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# The Expression of Human Epididymis Protein 4a (HE4) in the Normal Gastric Epithelia and Its Role in the Development of Intestinal Metaplasia and Gastric Cancer

**Betul Celik[1*], Tangul Bulut[1] and ArzuDidem Yalcin[2]**

*[1]Department of Pathology, SB University Antalya Hospital, Antalya, Turkey.*
*[2]Department of Allergy and Immunology, SB University Antalya Hospital, Antalya, Turkey.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author BC designed the study, managed the literature searches, wrote the protocol and wrote the first draft of the manuscript. Authors TB and BC performed the statistical analysis. Authors BC, TB and ADY managed the analyses of the study. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** Biomarkers that allow early diagnosis of gastric carcinoma (GC) patients are limited. Human epididymal protein 4 (HE4) is a novel biomarker for epithelial ovarian and lung cancer. This study was designed to evaluate the role of HE4 in the development of intestinal metaplasia (IM) and gastric carcinoma.
**Methods:** A total of 41 patients with a diagnosis of GC and 48 patients with a diagnosis of IM were enrolled. Gastric cancer samples were taken from patients who underwent gastric resection due to GC. For IM, biopsies obtained from patients who underwent endoscopic examination for non-tumoral reasons were used. Intestinal metaplasia adjacent to GC was also examined separately. For HE4 expression, immunohistochemistry was used and the results were compared to

**Corresponding author: E-mail: bet_celik@yahoo.com;*

demographic, clinic, pathologic and prognostic parameters.
**Results:** The patients were 38–90 years-old (mean: 65.6). Tumor localization were antrum in 37.8%, corpus in 27%, lesser curvature in 21.6%, greater curvature in 5.4%, and cardia in 8.1% of patients. Tumor sizes ranged between1-13 cm (mean 5.54). There were 37.8% well to moderately, and 62.2% poorly differentiated carcinoma. Pathological T evaluations were as follows: pT1=13.5%; pT2=8.1%; pT3=59.4%; pT4=18.9% patients. The expression of HE4 was seen in 50% of tumors. Among the tumor samples that harbor intestinal metaplasia, HE4 expression in metaplastic cells was seen in 61.1% of the cases. Diffuse type carcinoma had more HE4 expression than intestinal type cancers and there was an inverse correlation between depth of tumor invasion and the presence of HE4 (p=0.037).
**Conclusions:** Human epididymal protein 4 was expressed in normal oxyntic glands and metaplastic cells as well as GC cells but not in the surface foveolar epithelium. It was expressed mostly in diffuse type carcinoma. HE4 may be involved in personalized treatment in gastric cancer.

*Keywords: HE4; stomach; carcinoma; intestinal metaplasia.*

## 1. INTRODUCTION

The role of histological parameters ie; atrophic gastritis, intestinal metaplasia (IM) and dysplasia in the development of gastric cancer is indisputable. Of these triple precursor lesion called Correa cascade, IM has a progression rate of 0.377% [1,2]. The development of intestinal metaplasia is actually a protective response to inflammation, but it increases the risk of neoplastic transformation as well [3,4]. The prevalence of intestinal metaplasia is high in countries where H. pylori infection and stomach cancer are common. As a consequence of this observation, IM is thought to be the result of a number of genetic events that are mostly caused by H. pylori infection, but the molecular mechanisms that transform normal epithelia to intestinal metaplasia is yet to be elucidated.

Although carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) have been used for gastric cancer diagnosis, ubiquitous biomarkers are rare and despite the treatment with oxaliplatin, cisplatin and paclitaxel, the majority of patients relapse following chemotherapy, and therefore, there is a need for tailored treatment strategies.

Human epididymal protein 4 (HE4) belongs to whey protein family that contains acidic 4-disulfide center [5,6]. It was discovered in the epididymis but has been shown to be over-expressed in ovarian and lung cancers as well [7,8]. When ovarian cancer cell cDNA is hybridized and then compared to the sequences in the community, the HE4 gene is found to be present [9]. For ovarian cancer screening, HE4 has been registered as the equivalent of CA125: When the serum cut-off value is kept at70 pmol/L, HE4 level is found to be statistically higher in patients with ovarian cancer compared to patients with benign gynecological diseases [10]. The value of HE4 in ovarian cancer diagnosis is higher than that of CA125 in logistic regression analyzes [11].

