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Refining the diagnostic utility of OLFM4 in gastric cancer precursors: a call for rigorous methodologies

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Abstract

This commentary offers a thoughtful discussion of the study by Wei et al. published in the journal on the role of Olfactomedin 4 (OLFM4) in incomplete intestinal metaplasia, a gastric precancerous condition. The original paper introduces OLFM4 as a novel biomarker with potential enhanced diagnostic efficacy compared to established markers. However, several methodological and interpretive considerations are noted. The histopathological findings could be refined by using higher magnification to better elucidate the cellular localization of OLFM4. Including high-resolution images for key stainings would enhance the study's robustness in expression profiling. The statistical approach could be strengthened by employing more rigorous, quantitative methodologies. Additionally, integrating immunofluorescence double-staining may improve the reliability of the results. Discrepancies in immunohistochemical signals across datasets suggest a need for further investigation into tissue section representativeness. Clarifying the term "precancerous lesions of gastric carcinoma cells" to align with widely accepted definitions would enhance clarity. The choice of the GES-1 cell model treated with MNNG could be reconsidered in favor of more established models such as organoids, air-liquid interface models, and gastric cancer-specific cell lines. The in vivo MNNG-alcohol combination model might require additional empirical support, given the limited and conflicting literature on this approach, to ensure an accurate portrayal of IM pathogenesis. The commentary concludes with a call for stringent and standardized methodologies in biomarker research to ensure the clinical applicability and reliability of biomarker studies, particularly in the context of gastric cancer detection and intervention.

Keywords Intestinal metaplasia, Gastric cancer, Gastric carcinogenesis, Diagnostic biomarkers

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We read the paper by Wei et al. [1]. with great interest. The study published in *Molecular Cancer* comprehensively analyzes Olfactomedin 4 (OLFM4) and its role in the progression of incomplete intestinal metaplasia (IIM), a pre-cancerous condition. Utilizing clinical samples, cellular and animal models, and patient-derived organoids, the authors demonstrate a significant overexpression of OLFM4 in IIM compared to complete intestinal metaplasia (CIM) and normal tissue.

The study elucidates that OLFM4, in conjunction with myosin heavy chain 9, promotes the ubiquitination of glycogen synthase kinase 3 beta, thereby activating the β-catenin signaling pathway. This molecular interaction significantly enhances the proliferation and invasiveness of precancerous gastric cells. Notably, OLFM4 is identified as a potentially superior diagnostic biomarker for IIM, outstripping traditional markers such as caudal type homeobox 2 (CDX2) and mucin 2 (MUC2) in diagnostic accuracy.

Wei et al. [1]. emphasize OLFM4's potential as a crucial marker for the early detection and targeted surveillance of high-risk gastric lesions, presenting significant implications for the early diagnosis and treatment of gastric cancer.

We commend Wei et al.'s comprehensive analysis of OLFM4 in IIM, underscoring its potential as a pivotal biomarker for early gastric cancer detection. However, the broader context of biomarker discovery in gastric precursors necessitates rigorous methodologies and standardized approaches to validate findings across diverse patient populations and experimental models. The variability in cellular and molecular profiles within IIM highlights the complexity of gastric carcinogenesis, urging for meticulous characterization of OLFM4's diagnostic utility across different stages of gastric carcinogenesis. Future studies should emphasize robust histopathological analyses integrated with validated experimental models to elucidate OLFM4's role in the transition from metaplasia to dysplasia and carcinoma.

Intestinal metaplasia (IM) arises in the context of atrophic gastritis and serves as a critical intermediate step in the pathogenesis of gastric cancer, representing a pivotal stage in its progression. IM is not a homogeneous entity and can be categorized into two subtypes: complete and incomplete IM [2]. CIM mimics the structure of small intestinal glands, characterized by the absence of gastric mucins, the presence of eosinophilic enterocytes with brush borders, well-defined goblet cells, and occasional Paneth cells. In contrast, IIM is more disorganized, with immature goblet cells and intermediate columnar cells at various stages of differentiation, often containing intracytoplasmic mucin. IIM features mixed, immature intestine-like cells in the superficial glandular unit, with basal cells showing characteristics of spasmolytic polypeptide-expressing metaplasia (SPEM) cell differentiation [2].

