Immunohistochemistry for CDX2 expression in non-goblet-cell Barrett's oesophagus

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ABSTRACT

Interest has increased in *CDX2* gene expression in oesophageal non-goblet-cell columnar metaplasia as recent investigations indicate such metaplasia possesses neoplastic potential. This study aims to assess expression of the transcription factor CDX2 specifically in non-goblet-cell cardia and fundic oesophageal metaplastic tissue, and to compare the location of CDX2 expression in non-goblet-cell specimens to that in goblet-cell specimens. A total of 43 patient specimens (20 fundic-type metaplasia, 42 cardia-type metaplasia and 18 intestinal metaplasia goblet cell-positive) were examined in this study. These were selected over six months from a patient database using the systematised nomenclature of human and veterinary medicine coding system (SNOMED). CDX2 was detected in patient specimens with an anti-CDX2 mouse monoclonal antibody. The types of mucosa in each specimen were confirmed by haematoxylin and eosin (H&E) staining. Fundic specimens were consistently CDX2-negative (0%). CDX2 expression was distinct in 55% of cardia and 100% of intestinal cases. Nearly all cardia-positive cases displayed focal expression (95.5%) and all intestinal cases displayed diffuse distribution of expression. Almost all cardia- and intestinal-positive specimens demonstrated epithelial expression (95.5% and 100%, respectively). The percentage of cardia-positive specimens with deep tissue expression was lower than in intestinal specimens (31.8% *vs.* 94.4%, respectively). This study confirms CDX2 as an early marker for Barrett's oesophagus in the absence of goblet cells as expression was noted in cardia metaplasia. *CDX2* appears to induce the transformation of the normal oesophageal mucosa to cardia type, which then differentiates to an intestinal type under the influence of gastro-oesophageal reflux disease.

KEY WORDS: Barrett esophagus.
CDX2 protein, human.
Immunohistochemistry.

Introduction

The incidence of oesophageal adenocarcinoma (OA) is focused in the Western world, were it is described as the fastest rising solid malignancy, with the greatest burden found in males from the United Kingdom,[1,2] where the incidence is six in 100,000 inhabitants.[3] Most carcinomas harbour a poor outcome because local and systemic invasion is unnoticed in the early phases. The five-year survival rate in patients with OA is less than 20% unless the condition is diagnosed at an early stage.[3–6]

Hypothetically, early intervention of OA is attainable as it normally develops through the creation of Barrett's oesophagus (BO).[7] Early OA is now more often detected as a result of more intensive monitoring of patients with BO.[3] Barrett's oesophagus is the term used to depict the changes from the normal squamous epithelium (SE) in the lower oesophagus to a columnar epithelial-lined oesophagus (CELO).[8] The condition has gained much interest from gastroenterologists and basic researchers because of its potential for advancement to OA.[2]

Currently there is agreement as to whether or not the presence of intestinal metaplasia (IM) containing goblet cells (GCs) is required in order to diagnose BO. The present definition by the World Health Organization states that "BE is restricted to cases with histologically confirmed intestinal metaplasia" with further description of BE being "characterised by two different types of cell, i.e., goblet and columnar cells". Thus, the detection of GCs remains the gold standard for diagnosis of BO. This stringent interpretation has been opposed by the new British Society of Gastroenterology (BSG) guidelines for the diagnosis and management of BO. They state that BO is defined as "an endoscopically apparent area above the OGJ that is suggestive of Barrett's which is supported by the finding of columnar-lined oesophagus on histology". The presence of areas of IM, although often present, is not a requirement for diagnosis.[9]

In addition to IM, two further types of epithelium can be recognised in BO, cardia-type mucosa (CTM) and fundic-type mucosa (FTM), both of which are devoid of GCs. Apart from in children, IM is the most prevalent kind in BO. Endoscopy can miss IM, resulting in omission of patients from surveillance.[10] This can happen when GCs are well hidden or if small biopsies with crush artefact are sent for diagnosis. In addition, it has been recently shown that CTM is a precusor of IM. Thus, the discovery of a marker to identify intestinal differentiation in the absence of GCs might be of clinical value.[11]

