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Mucins as contrast agent targets for fluorescence-guided surgery of pancreatic cancer

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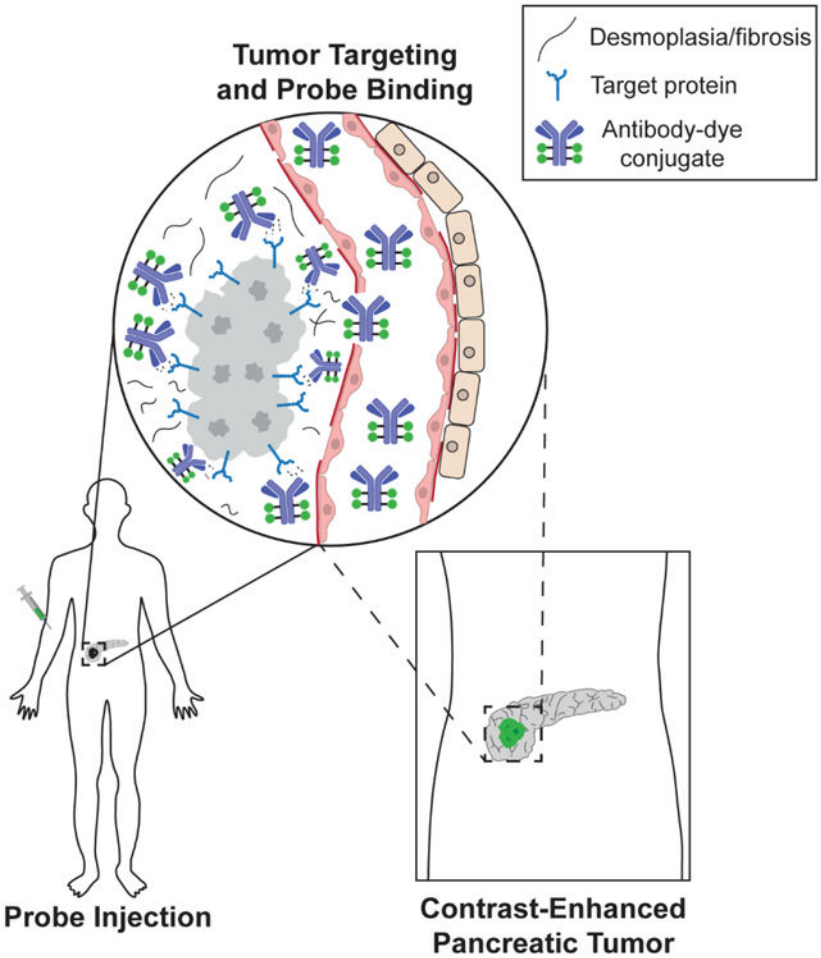
Aaron M. Mohs: NIH funding related to fluorescence-guided surgery targeting mucins. Patents related to contrast agents and imaging systems for fluorescence-guided surgery.

Declaration of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abstract

Pancreatic cancer is difficult to resect due to its unique challenges, often leading to incomplete tumor resections. Fluorescence-guided surgery (FGS), also known as intraoperative molecular imaging and optical surgical navigation, is an intraoperative tool that can aid surgeons in complete tumor resection through an increased ability to detect the tumor. To target the tumor, FGS contrast agents rely on biomarkers aberrantly expressed in malignant tissue compared to normal tissue. These biomarkers allow clinicians to identify the tumor and its stage before surgical resection and provide a contrast agent target for intraoperative imaging. Mucins, a family of glycoproteins, are upregulated in malignant tissue compared to normal tissue. Therefore, these proteins may serve as biomarkers for surgical resection. Intraoperative imaging of mucin expression in pancreatic cancer can potentially increase the number of complete resections. While some mucins have been studied for FGS, the potential ability to function as a biomarker target extends to the entire mucin family. Therefore, mucins are attractive proteins to investigate more broadly as FGS biomarkers. This review summarizes the biomarker traits of mucins and their potential use in FGS for pancreatic cancer.

Graphical Abstract



Keywords

optical surgical navigation; intraoperative molecular imaging; mucins; biomarkers; fluorescence

1. Introduction

Biomarkers are an integral tool in diagnosing and treating cancer [1]. They can be employed as predictive or prognostic tools to aid in diagnosis and therapy selection [2]. Biomarkers such as PSA, CA125, CA19-9, transthyretin, APOA1, β2-microglobulin, transferrin, follicle-stimulating hormone, and human epididymis protein 4 have been utilized as clinical diagnostic markers for prostate, pancreatic, and ovarian carcinomas [2]. However, biomarkers lack widespread implementation in the clinic due to test variation, issues in clinical validation, and low specificity and sensitivity [3]. Biomarkers may be utilized for intraoperative imaging of tumor resections. Surgical navigation for tumor resection can encompass many different imaging modalities, such as near-infrared fluorescent (NIRF) imaging, photoacoustic imaging, surface-enhanced Raman spectroscopy, and multimodal imaging [4]. The use of NIRF contrast agents for fluorescence-guided surgery (FGS) is expanding for real-time surgical imaging of tumors since it is cost-effective, easily accessible in surgery for intraoperative imaging, and provides excellent tumor-to-background contrast [5-7]. Optimal biomarkers for FGS show a distinguishable difference in expressed protein or other pathophysiological parameters, like decreased extracellular pH, between normal and malignant tissues. Mucin proteins are optimal targets for FGS based on their differential expression in malignant tissue; therefore, they have been investigated as potential FGS targets [5, 8-21]. Additionally, mucins may have a role as general biomarkers, as their expression levels are easily monitored and often correlated with a disease stage or prognosis. Two cleaved mucin proteins, CA125 and CA19-9, have been utilized as clinical biomarkers to distinguish disease progression and recurrence in ovarian and pancreatic cancers [2, 22]. Currently, twenty-one members of the mucin family are classified into two main groups, transmembrane, and secreted mucins. Transmembrane mucins are located in the cell membrane and have cytoplasmic, transmembrane, and extracellular domains [23]. In healthy tissues, they play a role in barrier formation and protein-receptor and cell-cell interactions [23-25]. These mucins are MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, MUC21, and MUC22 [9, 23-35]. Conversely, secreted mucins coat the epithelial surface of the cell [24]. These mucins create a barrier around healthy cells to protect against infiltrating invaders such as bacteria and viruses [23, 32]. Most secreted mucins are classified into one of two groups, gel-forming and soluble. MUC2, MUC5AC, MUC5B, and MUC6 are gel-forming secreted mucins [24, 35], while soluble, secreted mucins are MUC7, MUC8, and MUC9 [26, 31, 35]. The final secreted mucin, MUC19, belongs to neither subgroup [23, 25, 31]. Due to their expression pattern and correlation with disease staging, mucins are optimal targets for FGS in pancreatic cancer.

2. Mucin Expression in Normal versus Malignant Tissue

FGS biomarkers rely on differential expression between normal and malignant tissue for effective contrast enhancement. Several mucins can potentially be FGS biomarkers due to their differential expression between normal and malignant tissues. While mucins are expressed in healthy tissue, malignant tissue can dysregulate the expression of mucins to aid in tumor development.

2.1. Mucin Expression in Normal Tissues

Mucins have a wide range of expression in normal tissue (Table I). For example, the pancreas has minimal MUC11 and MUC17 expression, as well as MUC3A, MUC5B, MUC6, and moderate MUC12 expression, while small pancreatic ducts moderately express MUC1 [23, 33, 34, 36-42]. The range of minimal to high expression allows researchers to choose the optimal contrast agent target in the tissue. According to the Human Protein Atlas (www.proteinatlas.org), MUC20 is normally expressed in the kidney, bladder, vagina, cervix, fallopian tube, ovary, prostate, thyroid gland, pituitary gland, small intestine, colon, stomach, liver, pancreas, salivary gland, and esophagus, while MUC22 is expressed in the esophagus and vagina [43]. While mucin expression is found in many normal tissues, the proteins are often minimally expressed. In normal tissue, mucins act as lubricants and function in cellular differentiation, adhesion, signaling, tissue protection, and immune responses [23, 24, 30, 31, 33, 44]. This expression pattern differs from the mucin expression pattern in many malignant tissues.

