Immunohistochemical demonstration of *c-erbB-2* oncoprotein in gastric adenocarcinoma: Comparison of cryostat and paraffin wax sections and effect of fixation

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Abstract

Aims—To investigate the effects of fixation on the immunohistochemical demonstration of *c-erbB-2* oncoprotein using paraffin wax and cryostat sections; to compare *c-erbB-2* expression in non-neoplastic and neoplastic gastric tissues.

Methods—Adjacent blocks of tumour and non-neoplastic tissue from four gastrectomy specimens were put into a panel of 10 fixatives including acetone, B5, Bouin's fluid, Carnoy's fluid, buffered formalin, formol dichromate, zinc formalin, 4% paraformaldehyde, periodate-lysine-paraformaldehyde (PLP) and periodate-lysine-paraformaldehyde-dichromate (PLPD) before embedding in paraffin wax for sectioning. Similar tissue blocks were snap frozen and cryostat sections were postfixed in these fixatives, either alone or in combination, before immunostaining.

Results—In paraffin wax embedded sections the best fixative was PLP, and in frozen tissues the best results were obtained after fixation of cryostat sections in buffered formalin followed by cold methanol and acetone. Applying these fixatives to samples from a further 16 gastrectomy specimens, strong membrane staining of *c-erbB-2* protein was found in the tumour in eight of 16 cases (50%) using paraffin wax sections, and staining was stronger in the better differentiated carcinomas. For frozen tissues, positive membrane staining was found in all gastric adenocarcinomas, but differential staining intensity associated with tumour differentiation could not be detected.

Conclusions—These results indicate that fixation and paraffin wax embedding affect the results of immunohistochemical demonstration of *c-erbB-2* in gastric cancer. The choice of fixative is critical in the demonstration and evaluation of *c-erbB-2* protein expression by immunohistochemistry in gastric carcinomas. Staining results also vary depending on whether frozen or paraffin wax embedded tissues are studied.

The human proto-oncogene, *c-erbB-2* (also called *HER-2* and *neu* gene) encodes a cell surface glycoprotein that is similar in structure to the epidermal growth factor receptor (EGF-R),¹⁻⁵ and has been mapped to band q21 of chromosome 17.⁶ It is the human analogue of the transforming gene *neu*, originally found in rat neuroblastoma cell lines derived from tumours induced by ethylnitrosourea.¹ The product of the *c-erbB-2* gene is a 185 kilodalton transmembrane glycoprotein, with an extracellular ligand binding domain and an intracellular domain with tyrosine kinase activity, which is involved in signal transduction.

Because of these structural similarities to the EGF-R, it has been suggested that the *c-erbB-2* gene product may function as a receptor for some as yet unidentified growth factor. In normal tissues *c-erbB-2* expression has been demonstrated predominantly in epithelial cells,⁷ but the physiological function of *c-erbB-2*, like that of other known peptide growth factor receptors, has not been clarified. A possible role for the *c-erbB-2* gene in the pathogenesis of human cancer has been implicated by its relatively frequent amplification in human carcinomas arising from breast,⁸⁻¹⁰ stomach,¹¹ ovary,⁹ salivary gland,¹² kidney,^{13,14} colon,¹⁴ and lung.¹⁵

Immunohistochemical staining of oncoproteins has been used as a semiquantitative and relatively simple method of assessing oncogene expression. The results reported by various authors are often conflicting. One possible explanation is the use of different fixation procedures by various workers. We therefore tested the effects of various fixatives on immunohistochemical staining intensity and cellular localisation of *c-erbB-2* protein, using paraffin wax embedded and cryostat sections. After selecting an optimal fixative we compared the expression of *c-erbB-2* in adenocarcinomas of the stomach with adjacent non-neoplastic stomach mucosal epithelium.

Methods

TISSUE PREPARATIONS

A total of 20 fresh gastrectomy specimens received in the Department of Pathology, University of Hong Kong, were studied; the specimens were all resections performed for

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Accepted for publication
7 September 1993