It has been proposed that SE initially differentiates into either FTM or CTM, with the latter potentially differentiating to GC metaplasia.[9,11] Understanding of the early mechanisms associated with the pathogenesis of BO could permit the production of preventative treatments.[12]

Caudal, which are *Drosophilia* (fruit fly) homeobox genes,

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are needed for early modelling of the intestine. *Caudal* homologues have been found in a considerable number of organisms, from *Caenorrhabditis elegans* to humans.[13,14] In humans, there are three *caudal* homologues, CDX1, CDX2 and CDX4. The last two are significant in intestinal epithelial development in their role as master switches.[12,13,15] For the purpose of the current study, the caudal genes for humans will be written as CDX and for non-human as Cdx.

Research has shown that neoplasia develops when cells do not complete differentiation. Neoplastic cells possess numerous characteristics resembling those of embryonic cells, such as high proliferating rate and incomplete differentiate. Additionally, neoplastic cells have a propensity for migration and angiogenesis. It is also understood that many genes vital for typical development also function in the mechanisms of human carcinogenesis.[13]

The molecular processes that result in transformation in the oesophagus from SE to IM are not completely understood. Recent work indicates the involvement of *CDX* genes.[16–18] Is it is thought that CDX2 activity can control epithelial cell proliferation and differentiation. In particular situations it can block tumour progression and mediates apoptosis.[13,19] Although diminished, CDX2 expression causes focal gastric differentiation in the colon, and abnormal expression of CDX2 in the upper gastrointestinal is regarded as a central occurrence in the pathogenesis of Barrett's metaplasia of the oesophagus and IM of the stomach.[20–22]

Gastrointestinal oesophageal reflux disease (GORD) is the main aetiological factor for BO and it has been hypothesised to trigger the expression of CDX2, which in turn makes stem cells in the oesophageal epithelium differentiate into intestinal-type cells.[12] In healthy adults, CDX2 is displayed in the small and large intestine but not in the normal stomach or oesophagus.[23,24]

It is well established, by immunohistochemical (IHC) studies, that Cdx2 is expressed by oesophageal GC columnar metaplasia. There is evidence to support the hypothesis that CDX2 expression in CTM could be able to predict the presence of undiagnosed IM in BO and so could be a potential marker for the presence of IM in the absence of GCs. Thus, it could be an early marker of Barrett's metaplasia.[9,11,25–29] As CDX2 is a nuclear protein, expression appears to be mostly limited to cells of intestinal origin and it is thought to play a significant role in the early differentiation of the intestinal epithelium via the transcription of an intestinal-specific gene. Thus, identification could represent a more sensitive technique for BO diagnosis than present standard histochemical evaluation for the presence of GCs.[25,30]

Past research into the variation of CDX2 expression in ONGCCM and GC BO is limited, particularly in relation to cardia-type and fundic-type metaplasia. Further work would enhance current understanding of the role of CDX2 in the pathogenesis of BO. Therefore, the aim of the present study is to perform CDX2 immunohistochemistry (IHC) on patient specimens containing CTM, FTM and IM confirmed by haematoxylin and eosin (H&E) staining in order to assess variation in gene expression in these mucosal areas. Additionally, it will analyse variations in the pattern and location of CDX2 expression in tissue specimens in relation to different types of mucosa found in BO to understand how they are related. This will support or refute the following hypotheses: i) CDX2 can be used as an early marker for BO in ONGCCM in the absence of IM; ii) in the oesophagus, SE develops in either CTM or FTM, with the latter having no neoplastic potential; iii) GORD activates CDX2 in a time- or/and dose-dependent manner in the oesophageal epithelium; iv) CDX2 in the various types of metaplasia found in BO may be a treatment marker; and v) CDX2 may have a role as a tumour suppressor or tumour inhibitor

Table 1. Mucosa profile of the cases used in the study.

