

# Gene amplification of ERBB2 and EGFR in adenocarcinoma in situ and intramucosal adenocarcinoma of Barrett's esophagus

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Gene Amplification of *ERBB2* and *EGFR* in Adenocarcinoma *in situ* and  
Intramucosal Adenocarcinoma of Barrett's Esophagus.

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Running title: gene amplifications in Barrett cancer

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### Abstract

We examined 11 cases of carcinoma arising from Barrett's esophagus consisting of two adenocarcinomas *in situ* (ACIS), two intramucosal adenocarcinomas, and seven overt invasive adenocarcinomas, and measured overexpression of p53 (implying a mutation of the *p53* gene), ERBB2, and EGFR by immunohistochemistry, and measured gene amplification of *ERBB2* and *EGFR* by fluorescence *in situ* hybridization (FISH). In all the cases of ACIS and the intramucosal adenocarcinomas, almost all cancer cells overexpressed p53, however the populations overexpressing of ERBB2 and EGFR varied in different cases: in one ACIS ERBB2 was coexpressed in all the cancer cells, in the other ACIS and the one intramucosal adenocarcinoma, ERBB2 was overexpressed in about 50% and only 10% of the p53-positive cells respectively, and EGFR was co-expressed in 50% in the other intramucosal adenocarcinoma. Protein overexpression of ERBB2 or EGFR corresponded to the amplification of their respective genes on a cell by cell basis. These gene amplifications, however, were not found in the seven invasive adenocarcinomas. Thus we speculate that the gene amplification occurred late in dysplasia-carcinoma sequence probably after the mutation of *p53*. Furthermore, new clonal expansion accompanied by tumor invasion might have extinguished the originally amplified genes in these tumors.

Key words: Barrett adenocarcinoma, ERBB2, EGFR, gene amplification, FISH

## Introduction

Esophageal adenocarcinomas are thought to develop in columnar-lined Barrett's esophagus<sup>1</sup>. Progression from the metaplastic columnar epithelium of Barrett's esophagus through dysplastic changes to adenocarcinoma most likely entails a series of genetic alterations. The molecular alterations that have been detected in the course of the dysplasia-adenocarcinoma sequence include mutations and overexpression of p53<sup>2</sup>, and/or amplification and overexpression of *ERBB2*<sup>3</sup>, *EGFR*, or *MYC*<sup>4</sup>. Recently, array-based comparative genomic hybridization (aCGH) or single nucleotide polymorphism (SNP) array have detected novel candidate genes involved in development and progression of Barrett's carcinoma. Among them, *SOX7*<sup>5</sup> and *SNRPN*<sup>6</sup> were identified as the most frequently amplified genes by two groups respectively. Clinically, the treatment of dysplasia in Barrett's esophagus ranges from close monitoring to surgical excision, mainly depending on the interpretation of the grade of the dysplasia<sup>7,8</sup>. Although some have argued that finding high-grade dysplasia upon repeat biopsy is a possible indication for surgery<sup>9</sup>, occasionally there is significant morbidity associated with esophagectomy<sup>10</sup>. Thus the diagnosis of dysplasia and adenocarcinoma *in situ* (ACIS) of Barrett's esophagus is a heavy decision. Furthermore, the diagnosis of dysplasia and its grade are subjective<sup>11</sup>, and unfortunately there are many interobserver differences in diagnoses. Although various approaches such as mucin histochemistry, flow cytometry, immunohistochemistry (IHC), and molecular biology have been tried<sup>12-17</sup>; to help in this difficult decision, no objective and practical adjunct to routine histologic diagnosis has been established.