In this study, we investigated the expression of HE4 in normal gastric epithelia, the cells of intestinal metaplasia and gastric carcinoma in order to elucidate the role of, if any, HE4 in the development of IM and gastric carcinogenesis. We also compared our findings with histological parameters and H. pylori presence.

## 2. MATERIALS AND METHODS

After obtaining approval from Antalya Education and Research Hospital Ethics Committee (#12/27, 30/06/2016), 48 gastric endoscopic antral biopsy that had IM graded as 3 positive (intense) (Group I) and one representative tumor block containing sufficient tumor tissue from 41 gastric adenocarcinoma patients (Group II) were chosen retrospectively. Exclusion criteria were tumors with <10 tumor cells, tumors from metastatic focuses and endoscopic biopsies without full thickness gastric biopsies.

Patient information and histopathological parameters of each patient were obtained from the relevant pathology reports and from the hospital data basis. For tumor pathology reports, the AJCC cancer staging system, 7th Ed and for endoscopic biopsy reports, Sydney System was used [12]. Immunohistochemical study was performed on paraffin embedded tissues that were fixed in formalin. HE4 immunostaining was applied manually. Tissue sections of normal human epididymis processed in a comparable manner provided as positive control. Negative controls were obtained by omitting the primary

antibody. Cytoplasmic staining was graded for intensity (0, negative; 1, weak; 2, moderate; and 3, strong) and percentage of positive cells [0 (0%), 1 (1–24%), 2 (25–49%) and 3 (50–100%)]. Protein expression was then defined as negative, weak (score 1–2), moderate (score 3–4) or strong (score ≥5).

### 2.1 Immunohistochemical Procedure

Formalin-fixed, paraffin-embedded sections were de-waxed with xylene and rehydrated through gradient ethanol into phosphate-buffered solution (PBS). Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for 10 minutes at room temperature. Tris-EDTA buffer (ab93684; Abcam, Cambridge, MA, USA) was used for antigen retrieval in a domestic microwave. Protein block was applied for 5 minutes before application of the rabbit polyclonal antibody to HE4 [anti-HE4 antibody (EPR16658) (ab200828), 1:2,000 dilution]. After 2 h incubation with the primary antibody, biotinylated goat anti rabbit IgG secondary antibody and Streptavidin Peroxidase were applied. Diaminobenzidine (DAB) was used as chromogen. Slides washed in PBS three times and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting.

### 2.2 Statistical Analysis

All data were evaluated by using SPSS for Windows ver. 11.5 (Chicago, INC.). We used Chi-Square test and Fisher-Exact test to compare groups for categorical data. Unpaired t test (if variable has normal distribution) and Mann-Whitney U test (if variable has abnormal distribution) for continuous variables.

Frequencies and percentages were given as descriptive statistics for categorical data. Mean ±SD (Median) were given for continuous data. P values < 0.05 were considered to be significant in all tests.

## 3. RESULTS

One case which was HE4 negative was neuroendocrine carcinoma and was excluded from the study. One cancer slide had no enough tumor cells but had IM. This case was included in Group I. During IHC staining, 3 samples from cancer slides and 7 samples from IM slides were washed off while antigen retrieval heating. There were not enough metaplastic cells (grades less than 1) in six cases. In total, 37 patients with a diagnosis of adenocarcinoma and 34 patients with a diagnosis of IM were eligible for the study. There were 14 female and 23 male patients, aged 38–90 years (mean: 65.6) in Group II. Tumor localization were antrum in 14 (37.8%), corpus in 10 (27%), lesser curvature in 8 (21.6%), greater curvature in 2 (5.4%), and cardia in 3 (8.1%) patients and tumor sizes ranged between 1-13 cm (mean 5.54). There were 3 (8.1%) well, 11 (29.7%) moderately, 13 (35.1%) poorly differentiated tumor and 10 (27%) signet cell carcinoma. Pathological T and N evaluations were as follows: pT1=5 (13.5%); pT2=3 (8.1%); pT3=22 (59.4%); pT4=7 (18.9%) patients and pN0=10 (27%); pN1=5 (13.5%); pN2=7 (18.9%); pN3=15 (40.5%) patients (Table1). The expression of HE4 was seen in 18 (50%) tumors in which there were four 1+ (11.1%); nine 2+ (25%); and five 3+ (13.9%) staining (Fig. 1 and 2). Interestingly, one endoscopic biopsy that harbored dysplastic epithelia which expressed HE4 protein (Fig. 3).