	C	C&I	F&I	C&F	C&F&I
Number of specimens in which each type of mucosa was analysed	8	9	1	16	9

C: cardia; F: fundic; I: intestinal.

Materials and methods

The project required the use of archived biopsy specimens embedded in paraffin wax and held in the Salford Royal NHS Foundation Trust (SRFT) cellular pathology department. It also required a search of the patient diagnostic database to find suitable specimens. This research project was given full approval by the National Research Ethics Service (NRES) Committee Northwest – Greater Manchester East. Following application to the NRES, the project was also fully approved by the SRFT research and development department. To comply with the terms of the approval, all cases in the project were subsequently anonymised using a random coding system before their use in the laboratory practical work.

A total of 125 oesophageal endoscopic biopsies, taken from the distal oesophagus, previously reported for diagnostic purposes over a six-month period during 2010 were selected retrospectively from the tissue archive at SRFT, based on the systematised nomenclature of human and veterinary medicine (SNOMED) coding system. Original sections were stained with H&E. In total, 42 patient specimens were included in the research and one biopsy was assessed for each patient. These were assessed for the presence of cardia-, fundic- and goblet-cell metaplasia, based on standard morphological criteria as outlined in previous research.[9]

Specimens containing combinations of the different types of metaplasia were permitted and those with < 50% non-goblet cells were excluded. Each type of mucosa was only analysed if ≥10% was present in any one patient specimen (Table 1).

Tissue specimens had been fixed in neutral buffer formaldehyde and processed into paraffin wax employing standard histological protocols. Two sections were cut at 3–4 μm and placed on adhesive-coated glass microscope slide (Superfrost Plus, Menzel-Glazer, Europe), one for CDX2 immunohistochemistry and another as the negative control. This was followed by three more sections cut at deeper levels, mounted on normal glass slides (Twinfrost, VFM, UK) for H&E confirmation of tissue morphology.

The test sections were dried overnight at 60°C. Antigen retrieval was conducted in 0.01 mol/L Tris-EDTA-Tween (pH 8.5) for 30 min in a 900 W microwave oven.