In Japan, Barrett's esophagus and esophageal adenocarcinoma are less common than in Western countries. This is in sharp contrast to the high frequency of gastric adenocarcinomas. In recent years, however, there has been a gradual increase in the detection of both Barrett's esophagus and esophageal adenocarcinomas, although the incidences of these conditions remain considerably lower than in Western countries<sup>18</sup>. Recently, we had an opportunity to diagnose a case of ACIS of Barrett's esophagus confidently by biopsy specimen with the detection of *ERBB2* amplification using fluorescence *in situ* hybridization (FISH). To understand the significance of this finding, we searched the surgical pathology file of the Pathology Section of Kanazawa University Hospital and retrieved ten adenocarcinomas arising from Barrett's esophagus, and examined the amplification of *ERBB2*, as well as *EGFR*, *MYC*, *SOX7* and *SNRPN*, and the overexpression of p53.

## Materials and methods

The first case (case 1) was a 70-year-old Japanese man who had an open ulcer in a Barrett's esophagus. The diagnosis of previous biopsies was regenerating mucosa with moderate atypia. The biopsy specimen taken around the ulcer was resubmitted, and a diagnosis of "ACIS" was made. After a thorough analysis of this case, we searched the surgical pathology files of the Pathology Section of Kanazawa University Hospital between 2005 and 2008 for cases of adenocarcinoma of distal esophagus, adenocarcinoma of gastroesophageal junction (GEJ), and Barrett's mucosa. Only 12 cases were found despite the fact that more than 400 gastric adenocarcinomas were registered in this period. Of the 12 cases, six esophagectomy and four endoscopic mucosal resection/submucosal dissection (EMR/ESD) specimens (cases 2- 11) (Table 1) were retrieved as adenocarcinomas arising in Barrett's esophagus, because the metaplastic epithelium (columnar epithelium with goblet cells) was histologically confirmed above the macroscopically identified GEJ.

No patients had received radiation or chemotherapy before surgery except case 11 who received pre-operative chemotherapy. This laboratory study using these specimens was approved by the Institutional Review Board at the Kanazawa University, and written informed consent was obtained from all patients.

### Diagnoses and grading

The depth of invasion of adenocarcinomas was defined according to the cancer staging manual of the American Joint Committee on Cancer<sup>19</sup>. "ACIS" was defined synonymously with severe dysplasia<sup>9,10</sup>, and "intramucosal carcinoma" was defined as a carcinoma that has penetrated through the basement membrane of the glands into the lamina propria but has not yet invaded through the muscularis mucosae into the submucosa<sup>20</sup>. According to these criteria the hematoxylin-eosin staining slides were reviewed by the three pathologists (AO, YZ and SS).

### IHC

Serial sections (4  $\mu$ m) cut from representative formalin-fixed, paraffin-embedded tissues and placed onto MAS<sup>TM</sup>-coated slides (Matsunami, Tokyo, Japan) were used for IHC detection and FISH analysis. A monoclonal antibody against the recombinant human wild-type p53 (DO-7, Dako, Glostrup, Denmark), a polyclonal antibody against the internal domain of the human ERBB2 protein (Nichirei, Tokyo, Japan; working

dilution, 1:100), and a monoclonal antibody against the internal domain of human EGFR (Novocastra Lab, Newcastle, UK; working dilution, 1:20) were used. For the detection of p53 and EGFR, a high-temperature antigen unmasking technique was used. IHC was done using an ELSB™ 2 System (Dako) and visualized with 3,3'-diaminobenzidine.

For the evaluation of ERBB2 and EGFR positivities, each tumor or portion of tumor was scored according to the criteria recommended by Dako for the HercepTest. For an evaluation of p53 staining, only nuclear immunostaining significantly higher than that of the control cells of the normal esophageal mucosa were considered to be positive.