Of the 34 endoscopic biopsies in which metaplastic cells successfully stained with HE4, there were six 1+ (17.6%); nine 2+ (26.5%) and eleven 3+ (32.4%) staining (Fig. 4). Overall HE4 expression was 76.5%, whereas 8 cases (23.5%) were negative with HE4 (Table 2). Pretty much the same HE4 expression rate in metaplastic cells in the vicinity of thetumorwas seen: Eleven out of 18 cases (61.1%) (Table 3). Besides metaplastic cells, we observed that while superficial foveolar tall columnar mucous epithelium almost never expressed HE4 protein, the long tubular gastric glands usually expressed HE4 protein (Fig. 5).

Regarding inflammatory activity and H. Pylori status, all but one of the biopsies had chronic (97%) and 26 (76.5%) had active inflammation. Helicobacter pylori were seen in the 18 (53%) of the biopsy. Level of tumor invasion was inversely correlated with the HE4 expression (p=0.037) but there was no statistical relationships between the presence of HE4 and age (p=0.56), gender (p=0.3), tumor location (p=0.86), tumor size (p=0.83), tumor grade (p=0.34), nodal status (0.083), chronicity (p=0.76), activity (p=0.6), and the presence of H. pylori (p =0.58).The data of our cases were given collectively in Table1.

**Tablo 1. Clinicopathological characteristics and the expression of Human Epididymis Protein-4 in Group II (gastric cancer patients)**

| | | | HE4 expression | | |
|---|---|---|---|---|---|
| | | | **Present, n (%)** | **Absent, n (%)** | **p** |
| Age | (Mean±SD) | | 64,17±16,58 | 67,17±17,78 | 0.559 |
| Gender | male | #23 | 13 (56,5) | 10 (43,5) | 0.298 |
| | female | #13* | 5 (38,5) | 8 (61,5) | |
| Tumor location | antrum | #14* | 6 (46,2) | 7 (53,8) | 0.860 |
| | corpus | #10 | 4 (40) | 6 (60) | |
| | lesser curvature | #8 | 5 (62,5) | 3 (37,5) | |
| | greater curvature | #2 | 1 (50) | 1 (50) | |
| | cardia | #3 | 2 (66.7) | 1 (33.3) | |
| Tumor size | (Mean±SD) | | 5,14±2,35(5,50) | 6,07±3,66(5,00) | 0,830 |
| Tumor grade | well | #3 | 2 (66.7) | 1 (33.3) | 0.34 |
| | moderately | #11 | 5 (45,5) | 6 (54,5) | |
| | poor | #13* | 4 (33,3) | 8 (72,7) | |
| | signet | #10 | 7 (70) | 3 (30) | |
| pT | 1 | #5* | 4 (100) | 0 (0) | 0.037 |
| | 2 | #3 | 0 (0) | 3 (100) | |
| | 3 | #22 | 12 (54,5) | 10 (45,5) | |
| | 4 | #7 | 2 (28,6) | 5 (71,4) | |
| pN | 0 | #10 | 7 (70) | 3 (30) | 0.083 |
| | 1 | #5 | 0 (0) | 5 (100) | |
| | 2 | #7* | 3 (50) | 3 (50) | |
| | 3 | #15 | 8 (53,3) | 7 (46,7) | |

**1 patient had less tumor cells but HE4 was positive in metaplastic cells*

**Table 2. Human Epididymis Protein-4 expression in the metaplastic cells obtained from endoscopic biopsies**

| | | | HE4 expression | | | |
|---|---|---|---|---|---|---|
| | | | **Present, n (%)** | **Absent, n (%)** | | **p** |
| Chronicity | yes | #33 | 25 (75,8) | 8 | (24,2) | 0.765 |
| | no | #1 | 1(100) | 0 | (0) | |
| Activity | yes | #26 | 19 (73,1) | 7 | (26.9) | 0.615 |
| | no | #5 | 4(80) | 1 | (20) | |
| *H. pylori* | yes | #18 | 14 (77.8) | 4 | (22,2) | 0.583 |
| | no | #16 | 12 (75) | 4 | (25) | |