The IIM gland is a hybrid of both gastric and immature intestinal goblet cell lineages, indicating high proliferative capacity and susceptibility to dysplastic changes and neoplastic transformation [2]. Despite the recognition of IM, and specifically IIM, as a precursor to gastric cancer, the precise cell lineage within the IIM gland that acts as the potential cell of origin for gastric cancer remains elusive.

Recent research by Kumagai et al. [3]. has shed light on this area by exploring the origins of gastric tumors, specifically intramucosal gastric carcinoma (IGC) and oxyntic gland adenoma (OGA), in a 71-year-old woman without *Helicobacter pylori* infection. The study demonstrates that both IGC and OGA originated from SPEM with a Kirsten rat sarcoma viral oncogene homolog (KRAS) (G12D) mutation. Additionally, mutations in tumor protein p53 and cyclin-dependent kinase inhibitor 2 A were identified in IGC, and a guanine nucleotide-binding protein alpha stimulating mutation was found in OGA. These findings suggest that KRAS-mutated SPEM cell lineages can give rise to distinct gastric tumors.

Huang and colleagues, using single-cell RNA sequencing (scRNA-seq), found high expression of *OLFM4* in intestinal stem cells within the IM region [4]. These cells, characterized by unique metabolic and genetic profiles, show increased activity in oxidative phosphorylation, myelocytomatosis (*MYC*) pathways, and ribosomal gene expression. The potential of *OLFM4*-expressing stem cells as a reservoir for intestinal-type gastric cancer is supported by their co-expression with markers like *leucine-rich repeat-containing G protein-coupled receptor 5* and *aquaporin 5* (*AQP5*), identified in antral gastric cancer stem cells.

Integrating IM scRNA-seq data with early-stage gastric cancer data indicates a close relationship between early gastric cancer cells and *OLFM4*-expressing intestinal stem cells. Spatial transcriptomics supports this, showing that regions with high *OLFM4* expression display malignant gastric cancer-like signatures [4]. This implies that *OLFM4*-expressing stem cells in IM may play a role in the clonal expansion and malignant transformation observed in gastric cancer progression.

An additional scRNA-seq study of IM has identified specific cell types, including goblet cells (expressing *MUC2*) and enterocytes (expressing *fatty acid-binding protein 1* and *apolipoprotein A-I*) [5]. When compared with early gastric cancer cells, a transcriptional resemblance is observed between cancer cells and both enterocytes and a metaplastic Wnt-driven stem cell subtype, notable for its expression of *OLFM4*, *ephrin type-B receptor 2*, and *SRY-box transcription factor 9*. The study noted a significant increase in antral *OLFM4*-expressing gland mucous cells as the gastric mucosa undergoes

intestinalization, suggesting a phenotypic transition towards an intestinal stem cell-like phenotype [5]. Collectively, while the initial expression of *OLFM4* in the gastric mucosa seems to be an early indicator of gastric carcinogenesis, and is associated with a reduction in gastric antral gland mucous cells and the emergence of antral IM lesions, the precise role of elevated *OLFM4* expression in the transition from IM to dysplasia or cancer remains to be fully elucidated.

Recent research employing both scRNA-seq and spatial transcriptomics has corroborated the significant upregulation of *OLFM4* in specific gastric cell clusters undergoing metaplasia. *OLFM4* has been identified as a marker for "linking cells", indicative of a transitional phase between gastric and intestinal cell types [6]. This finding underscores *OLFM4*'s role in the cellular differentiation processes underlying IM progression. *OLFM4* may play a pivotal role in the early stages of IM development, potentially bridging the transition from gastric to intestinal phenotypes and contributing to the maintenance of stemness and the formation of metaplastic tissue.

However, the expression of *OLFM4* extends to intestinal-related lineages within IM, including differentiated enterocytes and goblet cells [6]. This necessitates further investigation into the role of elevated *OLFM4* expression in these intestinalized cell lineages and its potential contribution to the progression from IM to dysplasia or carcinoma.

