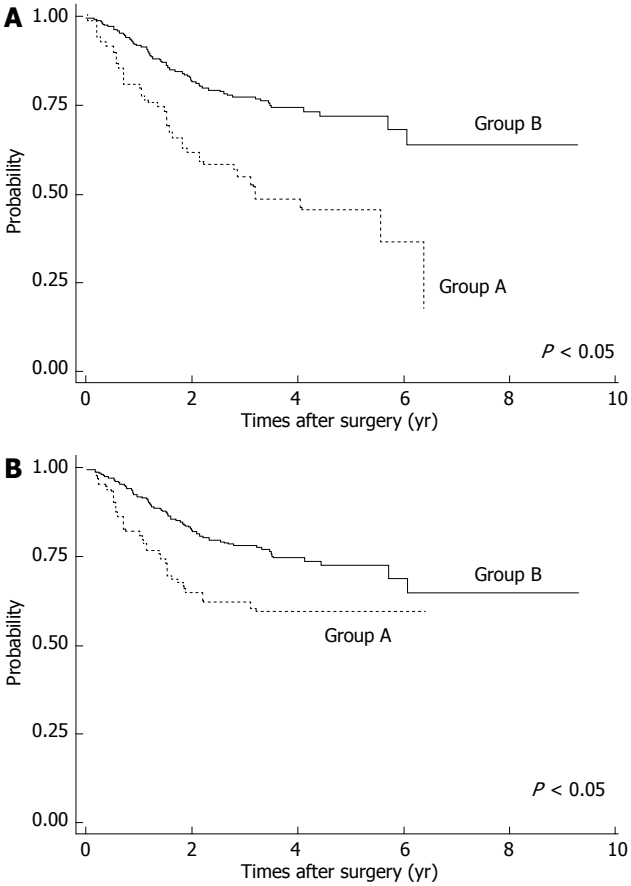


**Figure 2 Overall survival (A) and cause-specific survival (B) in two aged group after IPTW method.** OS and CSS were significantly lower in Group A than Group B ($P$ < 0.05). OS: Overall survival; CSS: Cause-specific survival; IPTW: Inverse probability of treatment weighting.

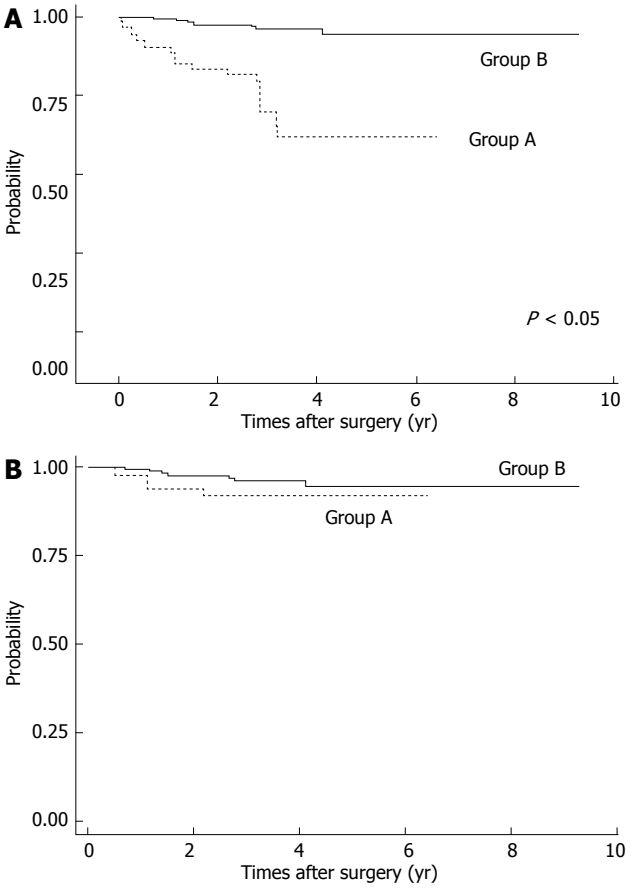


**Figure 3 Overall survival (A) and cause-specific survival (B) by age group among patients with pStage I gastric cancer who underwent gastrectomy.** OS was significantly lower significantly lower in Group A than Group B after IPTW method ($P$ < 0.05). OS: Overall survival; CSS: Cause-specific survival; IPTW: Inverse probability of treatment weighting.

features and survival of patients aged 80 years and older, compared with patients aged 79 years and younger after IPTW.