Table 1 Duration of fixation of paraffin wax and frozen sections in 10 fixatives

Fixative	Duration of fixation	
	Paraffin wax section (hours)	Frozen section (minutes)
Acetone	3	10
B5	2	15
Bouin	3	15
Carnoy	3	10
Neutral buffered formalin	20	15
Formol dichromate	20	15
Zinc formalin	20	15
Paraformaldehyde	20	15
PLP	20	15
PLPD	20	15

gastric cancer. Tissue blocks (5 mm in diameter) were obtained from both the tumour and adjacent non-neoplastic mucosa and put in the following fixatives before embedding in paraffin wax for sectioning: (a) acetone; (b) B5; (c) Bouin's fluid (Bouin); (d) Carnoy's fluid (Carnoy); (e) 10% neutral buffered formalin; (f) formol dichromate; (g) zinc formalin; (h) 4% paraformaldehyde; (i) periodate-lysine-paraformaldehyde (PLP); and (j) periodate-lysine-paraformaldehyde-dichromate (PLPD). Paraffin wax sections were cut at 3 μ m, mounted, and dried at 37°C overnight before immunostaining. Similar tissue blocks were also snap frozen and cryostat sections were cut at 6 μ m, air-dried at room temperature for two hours, and then fixed in the above fixatives, either alone or in combination, before immunostaining. The duration of fixation in different fixatives for paraffin wax and frozen sections is shown in table 1. Using the first four cases, the fixative that gave the highest sensitivity in paraffin wax sections compared with frozen sections, best membrane localisation, high signal to background ratio, and best preservation of morphology, was chosen for the detailed study of the remaining cases.

HISTOLOGICAL EVALUATIONS

The selected blocks were assessed histologically and the presence or absence of adenocarcinomatous tissue was confirmed. There were 10 cases of intestinal type (of which one was also mucinous), four cases of diffuse type (of which one was also mucinous), and two cases of mixed intestinal and diffuse type. The carcinomatous tissue present in the

Table 2 The effect of different fixatives on immunohistochemical staining of *c-erbB-2* oncoprotein in four cases of gastric carcinoma

Fixative	Paraffin wax		Frozen	
	Normal	Tumour	Normal	Tumour
Acetone	—	f+	+	++
B5	—	—	—	—
Bouin	—	—	—	—
Carnoy	—	—	—	+
Neutral buffered formalin	—	f+	f+	+
Formol dichromate	—	—	—	—
Zinc formalin	—	f+	—	—
Paraformaldehyde	—	f+	f+	+
PLP	f+	++	f+	+
PLPD	+	+	—	+

— = negative or weak staining of <5% tumour cells; f+ = focal moderate staining of 5–25% tumour cells; f++ = focal strong staining of 5–25% tumour cells; + = moderate staining of >25% tumour cells; ++ = strong staining of >25% tumour cells.

chosen blocks was graded using a modified Broder's classification. A predominance of solid area was regarded as poorly differentiated, and a predominance of well formed glands or a papillary pattern was regarded as well differentiated. Moderately differentiated areas were those with poorly formed glandular lumina, or predominantly cribriform in appearance.

ANTIBODIES

The mouse monoclonal antibody recognising the extracellular domain of *c-erbB-2*, developed from mice immunised with NIH/3T3 cells transfected with a full length cDNA clone of human *c-erbB-2*, was obtained from Triton Biosciences Inc (Alameda, California USA). It specifically precipitates the p185 protein in metabolically labelled cell lysates and does not cross react with the EGF receptor protein (170 kilodaltons) or any proteins in non-transfected NIH/3T3 cells.¹⁶

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method.¹⁷ Briefly, the sections were sequentially incubated with 10% normal rabbit serum for 10 minutes, monoclonal antisera (diluted 1 in 20) overnight at 4°C, rabbit antimouse immunoglobulin (diluted 1 in 50) for 30 minutes, and mouse APAAP complex (diluted 1 in 100) for 30 minutes. The intensity of the reaction product was enhanced by repeating rabbit antimouse immunoglobulin and the APAAP steps for 10 minutes each. The sites of alkaline phosphatase reactivity were visualised by incubation with naphthol AS-BI-phosphate and hexazotized new fuchsin in TRIS-buffered saline, pH 8.7, for 10 minutes. Finally, the sections were washed and counterstained with Mayer's haematoxylin, dehydrated, cleared, and mounted in Permount.

An unrelated mouse monoclonal antibody, UCHL1, was used in place of the primary antibody as a negative control. Paraffin wax sections of breast carcinoma were used as positive controls.

Normal rabbit serum, UCHL1, rabbit antimouse immunoglobulin and APAAP complex were purchased from Dakopatts A/S, Copenhagen, Denmark.