2.2. Mucin Expression in Malignant Tissue

Several malignant tissues have differential or new mucin expression compared to normal tissue expression (Fig. 1). These patterns are beneficial in determining potential mucin biomarkers for FGS. Pancreatic ductal adenocarcinoma (PDAC) expresses MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC12, MUC13, MUC16, MUC17, MUC20, and MUC21 [14, 24, 27, 28, 30, 33, 35, 37, 39, 42, 44, 51-53, 67, 75, 76, 79, 80, 83, 90, 93, 95, 104-126]. MUC6 is similarly expressed in pancreatic cancer compared to normal pancreatic tissue [37, 39, 104]. MUC2 is also expressed in intraductal papillary mucinous neoplasm of the pancreas and pancreatic colloid carcinoma, a noninvasive form of pancreatic cancer [26, 34, 120]. Small bowel carcinoma expresses MUC1, MUC2, MUC3A, MUC4, MUC5AC, and MUC6 in varying intensities [70, 127]. Specifically, MUC2, MUC3A, and MUC5AC are upregulated, while MUC4 and MUC6 display similar expression [70, 127]. Colon adenocarcinoma aberrantly expresses MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC19, MUC20, and MUC21 [15, 24, 30, 32, 36, 44, 47, 51-53, 62, 66-68, 72, 75, 79, 80, 82, 91, 99, 107, 109-111, 119, 128-137]. Specifically, MUC2, MUC11, MUC12, and MUC17 are downregulated or have similar expression in colon cancer compared to normal colon tissues [36, 51, 62, 66, 110, 130, 132, 136, 137]. Gao et al. found that the mRNA expression of MUC4 was downregulated in colon cancer compared to normal tissue [79]. However, other researchers found that MUC4 protein expression was upregulated in colon cancer compared to normal tissue [15]. Esophageal cancer differentially expresses MUC1, MUC4, MUC16, and MUC19 [44, 59, 81, 99,

113, 119]. In gastric carcinoma, MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC13, MUC16, MUC17, and MUC20 are aberrantly expressed [30, 32, 44, 49, 51-53, 60, 69, 75, 97, 109-111, 114, 119, 131, 133, 136]. MUC1, MUC5AC, and MUC6 have similar expression in gastric carcinoma compared to normal tissue [49, 52, 53, 69, 136]. MUC16 is expressed in gastrointestinal cancer [95]. Lung cancer expresses MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC12, MUC13, MUC15, MUC16, MUC17, MUC19, MUC20, MUC21, and MUC22 [23, 24, 30, 35, 44, 48, 61, 75, 79, 80, 85, 93, 95, 99, 103, 107, 111, 113, 114, 128, 131, 138, 139]. MUC1, MUC3A, MUC4, MUC6, MUC7, MUC9, MUC12, MUC13, MUC15, MUC16, and MUC20 are differentially expressed in RCC [35, 44, 51, 55, 79, 88, 129, 140-142]. Specifically, MUC1, MUC3A, MUC12, and MUC13 are upregulated in RCC, while MUC6, MUC15, and MUC20 are downregulated in RCC [55, 140, 141]. In RCC, MUC4 and MUC9 have similar expression in malignant tissues compared to normal tissue [55]. Salivary gland carcinoma differentially expresses MUC1, MUC4, MUC5AC, MUC5B, MUC6, and MUC19 with MUC1, MUC4, MUC5AC, MUC5B, and MUC19 upregulated compared to normal tissue [35, 54, 77, 101]. Thyroid cancer expresses MUC20 [109]. Breast carcinoma expresses MUC1, MUC2, MUC3A, MUC4, MUC5B, MUC6, MUC7, MUC16, and MUC19 [24, 30, 35, 44, 51-53, 63, 67, 78, 79, 86, 93, 98, 99, 107, 110, 111, 113, 115, 116, 143-146]. Ovarian carcinoma differentially expresses MUC1, MUC2, MUC4, MUC5AC, MUC6, MUC9, MUC13, MUC16, and MUC20 [24, 27, 30, 32, 35, 45, 52, 67, 75, 79, 87, 93, 95, 102, 104, 109, 111, 112, 115, 116, 133, 135, 147, 148]. Compared to normal ovarian tissue, MUC1, MUC4, MUC13, MUC16, and MUC20 are upregulated in ovarian carcinoma [30, 32, 45, 75, 79, 93, 102]. MUC1, MUC5B, MUC8, and MUC20 are upregulated in endometrial cancer [45, 109, 135, 149]. MUC16 is expressed in uterine cancer [95, 150]. MUC1 and MUC4 are significantly upregulated in cervical cancer; however, there is no significant increase in MUC5B, MUC8, and MUC16 expression in cervical cancer [44, 45, 79, 113, 133]. Prostate cancer downregulates MUC1 and MUC4 expression and upregulates the expression of MUC3A [24, 35, 46, 50, 79, 107, 111, 151, 152]. Bladder cancer expresses MUC1, MUC4, MUC5B, MUC7, MUC16, and MUC20, while gallbladder cancer expresses MUC1, MUC4, MUC5AC, MUC6, and MUC16 [35, 56, 75, 79, 88, 153]. Specifically, MUC4 is downregulated in bladder cancer [56]. Cholangiocarcinoma expresses MUC1, MUC2, MUC4, MUC5AC, MUC6, and MUC16 [76, 112, 153-156]. Hepatocellular carcinoma expresses MUC1 [44]. Appendiceal cancer expresses MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC16, and MUC17 [44, 129, 137]. Thymoma expresses MUC4 [79]. Brain cancers such as meningioma and glioblastoma express MUC4 [115, 157]. Head and neck squamous cell carcinoma express MUC4 [113, 116]. Melanoma expresses MUC4 and MUC19 and downregulates the expression of MUC20 compared to normal skin tissue [75, 99, 158]. Specific mucin staining intensity in cancer compared to normal tissue can be found in Figure 2. Based on mucin expression in malignant versus normal tissue, many malignant tissues aberrantly express mucins making these proteins optimal FGS targets.

Altered glycosylation is one distinct difference between mucin expression in normal and malignant tissues. Glycosylation is a common post-translational modification on proteins that results in the attachment of O or N-linked oligosaccharides to the protein contributing to its final structure and function [24, 159]. Mucins are generally under-glycosylated in

malignant tissue compared to normal tissue [34, 47, 69, 87, 160]. For example, MUC1 is normally found with long glycosylation chains in normal mammary cells; however, in breast cancer, these glycosylation chains are truncated, changing the mucin's structure [47]. Similarly, MUC1 glycosylation decreases from normal colon tissue to colon cancer [47]. However, under-glycosylated and glycosylated forms of MUC1 were overexpressed in pancreatic cancer and precursor lesions [34]. This is significant as altered mucin glycosylation has been correlated with the progression of tumor cell pathways [161]. Additionally, there is evidence that high levels of glycosylation in normal tissue impede an antibody from binding to the mucin protein; therefore, antibodies may target under-glycosylated mucins more efficiently [47]. The location of mucin glycosylation may also alter binding sites allowing some antibodies to bind, while impeding the binding of others [47]. Therefore, mucin glycosylation is an important consideration in the expression and targeting of mucins.