The optimal cut-off age for gastrectomies in elderly patients is controversial. The WHO classification defines "elderly" as older than 65 years old, "young-old" as 65-75 years old and "old-old" as older than 75 years[23]. In previously published studies of gastric cancer surgery in older patients, age thresholds ranged from 65 to 80 years old, so "elderly person" was not defined with regard to stomach cancer[4,5,7,8,11-17,24]. In the present study, we therefore used a survival ROC curve in patients with gastric cancer in terms of OS to determine the borderline age for gastrectomies, and concluded the optimal cut-off age is 79.2 years old, regardless of low AUC. Therefore, we divided the gastric cancer patients into two groups: 80 years and older (Group A, elderly group) and 79 years and younger (Group B, general population) in this study.

In general, morbidity and mortality of gastric cancer

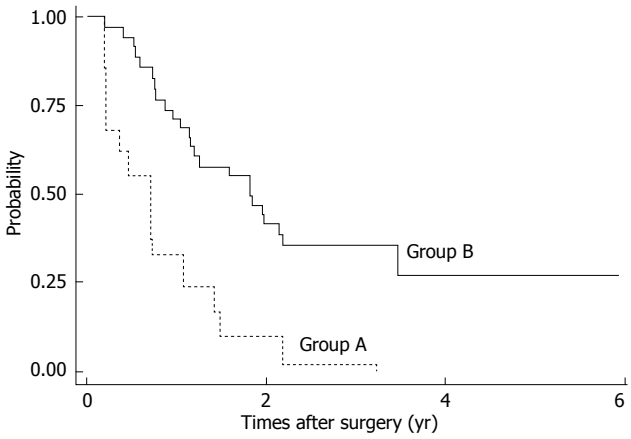


**Figure 4 Cause-specific survival and overall survival by age group among patients with pStage Ⅳ gastric cancer who underwent gastrectomy; after IPTW method.** CCS and OS were significantly lower in Group A than Group B ($P < 0.05$). OS: Overall survival; CSS: Cause-specific survival; IPTW: Inverse probability of treatment weighting.

patients after gastrectomy is controversial; mortality rates for elderly patients with gastric cancer who undergo gastrectomies range from 2% to 8.3% in the published data, which is compatible with our results[3-9,11,15]. Most reports did not find significant differences between the age groups, despite varying definitions of "elderly". In the present study, surgical mortality was significantly higher in Group A (4.8%) than in Group B (0.9%), possibly because the mortality rate of Group B was less than 1% in our institution. Among postoperative complications, respiratory complications were more frequent in Group A in the present study. Although postoperative respiratory complications in elderly patients have been reported, only two reports noted a high complication rate specifically in elderly patients with gastric cancer[4,6,8,11,15]. Postoperative respiratory complications of elderly gastric cancer patients might be associated with surgical mortality; they therefore warrant more careful postoperative attention.

In analyzing survival of patients with gastric cancer, we matched the two age groups using propensity scores; IPTW is considered to be a reliable statistical method for evaluating propensity scores[25]. Among patients with pStage Ⅰ disease, OS was significantly lower in Group A, but CSS was not significantly different. Lower OS for elderly pStage Ⅰ patients was due to surgical mortality, other causes of death, and death from other cancers. Therefore, careful observation after gastrectomy might improve survival of elderly patients with gastric cancer.

In multivariate analysis, we found that extent of lymph node dissection was independent prognostic factors in elderly patients with gastric cancer. Also, postoperative complications, especially respiratory complication and hospital death were more common in elderly group. However, relationships between extent of lymph node dissection and postoperative morbidity, mortality and prognosis in elderly gastric cancer patients are controversial in the literature[3,4,7,11].

Most of these reports showed that more extended lymphadenectomy in elderly patients did not affect postoperative complication rates or prognosis.

**Table 4 Multivariate analysis of overall survival in Group A**

| | Stepwise method ($P < 0.1$) | | |
|---|---|---|---|
| | HR | 95%CI | *P* value |
| pStage | 2.014 | 1.516-2.675 | 0.01 |
| Histology (Lauren) | 2.039 | 1.117-3.720 | 0.02 |
| Extent of LN dissection | 0.528 | 0.343-0.813 | 0.004 |

Only Eguchi *et al*[4] reported the extent of lymph node dissection in elderly gastric cancer patients to have influenced postoperative complications. Our findings indicate that more extended lymphadenectomy might improve survival in these patients if postoperative complications could be avoided.