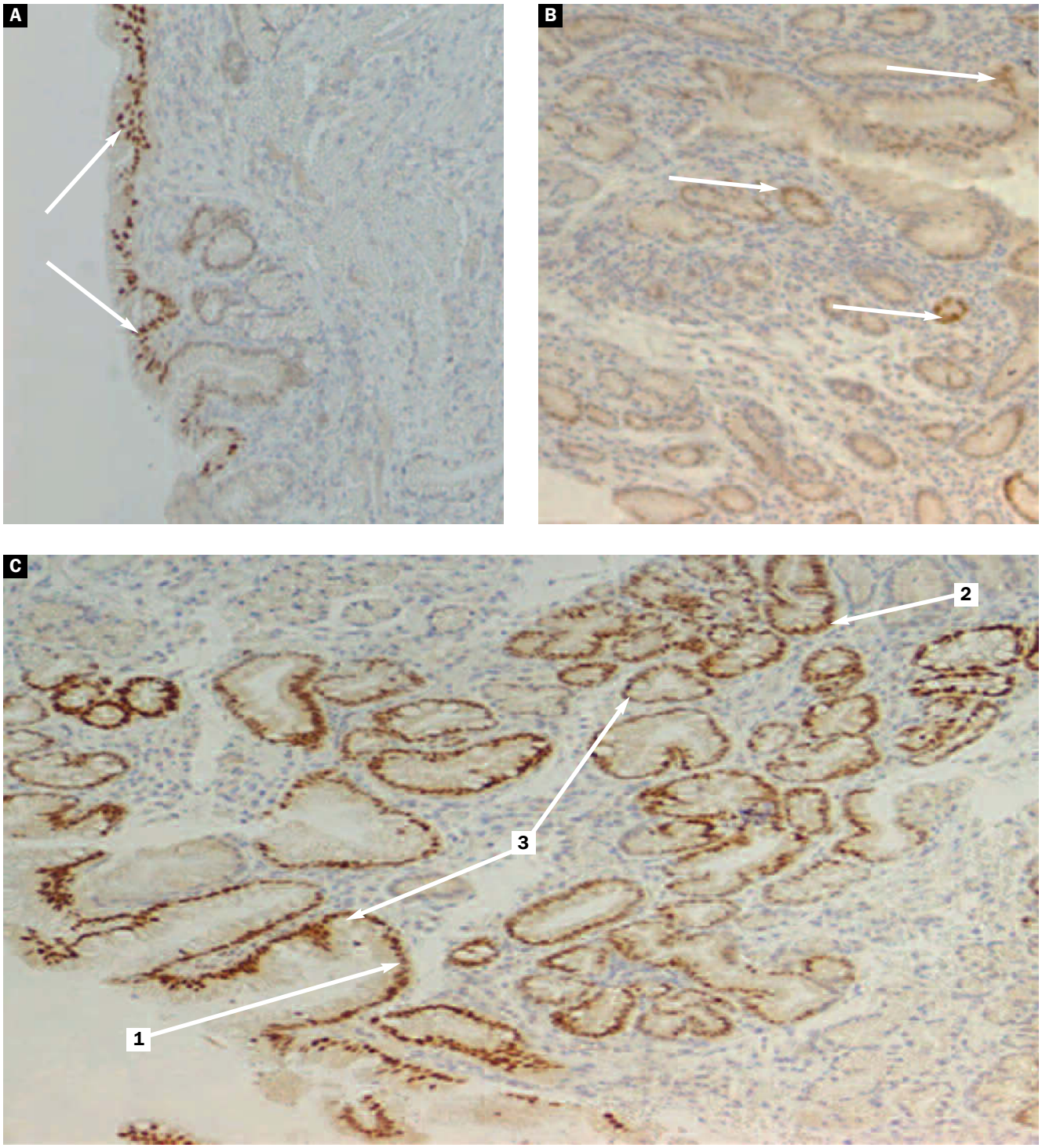

Fig. 1. Light micrographs of CDX2 expression (arrows) following immunohistochemistry in CDX2-positive cardia and intestinal specimens (original magnification x400. **A)** Focal positivity in surface epithelium of CTM and **B)** deep CTM tissue. **C)** Diffuse positivity in 1) epithelial and 2) deep IM tissue with 3) goblet cells. Note the lack of goblet cells in A and B compared to C in both the epithelium and deep tissue.

Immunohistochemistry staining was conducted on an automated immunostainer (Biogenex i6000; Biogenex, Milton Keynes, UK). CDX2 was detected with a monoclonal antibody (clone AMT28, diluted 1:30 [NCL-CDX2, Leica Microsystems, Milton Keynes, UK]). Endogenous peroxidase activity was blocked using peroxidase blocking solution (S2023; Dako, Glostrup, Denmark). Primary antibody was detected with the Dako REAL EnVision detection system (K5007), following the manufacturer's protocol. Washes between stages was conducted in TBS/Tween wash buffer [pH 7.6]. The sections were then counterstained lightly in Mayer's haematoxylin (01560BBE; Surgipath Europe), producing a pale blue nuclei, rehydrated in alcohol, cleared in xylene, and a coverslip was applied using Pertex (Histolab, Products AB, Gothenberg, Sweden).

Staining (H&E) was performed on a Leica XL automated

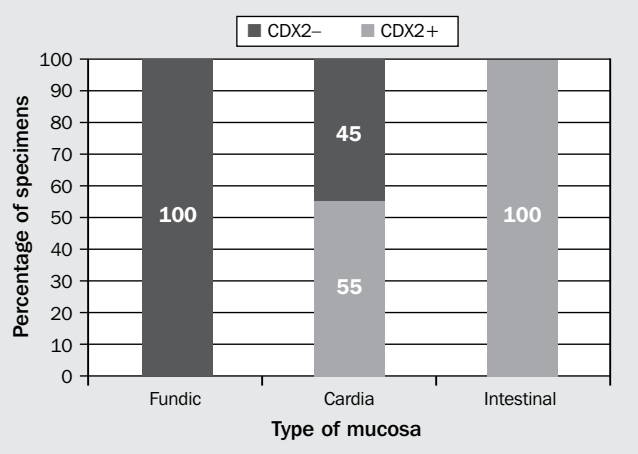

Fig. 2. χ^2 analysis comparing the number of cardia, fundic and intestinal specimens that were CDX2-positive.

