Expressions of C-erbB2 and C-myc in Esophageal and Gastric Cardia Multistage Carcinogenesis from the Subjects at High-risk Area in Linxian, Northern China

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Abstract: Linxian and nearby counties in Henan Province, northern China have been well-recognized as the highest incidence area for both esophageal squamous cell carcinoma (SCC) and gastric cardia adenocarcinoma (GCA). The molecular mechanism for SCC and GCA is largely unknown. Recent studies indicate that aberrations of DNA copy numbers at 8q and 17p in which the genes of C-myc and C-erbB2 reside, are very common events in SCC and GCA from the patients in Linxian. The present study was undertaken to characterize the changes of C-erbB2 and C-myc in protein level on the subjects with different esophageal and gastric cardia precancerous and cancerous lesions from Linxian. In the study, 144 samples were collected, including 30 SCC and 30 GCA from Linxian Esophageal Cancer Hospital and 84 biopsies from symptom-free subjects (16 cases with normal esophageal epithelia (ENOR), 34 with esophageal basal cell hyperplasia (BCH), 8 with esophageal dysplasia (EDYS), 7 with normal gastric cardia epithelia (GNOR), 6 with chronic superficial gastritis (CSG), 10 with chronic atrophic gastritis (CAG), and 3 with gastric cardia dysplasia (GDYS)). The avidin-biotin-peroxidase complex (ABC) method was performed for the expression of C-erbB2 and C-myc. No immunoreactivity was observed for C-erbB2 in normal esophagus, BCH and EDYS. However, 50% of SCC was positive for C-erbB2 immunostaining. In contrast, positive immunostaining for C-myc was observed in normal esophageal epithelia and different lesions. With lesions progressed from ENOR-EDYS-SCC, the positive immunostaining rates for C-myc increased apparently. In gastric cardia, positive immunoreactivity for both C-erbB2 and C-myc was observed in normal gastric cardia epithelia and different lesions. With the lesions progressed from GNOR-CSG-CAG-GDYS-GCA, an apparent increasing tendency was observed for both C-erbB2 and C-myc. In gastric cardia multistage carcinogenesis, the positive immunostaining rate for C-erbB2 and C-myc was much higher than in esophagus. The present results demonstrate that the immunostaining patterns for C-erbB2 and C-myc are different between esophageal and gastric cardia carcinogenesis from the population at same high-risk area in Linxian. Overexpression of both C-erbB2 and C-myc is a common event in gastric cardia multistage carcinogenesis, which may be a promising early biomarker for gastric cardia carcinogenesis. C-erbB2 may be a late event for esophageal carcinogenesis. [Life Science Journal. 2006;3(3):6-12] (ISSN: 1097-8135).

Keywords: squamous cell carcinoma; gastric cardia adenocarcinoma; precancerous lesion; C-erbB2; C-myc

Abbreviations: BCH: basal cell hyperplasia; CAG: chronic atrophic gastritis; CGH: comparative genomic hybridization; CSG: chronic superficial gastritis; EC: esophageal carcinoma; EDYS: esophageal dysphasia; ENOR:

normal esophageal epithelium; GDYS: gastric cardia dysphasia; GCA: gastric cardia adenocarcinoma; GNOR: normal gastric cardia epithelium; SCC: esophageal squamous cell carcinoma

1 Introduction

Linxian and nearby counties in Henan, northern China have been well-recognized as the highest incidence areas for esophageal squamous cell carcinoma (SCC)[1], and gastric cardia adenocarcinoma (GCA) seems to occur together with SCC in these areas[2] and in other countries[3]. SCC and GCA have a very poor prognosis and remain the leading cause of cancer-related death in Linxian. In clinic, more than 80% of the patients with SCC and GCA are diagnosed at late stage in these areas, whose five-year survival rates are less than 10%. In contrast, the five-year survival rates for the early SCC and GCA are more than 90%[4]. Apparently, early detection and high-risk subject screening is of great importance in decreasing the mortality rate for SCC and GCA. The early indicator for the subjects predisposed to SCC and GCA is the epithelial cell hyperproliferation, morphologically, manifested as basal cell hyperplasia (BCH), dysplasia (DYS) and carcinoma *in situ* (CIS) in esophagus[5] and chronic atrophic gastritis (CAG) with intestinal metaplasia (IM), DYS and CIS in gastric cardia[6]. All or part of these lesions could be considered as precancerous lesions for SCC and GCA[2]. These lesions are unstable, i.e., they may progress to more severe type, or stay in the same stage for long time, or return to less severe type, even to normal, which is difficult to explain based on morphological changes only[7]. Thus it becomes crucial to characterize the molecular changes in the early stage of SCC and GCA carcinogenesis to identify the biomarker for high-risk subject screening and early diagnosis. However, the underlying key molecular changes for multistage carcinogenesis of SCC and GCA are largely unknown.