3. Mucins and Pancreatic Cancer Diagnosis, Stage and Prognosis

Over the years, mucins have been investigated for their correlation with a pancreatic cancer diagnosis, staging, and prognosis. Mucins, such as MUC1, MUC4, MUC5AC, and MUC16, have shown the ability to detect pancreatic cancer with high sensitivity and specificity. For example, CA19-9, cleaved from MUC1, is a widely used biomarker for pancreatic cancer detection and is recommended for use by the United States Food and Drug Administration [42, 124]. Many studies have investigated CA19-9 and CA125, cleaved from MUC16, alone or in combination for their sensitivity and specificity in diagnosis and prognosis [95]. CA19-9 was shown to be highly specific and sensitive for pancreatic cancer, with a diagnostic sensitivity and specificity of around 80% and a prognostic sensitivity at 80% [95]. Additionally, CA125 showed high sensitivity and specificity in diagnosis (SN: 60-70% and SP: 80%) and prognosis (SN: 74.2% and SP: 100%) [95]. In combination, CA19-9 and CA125 showed diagnostic sensitivity and specificity at 80% and 90%, respectively [95]. Another study showed a diagnostic sensitivity of 68% for CA125 in pancreatic cancer [125]. Similarly, MUC16 was shown to have a diagnostic sensitivity of 62.9% and a specificity of 100% for pancreatic cancer [27]. CA19-9 has been investigated with MUC5AC to increase its diagnostic sensitivity and specificity. Kaur et al. found that the MUC5AC and CA19-9 combination increased the diagnostic sensitivity from 79% to 83% and the diagnostic specificity from 43% to 83% when distinguishing pancreatic cancer from benign controls and chronic pancreatitis [106]. Likewise, they found a similar increase in diagnostic significance when distinguishing early-stage pancreatic cancer from benign controls and chronic pancreatitis (SN: 67% to 83% and SP: 67% to 83%) [106]. Zhang et al. found an increased diagnostic specificity when distinguishing early and late-stage pancreatic cancer from benign controls using MUC5AC and CA19-9 combined (SP: 61.8% to 88.6%) [105]. Finally, MUC4 showed a diagnostic sensitivity of 74% and a specificity of 100% for pancreatic cancer [27]. These studies indicate the ability of mucin biomarkers to diagnosis pancreatic cancer with high sensitivity and specificity. Other diagnostic uses of mucins have been reviewed in Cox et al. and Kaur et al. [62, 126].

Mucin upregulation is correlated with increased pancreatic cancer stage and poor prognosis. MUC1, MUC2, MUC3A, MUC4, MUC5AC, MUC5B, MUC6, MUC9, MUC13, MUC16,

MUC17, MUC20, and MUC21 are all correlated with either stage or prognosis in the development of pancreatic cancer. In particular, high expression of mucins, MUC1, MUC4, MUC5AC, MUC15, MUC16, MUC17, MUC20, and MUC21 was significantly correlated with a worse prognosis and overall survival [42]. FGS utilization in pancreatic cancer has the potential to target multiple mucins to improve the amount of tumor resected.

MUC1 expression increases as pancreatic tumors progress [33, 39, 104, 120], contributing to the poor prognosis of late-stage PDAC patients [33, 104, 120]. Rachagani et al. found that MUC1 expression increased as pancreatic lesions in Kras mice progressed from PanIN-I to PDAC [33]. MUC1 expression is differentially upregulated in pancreatic lesions compared to normal tissue, with stronger staining seen in PanIN-3 and PDAC tissues [39]. Overall, Higashi et al. concluded that MUC1 expression is upregulated in all stages of PDAC [112].

MUC2 correlation with stage and prognosis is more commonly seen in pancreatic colloid carcinoma than in PDAC. As pancreatic lesions progress to PDAC, MUC2 upregulation is only found in PDAC stages [39]. Specifically, MUC2 expression is correlated with late-stage PDAC and poor prognosis [104, 112]. Conversely, in pancreatic colloid carcinoma, MUC2 expression increases from early intraductal papillary mucinous neoplasms (IPMNs) to colloid carcinoma [120]. MUC2 expression in pancreatic colloid carcinoma is associated with a better prognosis [120].

MUC3A expression in pancreatic lesions increases from the precancerous stages of PanIN-I to PanIN-III with a decrease in expression in PDAC [40]. This indicates that MUC3 is correlated with PanIN stages of pancreatic cancer development. High MUC3A expression is correlated with a poor PDAC prognosis [107, 129].

Similarly, MUC4 expression increases as pancreatic cancer progress from PanIN-I to PDAC with minimal to no expression in normal pancreas and pancreatitis [27, 37, 40, 122]. In a Kras mouse model, Rachagani et al. determined that MUC4 expression increased significantly early in the development of the model with minimal increase in expression seen in later stages of tumor development [33]. MUC4 upregulation in PDAC is an indicator of a poor prognosis [33, 75, 104].

In pancreatic cancer, MUC5AC is correlated with later stages of tumor progression [39]. Kim et al. found that MUC5AC has strongly increased expression from the PanIN-IA stage to PDAC [39, 106]. Within PDAC stages, MUC5AC slightly increased in expression from stage IA to stage IV [106]. Since MUC5AC is a secreted mucin, Zhang et al. compared the serum levels of MUC5AC in healthy controls, pre-pancreatic cancer stages, and pancreatic cancer [105]. They found that MUC5AC serum levels were significantly upregulated in pancreatic cancer compared to the controls and precancerous tissues [105]. In a Kras pancreatic cancer mouse model, MUC5AC expression increased during the development of the tumor [33]. While MUC5AC correlates with tumor stage, the correlation between mucin upregulation and the prognosis is inconsistent. MUC5AC upregulation is correlated with a poor prognosis of pancreatic cancer [33]. However, Higashi et al. found that MUC5AC expression in late-stage PDAC is correlated with better prognosis [112]. High MUC5AC mRNA expression is also correlated with a better prognosis in PDAC [83].

MUC5B expression in pancreatic cancer is correlated with tumor development and prognosis. MUC5B has been shown to be upregulated in pancreatitis compared to PDAC [37]. Researchers found that MUC5B upregulation in PDAC indicates poor prognosis [104].

Studies have indicated that MUC13 expression is upregulated in all stages of PDAC [108]. However, MUC13 nuclear stain is correlated with increasing stages from stage I to stage IV [108]. MUC13 upregulation is associated with poor prognosis as the suppression of MUC13 in animal models decreased the size of the tumor and increased the survival rate [90].

As PDAC progresses, MUC16 upregulation is higher in the advanced stages of cancer [112, 125]. MUC16 expression increases as pancreatic cancer progress from PanIN-I to PDAC [117, 118]. MUC16 upregulation in pancreatic cancer is correlated with poor prognosis [104, 112, 117, 119].

Some mucins are not correlated with either pancreatic cancer stage or prognosis. MUC6 expression is associated with disease stage but does not correlate with prognosis. Chronic pancreatitis has higher MUC6 expression than PDAC stages [37]. MUC6 expression in the development of cancer decreases from PanIN-IA to PDAC [39]. Specifically, MUC6 expression decreases significantly as PDAC stages progress from stages I and II to stages III and IV [112]. MUC6 expression in PDAC is similar to the protein's expression in normal pancreatic ducts [39]. Conversely, there is no significant correlation between MUC6 expression and PDAC disease prognosis [42, 83]. While MUC6 expression is not significantly correlated with disease prognosis, MUC9 correlation with disease stage progression is unknown. In tumor prognosis, MUC9 is associated with a better prognosis [104]. MUC17, MUC20, and MUC21 have no correlation with stage progression [104]. Conversely, upregulation of these mucins is correlated with poor prognosis in PDAC [104]. Unlike the other mucins expressed in pancreatic cancer, the relationship between MUC12 expression and the prognosis and stage of pancreatic cancer is unknown [42].

Pancreatic tumor resection occurs in early stages of tumor development; therefore, it is important to know the pattern of mucin expression in tumor staging. Mucins with increasing or high expression in early tumor stages may serve as FGS targets in pancreatic cancer. Since mucin expression differs in pancreatic cancer staging, these proteins could be investigated for expression colocalization to target multiple expression patterns in a patient. Like tumor stage expression, prognosis correlation can be a tool for FGS by potentially indicating the growth of the tumor.

The correlation of mucin family members with pancreatic cancer stage or prognosis can be found in Table II.

4. Fluorescence-Guided Surgery

Surgical resection is the only potentially curative treatment for pancreatic cancer [8, 14, 17, 21]. One limiting factor in the resection of pancreatic cancer is the inadequacy of available tools to guide the extent of resection. Currently, surgeons rely on intraoperative tactile and visual cues to achieve a complete resection of the tumor [4, 5, 8, 14]. Pancreatic cancer is especially challenging to resect due to high desmoplasia, tumor growth, and metastasis at

diagnosis [8, 11, 28, 162]. Desmoplasia is highly cross-linked stromal tissue that comprises a majority of the tumor composition and contributes to tumor growth and metastatic rates [162]. These challenges contribute to high rates of incomplete resections seen in 50-80% of patients that undergo surgery [14]. Currently, patients eligible for surgical resection have high rates of recurrent disease, with 60% of patients developing recurrent pancreatic cancer within 2 years of resection [14]. The presence of metastatic disease often contraindicates tumor resection; however, it is difficult to perceive metastatic disease in bright-field surgical imaging [11, 163]. Clinicians may treat patients with neoadjuvant chemotherapy prior to surgery to potentially downstage the tumor and decrease metastatic disease [12, 163-165]. However, patients may still develop recurrent diseases after the addition of neoadjuvant chemotherapy [12]. Therefore, visual tools to aid resection may improve overall survival post-resection.