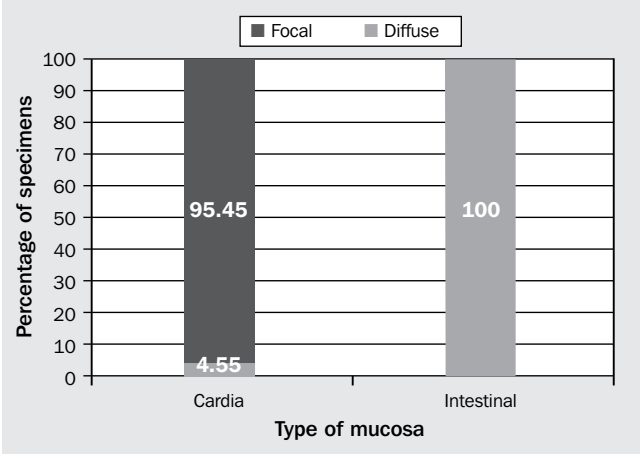

Fig. 3. χ^2 comparison of distribution of staining in CDX2 immunohistochemical-positive cardia and intestinal cases.

stainer. Section were dewaxed by placing in xylene and then dehydrated with alcohol. This was followed by staining in haematoxylin for 8 min. Differentiation in 0.5% acid alcohol was followed by Scott's tap water, and the sections were then placed in eosin for 4 min. Finally they were dehydrated and cleared, mounted and coverslipped as per the IHC protocol. Washes between reagents were performed with distilled water.

Small strips of CDX2-positive (colon) and CDX2-negative (uterus) controls were cut at 3–4 µm and placed with each patient test section. The controls were treated in exactly the same way as the tests, although antibody was replaced by TBS/Tween wash buffer (pH 7.6). Cases were scored as either CDX2-positive or CDX2-negative by light microscopy (Leica DM2000). A positive result was recorded even if staining was only seen focally for CDX2. All cases were checked against the original, and diagnostic sections were taken at three deeper levels and stained with H&E to ascertain if goblet cells were present. Only localised brown nuclear staining was regarded as CDX2 expression.

Qualitative analysis was performed using a Minitab 16 software package. Fisher's exact test (FET) was used to assess variation in the location of CDX2 expression between different mucosa. χ^2 was utilised for all other statistical analyses. The normal 5% threshold was employed to ascertain statistical significance.

Results

Light microscopy examination found that some CTM, none of the FTM and all of the IM mucosa displayed distinct CDX2-positive brown nuclear staining following IHC (Fig. 1A–C). A significantly greater percentage of cardia specimens were CDX2-positive in relation to fundic specimens (55% *vs.* 0%, respectively; $P=0.001$). A significantly greater proportion of intestinal specimen were CDX2-positive in comparison to cardia-specimens (100% *vs.* 55%; $P=0.001$) (Fig. 2). There was a statistically significant greater percentage of intestinal specimens than cardia specimens demonstrating diffuse distribution of CDX2 staining (100% *vs.* 4.6%, respectively; $P=0.001$) and a statistically significant lower percentage of intestinal specimens than cardia specimens demonstrating focal distribution of CDX2 staining (Fig. 3). The percentage of intestinal specimens that showed CDX2 reactivity in the epithelium was greater than in cardia specimens, although not statistically different (100% *vs.* 95.5%, respectively) (Fig. 4). The percentage of intestinal specimen that showed CDX2 reactivity in deep tissue was almost statistically greater when compared to cardia specimens (94.4% *vs.* 31.8; $P=0.053$) (Fig. 5). The CDX2-negative tissue control (uterus) showed no CDX2 expression. All patient tissue sections treated with buffer instead of antibody showed no visible brown staining. Immunohistochemistry for CDX2 displayed high-quality nuclear localisation in epithelial cells on examination of the positive control sections (colon).