Recent studies with comparative genomic hybridization (CGH) demonstrate that aberrations in DNA copy number at chromosome 8q and 17q, in which the genes of C-myc and C-erbB2 reside, are frequently observed in SCC and GCA tissues from the patients in Linxian[8]. C-myc, an oncogene, belongs to a family of nuclear phosphoproteins. Myc family of proteins influences the expression of around 10% of all human genes[9]. The expression of C-myc protein is an important factor in cell proliferation via activating the cell division cycle gene cdc25A, the product of which catalyses the dephosphorylation of the cyclin-E / cyclin dependent / kinase 2c (CDK2) complex[10]. The expression of C-myc also induces apoptosis via interaction with a number of apoptotic pathways[11]. Amplification of C-myc both in mRNA and protein level has been found frequently in SCC[11-13], especially in advanced stages of SCC. Antisense myc gene introduced into esophageal cancer cell line (EC8712) is capable of inhibiting cell proliferation and malignancy[14]. Evidence for the expression of C-myc in GCA is very limited. Luo *et al* reported a 62% of positive immunostaining for C-myc in sporadic GCA from Chinese people[15]. But, the expression pattern of C-myc is largely unknown both in esophageal and gastric cardia precancerous lesion.

The C-erbB2 (HER-2/neu) oncoprotein is a 185-kDa transmembrane receptor[16]. Over expression of C-erbB2 has been found not only in breast and ovarian, but also in gastric and many other human cancers[17]. The C-erbB2 amplification has been known as independent predictor for neoplastic recurrence and overall survival rate[18,19]. The accumulated evidences indicate that C-erbB2 aberrant expression occurs more frequently in primary esophageal adenocarcinoma, but not in SCC[20-22]. The expression pattern for C-erbB2 in gastric cardia carcinogenesis is not clear. To define whether C-myc and C-erbB2 is the target gene in multistsge carcinogenesis of SCC and GCA at 8q and 17q aberrations as indicated by CGH from the SCC and GCA patients at Linxian, the highest incidence area for both SCC and GCA in northern China, the present study was undertaken to characterize C-myc and C-erbB2 expression in both esophageal and gastric cardia multistage carcinogensis from the patients at same high incidence areas for both SCC and GCA in Linxian, northern China.

2 Materials and Methods

2.1 Endoscopic examination and biopsy

Esophageal endoscopic examination and biopsy were performed on 84 symptom-free subjects who volunteered to participate in a routine endoscopic screening for esophageal cancer (EC) in Linxian, the highest incidence area for EC in Henan Province, northern China. No selection process was involved. Of these subjects, there were 45 males (35 – 71 years of age with a mean ± SD of 51 ± 10 years) and 39 females (32 – 71 years of age with a mean ± SD of 49 ± 11 years). Esophageal endoscopic examination was performed with Olympus GIF-V70 (Olympus Com., Japan).

Esophageal biopsies were taken from each subject at the middle third of the esophagus (30 – 32 cm from incisor teeth). Gastric cardia biopsies were taken within 2 cm lower from the esophageal and gastric cardia junction. Additional biopsies were taken when there were macroscopic lesions. The biopsy specimens were fixed in 85% alcohol, embedded in paraffin, and sectioned at 5 μm.

2.2 SCC and GCA specimen collection and processing

A total of 30 surgically resected primary SCC specimens (52 – 72 years of age with a mean ± SD of 56 ± 11 years) and 30 surgically resected primary GCA specimens (50 – 71 years of age with a mean ± SD of 53 ± 10 years) were collected from Linxian Esophageal Cancer Hospital from October to December, 2005. All the patients had received neither chemotherapy nor radiotherapy before surgery. All the tissues were fixed with 85% alcohol, embedded with paraffin, and sectioned at 5 μm. Five adjacent ribbons were collected for histopathologic and immunohistochemical analysis.