### FISH

FISH analyses of the gene amplification were applied to all tumors. For FISH probes, bacterial artificial chromosomes RP11-62N23, RP11-339F13, RP11-49I23 specific for *ERBB2* locus (17q12), *EGFR* locus (7p12), and *SOX7* locus (8p23.1) respectively, and P1 artificial chromosome clone RP1-80K22 specific for *MYC* locus (8q24.12-q24.13) were used. RP11-49I23 was available from BACPAC Resources (Oakland, CA, USA). These probes were labeled with SpectrumOrange™ using a nick translation kit (Abbott, Abbott Park, IL, USA). In order to standardize the chromosome number a SpectrumGreen™-labeled pericentromeric probe (Abbott), which was specific to the chromosome on which the gene was located, was cohybridized. *SNRPN* specific probe was purchased from VYSIS as microdeletion detecting kit (Tri Color Probe 1N12-10, *SNRPN* SpectrumOrange™/CEP15 SpectrumAqua™/*PML* SpectrumGreen™). FISH was performed using standard methods as described elsewhere<sup>21</sup>. The tissue sections were counterstained with 4',6-diamidino-2'-phenylindole dihydrochloride and p-phenylenediamine in phosphate-buffered saline and glycerol (DAPI II) (Abbott) and examined under a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter™ set (Abbott) to discriminate DAPI II, SpectrumOrange™, and SpectrumGreen™, and a filter specific to SpectrumAqua™.

For the evaluation of gene amplification, each tumor was scored according to the criteria recommended by Abbott for PathVision™. A cell with a definite cluster of signals or a total of more than 10 signals was scored as exhibiting high-level amplification<sup>21</sup>. FISH images were taken using a CCD camera and recorded on a personal computer.

## Results

### Case 1

The biopsy specimen taken around the ulcer (Fig. 1) showed intestinal-typed atypical epithelium highly suggestive of ACIS (Fig. 2A). By IHC, atypical cells were found to have p53-positive nuclei and ERBB2 2+ cytoplasmic membranes (Fig. 2B, C). FISH revealed that the ERBB2-positive cells had clustered signals of *ERBB2* (Fig. 2D). No amplification of the *EGFR* and *MYC* by FISH was found. We diagnosed this as ACIS arising in a Barrett's esophagus. After two weeks of medication for peptic ulcer, the lower 11 cm of the esophagus was resected (Fig. 3A). An ulcer scar interrupting the muscle coat was found 2.3 cm from the GEJ. The mucosa between the GEJ to squamocolumnar junction showed immature intestinal metaplasia with islands of squamous mucosa and esophageal glands. The mucosa measuring 2.5 × 2.3 cm surrounding the ulcer scar was finely granular. Histologically, corresponding to the granular mucosa, ACIS was found with patches of regenerating mucosa (Fig. 3B). No metastasis in regional lymph nodes was found. Identical to the results in biopsy specimen, almost all adenocarcinoma cells were positive for both p53 and ERBB2 (Fig. 3C), and were amplified for *ERBB2* (Fig. 3D). No amplification of *EGFR*, *MYC*, *SOX2* and *SNRPN* was found.

#### Cases 2–11

By examining the slides of resected specimens, interobserver agreements was achieved in all the cases except case 2, for which the diagnosis was separated into ACIS and intramucosal adenocarcinoma with limited infiltration to lamina propria mucosae by two versus one. We looked for overexpression of ERBB2, EGFR, and p53, and amplification of *ERBB2*, *EGFR*, *MYC*, *SOX2* and *SNRPN* in these cases using resected specimens and biopsy specimens for which the diagnosis of adenocarcinoma was made. Parts of the results are summarized in Table 1.

In case 2 and an intramucosal adenocarcinoma (case 3), almost all cancer cells were p53-positive (Fig. 4A) and approximately 50% and 10% of them were also positive for ERBB2 and occupied a part of the tumor (Fig. 4B&C). In FISH using the adjacent section, ERBB2-positive cells were found to have one or two clusters of numerous *ERBB2* signals as shown in Fig. 4D. *EGFR*, *MYC*, *SOX2* and *SNRPN* were not amplified.

In case 4, the other case of intramucosal adenocarcinoma, although almost all cancer cells overexpressed p53, overexpression of EGFR was confined to only 20% of them and occupied a proximal area of the tumor (Fig. 5A–C). Corresponding to the protein overexpression, one or two clustered signals of *EGFR* were found by FISH (Fig. 5C, D). Neither *ERBB2*, *MYC*, *SOX2*, nor *SNRPN* were amplified. Comparing IHC and FISH

specimens, protein overexpression and gene amplification of *ERBB2* and *EGFR* corresponded on a cell by cell basis respectively in cases 2 and 3 (Fig. 5C, D).