Discussion

The specimens in the present study were probably not representative of the general population of BO sufferers as the selection process was designed to ensure that a higher number of non-GC cases were included. Normally, there is a higher proportion of IM in BO in comparison to CTM or/and FTM. This was done because the main aim was to assess CDX2 reactivity in non-goblet types of mucosa. A smaller number of such cases may not have given a good representation of the level of CDX2 expression such mucosa possesses. Also, including a higher number of CTM specimens ensured that the trend found in the pattern and distribution of CDX2 positivity was representative of all BO cases. It was thought during the planning of the study that most of the IM cases found in the study were likely to be all CDX2-reactive based on past research.

The present study has demonstrated CDX2 expression in CTM, but no expression was seen in FTM. Previous studies have demonstrated expression for CDX2 using IHC in non-GC metaplasia.[11,26,27,29] The present study demonstrated CDX2 expression in ONGCCM-containing epithelium in 30%, 38%, 34% and 43% of cases, respectively. It has been proposed that SE initially differentiates into CTM, which in turn develops into FTM with no malignant potential or to IM with the danger of further progression to adenocarcinoma. A proposed alternative hypothesis would be for SE to differentiate into either FTM or CTM, with the latter potentially differentiating to IM.[9,11] The hypothesis that SE

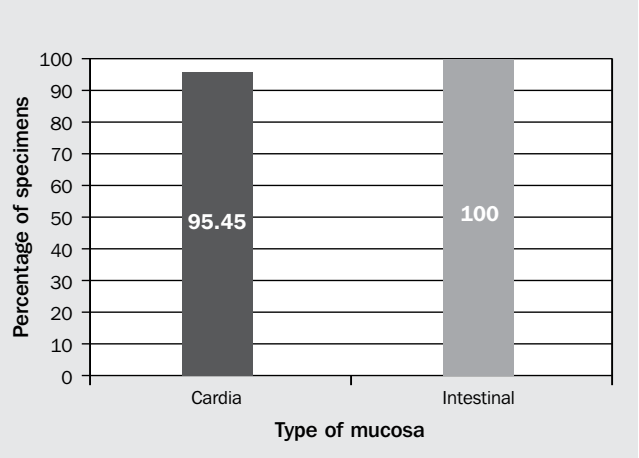

Fig. 4. Fisher's exact test of reactivity in epithelial tissue from CDX2 immunohistochemical-positive cardia and intestinal cases.

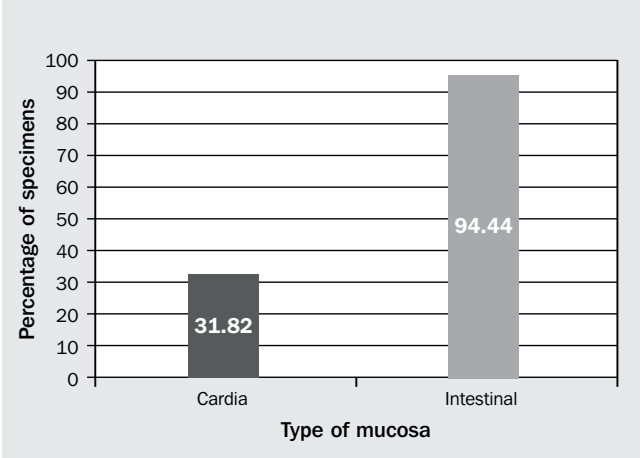

Fig. 5. Fisher's exact test of variation of deep tissue reactivity in CDX2 immunohistochemical-positive cases.

differentiates into either FTM, with non-neoplastic potential, or into CTM, which potentially differentiates to IM, is supported by the demonstration in the present study of CDX2 expression in some cases of CTM, by the lack of CDX2 expression in FTM cases, and by the location and distribution displayed in CDX2 gene expression. It also supports the theory that CTM develops into IM under the influence of CDX2.