2.3 Histopathological analysis

Histopathological diagnosis for esophageal epithelia was made based on the changes in cell morphology and tissue architecture using previously established criteria[2]. In brief, the normal esophageal epithelium contained one to three proliferating basal cell layers; the papillae were confined to the lower half of the whole epithelium thickness. In BCH, the proliferating basal cells surpassed 15% of the total epithelial thickness. Dysplasia was characterized by nuclear atypia (enlargement, pleomorphism, and hyperchromasia), loss of normal cell polarity, and abnormal tissue maturation. SCC was characterized by confluent and invasive sheets of cohesive, polymorphous cells with hyperchromatic nuclei. The following histopathological classification was used for the gastric cardia epithelia: chronic superficial gastritis (CSG), inflammation manifested by mild lymphocyte and plasma cell infiltration; chronic atrophic gastritis (CAG), glandular morphology disappeared partially or completely absent in the mucosa and replaced by connective tissue, interglandular space infiltrated mainly by plasma cells and lymphocytes; gastric cardia dysplasia (GDYS), neoplastic features including nuclear atypia and /or architectural abnormalities confined to the gastric cardia epithelium, without invasion; gastric cardia adenocarcinoma (GCA), invasion of neoplastic gastric cells through the basement membrane[6].

2.4 Immunohistochemical staining

Anti-C-erbB2 antibody is a monoclonal mouse antiserum against the human C-erbB2 (DAKO, Carpinteria, CA, USA). Anti-C-myc antibody is a polyclonal rabbit antiserum against human C-myc (Oncogene Science, Manhasset, NY, USA). The avidin-biotin-peroxidase complex (ABC) method was used for the immunostaining of C-erbB2 and C-myc. In brief, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum (Vectastain Elite Kit; Vector, Burlingame, CA, USA), the sections were incubated overnight at 4 ℃ with a diluted solution of the primary antibodies (1:200 for C-erbB2 and 1:150 for C-myc). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain Elite Kit, Burlingame, CA, USA). Normal serum blocking and omission of the primary antibodies were used as negative controls. Clear nuclear staining was the criterion for a positive reaction of C-myc. C-erbB2 positive immunoreactivity was localized at cytoplasm.

2.5 Statistical analysis

The χ^2 test was used for the percentage of lesions with positive immunostaining. Spearman correlation test and linear tendency test were used for the correlation between positive rates and different severities of the lesions ($P < 0.05$ was considered significant).

3 Results

3.1 Histopathological findings

Of the 84 biopsies, 58 esophageal mucosa and 26 gastric cardia mucosa were identified, respectively. Histopathological examination showed that, of the 58 esophageal biopsies, 16 biopsies were identified with normal epithelia (ENOR) (28%), 34 with esophageal basal cell hyperplasia (BCH) (59%), 8 with esophageal dysphasia (EDYS) (13%). In 26 gastric cardia biopsies, there were 7 biopsies identified with normal gastric cardia epithelia (GNOR) (27%), 6 with chronic superficial gastritis (CSG) (23%), 10 with chronic atrophic gastritis (CAG) (36%), 3 with gastric cardia dysplasia (GDYS) (11%). Histopathologically, all the surgically resected esophageal specimens were confirmed as SCC, and all the gastric cardia cancer specimens were confirmed as GCA.

3.2 Immunohistochemical staining for C-myc and C-erbB2

In esophagus (Table 1): positive immunoreactivity for C-myc was observed both in esophageal precancerous and cancerous lesions (Figure 1). With the lesions progressed from BCH-EDYS-

SCC, the positive immunostaining rate for C-myc increased. A good correlation between the C-myc positive staining rate and lesion progression was observed ($P < 0.05$). However, the positive immunoreactivity for C-erbB2 was identified only in SCC (Figure 2). All the normal esophagi and the precancerous lesions were negative for C-erbB2 expression.

Table 1. Immunoreactivity of C-erbB2 and C-myc in esophageal multistage carcinogenesis*

Histological types	C-erbB2		C-myc**	
	Cases of samples examined	Samples with positive staining (n (%))	Cases of samples examined	Samples with positive staining (n (%))
ENOR	12	0 (0)	16	1 (6)
BCH	34	0 (0)	14	3 (21)
EDYS	8	0 (0)	4	1 (25)
SCC	30	15 (50)	27	16 (59)

* Part of the slide tissue lost during the immunohistochemistry processing. ** BCH *vs*. EDYS, $P<0.05$ (χ^2 test).