4.1. FGS Contrast Agents

While many options exist for clinical cancer imaging, there are challenges associated with the translation to intraoperative imaging. Clinical imaging modalities such as CT, MRI, and PET have limited use as real-time surgical imaging techniques since they are expensive and not adaptive to the surgical theatre [5, 13, 166]. To visualize tumors intraoperatively, FGS uses a contrast agent-conjugated probe, e.g., an antibody or vitamin, to target a protein differentially expressed in malignant tissue compared to normal tissue [4, 9, 167]. Fluorescent dyes such as Cy5.5, indocyanine green (ICG)-sulfo-OSu, fluorescein, AlexaFluor 488, DyLight 650, and IRDye800CW have been employed as conjugated contrast dyes for FGS (Fig. 3A) [5, 8-16]. While any fluorescent dye may be used, NIRF dyes are the most common. Dyes in the near-infrared (NIR) window are optimal due to their low scattering, minimal background fluorescence, and higher tissue penetration [4, 5, 9]. Reactions such as NHS ester coupling, cysteine residue engineering, or maleimide coupling are used to conjugate the fluorophore to the targeting moiety (Fig. 3B) [168-173]. Once conjugated, these probes can be implemented in FGS, which have been reviewed in detail elsewhere [5, 174].

4.2. Targeting Mucins for FGS

Mucins have potential use in targeting pancreatic cancer as their expression is often upregulated in PDAC tumor stages compared to normal tissue and pancreatitis. Thus, they may serve as intraoperative biomarkers for targeted contrast agents. A limited number of mucins have been investigated for FGS of pancreatic cancer, including MUC1, MUC4, MUC5AC, and MUC16. For example, MUC5AC can be targeted for intraoperative imaging of PDAC since there is a significant difference in expression in PDAC compared to chronic pancreatitis, healthy pancreas, and healthy duodenum [175].

MUC1 and its cleaved portion, CA19.9, have been targeted by many research groups as a biomarker target for imaging of pancreatic cancer. Houghton et al. explored using two antibody probes to target CA19-9 in pancreatic cancer [17]. They found that the fluorophore-conjugated probe and the dual radio and fluorophore-labeled probe bound specifically to the CA19-9-positive tumor, grown with a pancreatic cancer cell line, with no binding in the CA19-9-negative tumor (Fig. 4A). They also observed minimal

background signal, producing at least a 25-fold stronger signal in the tumor than background organs. Additionally, Houghton et al. investigated the efficacy of their antibody probe in an orthotopic pancreatic cancer model as well as in targeting metastases. In addition to the subcutaneous model, their antibody probe specifically targeted the orthotopic tumor and identified metastatic lesions in the mouse [17]. McElroy et al. studied an antibody-fluorophore conjugate targeting CA19-9 in pancreatic cancer for surgical benefits using an orthotopic tumor model [11]. They found that the antibody probe targeted the tumor and metastases with no binding in the healthy pancreas or stroma. Hiroshima et al. targeted treated and untreated pancreatic cancer utilizing a CA19-9-targeting antibody conjugated with DyLight 650 [12]. They found the antibody bound to both the untreated and treated orthotopic patient-derived xenograft tumors *in vivo* [12]. Wu et al. targeted MUC1 by utilizing an antibody designed to bind to the extracellular region of the MUC1 C-terminal domain of the MUC1 protein [19]. They found that the antibody tagged with DyLight 755 accumulated specifically in the subcutaneous pancreatic cell line tumor model with minimal background signal (Fig. 4B). The antibody-dye signal was strongly visible in the mouse model 48 hours after injection. Park et al. utilized a commercially available MUC1 antibody to target subcutaneous and orthotopic pancreatic tumors [18]. They investigated the efficacy of the antibody-dye conjugate in two pancreatic cancer cell lines grown subcutaneously and orthotopically. They observed that the antibody-dye conjugate bound to all tumor models with higher tumor signal compared to background noise.

Additionally, Turner et al. investigated the targeting of both MUC4 and MUC5AC in pancreatic cancer for surgical imaging [14, 176]. They have conducted preliminary studies targeting a subcutaneous pancreatic cancer tumor model using MUC4-IR800, which targets MUC4 expression in the tumor [176]. They found that the tumor-to-background ratio was greater than 2, leading to more studies. Investigating MUC5AC, they used an antibody-dye conjugate, MUC5AC-IR800, to target the tumor for *in vivo* imaging of an orthotopic patient-derived xenograft model (Fig. 4C) [14]. The antibody-dye conjugate targeted the tumor with little background signal. Additionally, the conjugate was able to target nearby tumor invasion into the abdominal wall. Liu et al. conducted preliminary research on an antibody probe to determine its binding to MUC5AC in PDAC [177]. The researchers investigated the binding of the humanized antibody, clivatuzumab, to bind to MUC5AC in pancreatic cancer cell lines. This antibody was found to bind to MUC5AC and confirmed with two validated MUC5AC antibodies.

Olson et al. used an antibody-conjugated probe, AR9.6-IRDye800, to target MUC16 for the surgical removal of pancreatic cancer [8, 21]. They showed that the antibody was able to target MUC16 in the model and detect metastatic disease. Further, Olson et al. showed the ability of the humanized novel antibody-probe to bind to MUC16-positive patient-derived xenograft pancreatic tumors (Fig. 4D) [8].

Overall, the researchers concluded that their antibody-dye contrast agents specifically targeted mucin expression found in PDAC. Liu et al. concluded that targeting MUC5AC with an antibody probe may be a successful candidate for future research in treating MUC5AC-expressing cancers, including PDAC. Ultimately, these probes show positive

results for future targeting and treatment of PDAC. However, the current research could be expanded to investigate other mucin-targeting probes in PDAC.

4.3. FGS in Clinical Trials for Pancreatic Cancer

To date, five clinical trials have investigated contrast agents and conjugates for targeted FGS of pancreatic cancer (Table III). Vahrmeijer et al. completed a clinical trial on the antibody-dye conjugate, SGM-101, with a contrast agent, BM104, targeting CEA expression in PDAC [178, 179]. In research published by Gutowski et al., they found that the antibody specifically targeted an orthotopic pancreatic with a tumor-to-background ratio of 3.5. Panitumumab-IRDye800 is another antibody-dye conjugate that has entered a clinical trial for pancreatic cancer [180]. Lu et al. researched this antibody-dye conjugate targeting EGFR for pancreatic cancer FGS [181]. They found that panitumumab-IRDye800 targeted the pancreatic tumor with liver background fluorescence in pancreatic cancer patients. Also targeting EGFR expression, cetuximab-IRDye800 entered clinical trials for FGS of pancreatic cancer [182]. Tummers et al. found that surgeons were able to identify the tumor enhanced by the cetuximab-IRDye800 conjugate [183]. VEGF-A has also been investigated in clinical trials for FGS in pancreatic cancer using bevacizumab-800CW [184]. However, the antibody-dye conjugate was unable to produce a sufficient tumor-to-background ratio [184, 185]. In addition to antibodies, other modalities have been investigated via clinical trials. Fields et al. conducted a clinical trial on cancer vision goggles in pancreatic cancer and other gastrointestinal tract tumors [186]. The goggles are designed to image a probe targeting phosphorylated Annexin A2 (pANXA2), LS301, in a patient [187]. This trial was terminated with no results posted. Finally, Vahrmeijer et al. investigated a peptide, cRGD, conjugated to a zwitterionic dye, ZW800-1, for FGS in pancreatic cancer [188]. In results published by Handgraaf et al., cRGD-ZW800-1 is specifically bound to an orthotopic pancreatic tumor with minimal background signal [189].