Thus, the study suggests that CDX2 could be used to detect BO when goblet cells are not present in biopsies. However, the fact that a considerably higher percentage of IM cases in the present study were CDX2-positive in comparison to CTM, it seems more like that BO would be detected on a biopsy containing goblet-cell metaplasia than non-goblet-cell metaplasia with IHC for CDX2. The belief that CTM rather than FTM possesses neoplastic potential could be investigated by a longitudinal study in which patients with oesophageal biopsies taken on endoscopy displaying either of these mucosal elements were followed over time to see if they develop oesophageal cancer.

Interestingly, a study has reported the discovery of a non-intestinal pathway to neoplastic development in BO, which is metaplasia–foveola dysplasia–adenocarcinoma.[31]

It was found in the study of oesophagogastrectomy cases that the foveola type was repeatedly devoid of markers for intestinal differentiation, although regularly displayed MUC5 IHC reactivity. In light of these findings, it may be useful to test the reactivity of MUC5 antibody against fundic-type metaplasia cases.

Various bile acids (BAs) found in the refluxate of GORD suffers can cause damage to the oesophageal epithelium and trigger the creation of metaplasia via mitochondrial alterations, oxidative stress or DNA injury. It has been demonstrated that BAs coupled to low pH promotes oxidative stress that could partly be linked to the pathogenesis of BO.[32] Various studies have linked different BAs to BO and the promotion of CDX2.[33] These risk factors for GORD are over-eating, pregnancy, and poor posture, as well as factor that make the sphincter lax or defective, such as smoking and alcohol consumption; however, alcohol and tobacco are believed by some to be minor risk factors.

Genetic factors could be involved but this is an area that needs more research.[6,32,34,35] A study of rat models has shown that high dietary animal fat altered BA composition and elevated the level of BA components in bile juice.[35] According to some authors, symptoms of GORD are poor forecasters of BO, and little or no link with heartburn symptom intensity has been reported, although length of symptoms may be more important.[31]

Kazumori *et al.* found that BAs in oesophageal epithelial cell lines (OECLs) produced elevated CDX1 transcription, and that transfection of the CDX1 vector in cultured OECLs promoted CDX2 protein manufacture,[37] suggesting that CDX1 preceded by CDX2 could have a vital role in the formation of BO. It has been shown in murine models that *Cdx1* is linked with gastric IM, and that ablation of *Cdx2* at embryonic development day 13.5 did not alter Cdx1 activity in the intestine. However, *Cdx1*-negative mutants displayed a small lowering of Cdx2 in the large intestine by approximately 30%.[38]

In the current study, a high percentage of CTM cases were CDX2-negative in comparison to IM. This could indicate that CTM does not have that a strong relationship to IM; however, it could be due to the formation of CTM, with CDX2-negative expression caused by CDX1 activation through GORD. The GORD insult in these specimens had not been long or/and strong enough to elicit CDX2 expression, which would indicate that the gene is required for intestinal differentiation and CDX1 cannot compensate in such a transformation in the oesophagus. However, there is no published information on CDX1 expression in cardia-type oesophageal tissue.

The dose- or/and time-related theory for activation of CDX2-induced BO by GORD may be supported by the findings of the current study because it would help to explain the lower level of significance between the number of CDX2-positive CTM and IM specimens with deep staining as opposed to surface CDX2-positive expression. It is possible that when GORD/CDX1 activates CDX2 in the specimens in detectable quantity, it does so first in the epithelium and then in deeper tissue. If the development of BO relies on the intensity of GORD, it would seem plausible that the level would differ between patients from which the specimens were taken. Additionally, the length of time the specimens were taken after activation of CDX2 in the epithelium would be expected to vary. Hence, it would take longer for CDX2 expression to appear in some cardia-type

deeper tissue specimens than in others. It might then be suggested, given the variation in the number of CDX2-positive CTM and IM specimens with diffuse expression, that once GORD-driven CDX2 has established localised expression in the cardia-type epithelium and then deep tissue, further GORD over time promotes expansion of CDX2-positive expression to a greater proportion of tissue and the subsequent development of IM.