In conclusion, our retrospective study indicated that optimal cut-off ages for elderly patients with gastric cancer was eighty years old, and suggests that even if curative surgery is performed for pStage Ⅰ disease in elderly gastric cancer patients, careful follow up is needed to stay abreast of other diseases, other cancers as outpatients. Additionally, more extensive lymph node dissection might improve prognosis of elderly patients with gastric cancer if postoperative complications could be minimized. However, postoperative complications lead to hospital death should be noted.

## COMMENTS

### Background

In the past decade, incidence of gastric cancer in elderly patients has increased in Japan. There was no randomized study compare the prognosis, morbidity and mortality of elderly gastric cancer patients and that of younger populations. Propensity score matching (PSM) and inversed probability of treatment weighting (IPTW) attempts to reduce bias due to confounding variables in estimates of treatment effects. They evaluated the clinicopathological features and surgical outcomes of gastric cancer treated in our department among patients aged 80 years and older, and compared them with those of patients aged 79 years and younger, after IPTW.

### Research frontiers

There were some retrospective studies compared the outcomes of elderly gastric cancer patients to that of general populations, but the effects of age on morbidity, mortality from gastrectomy and/or prognosis are controversial, as no randomized studies have been conducted to our knowledge.

### Innovations and breakthrough

PSM and IPTW attempt to reduce bias due to confounding variables in estimates of treatment effects. Quasi randomization is possible when they compared elderly group and younger group, statistically.

### Applications

The clinical significance of elderly gastric cancer patients received gastrectomy were evaluated and revealed the higher postoperative complications and mortality in elderly patients, and more extensive lymph node dissection might improve prognosis of elderly patients with gastric cancer.

### Peer-review

This is interesting to report the effects of age on survival and morbidity in gastric cancer patients undergoing gastrectomy. The author of this manuscript evaluated the gastric cancer patients received gastrectomy in elderly compared to that in younger population. Notably, this manuscript was compared the

results of these patients used propensity score.

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CASE REPORT

# Gastric plexiform fibromyxoma resected by endoscopic submucosal dissection after observation of chronological changes: A case report

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Author contributions: Kawara F, Tanaka S and Morita Y performed endoscopic resection and wrote the manuscript; Kawara F, Ohara Y and Okabe Y performed follow-up endoscopy and endoscopic ultrasound; Yamasaki T, Yokozaki H and Hirose T performed histopathological examinations; Hoshi N, Toyonaga T, Umegaki E and Azuma T contributed to the literature review and manuscript editing.

Institutional review board statement: This case report was exempt from approval by the Ethics Committee of Kobe University Hospital.

Informed consent statement: Informed consent was obtained from the patient.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.



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## Abstract

A 66-year-old man was diagnosed with a gastric submucosal tumor. Endoscopic ultrasound (EUS) revealed an iso/hypoechoic mass in the third layer. No malignant cells were detected in a histological examination. Yearly follow-up endoscopy and EUS showed the slow growth of the tumor. Endoscopic submucosal dissection (ESD) was performed and a glistening tumor was resected. The lesion showed a multinodular plexiform growth pattern consisting of spindle cells with an abundant fibromyxoid stroma that was rich in small vessels. The tumor was diagnosed as plexiform fibromyxoma (PF) by immunohistochemistry. Although difficulties are associated with reaching a diagnosis preoperatively, chronological changes on EUS may contribute to the diagnosis of PF. ESD may also be useful in the diagnosis and treatment of PF.

**Key words:** Plexiform fibromyxoma; Plexiform angiomyxoid myofibroblastic tumor; Endoscopic ultrasound; Endoscopic submucosal dissection; Gastrointestinal stromal tumor

**Core tip:** Plexiform fibromyxoma (PF) is a very rare gastric submucosal tumor. Therefore, difficulties are associated with diagnosing PF preoperatively, particularly in a differential diagnosis of gastrointestinal stromal tumors with cystic changes. We suggest that the chronological changes observed by endoscopic ultrasound contribute to the preoperative diagnosis of PF. Furthermore, endoscopic submucosal dissection needs to be considered for the diagnostic treatment of PF without muscle invasion.