By examining biopsy specimens in which diagnosis of adenocarcinoma was made retrospectively, amplification of *ERBB2* and *EGFR* was found in case 2 and 4 respectively, but amplification was not detected in case 3. In the seven advanced cancers, p53 was overexpressed in cases 5, 7, 8, and 11, however overexpression of *ERBB2* and *EGFR* was not observed and no gene amplification was found.



## Discussion

It is now generally accepted that Barrett's esophagus predisposes patients to esophageal adenocarcinomas<sup>1</sup>. Early diagnosis of dysplasia or invasive adenocarcinoma is crucial for optimal treatment. However, concerning morphological recognition of dysplasia and occasionally adenocarcinoma, sometimes considerable interobserver- and intraobserver-variability exist. In an interobserver variability study performed by 12 pathologists, Montgomery et al. reported that majority diagnosis was not attained in 39 of 138 endoscopic biopsy specimens obtained from Barrett's esophagus. As found in the present case, biopsy specimens taken from the edges of ulcers may be hardly distinguishable from dysplasia from reactive or regenerative hyperplasia<sup>22</sup>. In another study of a blinded review of endoscopic biopsy specimens and samples of surgical specimens, fortunately the best inter-observer agreement among the eight participants averaged 86%, which was achieved in comparing the combinations of high grade dysplasia and intramucosal carcinoma with all other diagnoses (negative, indefinite, and low-grade dysplasia)<sup>11</sup>. However, considering that esophagectomy may be decided by this diagnosis, this figure is not satisfactorily high.

Overexpression of p53 protein detected immunohistochemically, implying a mutation in the *p53* gene, has been reported in various neoplastic lesions of Barrett's esophagus by a number of investigators<sup>23</sup>. The present study also showed that p53 was overexpressed in all the four mucosal cancers. This suggests that p53 overexpression is a sensitive objective marker for dysplasia and adenocarcinoma. However, concerning its specificity to adenocarcinoma, there remains some problems because nuclear accumulation of p53 is as high in low-grade dysplastic lesions as in high-grade lesions or in adenocarcinomas<sup>2</sup>. Furthermore, mutation and accumulation of p53 are frequently discordant in low-grade dysplasia, although they are mostly concordant in adenocarcinomas and high-grade dysplastic lesions<sup>2</sup>. These results indicate that p53 staining may have potential value for confirming a suspected diagnosis of a wide range of lesions from low grade dysplasia to more advanced lesions in the sequence.

Amplification of *ERBB2* (located on 17q11.2-q12 and 17q21.1) in Barrett's esophagus and/or esophageal adenocarcinomas has been examined by Southern blotting<sup>14</sup>, differential PCR<sup>14,24</sup>, CGH<sup>25</sup>, or most recently SNP assay<sup>5</sup>. However, FISH is the best method to detect gene amplification because one can define the genetic change on cell by cell basis as shown in the present study. Using FISH, Walch et al. found high grade dysplasia to be amplified for the *ERBB2* locus; however, gene amplification was

not detected in low grade intraepithelial neoplasia, intestinal metaplasia, or squamous epithelium, indicating that the alteration of *ERBB2* is a late event in the carcinogenesis of Barrett's esophagus<sup>26</sup>. Our finding that p53-positive populations contained both *ERBB2*-positive and -negative populations, or *EGFR*-positive and -negative populations, strongly suggests that amplification of *ERBB2* or *EGFR* in those tumors occurred after a missense mutation of the *p53* gene. In our previous FISH study of adenocarcinomas of the stomach, similar to case 4 in the present study, we found a mucosal cancer in which p53-positive populations contained both *EGFR*-positive and -negative subpopulations<sup>27</sup>. These studies suggest that karyotypic instability by loss-of-function of p53 precedes the associated amplification of *ERBB2* or *EGFR*. In another FISH study, we found that *MYC* and *ERBB2*, and *MYC* and *EGFR*, were non-incidentally co-amplified in gastric adenocarcinoma<sup>21</sup>. We recently reported on a mucosal adenocarcinoma of the gallbladder with two populations of tumor cells with the coexistence of amplified *MYC* and *ERBB2*, and *MYC* and *EGFR*<sup>28</sup>. Thus we thought that amplification of *MYC* is another genetic alteration preceding *ERBB2* or *EGFR* amplification; however, in the present study, amplification of *MYC* was not found.