EVALUATION OF THE STAINING RESULTS

A semiquantitative analysis was carried out. The staining reactions were recorded as follows: — = negative or weak staining of less than 5% tumour cells; f+ = focal moderate staining of 5–25% tumour cells; f++ = focal strong staining of 5–25% tumour cells; + = moderate staining of more than 25% tumour cells; ++ = strong staining of more than 25% tumour cells. The staining was considered optimal when: (i) background was low; (ii) localisation to membrane was sharp; (iii) signal was strong.

CHOICE OF FIXATIVE

The results of immunohistochemical staining

Figure 1 Frozen section of moderately differentiated gastric adenocarcinoma fixed in neutral buffered formalin followed by cold methanol and acetone, showing strong membrane staining for c-erbB-2 protein.

of c-erbB-2 oncoprotein in paraffin wax and frozen sections are summarised in table 2. In paraffin wax embedded sections, the best result was found after fixation in PLP for two to 20 hours and the reaction was mainly localised to the cell membrane of the tumour areas. Focal moderate membrane staining was also obtained after fixation in acetone, neutral buffered formalin, zinc formalin and paraformaldehyde. Moderate staining was also found after fixation in PLPD. Other fixatives such as B5, Bouin, Carnoy and

formol dichromate were unsuitable and they showed negative staining.

In frozen sections the best results were obtained after fixation in neutral buffered formalin followed by cold methanol and acetone and the reaction was localised to the cell membrane (fig 1). Acceptable results were also obtained after fixation in neutral buffered formalin, paraformaldehyde and PLP, and the reaction was localised to cell membrane. Fixation in Carnoy and PLPD showed moderate diffuse cytoplasmic staining. Although strong membrane staining was shown after fixation in acetone, the result was unacceptable due to poor tissue morphology. Other fixatives such as B5, Bouin, formol dichromate and zinc formalin provided negative results.

In both methods focal membrane staining of normal gastric epithelia, mainly located at the surface, was also noticed on some of the sections (fig 2). Negative staining with UCHL1 confirmed the specificity of this result.

COMPARISON OF CARCINOMA WITH ADJACENT NON-NEOPLASTIC MUCOSA

The results of c-erbB-2 oncoprotein expression in paraffin wax and frozen sections of 16 cases gastric adenocarcinomas are shown in table 3. In frozen tissues positive membrane staining was found in all gastric carcinomas. Enhanced oncoprotein expression (compared with the adjacent non-neoplastic mucosa) was identified in 13 out of 16 cases (81%). There was no correlation between the intensity of staining and tumour differentiation, as assessed on frozen sections.

In paraffin wax embedded tissues the staining results differed from those in frozen sections. Positive membrane staining was found in only eight of 16 (50%) of gastric carcinomas, of which seven cases expressed more than the adjacent non-neoplastic mucosa. In most of the positive staining tumours the membrane staining was heterogeneous in its intensity, with some areas more intense than others. Sometimes the staining was demonstrated intracytoplasmically in addition to obvious membrane staining. Most positive cases with prominent staining (++) were moderate to well differentiated adenocarcinomas (fig 3). In contrast to this, poorly differentiated carcinomas showed weaker staining or none at all (fig 4).

Discussion

Porter *et al*¹⁸ reported that there was no difference in the detection of c-erbB-2 oncogene product in frozen and paraffin wax sections of breast lesions. However, there was a poor correlation in c-erbB-2 immunoreactivity between the paraffin wax and frozen sections in our study; agreement between the two types of sections was found in only 50% of gastric cancers. Eight negative cases were found in paraffin wax sections, but not in frozen sections of gastric cancers. The localisation of staining in tumour tissues was similar in both paraffin wax and frozen sections.

Figure 2 Normal gastric epithelium fixed in PLP (two hours), paraffin wax section showing negative staining for c-erbB-2 protein.

Table 3 Comparison of *c-erbB-2* oncoprotein staining in paraffin wax and frozen sections of gastric adenocarcinoma

Case	Degree of differentiation	Paraffin wax		Frozen	
		Normal	Tumour	Normal	Tumour
G1	Well	f+ +	++	f++	++
G2	Moderate	f+	++	f++	++
G3	Moderate	f+	—	f++	++
G4	Moderate	—	—	f++	+
G5	Moderate	—	—	f++	+
G6	Moderate	f+	++	—	++
G7	Moderate	f+ +	++	f++	++
G8	Moderate	f +	++	f+	++
G9	Moderate	—	++	f++	++
G10	Poor	f+	—	f++	++
G11	Poor	—	—	f++	+
G12	Poor	+	+	++	++
G13	Poor	+	—	++	+
G14	Poor	f+	—	—	+
G15	Poor	f+	—	f++	++
G16	Poor	f+	+	++	++

(For key and abbreviation — to ++, please see table 2).