Kawara F, Tanaka S, Yamasaki T, Morita Y, Ohara Y, Okabe Y, Hoshi N, Toyonaga T, Umegaki E, Yokozaki H, Hirose T, Azuma T. Gastric plexiform fibromyxoma resected by endoscopic submucosal dissection after observation of chronological changes: A case report. *World J Gastrointest Oncol* 2017; 9(6): 263-267 Available from: URL: http://www.wjgnet.com/1948-5204/full/v9/i6/263.htm DOI: http://dx.doi.org/10.4251/wjgo.v9.i6.263

## INTRODUCTION

Plexiform fibromyxoma (PF), also known as a plexiform angiomyxoid myofibroblastic tumor (PAMT), is a very rare gastric submucosal tumor (SMT) with a unique plexiform growth pattern of bland spindle cells[1-3]. Few studies have described the endoscopic ultrasound (EUS) characteristics of PF, and its chronological changes also remain unclear. We herein report a case of PF resected by endoscopic submucosal dissection (ESD) after a 4-year follow-up period.

## CASE REPORT

A 66-year-old man was referred to our institute for the management of a gastric tumor. An endoscopic examination revealed a SMT, approximately 20 mm in diameter, located in the antrum (Figure 1A). EUS showed an iso/hypoechoic mass in the third layer (Figure 1B). Computed tomography (CT) displayed a poorly enhanced lesion (Figure 2). Endoscopic biopsy and endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) were performed. Histological findings showed no malignant cells, and no further diagnosis was made.

Yearly follow-up endoscopy revealed the slow growth of the tumor, which became pedunculated and showed transpyloric prolapse (Figures 1C-F). EUS revealed gradual increases in the solid and multicystic components without muscle invasion. Based on these findings, our preoperative diagnosis was a hamartomatous inverted polyp[4-6]. In order to avoid outlet obstruction and reach a histological diagnosis, ESD was performed (Figure 3).

On dissection, a glistening, 40 mm × 30 mm tumor covered with normal gastric mucosa was identified. Microscopically, the lesion showed a multinodular plexiform growth pattern, and consisted of bland spindle cells

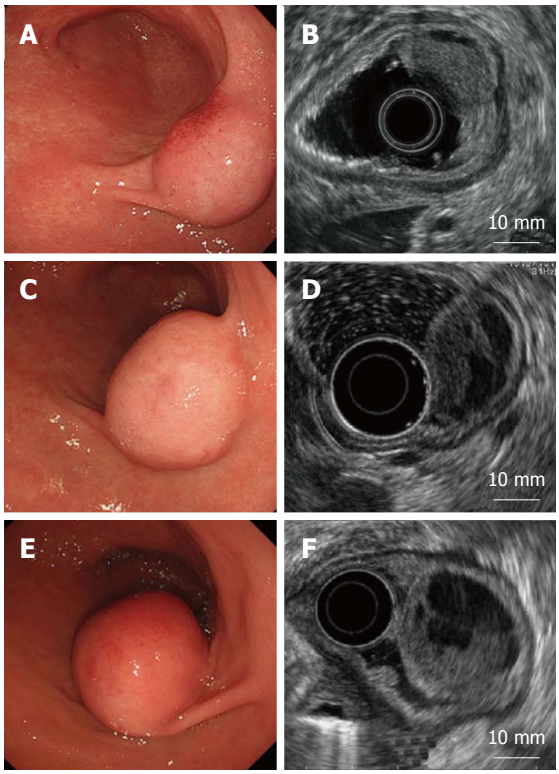


**Figure 1 Endoscopic and endoscopic ultrasound findings.** A: A submucosal tumor covered with a normal mucosa; B: An iso/hypoechoic mass with cystic components in the third layer; C, D: One year later; E, F: Four years later. The tumor increased in size and became pedunculated. Solid and multicystic parts both grew larger without muscle invasion.