While these biomarkers have progressed in the FGS field, there are still areas to improve. In particular, VEGF-A is not expressed at the membrane limiting its use in FGS, while EGFR is widely expressed in normal tissue [179]. Additionally, background tissues such as the stomach and duodenum normally express EGFR [183]. Mucins in pancreatic cancer have an advantage as many-are membrane-bound proteins with minimal expression in the pancreas or in surrounding background organs. There is a broad field for FGS of pancreatic cancer, where additional mucin biomarkers will have a clear role in a growing array of targets.

4.4. Imaging Systems and Agents

Instrument standardization is needed to grow FGS in clinical research [4, 190]. FGS instruments contain NIRF channels with detection ranging from low pM in a hand-held instrument to the nM range in stationary instruments and depths around 5 mm [190-193]. These instruments must be highly sensitive and specific in order to reduce background noise and achieve accurate intraoperative images [194]. Pre-operative imaging modalities, like PET, could be used in conjunction with FGS to aid imaging, as PET is more sensitive and specific compared to NIR imaging [4].

Multimodal imaging systems allow clinicians and surgeons to compare preoperative images with real-time intraoperative imaging. In addition, most clinically available laparoscopic and robotic systems have near-infrared fluorescence modes that allow for minimally invasive assessment of tumor extent. This may aid clinicians and surgeons in their decisions on the best course of treatment. Furthermore, these probes may be useful for the delivery of treatment payload to a tumor. As the application of FGS expands into the clinical field, researchers may use other materials or macromolecules besides antibodies to target mucin biomarkers for surgical resection in pancreatic cancer and other cancers. For example, a research team targeted MUC1 by using a Cy5.5-tagged iron oxide crosslinked nanoparticle with a MUC1-targeting peptide to detect tumors and monitor tumor growth [9, 20]. Moore et al. found that their nanoparticles could target MUC1+ tumors *in vivo*, produce high-resolution T2-weighted MRI images using the iron oxide core, and capture real-time NIRF imaging with the Cy5.5 dye. The introduction of the iron oxide core at the tumor site induced a change in T2 relaxation states in the tumor thus distinguishing it from nearby normal tissue. This research investigated the widespread application of the nanoparticles in many MUC1-positive tumors, specifically focusing on the probe's ability to detect with minimal background signal and monitor subcutaneous and orthotopic models of pancreatic cancer [9, 20]. Mucin targeting also has applications in preoperative settings. Sharma et al. researched an immunoPET antibody conjugate to target ovarian and pancreatic cancer [195]. They showed that their probe was able to specifically target the tumor *in vitro* and *in vivo*. This highlights the use of mucin-targeted probes for PET imaging and the potential to create multimodal antibody probes. The use of multimodal nanoparticle probes provides opportunities for research on the use of other nanoparticle probes and quantum dots in FGS. Other materials, such as antibody fragments and peptides, may be utilized as imaging modalities for FGS [170, 196, 197]. There are many opportunities to utilize these materials to improve and expand the field of FGS.

5. Opportunities and Key Points

Utilizing mucins as targets for FGS is an emerging field for surgical navigation. A limited number of the targetable mucins have been explored or are currently being explored for FGS. Additionally, the expression colocalization of mucins has yet to be explored. Targeting mucin colocalization may increase the number of patients benefitting from FGS. Mucin probes are not just limited to one imaging modality. They can be utilized in preoperative, intraoperative, and postoperative imaging due to dual imaging modalities such as NIR/PET or NIR/MRI.

One consideration for mucin-targeting is the presence of mucin expression in pancreatic cancer risk factors, such as smoking and pancreatitis [198, 199]. Momi et al. found that MUC4 expression increased in smoke-exposed pancreatic cancer [200]. Conversely, mucin expression in pancreatitis tends to be minimal, with much lower expression compared to pancreatic cancer with some exceptions [21, 27, 37, 42, 104-106, 108, 118, 122, 126]. In particular, Andrianifahanana et al. found that the total RNA of MUC5B and MUC6 was increased in chronic pancreatitis patients compared to total the RNA from PDAC patients [37]. These results indicate that mucin expression does vary with pancreatic cancer risk

factors. However, the expression fluctuation should not greatly impact the use of mucins in FGS for pancreatic cancer resection.

In addition to target investigation, the development of FGS models is an important consideration for future studies. For accurate results, models should be as similar to the clinical field as possible. Many mouse models, such as tumor cell line subcutaneous and orthotopic xenografts, patient-derived subcutaneous and orthotopic xenografts, and genetically engineered mouse models, are available. Of the models, patient-derived orthotopic xenografts are the most clinically relevant, as these tumors more closely mimic patient disease [4, 12, 15, 201].

While FGS has great potential for increasing the success rates of tumor resection, limitations hinder the use of FGS in clinical applications. Currently, FGS is limited in its preoperative imaging application, the imaging depth of NIRF agents, use in ambient light environments, contrast agent target stability, and tumor features such as desmoplasia [4, 5, 8, 166, 194]. These limitations pose challenges for researchers in the development of FGS contrast agents and their clinical application. The challenges of tumor composition in patients present a limitation in both the development of agents and the clinical application. Tumor features such as high stromal content in pancreatic cancer may limit the penetration of NIRF agents [5]. In contrast-agent development, antibody-conjugated probes present unique limitations. These probes clear slowly from the body and often show high liver uptake contributing to increased background signal and limited tumor uptake due to size [5, 8, 166, 202-204]. With regard to the studies highlighted in this paper, slow probe clearance leads to later imaging times post-injection and higher background tissue and liver fluorescence [8, 14, 18, 21]. These limitations must be considered in future studies.

There are also limitations in targeting mucins. Transmembrane mucins such as MUC1 or MUC4 can generate truncated forms increasing the variation in targets [47]. These glycoforms have varying expression levels in normal and malignant tissue [34]. Due to glycoform expression differences, some may be more reliable as targets in malignant tissue [53]. This challenge increases the difficulty in determining the optimal antibody to bind to the mucin glycoform found in each cancer. Additionally, mucin expression varies between patients [15, 21, 37, 90, 97, 109, 116]. It is important to note this limitation, as the FGS contrast agents targeting mucins may not be universally effective in tumors.

6. Summary

We have described the differential mucin expression in malignant tissue, FGS applications for mucin targeting in pancreatic cancer, and opportunities for this investigational field to expand and advance. Malignant tissue differentially expresses mucins compared to normal tissue. The expression pattern of mucins from normal to malignant tissue may indicate using these proteins as biomarkers for FGS. Mucins are a particularly strong candidate for FGS-targeting in pancreatic cancer. The correlation of mucins with pancreatic cancer stage and progression may aid in determining the optimal mucin expression to explore in pursuing FGS biomarkers. Current research investigating mucins as biomarkers for FGS in pancreatic cancer has shown the promising use of mucins to aid surgical removal.

Mucin expression is readily targetable and capable of producing relatively high tumor-to-background ratios. This research may help propel mucins into clinical trials and further clinical use as FGS biomarkers. In all, mucins contain ideal characteristics, such as diverse differential expression in early to late stages of malignant tissue and ease of targeting and detection, which are often present in optimal FGS contrast agents and biomarkers.

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Highlights

- Several mucins are differentially or uniquely expressed in pancreatic adenocarcinoma and could serve as targets for contrast agents that use the high pancreatic cancer expression.
- When linked to a suitable fluorophore, targeting molecules, e.g. antibodies, can fluorescently enhance pancreatic cancer for the purpose of intraoperative imaging.
- Intraoperative imaging, or fluorescence-guided surgery, can potentially increase the rate of complete tumor resection.
- Mucin-targeting fluorescent contrast agents for fluorescence-guided surgery are in preclinical development, show promise towards eventual clinical development, and could be an important complement to other surgical imaging probes already in clinical trials.