Because Porter *et al* used routine formalin fixed paraffin wax embedded sections in their study, and PLP fixed paraffin wax sections were used in our study, the discrepancy between our results and Porter's may have been due to the different fixatives and the antibody used. The different detection of *c-erbB-2* protein in paraffin wax and frozen sections may due to the fragile antigenic sites for the protein, as these may be destroyed or diminished when exposed to fixation and heat during paraffin wax processing.

Although neutral buffered formalin was a poor fixative for demonstrating *c-erbB-2* protein in gastric carcinoma, interestingly, strong membrane staining was demonstrated in our neutral buffered formalin fixed control (breast carcinoma) paraffin wax sections. The different effect of the same fixative on the two tumour tissues is not readily explained. It may have been due to the quantitative or qualitative differences in the *c-erbB-2* protein

Figure 3 Paraffin wax section of moderately differentiated gastric adenocarcinoma fixed in periodate-lysine-paraformaldehyde (two hours), showing strong membrane staining for *c-erbB-2* protein.

in gastric and breast tissues or, as suggested by Falck *et al*, breast cancers in a positive tumour uniformly overexpress the *c-erbB-2* proteins, while in gastric cancers, the expression can be patchy or very focal.¹⁹

Previous studies by different investigators have shown discrepant results for the expression of *c-erbB-2* oncoprotein in gastric tumours. Houldsworth *et al*,²⁰ Yonemura *et al*,²¹ Falck *et al*,¹⁹ and Lemoine *et al*,²² using formalin fixed paraffin wax sections, showed that positive staining was found in 10%, 12%, 19%, and 26% of gastric carcinomas, respectively. This low level of positivity, compared with our results, indicates that using routine formalin fixed paraffin wax embedded material may underestimate the percentage of positivity in tumours studied. Southern blotting has shown *c-erbB-2* amplification in 6-22% of gastric carcinomas in various studies (Park *et al*²³; Yokota *et al*^{11 13}; Ranzani *et al*²⁴). In another study, using western blotting, Kameda *et al*²⁵ reported that 55% of cases showed higher expression of *c-erbB-2* protein in extracts of tumours compared with normal tissue. These studies support our finding that *c-erbB-2* protein is frequently overexpressed in gastric cancer.

Focal to membrane staining was found in "normal" gastric epithelium in paraffin wax sections, even when the tumour was negative, but this staining pattern was not observed in frozen sections in our study.

Our results demonstrate that the sensitivity of immunostaining is dependent on fixation procedure and the detection of *c-erbB-2* in 50% of our cases using PLP fixed paraffin wax sections is more in keeping with the finding by Kameda *et al* using western blotting.²⁵ Using frozen sections, we not only detected the presence of *c-erbB-2* in all 16 cases of gastric carcinomas, but we have also demonstrated the consistent enhanced expression of the oncoprotein in tumour tissues.

A higher prevalence of membrane staining was found in moderate to well differentiated carcinomas than in poorly differentiated adenocarcinoma in PLP fixed paraffin wax sections in our study. This is similar to the result reported by Jain *et al*.²⁶ There is no correlation, however, between the differentiation of the tumour and the intensity of staining in frozen sections, in contrast to the results using paraffin wax sections. This suggests that there may be a difference in resistance to loss of antigenicity of *c-erbB-2* protein following fixation in tumours with a different degree of differentiation. The reason for this is not known and deserves further investigation.

We conclude that the choice of fixative is critical in the demonstration and evaluation of *c-erbB-2* protein expression shown by immunohistochemistry in gastric carcinomas. Staining results also vary, depending on whether frozen or paraffin wax embedded tissues are studied. Correlation of staining intensity with tumour differentiation is also influenced by such factors. This must be taken into consideration when collecting and comparing data.

Figure 4 Paraffin wax section of poorly differentiated gastric adenocarcinoma fixed in periodate-lysine-paraformaldehyde (two hours), showing weak membrane staining for c-erbB-2 protein (arrowhead).

We thank Mrs Clara Chiu for preparing the manuscript and Mr C H Ning for photographic assistance. This work was supported by the University of Hong Kong Research Grants Account Code: 337/046/0005.

The article forms part of a thesis to be submitted to the IMLS for Fellowship examination.

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