**Table 3. Human Epididymis Protein-4 expression in the metaplastic cells in the vicinity of the tumor**

| | | HE4 expression in metaplastic cells | | |
|---|---|---|---|---|
| | | **Present** | **Absent** | **p** |
| | | **n (%)** | **n (%)** | |
| Tumor location | antrum #11 | 6 (54,5) | 5 (45,5) | 0.478 |
| | corpus #5 | 3 (60) | 2 (40) | |
| | cardia #2 | 2 (100) | 0 (0) | |

## 4. DISCUSSION

Human epididymis 4 (HE4) protein is a secretory protease inhibitor [13] and involves in the innate immunity of the respiratory tract and nasal cavity [6].It was originally discovered in the epididymis but is now shown to over-expressed in different types of carcinoma: Silencing of HE4 in ovarian cancer cells results in the inhibition of proliferation and the decrease of invasion [14,15]. On the other hand, over-expression of HE4 results greater adhesion and migration of

cancer cells. Besides these in-vitro studies, in vivo studies also demonstrated that serum level of HE4 increases in ovarian cancer patients and this increase is associated with worse progression-free survival (PFS), and it is an independent prognostic parameter for PFS [16]. Besides ovarian carcinoma patients, HE4 is also detected in the serum or pleural effusion of patients with lung cancer and its utility for lung cancer diagnosis has been investigated in different studies [17-19].

There are few studies conducting on gastric cancer: Gastrin-deficient mice have a little HE4 expression in the normal gastric mucosa [20]. Silencing of HE4 expression in gastric cancer cells leads to increased apoptosis and decreased proliferation [21].

Two types of gastric metaplasia develop in the human stomach and considered putative pre-neoplastic lesions: Goblet cell intestinal metaplasia and pseudopyloric metaplasia [22]. In intestinal metaplasia, the metaplasticepithelium

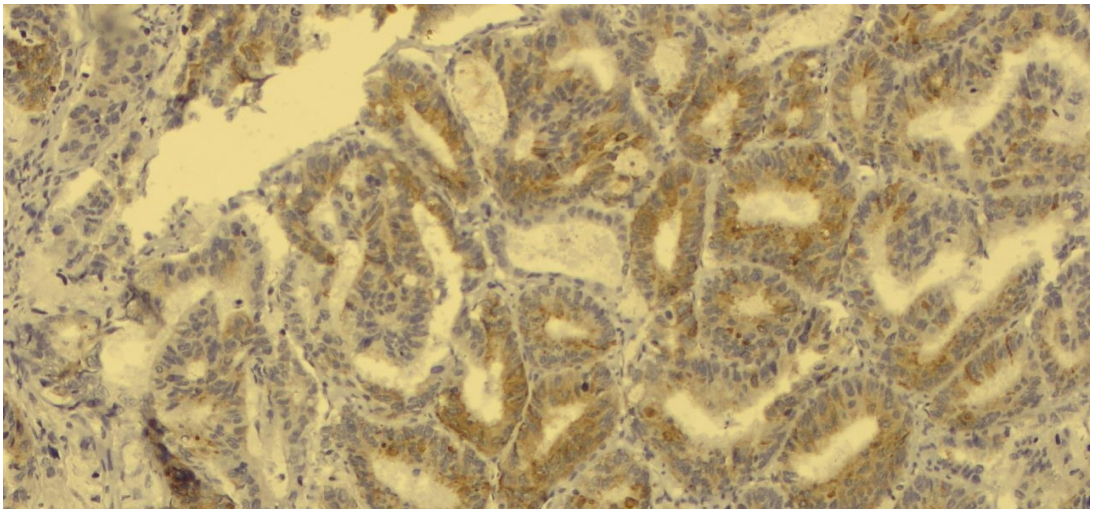

**Fig. 1. Strong and diffuse Human Epididymis Protein-4 expression in seen in this moderately differentiated adenocarcinoma cases (anti-HE4, x10)**