*ERBB2* amplification was not found in the seven invasive adenocarcinomas. A CGH study comparing Barrett's adenocarcinomas and their adjacent precursors detected an intriguing case, in which the amplicon of the cancer cells did not contain the *ERBB2* locus which was highly amplified in adjacent high grade dysplasia<sup>25</sup>. This and our results may indicate that new clonal expansion accompanied by tumor invasion might have extinguished the originally amplified genes in these tumors, and the tumor becoming progressively more invasive do not require originally amplified *ERBB2* or *EGFR*. However, further investigation of this hypothesis is needed.

By aCGH Albrecht et al. detected most frequent copy number gain in Barrett's adenocarcinoma was on 15q12, the *SNRPN* locus (61%), and they confirmed amplified *SNRPN* genes in single case by FISH<sup>6</sup>. More recently using SNP, Wiech et al. detected frequent copy number gain (26%) and the highest level of amplification on 8p23.1, the *SOX7* locus<sup>5</sup>. Albrecht et al.<sup>6</sup> also demonstrated a FISH picture showing *SNRPN* was involved in amplification. These prompted us to do FISH analysis of our cases using the DNA probes of the same sources of theirs. However, we could not detect the amplification of *SNRPN* or *SOX7* in our cases. This could be explained by different sensitivity of array hybridization assay and FISH: in most amplification-positive tumors by the array assay the amplification level was so low that can be detected clearly by FISH. Alternatively this discrepancy may be due to racial difference between Japanese and Caucasians<sup>12</sup> because in the series of Wiech et al. the frequency of amplification of

*ERBB2* locus was very low<sup>5</sup>. However, although both the studies examined Caucasians patients, the frequent amplification of *SNRPN* was not detected in a series of Wiech et al.<sup>5</sup>, and to the contrary that of *SOX7* was not found in the work of Albrecht et al.<sup>6</sup>. Thus additional data seem to be necessary to resolve the discordance

Recently, instead of esophagectomy, less invasive therapies such as endoscopic ablation or mucosal resection have become available for the treatment of severe dysplasia/ACIS<sup>29</sup>. In our case 1, however, we chose esophagectomy because the existence of an ulcer technically prevented EMR, and an ulcerated high-grade dysplasia is often a marker for adjunct invasive disease<sup>10</sup>. In older debilitated patients who may not tolerate esophagectomy, amplification of *ERBB2* or *EGFR* may be beneficial because molecularly targeted therapies against *ERBB2* or *EGFR* could be another clinical option. The humanized monoclonal antibody, trastuzumab, is most effective in treatment of breast cancer with *ERBB2* amplification. Now FISH tests for *ERBB2* amplification are becoming available even in diagnostic laboratories, and FISH probes for *EGFR* are also available commercially. Thus, FISH tests for gene amplification of *ERBB2* and *EGFR* should be applied for biopsy specimens as an objective and practical adjunct to routine histologic diagnosis of adenocarcinoma of Barrett's esophagus.