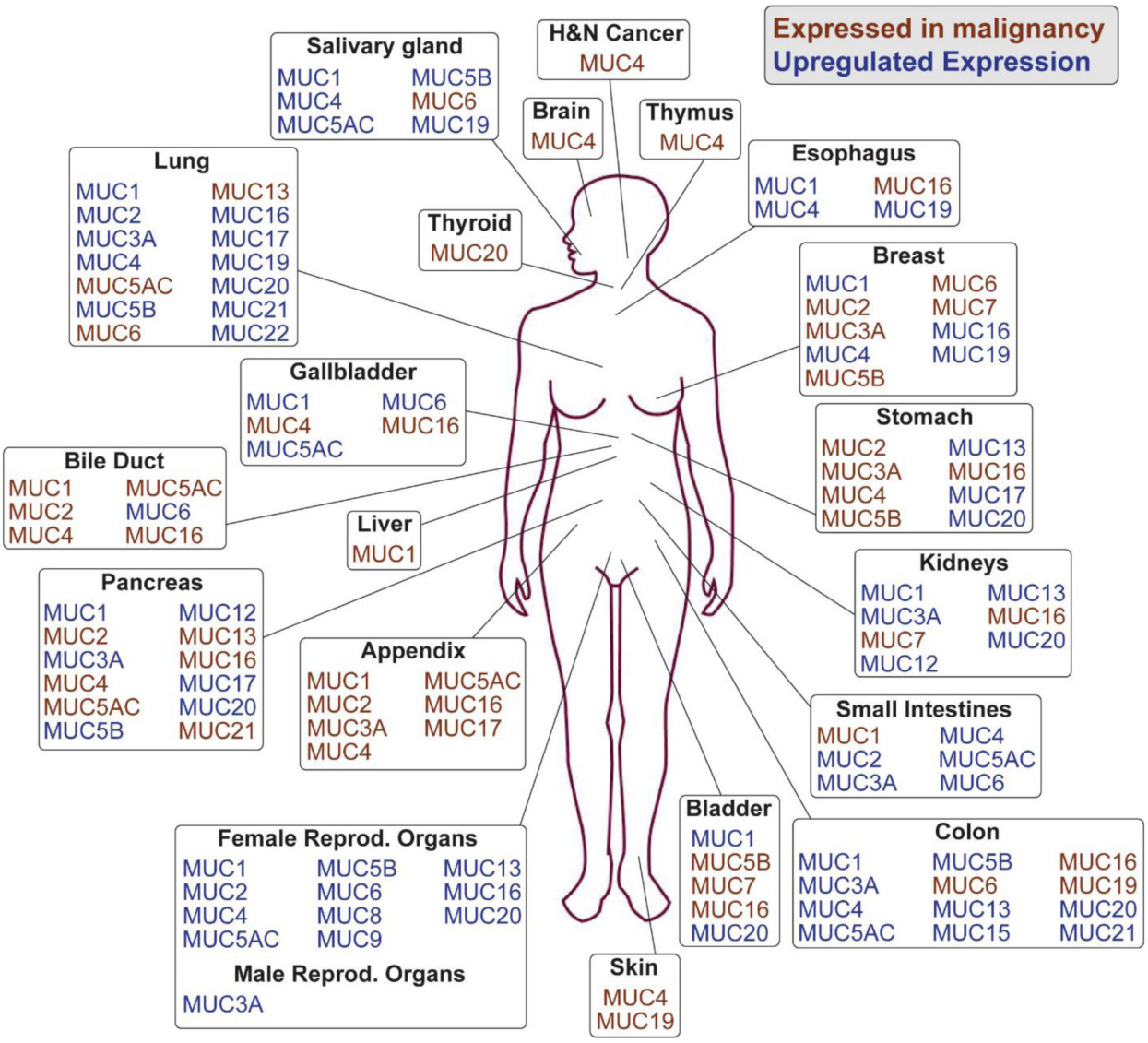

Figure 1. Mucin expression pattern in malignant tissue compared to normal tissue.

Mucin expression displayed by organ. Mucins that are uniquely expressed in malignancy are written in brown. These mucins have optimal expression patterns as they are known to be expressed in malignant tissue with no known expression in normal tissue. Mucins in blue are also potential FGS biomarkers with upregulated expression in malignant tissue compared to normal tissue.

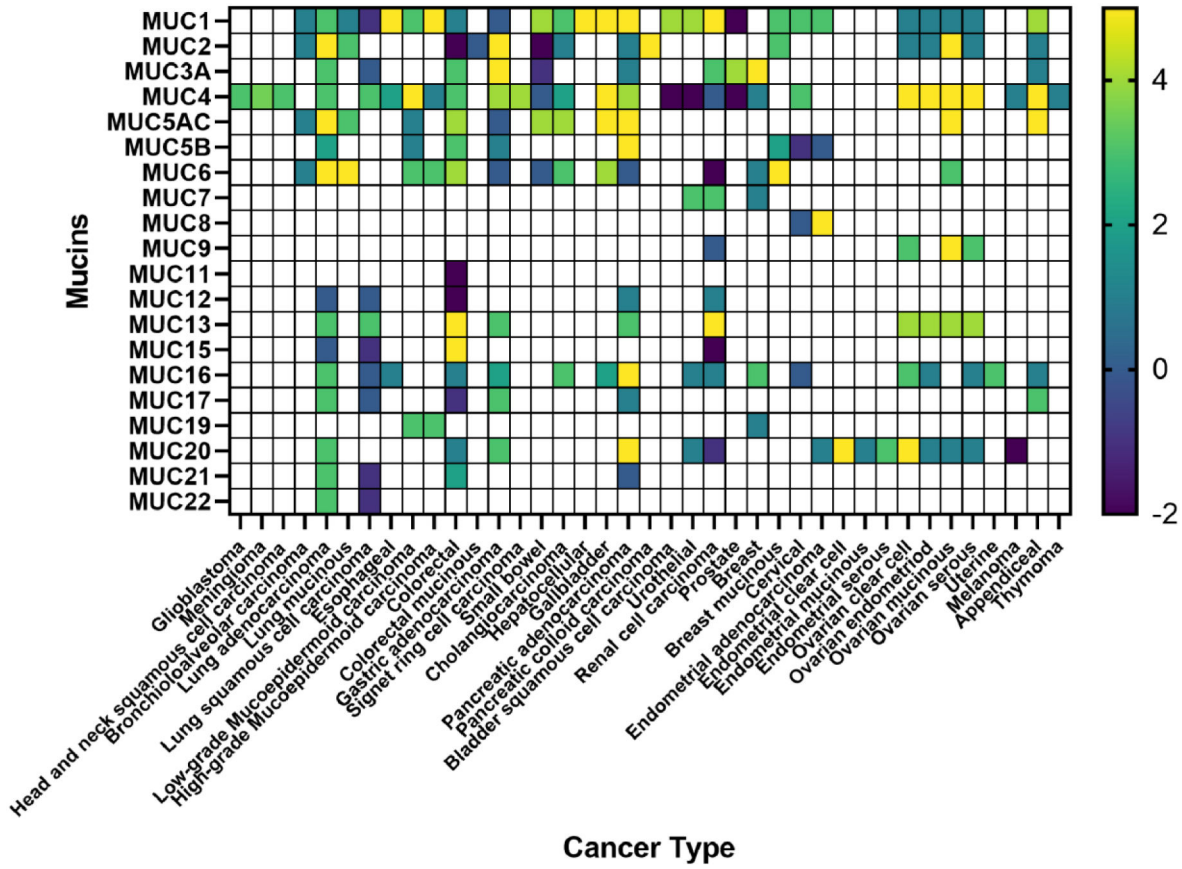

Figure 2. Heatmap of mucin expression in cancers.

Mucin expression in cancer is arranged alphabetically in each organ system. Mucin expression was scored by the intensity of stain and percent of expression tumors on a scale of −2 to 5. Legend: 0 represents similar mucin expression in malignant and normal tissue. 1 represents the weak intensity of staining in tumors and mucins with staining in a few tumors. 2 and 3 represent moderate mucin staining intensity in a few or many tumors. 4 and 5 represent high mucin staining intensity in few or many tumors. −1 and −2 represent malignancy mucin expression below normal tissue expression either slightly or dramatically. The white boxes indicate that the mucin was not found expressed in cancer or had unknown expression intensity.