Most research has focused on the RNA-level expression patterns of *OLFM4* in antral IM using scRNA-seq. There is a lack of published data documenting OLFM4 protein overexpression in intestinal stem cells or intestinalized cell lineages within IM glands. The expression profile of OLFM4 in corporal IM remains understudied, presenting an opportunity for future research to delineate its role across various gastric regions. Understanding OLFM4's distribution and influence in different gastric areas could provide critical insights into disease progression and region-specific therapeutic strategies.

Wei et al.'s study commendably expands on OLFM4 research by examining its protein expression in IIM [1]. The study identified OLFM4 as a novel IIM biomarker through histopathological examinations, including hematoxylin and eosin (HE), Alcian Blue-Periodic Acid-Schiff (AB-PAS) staining, and immunohistochemical assessments of CDX2, MUC2, and OLFM4.

However, the histopathological findings in the study could be improved by including higher magnification images to better elucidate the cellular localization of OLFM4. Additionally, high-resolution images for both HE and AB-PAS stainings are necessary for the precise identification of goblet cells, a hallmark of IM. The provided images currently offer a limited depiction of columnar cells with a blend of neutral and acid mucins, which may be insufficient for accurate diagnosis and comprehensive characterization of IM.

In the study, Fig. 2c of the training set shows a pronounced immunohistochemical signal for OLFM4 in IM tissue, while signals for established IM markers CDX2 and MUC2 appear weaker. Conversely, in the validation set (Figure S2a), signals for CDX2 and MUC2 are more robust, while the OLFM4 signal is subdued. This discrepancy suggests variability in tissue section representativeness, which could impact the reliability of the comparative expression analysis.

The authors differentiate between CIM and IIM in Fig. 2e and S2b by examining OLFM4 expression patterns. However, Fig. 2e shows strong OLFM4 signals in regions with magenta staining, which are indicative of neutral mucins more characteristic of normal gastric epithelium than IM, potentially suggesting misclassification. In Figure S2b, IIM sections with high CDX2 and MUC2 expression show diminished OLFM4 signals, raising further questions about the reliability of OLFM4 as an IIM marker.

The study evaluates CDX2, MUC2, and OLFM4 immunohistochemical scores based on the proportion of stained areas, which is a subjective method. Methodologically rigorous studies often advocate for a more quantitative approach, assessing the extent of positive staining within IM glands and reporting the percentage of positive IM glands [7].

Implementing immunofluorescence double-staining for OLFM4 with either CDX2 or MUC2, along with high-resolution imaging, could facilitate a more precise evaluation of co-expression patterns and enhance the reliability of staining outcomes.

Additionally, double-staining OLFM4 with defensin alpha 5 (DEFA5), a Paneth cell marker, or CD10, a marker for columnar enterocytes, within CIM glands is recommended [7]. In IIM tissues from the corpus, IIM glands show SPEM cells at the bases (AQP5-positive) and trophoblast cell surface antigen 2 (TROP2)-positive cells in the upper regions, while CIM glands predominantly feature DEFA5-positive Paneth cells at their bases [2]. Since AQP5 marks SPEM cells in IIM and TROP2 indicates dysplasia, using immunofluorescence double-staining of OLFM4 with these markers is recommended [2, 8]. This approach would facilitate the precise localization of OLFM4 within specific cellular lineages and improve understanding of its role within the glands. These refined methodologies could provide a more objective and accurate assessment of OLFM4's diagnostic efficacy.

Wei et al.'s study [1] does not include higher magnification images and does not specify whether IM tissues were from the corpus, antrum, or both. This distinction is important because IIM in the corpus contains SPEM cells with greater hybrid characteristics and lineage

confusion compared to the antrum [2]. This detail significantly affects the interpretation of OLFM4's role in IM progression. The absence of this information leaves unresolved questions about whether intestinalized cell lineages within IIM glands or SPEM cell lineages serve as *de novo* sources of dysplastic or gastric cancer cells under the influence of overexpressed OLFM4. Addressing these concerns is crucial for a precise and comprehensive investigation of OLFM4's diagnostic and pathological significance in IM.