Histological research in males examined by gastric endoscopy prior to *Helicobacter pylori* eradication, and after a median 72 months following eradication, showed that IM was not easily remedied in the setting of high-virulence *H. pylori* eradication.[39] It has been demonstrated that cure before the development of IM reduced inflammation and could block intestinal-type gastric cancer.[21] *H. pylori* cure in gastric metaplasia has resulted in lowered CDX2 expression in non-GC glands.[40] In consideration of the findings in the present study, it may indicate that removal of GORD (i.e., via lifestyle modification) and/or its treatment at a CTM stage may result in a better outcome (i.e., less chance of progression to dysplasia and OA) than at an IM stage, and that earlier detection could be by IHC for CDX2.

Kruppel-like factor-4 (KLF4), a principal protein that controls the differentiation of the epithelium of the gastrointestinal tract, is another transcriptional factor displayed in Barrett's epithelium that is activated by bile acids. It has been demonstrated that KLF4 and CDX2 cooperatively promote their activity, and KLF4 stimulates the mucin protein MUC2 linked to IM. It has also been demonstrated that the inflammation-linked route, via NF-κB, is associated with this mechanism. KLF4 and CDX2 are potential molecular markers for the inhibition of BO, and treatment aimed at these proteins might produce a clinical therapy for BO.[16]

The fact that the number cases displaying CDX2 reactivity increased through CTM to IM would suggest that CDX2 has a tumour-promoting role. Additionally, goblet CELO appears to be harder to treat than ONGCCM, so it would seem that *CDX2* up-regulation results in a phenotype predisposed to cancer development. It would be expected that if CDX2 was a cancer suppressor then as transcription intensified it should results in a more treatable type of mucosa, but this does not seem to be the case. However, metaplasia seems to be a protective reaction to the agents that trigger severe inflammation. In BO, the intestinal-type epithelium is less susceptible to acid injury than is SE,[12] and research indicates a role for CDX2 in tumour inhibition in the colon.

Immunohistochemical studies have shown that CDX1 and CDX2 activity is lowered in oesophageal adenocarcinoma, indicating that the genes could inhibit carcinoma.[13] This has demonstrated a significant decrease in CDX2 expression from Barrett's metaplasia through dysplasia and adenocarcinoma. The authors proposed that the difference could be in line with function as a tumour suppressor gene, although an alternative and maybe a more likely explanation might be that CDX2 acts as a differentiation marker that tends to be down-regulated with reduced differentiation.[25]

It is possible that *CDX2* and *CDX1* are dormant in the oesophagus but active in the intestine following birth. In adults, GORD may activate *CDX1* and *CDX2* to promote the development of intestinal-type tissue in the oesophagus and reverse the embryonic process.

Conclusions

This study confirms CDX2 as an early marker for BO in the absence of GCs, as expression was noted in CTM specimens. This could be important in BO cases where GCs are not present or are hidden in biopsies. CDX2 appears to induce the transformation of the SE to CTM, which then differentiates to IM under the influence of GORD in a time- and dose-dependant manner. Lack of CDX2 expression in FTM cases suggests that in the oesophagus this mucosa does represent end differentiation, with no neoplastic potential; however, there is evidence to suggest that a non-intestinal neoplastic pathway could be a precursor to OA. Detection of CTM could be important in terms of an early and effective therapy for reduction of metaplasia of the stomach and oesophagus. Increasing CDX2 expression, from early stages of BO to more advanced and possibly harder-to-treat stages of BO, could indicate that it is a tumour promoter gene, but other research supports the hypothesis that it is just a differentiation marker. □

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