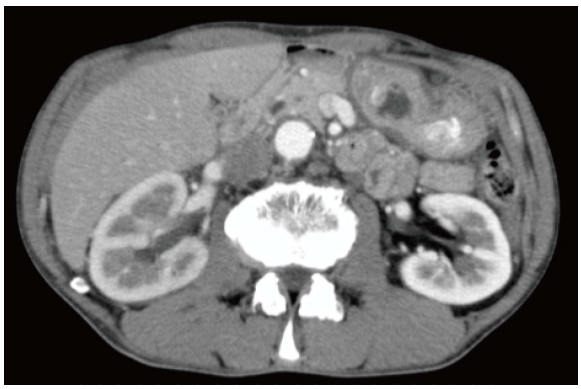

**Figure 2 Computed tomography of the patient.** A computed tomography scan revealed a poorly enhanced tumor in the antrum.

separated by abundant intercellular myxoid or fibromyxoid matrix. The stroma was rich in small vessels (Figure 4). Immunohistochemical tests revealed that tumor cells were focally positive for smooth muscle actin (SMA), muscle-specific actin (HHF35), and calponin, but were negative for c-kit, CD34, DOG-1, desmin, the S-100 protein, CD10, and h-caldesmon. The Ki-67 labeling index was approximately 2% (Figure 5). The pathological assessment led to a diagnosis of PF. Resected margins were histologically tumor-free. Although vascular invasion was positive, the patient did not undergo surgery due to the reportedly good prognosis of PF[1,7], and remained under careful observation

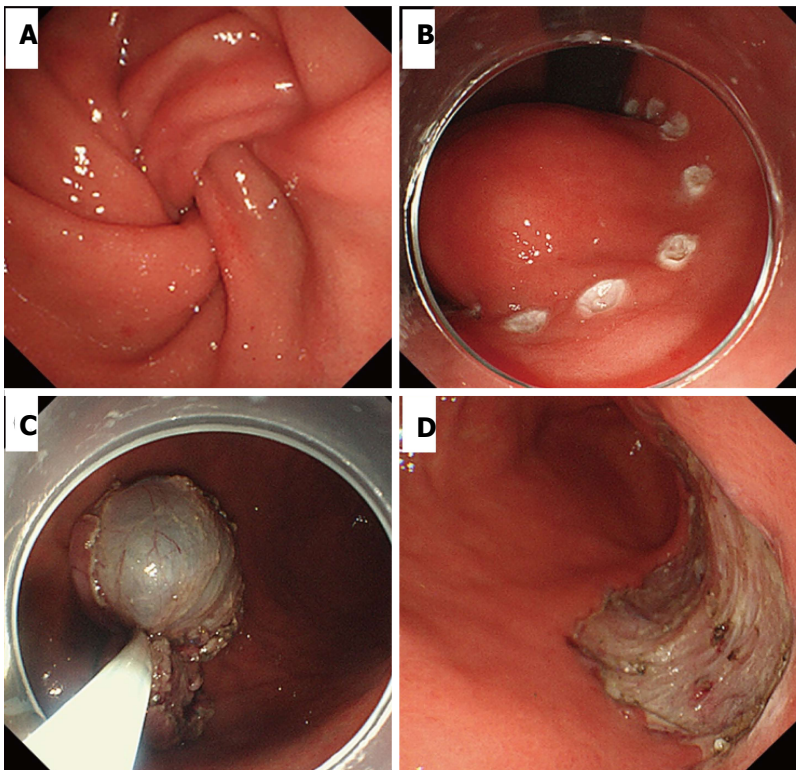


**Figure 3 Endoscopic submucosal dissection.** A: Tumor prolapse into the duodenum from the pylorus; B: Circumferential marking around the mass; C: Resected tumor retrieved using a snare; D: The ulcer bed after endoscopic submucosal dissection.