Given the low annual carcinogenic rate of IM (0.09–1.7%), identifying high-risk IM that progresses to cancer is of paramount importance for understanding gastric cancer development. Characterizing these lesions could uncover pathways for preventing gastric cancer. As IM glands expand and acquire copy number aberrations, leading to field cancerization, they may be predisposed to malignant transformation via subsequent genetic alterations. Assessing OLFM4 expression and its immunohistochemical scores in IM areas adjacent to dysplasia or early gastric cancer is crucial for understanding OLFM4's role in the transition from IM to cancer.

The authors introduce the term "precancerous lesions of gastric carcinoma (PLGC) cells" based on single-cell RNA sequencing data, which serves as the basis for subsequent in vitro and in vivo experiments. However, the term "PLGC cells" is not widely recognized in international pathology. The study reports that these "PLGC cells" display lower differentiation and elevated OLFM4 expression. While histopathological analyses suggest OLFM4 as a biomarker for IIM, the use of the undefined term "PLGC cells" raises questions about its relationship with IM, particularly IIM.

The introduction of "PLGC cells" introduces ambiguity due to a lack of validation and international consensus. A comprehensive explanation and justification for this term, along with its relationship to recognized pathological stages and biomarkers, would be beneficial for broader acceptance and practical utility of the study's conclusions."

The study's in vitro model using GES-1 cells exposed to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) raises concerns due to its unconventional nature and limited scientific consensus. Historically, MNNG was used to induce gastric tumors in animal models before the discovery of *Helicobacter pylori*. In rats, MNNG exposure typically results in erosive lesions, disrupted glandular structures, and mucosal proliferation [9]. Lifelong administration generally leads to adenomatous tumors in the glandular stomach epithelium [9], contrasting with the mucosal atrophy, intestinal metaplasia, and eventual intestinal-type adenocarcinomas described in the Correa pathway, which is the broader context of this study. CDX2 expression was not observed in GES-1 cells stimulated with MNNG, as determined by immunofluorescence [10].

This experimental approach with GES-1 cells is primarily utilized by a select group of researchers in China, particularly in fields such as herbal medicine and complementary and alternative medicine. A search in the Science Citation Index Expanded of the Web of Science Core Collection (WOSCC) by Clarivate Analytics revealed only 27 publications referencing both "GES-1 cells" and "MNNG," all originating from China. Introduced in 2014, this methodology has produced sporadic and low publication volumes, averaging fewer than five per year. The primary research domains adopting this cell model include pharmacology and pharmacy (14 publications), integrative complementary medicine (11), plant sciences (10), biochemistry and molecular biology (5), research and experimental medicine (4), chemistry (3), food science and technology (3), toxicology (3), cell biology (2), and oncology (2).

In contrast, the emergence of more sophisticated and representative cell models, such as organoids and air-liquid interface (ALI) cultures, offers enhanced fidelity in replicating IM processes. For instance, Koide et al. [11]. demonstrated the efficacy of human gastric organoids derived from induced pluripotent stem cells in modeling IM by inducing specific gene expressions like CDX2. Similarly, Min J et al. [12]. developed Meta3 organoids from Mist1-CreERT2$^{Tg/+}$; LSL-K-ras(G12D)$^{Tg/+}$ (Mist1-Kras) transgenic mice, which exhibit high proliferative capacity and express caudal type homeobox 1, effectively mirroring IM features. Additionally, Liu et al. [13]. introduced an ALI model that enables sustained culture and detailed analysis of IM cells, identifying key biomarkers linked to cancer pathways and enhancing our understanding of IM in gastric cancer.

Molecular profiling studies have identified KATO III and AGS cells as ideal for studying IM due to their robust expression of IM markers [14]. AGS cells, exposed to *Helicobacter pylori* infection, upregulated IM markers such as CDX2 and MUC2, providing a robust in vitro IM model [15].