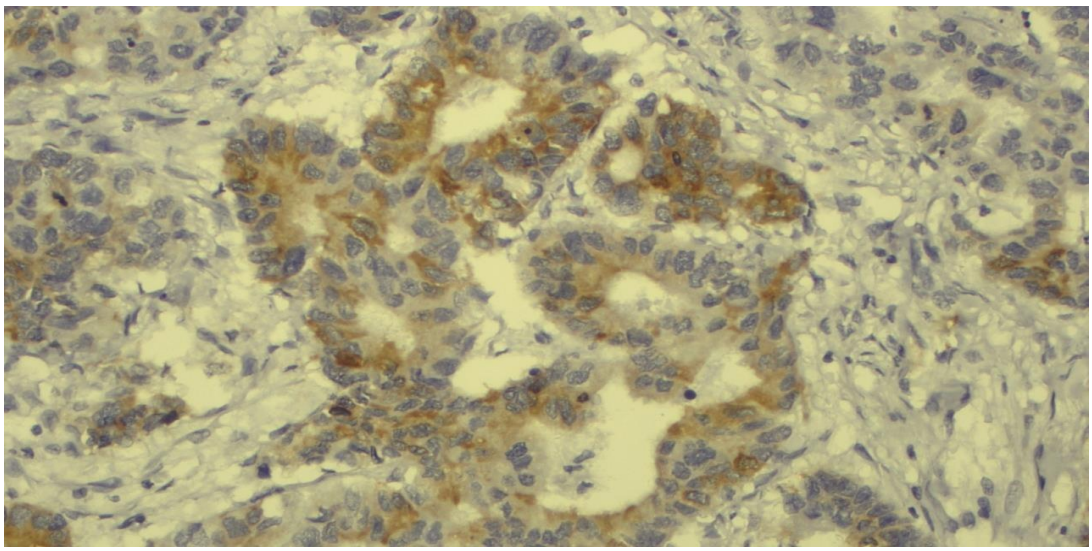

**Fig. 2. Closer view highlights luminal as well as cytoplasmic staining in tumor cells (anti-HE4, x20)**

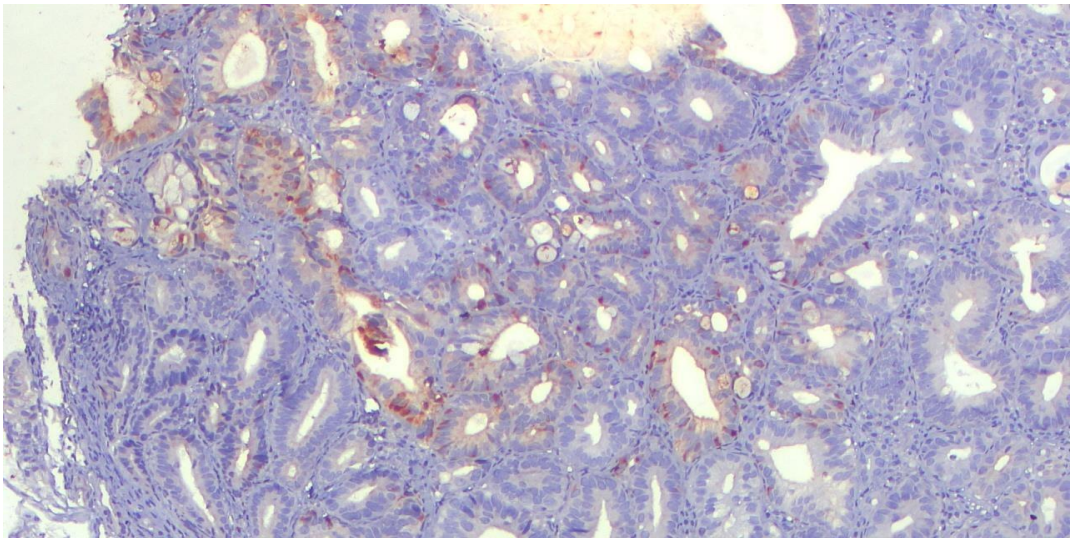

**Fig. 3. Back to back glands with crowded nuclei are the hallmark of this displastic focus. Note HE4 expression in pre-neoplastic cells (anti-HE4, x10)**