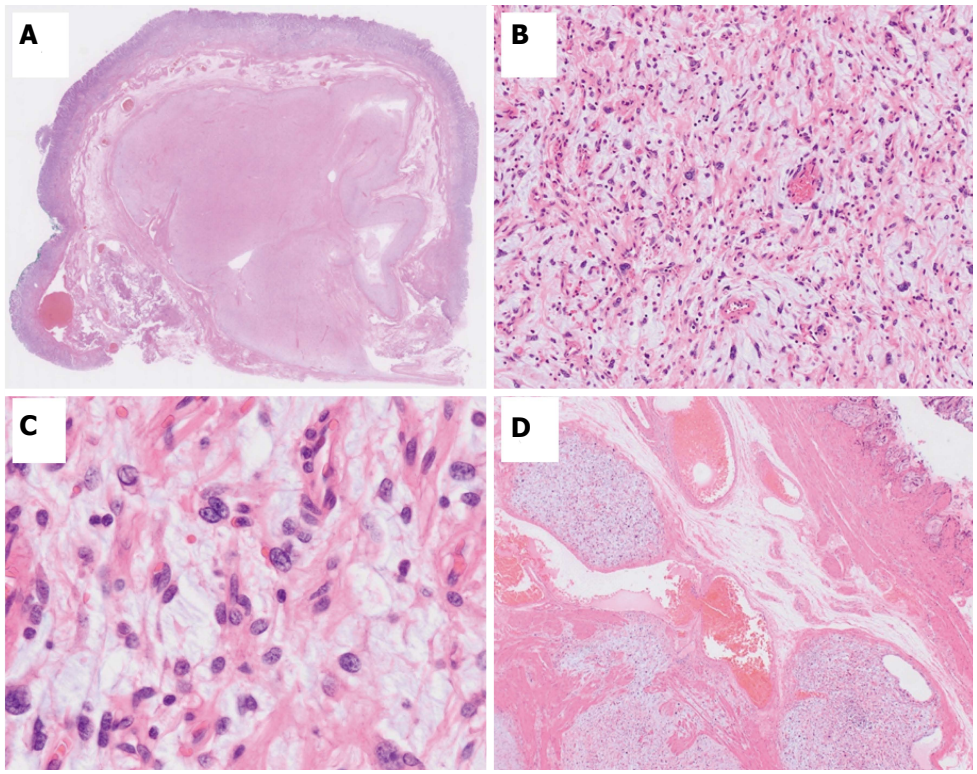


**Figure 4 Histological appearance of the tumor. The margins were histologically tumor-free.** A: The tumor showed a plexiform growth pattern; B, C: The tumor consisted of spindle-shaped cells with an abundant myxoid or fibromyxoid stroma; D: Some tumor cells intruded into the vessel space.

by endoscopy and CT follow-up. There was no recurrence or metastasis in the 12-mo follow-up.

## DISCUSSION

Gastric PF is a new benign mesenchymal tumor that has been adopted by the 2010 WHO classification of tumors of digestive system[8]. The term PAMT is also used for this type of tumor. The distinction between these terms has been controversial[7,9]. Previous studies reported that most cases of this tumor are found in the antrum, with approximately half extending into the extragastric

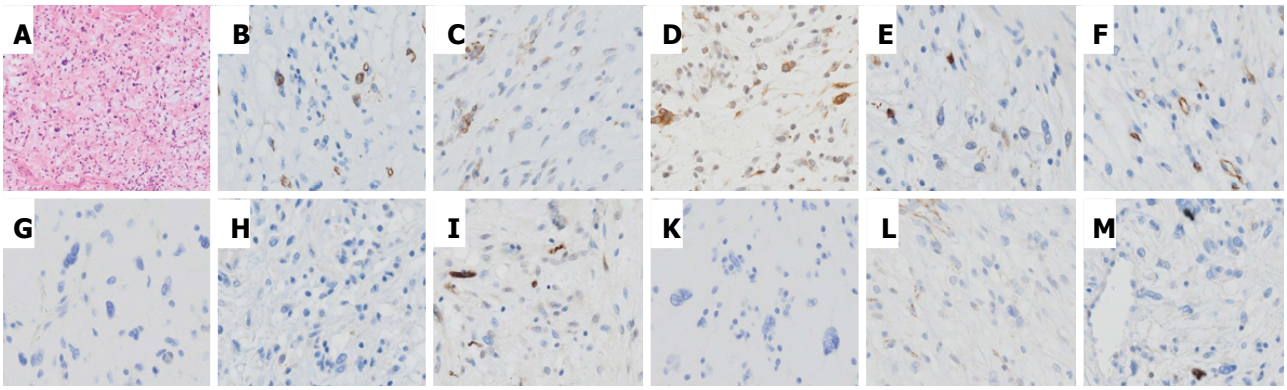


**Figure 5 Hematoxylin and eosin.** A: Histological appearance with hematoxylin and eosin (HE) staining; B-L: Immunohistochemically, tumor cells were focally positive for SMA (B), HHF35 (C), and calponin (D), but negative for c-kit (E), CD34 (F), DOG-1 (G), desmin (H), the S-100 protein (I), CD10 (K), and h-caldesmon (L); M: The Ki-67 labeling index was 2% at most.