The in vivo methodology employed in this study to induce preneoplastic IM through the combination of MNNG and alcohol raises important questions regarding its validity and effectiveness, as highlighted by conflicting reports in the literature [1]. Comparing the methodologies for inducing gastric cancer with MNNG and alcohol reveals significant variations across different studies. Iishi et al. [16]. administered MNNG at 50 μg/ml in drinking water for 20 weeks, followed by intraperitoneal injections of 20% ethanol every other day until week 52. This sequential approach resulted in a significant increase in gastric cancer incidence, suggesting ethanol's role in promoting carcinogenesis through increased cell

proliferation in the antral mucosa. In contrast, Cerar et al. [17]. used a higher MNNG concentration of 100 µg/ml in drinking water combined with 11% ethanol or wine for six months. This simultaneous administration demonstrated a protective effect of ethanol, significantly reducing the incidence of glandular stomach and duodenal carcinomas.

Wei et al. [1]. administered MNNG at 170 µg/ml with 5% alcohol by gavage every two days for 24 weeks, combining a high MNNG concentration with intermittent fasting. Iishi et al.'s method involved sequential administration, where MNNG was given first, followed by ethanol through a different route (intraperitoneal). Conversely, Cerar's study models dietary scenarios, highlighting ethanol's protective potential against carcinogenesis. Wei et al.'s study aligns more closely with Cerar's method by concurrently administering MNNG and ethanol orally (drinking solution or gavage). However, the combination of a high MNNG concentration and intermittent fasting introduces additional variables.

A search within the Science Citation Index Expanded of the WOSCC found no corroborating studies using the combination of high MNNG concentration, alcohol, and intermittent fasting. Even in the study by Iishi et al. [16], where at the conclusion of the 52-week experiment, 72% of rats developed gastric cancer (averaging 0.8 cancers per rat) and 100% exhibited atypical glandular hyperplasia (dysplasia), the timeline for specific preneoplastic conditions such as atrophy and IM was not explicitly detailed. Additionally, the lack of characterization of the temporal dynamics and specific pathologies induced by this combination, coupled with the faint expression levels of CDX2 and MUC2 observed in Figure S5d, raises concerns about the study's methodology. These issues suggest that the 24-week endpoint for IM analysis might not be sufficient, potentially affecting the foundational assumptions of the study [1].

To enhance reliability, more robust rodent models should be considered, such as Mist1-Kras mouse, aged mice on the amphiregulin-null background, and gerbils infected with *Helicobacter pylori*, among others [8]. These models faithfully recapitulate IM induction and are better suited for investigating its mechanisms and progression.

We recommend that the scientific community adopt rigorous and standardized approaches when examining biomarkers like OLFM4 in IM and gastric carcinogenesis. Utilizing well-validated models and reproducible histopathological analyses is essential for obtaining reliable and clinically applicable results. Early detection and precise characterization of high-risk lesions are crucial for improving gastric cancer outcomes. Employing well-recognized models and techniques will enhance research validity and facilitate clinical translation.

Abbreviations

OLFM4	Olfactomedin 4
IIM	Incomplete Intestinal Metaplasia
CIM	Complete Intestinal Metaplasia
CDX2	Caudal type homeobox 2
MUC2	Mucin 2
SPEM	Spasmolytic polypeptide-expressing metaplasia
IGC	Intramucosal gastric carcinoma
OGA	Oxyntic gland adenoma
KRAS	Kirsten rat sarcoma viral oncogene homolog
scRNA-seq	Single-Cell RNA sequencing
MYC	Myelocytomatosis
AQP5	Aquaporin 5
HE	Hematoxylin and eosin
AB-PAS	Alcian Blue-Periodic Acid-Schiff
DEFA5	Defensin alpha 5
TROP2	Trophoblast cell surface antigen 2
PLGC	Precancerous lesions of gastric carcinoma
MNNG	N-Methyl-N'-Nitro-N-Nitrosoguanidine
WOSCC	Web of Science Core Collection
ALI	Air-liquid interface

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Author contributions
X.T. conceptualized the study, acquired funding, and supervised the project; T.Z. prepared the original draft. All authors reviewed and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This comment does not involve any new research involving human participants or animals. Therefore, ethics approval and consent to participate are not applicable.

Consent for publication
This comment does not include any individual person's data in any form. Therefore, consent for publication is not applicable.

Competing interests
The authors declare no competing interests.

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