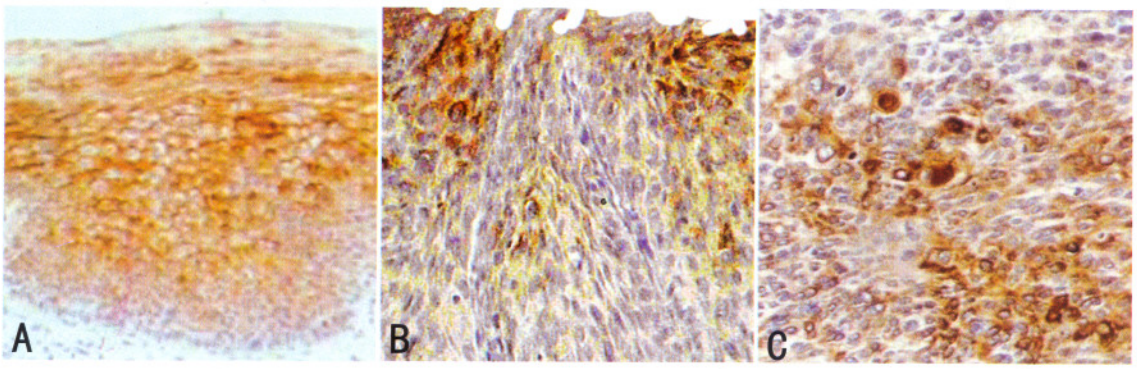

Figure 1. Microphotograph for C-myc immunostaining in esophageal basal cell hyperplasia (A: ×200), dysphasia (B: ×200) and squamous cell carcinoma (C: ×200). Immunoreactivity is mostly located in the nuclia. The positive cells were invariably associated with cell proliferative activity.

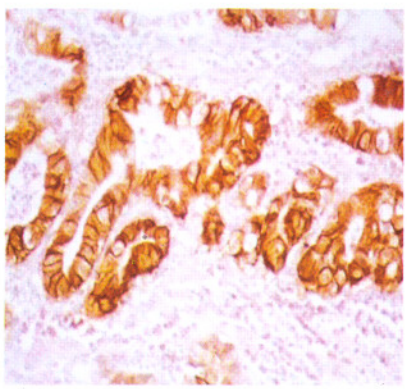

Figure 2. Microphotograph for C-erbB2 immunostaining in esophageal squamous cell carcinoma. Immunoreactivity is mostly located in the cytoplasm and cell membrane. The positive cells are in variably associated with cell proliferative activity (×400).

In gastric cardia (Table 2): positive immunoreactivity for both C-myc and C-erbB2 was observed in gastric cardia precancerous and cancerous lesions (Figure 3 and Figure 4). The positive immunostaining rate apparently increased with the lesions progressed from CSG-CAG-GDYS-GCA. The positive immunostaining rate for C-erbB2 and C-myc was very low in normal gastric cardia epithelia, and increased significantly in EDYS and GCA ($P<0.05$).

It was noteworthy that the positive immunostaining rate for C-erbB2 and C-myc in gastric cardia multistage carcinogenesis was higher than in esophageal carcinogenesis. Furthermore, the "diffuse" immunostaining pattern in C-erbB2 was predominant in gastric cardia carcinogenesis, in contrast, the "focal" immunostaining pattern was frequently observed in esophageal carcinogenesis.

Table 2. Immunoreactivity of C-erbB2 and C-myc in gastric cardia carcinogenesis*

Histological types	C-erbB2		C-myc**	
	Cases of samples examined	Samples with positive staining (n (%))	Cases of samples examined	Samples with positive staining (n (%))
GNOR	7	2 (9)	5	2 (40)
CSG	6	5 (83)	5	4 (80)
CAG	10	9 (90)	6	5 (83)
GDYS	3	3 (100)	2	2 (100)

* Part of the slide tissue lost during the immunohistochemistry processing. ** Normal *vs*. CAG, Normal *vs*. GCA, $P<0.05$.