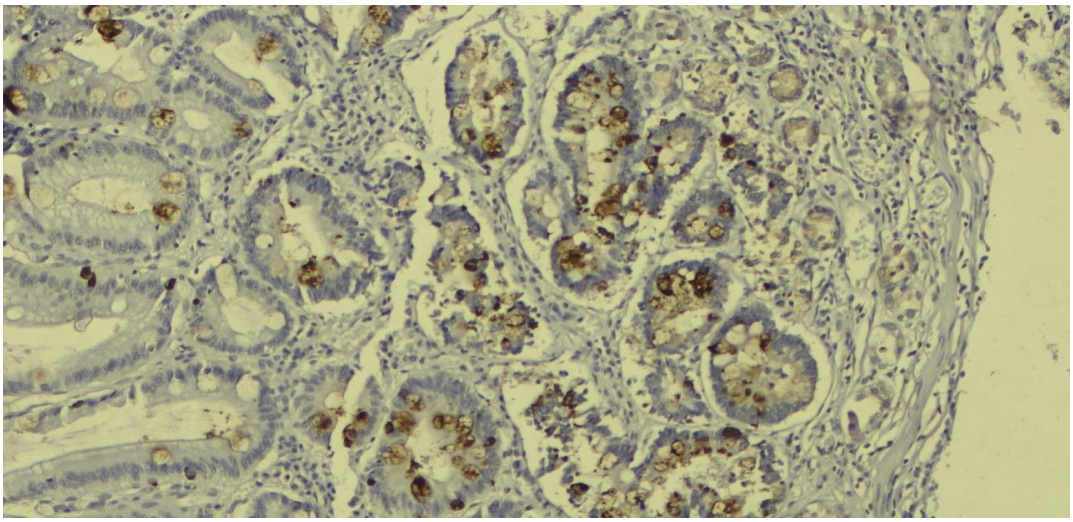

**Fig. 4. Metaplastic cells in the form of Goblet cells in the foveolar epithelium. Deep gastric glands have strong HE4 expression (anti-HE4, x10)**

is seen morphologically as goblet cells and absorptive enterocytes and according to enzyme histochemistry, sulfomucin-containing type is associated with increased risk of gastric cancer [23]. Pseudopyloric metaplasia, also called fundicantralisation or spasmolytic polypeptide expressing metaplasia (SPEM), on the other hand, is characterized by the antral type metaplastic transformation of the oxyntic mucosa. Contrary to true pyloric glands present in the antrum, these cells does not produce gastrin (hence the name pseudopyloric) but expresses spasmolytic polypeptide [24].

Our study localized HE4 in the oxyntic mucosa. To our knowledge, localization of HE4 in the oxyntic mucosa has never been observed before. Nozaki et al. indicate that HE4 is not detected in the normal human fundic (oxyntic) mucosa but it is positive in all metaplastic lesions, including pseudopyloric metaplasia and intestinal metaplasia [25]. Our study also confirmed its presence in the metaplastic cells but contrary to their observation, we detected diffuse and strong HE4 expression in oxyntic mucosa (Fig. 5). Even IM cells present in the cardiac mucosa expressed HE4. HE4 was absent on the surface