soft tissues or proximal duodenum[2,7]. The diagnosis of PF is based on its histological features, including immunohistochemical findings[1]. Its histology indicates a plexiform growth pattern composed of spindle cells, fine small vessels, and a myxoid matrix. Tumor cells are typically immunoreactive for SMA and HHF35, whereas c-kit, CD34, DOG-1, and the S-100 protein are nearly completely negative. Focal immunoreactivity for CD10, caldesmon, or desmin has occasionally been detected[1,3,7].

In the present case, endoscopy and EUS showed that the tumor grew gradually, with increases in the solid and multicystic components. Spindle cells, with a rich vascular myxoid stroma, were considered to be detected as an isoechoic lesion and fluid leakage was observed as a hypoechoic lesion.

Previous studies reported the lack of recurrence or metastasis of PF after excision[1,7]; however, Miettinen *et al*[1] demonstrated that some plexiform elements showed intravascular involvement, suggesting that PF occasionally spreads through vessels. Since our case also exhibited vascular invasion, follow-up examinations were carefully performed. Since no patients have developed recurrence, annual endoscopy and CT are considered to be sufficient to monitor patients.

Although PF is considered to be benign, distal or partial gastrectomy is generally performed under the assumption of the presence of GIST[7]. Although GIST typically appears as a solid mass, few studies have described myxoid GIST that also shows a plexiform growth pattern[10], and some cases of GIST have shown cystic changes as a result of degeneration or necrosis[11-13]. Thus, it may be difficult to distinguish PF from these GIST by performing EUS only once. The chronological changes observed in the present case may contribute to a preoperative diagnosis of PF and the elucidation of its growth process. In this case, even though contrast-enhanced EUS was not performed, it may also be useful for reaching a differential diagnosis[14,15]. The distinction of PF from a hamartomatous inverted polyp is also important. EUS-FNA is the first choice for a definite diagnosis of SMT[16]. Nevertheless, ESD remains an important option for diagnostic treatment, including that for cases of gastric SMT of the submucosal layer[6,17]. Since EUS-FNA revealed no abnormalities in the present case, ESD was selected as a second choice. We performed *en bloc* ESD, which allowed for the diagnosis of PF. To the best of our knowledge, this is the first case report to describe the successful resection of PF by ESD. Further studies are needed in order to establish the appropriateness of ESD for PF.

## COMMENTS

### *Case characteristics*
A 66-year-old man presented with a gastric tumor located in the antrum.

### *Clinical diagnosis*
Gastric submucosal tumor.

### *Differential diagnosis*
A hamartomatous inverted polyp, myxoid gastrointestinal stromal tumor (GIST), and GIST with cystic degeneration.

### *Laboratory diagnosis*
Laboratory test results were within normal limits.

### *Imaging diagnosis*
Endoscopic ultrasound revealed an iso/hypoechoic mass of 20 mm in diameter in the third layer, and it showed gradual increases in the solid and multicystic components without muscle invasion.

### *Pathological diagnosis*
Plexiform fibromyxoma.

### *Treatment*
Endoscopic submucosal dissection was performed as a diagnostic treatment.

### *Related reports*
Few studies have described plexiform fibromyxoma, also known as a plexiform angiomyxoid myofibroblastic tumor. Patients with plexiform fibromyxoma have generally undergone distal or partial gastrectomy.

### *Term explanation*
Plexiform fibromyxoma is a new mesenchymal tumor entity that shows a unique

plexiform growth pattern of bland spindle cells.

### *Experiences and lessons*

Plexiform fibromyxoma needs to be considered in a differential diagnosis of gastric submucosal tumors, and follow-up endoscopic ultrasound (EUS) may be able to distinguish plexiform fibromyxoma from other gastric submucosal tumors.

### *Peer-review*

The rarity of the case could be enriched with a brief review of the literature, due to the scarce number of papers reporting similar tumors. Moreover it could be interesting to expand data about EUS, for example explaining the characteristics of elastometry and eventual contrast enhancement. The quality of the article is augmented by the images, which are impressive and clear. Overall it is a good paper.

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