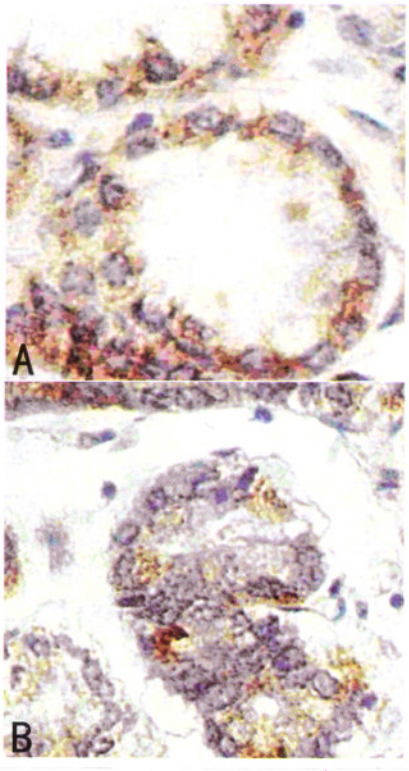

Figure 3. Microphotograph for C-myc immunostaining in gastric cardia dysphasia (A: ×400) and chronic atrophic gastritis (B: ×400). Immunoreactivity is mostly located in the nuclia. The positive cells are in variably associated with cell proliferative activity.

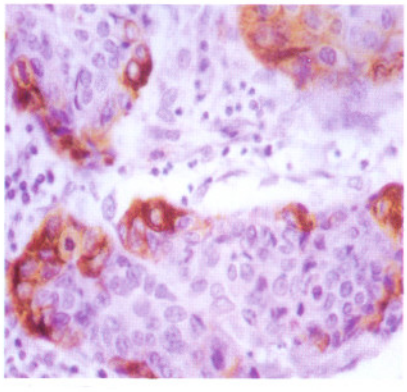

Figure 4. Microphotograph for C-erbB2 immunostaining in gastric cardia adenocarcinoma. Immunoreactivity is mostly located in the cytoplasm and cell membrane. The positive cells are in variably associated with cell proliferative activity (×200).

4 Discussion

The present study demonstrates that over-expression of C-erB2 and C-myc is a very early frequent event in gastric cardia multistage carcinogenesis. These aberrant protein expressions are well correlated with gastric cardia epithelial lesion progressions, suggesting that C-erbB2 and C-myc may play an important role in gastric cardia multistage carcinogenesis. These results are consistent with our CGH work[4], indicating that CGH is a good technique in narrowing down the scope for identifying the key related genes with cancers. The present results also indicate that C-erbB2 and C-myc aberrant expression may be a promising early biomarker to predict the gastric cardia carcinogenesis. The recent studies by our group and other laboratories have showed that autoantibodies to C-myc could be detected through cancer patient's blood serum, including esophageal and gastric cardia cancers, and could increase the early detection of these cancers[23-27].

An interesting result in this study is that aberrant C-erbB2 expression occurs only in SCC, none in normal esophagus and esophageal precancerous lesions, suggesting that C-erbB2 may be a late event for esophageal carcinogenesis. Accumulated evidences have demonstrated that aberrant C-erbB2 expression occurs more frequently in adenocarcinoma, e.g. in GCA and Barrett's esophagus-related esophageal adenocarcinoma, not in SCC[20]. These different expression patterns may be related with the different tissue types occurring of tumor cells, which could explain the different immunostaining patterns observed in GCA and SCC for C-erbB2 and C-myc in this study.

Many studies suggest that tumor occurring and progression are the result of a multistage and progressive process which may be related with the deactivity of tumor suppressor gene and the activity of the tumor oncogene in different stages. The present studies demonstrate that, in the multistage progression of the esophageal carcinogenesis, there is few expression of C-erbB2 in the early stage but some in SCC; however, the overexpression of C-myc is positive during the esophageal multiple carcinogenesis, suggesting the possibility of multiple genetic changes involved in esophageal carcinogenesis.

Historically, EC and GCA have been considered as a single clinical entity for incidence and mortality-rate calculations in Linxian because of the common syndrome of dysphagia[28]. The similar geographic distributions of SCC and GCA in China suggest that there may be similar risk factors and genetic changes involved in these two cancers. GCA is an under-studied subject. The molecular changes in the early stage of GCA carcinogenesis have not been characterized. There is evidence, however, that GCA differs from cancer of the rest of stomach in terms of time trend, risk factors and histopathogenesis[29]. Because of the common occurrence both of SCC and GCA in Henan, it is of great interest to know whether the molecular changes observed in SCC also occur in GCA. The

present results demonstrate that the aberrant expressions of C-erbB2 and C-myc occur similarly in SCC and GCA, however, the immunostaining pattern for C-erbB2 and C-myc in precancerous lesions of the esophagus and gastric cardia is different, especially in C-erbB2. The significance of these observations needs to be further analyzed.

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