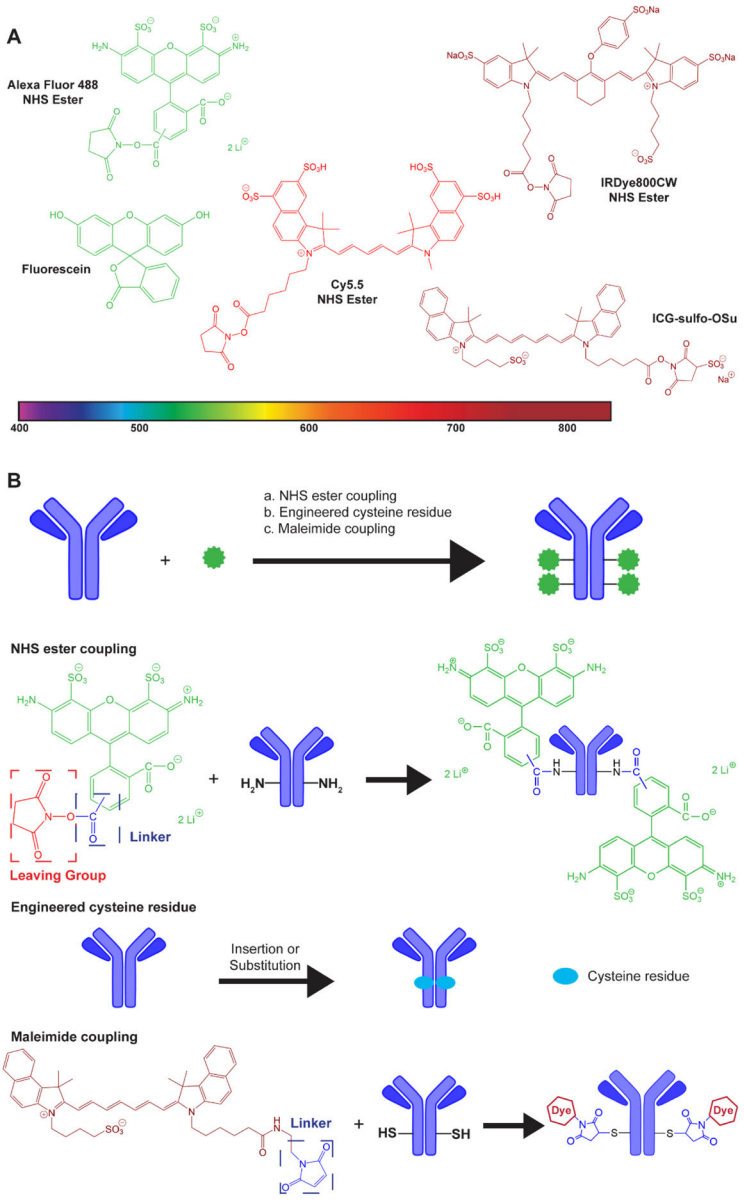

Figure 3. FGS contrast agent components.

A. Examples of fluorescent dyes for conjugation to a biomolecular targeting agent are Alexa Fluor 488, fluorescein, Cy5.5, IRDye800CW, and ICG-sulfo-OSu. These dyes fluoresce in the visible or NIR channels. **B.** Antibody-dye conjugation reactions. Common methods, including NHS ester coupling, engineering of a cysteine amino acid, and maleimide coupling, are examples of methods to form a fluorescent dye-conjugated probe.

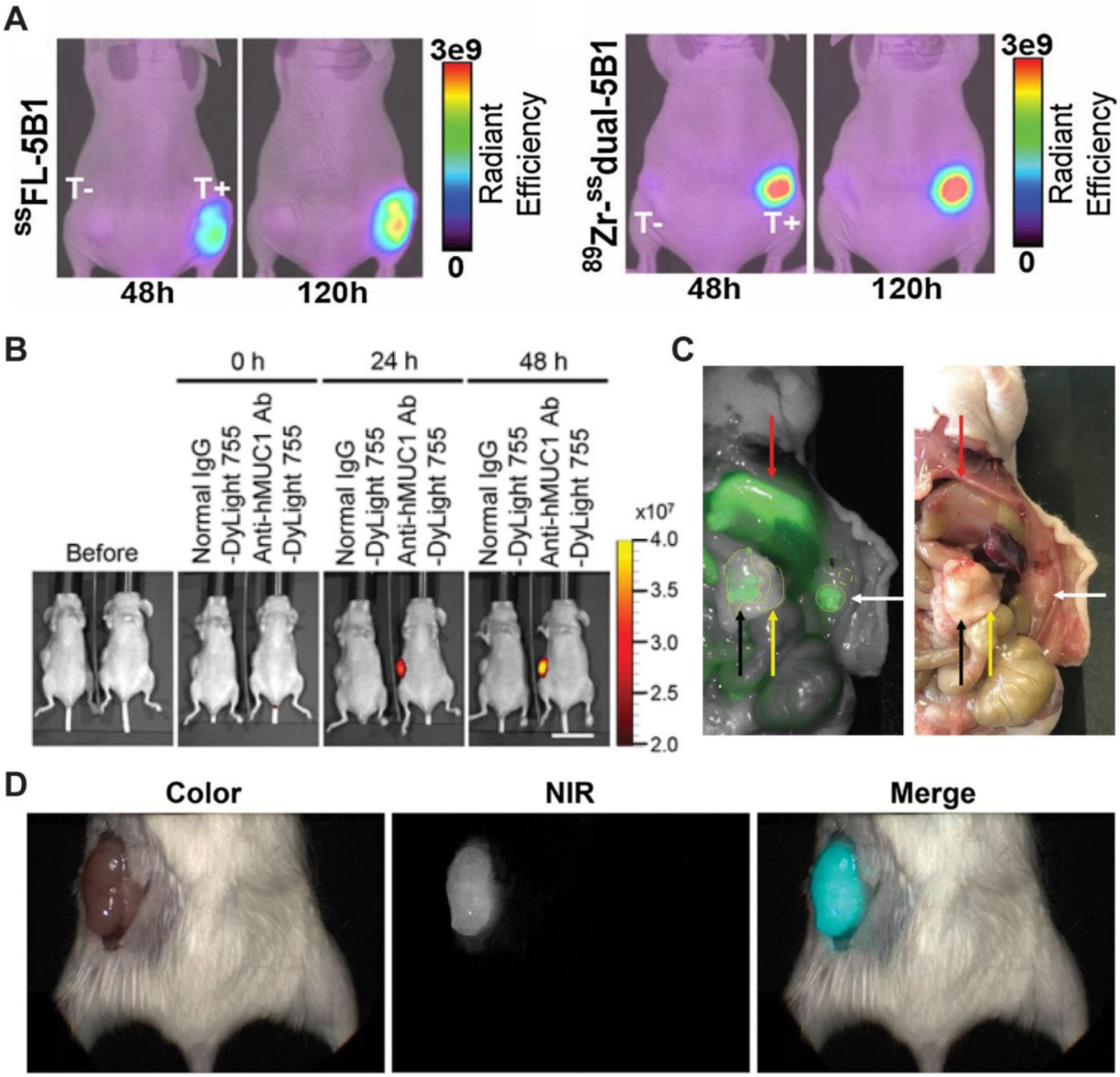

Figure 4. ***In vivo*** **targeting of mucins for FGS.**

A. *In vivo* imaging of subcutaneous CA19-9 positive and negative tumors of a fluorescent-labeled probe (left) and a dual-labeled probe (right). *Adapted with permission from Houghton JL, Zeglis BM, Abdel-Atti D, et al., (2015) Site-specifically labeled CA19.9-targeted immunoconjugates for the PET, NIRF, and multimodal PET/NIRF imaging of pancreatic cancer. PNAS.* **B.** Time-lapse imaging of the targeting ability of a conjugated MUC1 antibody compared to a conjugated IgG antibody in a subcutaneous pancreatic cancer model. *Adapted with permission from Wu G, Maharjan S, Kim D, et al., (2018) A novel monoclonal antibody targets Mucin1 and attenuates growth in pancreatic cancer model. Int JMol Sci.* **C.** *In vivo* imaging of antibody targeted to MUC5AC in an orthotopic pancreatic tumor model. The black arrow denotes pancreatic tumor fluorescence. The yellow arrow denotes a non-fluorescent normal pancreas. The white arrow denotes a fluorescent metastatic pancreatic tumor in the peritoneum. The red arrow denotes background

fluorescence in the stomach. *Adapted with permission from Turner MA, Hollandsworth HM, Nishino H, et al., (2022) Fluorescent Anti-MUC5AC brightly targets pancreatic cancer in a patient-derived orthotopic xenograft. In Vivo.* **D.** Humanized antibody targeting MUC16 in a subcutaneous patient-derived xenograft tumor model of pancreatic cancer. *Adapted with permission from Olson MT, Aguilar EN, Brooks CL et al., (2022) Preclinical evaluation of a humanized, near-infrared fluorescent antibody for fluorescence-guided surgery of MUC16-expressing pancreatic cancer. Mol Pharm. Copyright 2022 American Chemical Society.*

Table I.