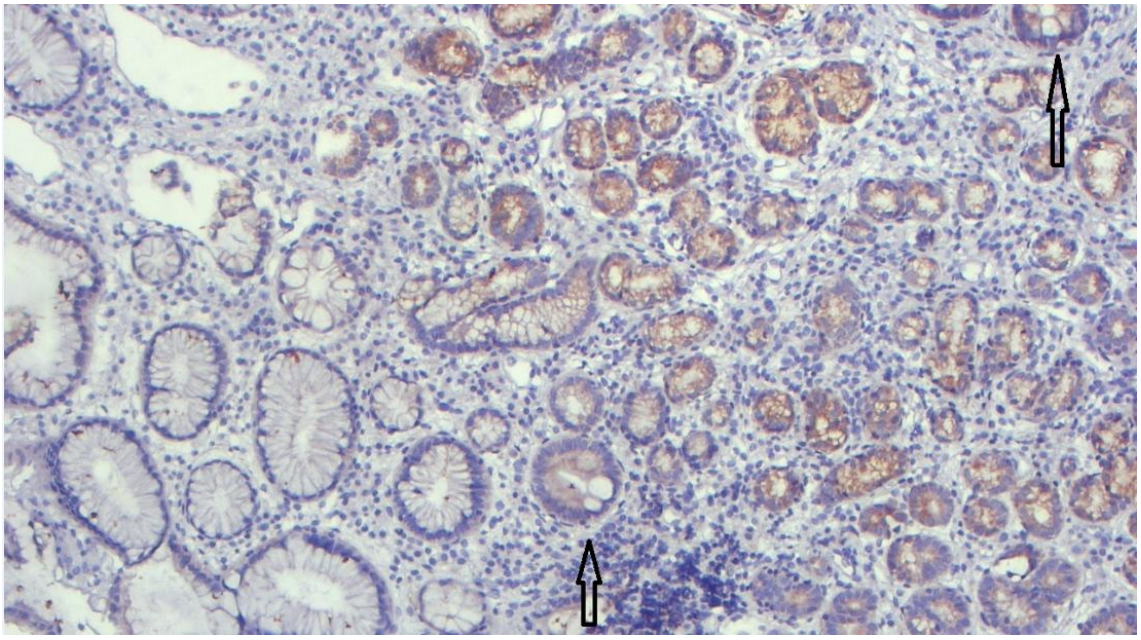

**Fig. 5. Superficial foveolar epithelium is devoid of HE4 expression, while the long tubular gastric glands have diffuse and strong expression. Some metaplastic cells also have weak expression (arrow) (anti-HE4, x10)**

epithelium. It's presence in metaplastic cells as seen in our study is consistent with the proposal that the origin of metaplastic cells are the HE4 positive stem cells located in the base of the gland instead of surface foveolar cells [26-29]. There is a need to sub-classify gastric cancers into types in which it originates: Foveolar, oxyntic, fundic, etc [30].

The HE4 expression in diffuse-type adenocarcinoma has been higher than intestinal type adenocarcinoma in a study (61% vs 25%) [31].Although not statistically significant, higher HE4 expression in diffuse-type adenocarcinoma than intestinal type adenocarcinoma was also observed in our study (70% vs 41%). We also observed that HE4 expression was inversely correlated with invasiveness and it has been statistically significant ($p< 0.05$). Oppositeresults were seen in studies conducted on ovarian, endometrial and lung cancers: Serum HE4 level had been correlated with myometrial invasion [32,33], treatment response of ovarian cancer [34]and lung cancer [35]. As a secreted protein, higher tumor volume would lead higher serum level of HE4, as tumor cells produce and secrete HE4. Higher serum level of HE4 in these tumors actually reflects higher tumor volume. If normal oxyntic mucosa possesses this protein, as observed in our study, higher tumor size would have had higher HE4 expression. This hypothesis was not proved in our study but the study of Guo et al has proven the hypothesis: HE4 was significantly correlated with Lauren classification, TNM stage, and tumor size [36].The opposite observation seen in our study, ie: inverse correlation with the depth of tumor invasion may be due to a number of cases present in T1tumors in our study group.

The cell-growth-related signals are AKT and ERK. Knockdown of HE4 in ovarian cancer cells result in the reduction in cell growth and increased sensitivity to cisplatin[37] and HE4-overexpressing ovarian clones display increased resistance to cisplatin and paclitaxel [38].The same result was observed in gastric carcinoma cells: Silencing of HE4 inhibits activation of AKT and ERK1/2 [36]. These are in vivo studies but it is obvious that HE4 involves in intestinal metaplasia, gastric carcinogenesis, biological behavior and treatment response. In vivo studies are needed to clarify this issue.

Our study has limitation. Besides small study population, we didn't investigate HE4 expression in the cell of SPEM, a novel candidate for gastric cancer development that gives rise to IM [39,40]. We also didn't test serum level of HE4 due to retrospective nature of the study.

## 5. CONCLUSION

In conclusion, HE4 is expressed in oxyntic mucosa and the cells in intestinal metaplasia. It is expressed in diffuse type carcinoma compared to intestinal-type carcinoma. Before making a conclusion regarding expression correlation with pT1, large scale studies are needed. HE4 may be good a candidate for personalized treatment.

## CONSENT

As per international standard or university standard written participant consent has been collected and preserved by the authors.

## ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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