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Table 1. Pathological Classification and Results of IHC and FISH Analysis of Adenocarcinoma Arising in Barrett's Esophagus

Case No.	Size (cm)	Depth of invasion	p53		HER2		EGFR	
			IHC		IHC	FISH	IHC	FISH
1	2.5 x 2.3	pTis	+ (100)		2+ (100)	LC	-	-
2	1.0 x 0.7	pTis#	+ (100)		2+ (50)	LC	1+	-
3	1.5 x 1.5 x 0.8	pT1a (LPM)	+ (100)		3+ (5)	LC	-	-
4	3.6 x 1.1	pT1a (LPM)	+ (100)		-	-	3+ (20)	LC
5	1.3 x 0.7	pT1b (SM)	+ (100)		-	-	-	-
6	1.4 x 1.0	pT1b (SM)	-		-	-	-	-
7	2.3 x 1.6	pT1b (SM)	+ (10)		-	-	-	-
8	1.8 x 0.5	pT1b (SM)	+ (100)		-	-	-	-
9	2.0 x 1.0 x 0.4	pT1b (SM)	-		-	-	-	-
10	3.4 x 2.8	pT2 (MP)	-		-	-	-	-
11	6.2 x 3.5	pT3 (AD)	+ (100)		-	-	-	-

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; LC, large clustered signal; LPM, lamina propria mucosae; SM, submucosa; MP, muscularis propriae; AD, adventitia; figures in parentheses, % of positive cells. #The diagnosis was separated to pTis and pT1a by two versus one.

## Figure legends

## Fig. 1

Case 1. Endoscopic finding. An open ulcer in the lower esophagus.

## Fig. 2

Case 1. Endoscopic mucosal biopsy. Tubules of atypical cells (A) are positive for p53 (B) and ERBB2 (C). FISH shows that the cancer nuclei have clusters of *ERBB2* signals (orange) (D).

## Fig. 3

Case 1. Resected esophagus. The ulcer scar was surrounded by fine granular mucosa (A). Representative histology of adenocarcinoma *in situ* (B). The adjacent sections were stained for ERBB2 (C). The region within the black rectangle corresponds to the field in Panel D. FISH shows that adenocarcinoma cells have clustered signals of ERBB2 (D).

## Fig. 4

Case 3. Mucosal carcinoma with p53 overexpression (A) has small area of ERBB2-overexpressing cancer cells (B). The region within the black rectangle in Panel B is magnified in Panel C. FISH analysis on the same field as Panel C shows that ERBB2-overexpressing cancer cells have the amplified *ERBB2* gene, however the cancer cells overexpressing p53 but no ERBB2 have no amplified *ERBB2* gene (D).

## Fig. 5

Case 4. Mucosal carcinoma with p53 overexpression (A&B). Cancer cells with overexpression and amplification of EGFR occupy the right area of the picture (C&D). The region within the black rectangle in Panel C corresponds to the field in Panel D.





Table 1. Pathological Classification and Results of IHC and FISH Analysis of Adenocarcinoma Arising in Barrett's Esophagus

Case No.	Size (cm)	Depth of invasion	p53	HER2		EGFR	
			IHC	IHC	FISH	IHC	FISH
1	2.5 x 2.3	pTis	+ (100)	2+ (100)	LC	-	-
2	1.0 x 0.7	pTis#	+ (100)	2+ (50)	LC	1+	-
3	1.5 x 1.5 x 0.8	pT1a (LPM)	+ (100)	3+ (5)	LC	-	-
4	3.6 x 1.1	pT1a (LPM)	+ (100)	-	-	3+ (20)	LC
5	1.3 x 0.7	pT1b (SM)	+ (100)	-	-	-	-
6	1.4 x 1.0	pT1b (SM)	-	-	-	-	-
7	2.3 x 1.6	pT1b (SM)	+ (10)	-	-	-	-
8	1.8 x 0.5	pT1b (SM)	+ (100)	-	-	-	-
9	2.0 x 1.0 x 0.4	pT1b (SM)	-	-	-	-	-
10	3.4 x 2.8	pT2 (MP)	-	-	-	-	-
11	6.2 x 3.5	pT3 (AD)	+ (100)	-	-	-	-

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; LC, large clustered signal; LPM, lamina propria mucosae; SM, submucosa; MP, muscularis propriae; AD, adventitia; figures in parentheses, % of positive cells. #The diagnosis was separated to pTis and pT1a by two versus one.