Mucin expression in normal tissue.

MUC1	Ovary, vagina, endometrium, endocervix, cervix, prostate, breast, mammary glands, colon, pancreas, stomach, gallbladder, esophagus, salivary gland, lung, trachea, kidney, bladder, eye, ear	[10, 23, 33, 34, 37-39, 42, 45-62]
MUC2	Endocervix, small intestine, large intestine, colon, salivary gland, trachea, bronchus, eye, ear	[23, 34, 37, 38, 45, 47-49, 54, 62-70]
MUC3A	Ovary, uterus, endocervix, prostate, small intestine, colon, pancreas, liver, gastrointestinal epithelium, gallbladder, lung, trachea, thymus, heart, kidney, ear	[23, 30, 36-38, 40-42, 47, 51, 55, 61, 63, 64, 70-73]
MUC3B	Small intestine, colon, gastrointestinal epithelium, gallbladder, ear	[23, 30, 36, 38, 40, 41, 47, 51, 64, 71, 73, 74]
MUC4	Ovary, uterus, vagina, cervix, endocervix, testis, seminal vesicle, prostate, breast, mammary gland, small intestine, colon, ascites fluid, esophagus, salivary gland, oral cavity, lung, bronchi, trachea, bladder, kidney, eye, lacrimal glands, ear	[15, 23, 27, 30, 35, 37, 38, 40, 45-47, 50, 54-58, 61-63, 69, 70, 75-81]
MUC5AC	Cervix, endocervix, small intestine, colon, stomach, gallbladder, saliva, salivary gland, lung, bronchi, trachea, eye, ear	[23, 34, 37, 38, 45, 47-49, 52-54, 57, 58, 60, 68-70, 82-85]
MUC5B	Cervix, endocervix, seminal fluid, large intestine, colon, pancreas, gallbladder, esophagus, saliva, salivary gland, bronchi, trachea, lacrimal gland, ear	[23, 37, 38, 42, 45, 47, 54, 57, 58, 63, 65, 68, 84-86]
MUC6	Endometrium, cervix, endocervix, male genital ducts, seminal fluid, small intestine, pancreas, stomach, gallbladder, bile duct, kidney, ear	[23, 34, 37-39, 45, 48-50, 53, 60, 63, 65, 68, 69, 83, 87]
MUC7	Salivary glands, trachea, respiratory tract, lacrimal gland, ear	[23, 37, 38, 54, 57, 88]
MUC8	Endometrium, cervix, placenta, testis, bronchus, trachea, ear, nose	[23, 38, 45]
MUC9	Ovary, cervix, bronchus, trachea, kidney	[23, 38, 55, 71, 89]
MUC11	Uterus, prostate, small intestine, large intestine, colon, pancreas, liver, appendix, lung, kidney	[36, 42, 47, 62, 65]
MUC12	Uterus, prostate, small intestine, large intestine, colon, pancreas, stomach, lung, kidney	[30, 36, 38, 42, 47, 51, 61, 62, 65]
MUC13	Ovary, small intestine, large intestine, colon, appendix, stomach, esophagus, respiratory tract, trachea, kidney, ear	[30, 32, 38, 47, 55, 61, 62, 72, 90]
MUC15	Ovary, placenta, prostate, testis, breast, small intestine, colon, salivary gland, lungs, thyroid gland, thymus, spleen, bone marrow, lymph node, kidney, ear	[30, 38, 55, 61, 62, 71, 91]
MUC16	Ovary, endometrium, uterus, fallopian tube, female and male reproductive organs, breast, epithelial surface of internal organs, epithelial surface of body cavity, gastrointestinal tract, bronchi, upper respiratory tract, trachea, eye, lacrimal glands, ear	[27, 30, 38, 57, 61, 92-96]
MUC17	Small intestine, colon, pancreas, stomach, lung, ear	[38, 42, 61, 62, 97]
MUC19	Breast, gastrointestinal tract, salivary gland, respiratory tract, trachea, bone marrow, lymph node, thymus, eye, lacrimal glands, ear	[38, 57, 98-101]
MUC20	Ovary, vagina, cervix, fallopian tube, placenta, prostate, small intestine, colon, liver, stomach, esophagus, salivary glands, lungs, bladder, kidney, thyroid gland, pituitary gland, ear, skin	[38, 43, 55, 61, 75, 102]
MUC21	Testis, large intestine, colon, lungs, thymus	[38, 61, 71, 103]
MUC22	Vagina, placenta, testis, esophagus, lungs	[38, 43, 61]

Table II.

Mucin correlation with pancreatic cancer stage and prognosis.

Mucin	Cancer	Stage	Prognosis	References
MUC1	PDAC	PanIN-I < PanIN-III < Stage I < Stage IV	Poor prognosis	[33, 34, 39, 104, 112, 120]
MUC2	PDAC	Stage IV	Poor prognosis	[104, 112]
	Pancreatic Colloid Carcinoma	Early IPMNs < high grade IPMNs < colloid carcinoma	Better prognosis	[120]
MUC3A	PDAC	PanIN-I < PanIN-III > Adenocarcinoma	Poor prognosis	[34, 40, 107, 129]
MUC4	PDAC	PanIN < Stage IV	Poor prognosis	[27, 33, 37, 40, 75, 104, 112, 121, 122]
MUC5AC	PDAC	PanIN-IA < PanIN-IB to PanIN-III < Stage I ≈ Stage IV; In serum: benign tissue ≈ chronic pancreatitis and Stage I < Stage IV	Poor prognosis; Good prognosis	[33, 39, 83, 105, 106, 112]
MUC5B	PDAC	Pancreatitis > PDAC	Poor prognosis	[37, 104]
MUC6	PDAC	Chronic pancreatitis > PanIN stages > Ia > IV	No correlation	[34, 37, 39, 83, 112]
MUC9	PDAC	Unknown	Better prognosis	[104]
MUC13	PDAC	Nucleus expression: Stage I < Stage IV	Poor prognosis	[90, 108]
MUC16	PDAC	Stage I (20%) < Stage IV (70%); PanIN-I < PanIN-III	Poor prognosis	[104, 112, 117-119, 125]
MUC17	PDAC	No correlation	Poor prognosis	[104]
MUC20	PDAC	No correlation	Poor prognosis	[104]
MUC21	PDAC	No correlation	Poor prognosis	[104]

Table III.

Characteristics of FGS clinical trials in pancreatic cancer

Identifier	Contrast Agent	Probe	Target	Imaging Channel	Clinical Trial Status	Reference
NCT02973672	SGM-101	Chimeric Antibody	CEA	NIR	Completed	[178]
NCT03384238	Panitumumab-IRDye800	Murine antibody	EGFR	NIR 800	Active, not recruiting	[180]
NCT02736578	Cetuximab-IRDye 800CW	Chimeric antibody	EGFR	NIR 800	Terminated (logistics)	[182]
NCT02743975	Bevacizumab −800CW	Humanized antibody	VEGF-A	NIR 800	Terminated	[184]
NCT04105062	Cancer goggles	LS301	pANXA2	NIR 800	Withdrawn	[186, 187]
NCT05518071	cRGD-ZW800-1	Peptide	Integrins (αvβ3, αvβ5, αvβ6)	NIR 800	Recruiting	[188]