Immunohistochemical expression of the c-kit proto-oncogene product in human malignant and non-malignant breast tissues

X Chui, H Egami, J Yamashita, T Kurizaki, H Ohmachi, S Yamamoto and M Ogawa

Department of Surgery II, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto, Kumamoto 860, Japan.

Summary The immunohistochemical expression of c-kit proto-oncogene product in 57 breast cancer tissues was studied using anti-c-kit proto-oncogene product antibody in comparison with 20 normal breast tissues and 58 benign breast tumours. In normal breast tissues, the c-kit proto-oncogene product was strongly expressed on cell membrane and/or cytoplasm of alveolar and ductal cells. The immunoreactive score (IRS) of c-kit proto-oncogene product in normal mammary epithelia was 6.22 ± 2.11 (mean ± s.d.). In benign breast diseases, the c-kit proto-oncogene product was detected heterogeneously with a reduced IRS (3.33 ± 2.44). In breast cancer tissues, the expression of the immunoreactive c-kit proto-oncogene product was often deleted and the average IRS was significantly reduced compared to those of normal breast tissues or benign breast disease tissues. Among benign diseases, the average IRS of intraductal papilloma was significantly reduced (1.34 ± 1.70) and the staining intensity and pattern were found to be similar to those seen in breast cancer. The results in this study suggested that the c-kit proto-oncogene product is correlated with the growth control or the differentiation of normal breast epithelium. Also, the loss of the expression of this protein may indicate the change of the signal transduction in relation to malignant transformation in human mammary epithelium.

Keywords: breast cancer; c-kit proto-oncogene product; immunohistochemistry

The c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor with a molecular weight of 145 000 Da, which is structurally similar to the platelet-derived growth factor receptor and the colony-stimulating factor-1 receptor (Qiu et al., 1988). Recent studies have demonstrated that the c-kit proto-oncogene product is expressed in a restricted number of human fetal, adult tissues and solid tumours (Natali et al., 1992a; Matsuda et al., 1993).

Analysis of the tissue expression of the c-kit proto-oncogene in the breast tissues has shown that normal epithelium contained large amounts of c-kit-specific RNA transcripts (Natali et al., 1992b). Immunohistochemically, homogenous expression of c-kit proto-oncogene product in normal mammary epithelia, and the loss of expression of this protein in breast cancer has been demonstrated using fresh frozen sections (Natali et al., 1992b). However, little is known about the biological significance of this protein in benign and malignant diseases of the breast. To elucidate the relationship between the loss of this protein and the malignant transformation of human breast tissue, the immunohistochemical expression of the c-kit proto-oncogene product in malignant and non-malignant breast tissues was examined using paraffin-embedded sections on a comparative basis.

demonstration of the c-kit proto-oncogene product, as described below.

Histopathological classification

The benign breast disease and the breast cancer were diagnosed according to the General Rules for Clinical and Pathological Recording of Breast Cancer (Japanese Breast Cancer Society, 1992). The breast cancer was classified into three types: four cases of non-invasive carcinoma, 50 cases of invasive ductal carcinoma and three of mucinous carcinoma. Four cases of non-invasive carcinoma were all non-invasive ductal carcinomas, and 50 cases of invasive ductal carcinoma consisted of 29 solid tubular, 10 papillotubular and 11 scirrhus carcinoma. Benign disease was classified into three types: 23 cases of fibrocystic change, 20 of fibroadenoma and 15 of intraductal papilloma.

Antibody

Commercially available anti-c-kit rabbit IgG (K963; IBL, Fujioka, Japan) was used. It was derived by immunising rabbit with the carbon-terminal peptide of c-kit (tyrosine kinase receptor) as immunogen (Matsuda et al., 1993).

Materials and methods

Tissues

Fifty-seven patients with primary breast cancer and 58 patients with benign breast disease were studied. All patients were female and the ages of patients with breast cancer and benign disease ranged from 29 to 73 years (average age 51.8 years) and from 22–89 years (average age 43.7 years) respectively. Furthermore, normal breast tissues were obtained from 20 surgical specimens of mastectomy patients. Tissues were fixed in 10% buffered formalin and embedded in paraffin. One serial section from each tissue sample was stained with haematoxylin and eosin for routine histological examination and others were treated for

Immunohistochemistry

ABC kits (Vector Laboratories, CA, USA) for rabbit IgG were used. Four micron tissue sections were deparaffinised with xylene and rehydrated with a series of ethanol solutions. Tissue sections were incubated in normal goat serum for 30 min, incubated overnight at 4°C with optimally diluted primary antibody and subsequently incubated with biotinylated anti-rabbit IgG and avidin–bien alkaline phosphatase complex (AK-5100; Vector Laboratories, CA, USA) for 60 min at room temperature. They were washed in 0.01 M phosphate-buffered saline (PBS, pH 7.2) between each incubation step. Then, the alkaline phosphatase substrate I (SK-5100; Vector Laboratories, CA, USA) in the presence of 1.25 mmol l⁻¹ levamisole (SP-5000; Vector Laboratories, CA, USA) was used for signal detection. All sections were counterstained with Mayer's haematoxylin. SCLC was used as a positive control for c-kit proto-oncogene product staining in which c-kit proto-oncogene product is known to

Correspondence: M Ogawa
Received 3 March 1995; revised 26 September 1995; accepted 19 October 1995
be detectable (Natali et al., 1992a). For the negative controls, the following procedures were employed: (1) sections were processed without the primary antibody, and (2) rabbit IgG (Zymed Laboratories, CA, USA) was used instead of the primary antibody.

**Evaluation for immunohistochemical reactivity**

Evaluation of the cell staining reaction was performed in accordance with the following immunoreactive score (IRS) proposed by Remmele and Stegner (1986) with slight modification as follows: IRS = SI (staining intensity) × PP (percentage of positive cells). SI was determined as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0, negative; 1, 1–20% positive cells; 2, 21–50% positive cells; 3, 51–100% positive cells. Ten visual fields from different areas of each specimen were chosen at random for the IRS evaluation and the average IRS was calculated as final value.

**Statistical analysis**

The data obtained were evaluated as follows: difference between the means of continuous variables was calculated using unpaired Student's t-test. Follow-up survival analysis was performed by the Kaplan–Meier method and comparison between individual subgroups was performed using the generalized Wilcoxon test. The probability level of <0.05 was taken as the limit of significant difference.

**Results**

**Normal breast tissue**

The expression of c-kit proto-oncogene product was observed homogeneously in the cytoplasm and/or on cell membrane of alveolar and ductal mammary epithelia in all specimens (Figure 1a). The average IRS of normal breast tissue was $6.22 \pm 2.11$ (mean ± s.d.) (Table I).

**Benign breast tissue**

In benign breast tissue, the expression was found to be distributed heterogeneously in the cytoplasm or on the cell membrane of tumour cells (Figure 1b–d). The average IRS of c-kit proto-oncogene product expression tended to be reduced as $3.33 \pm 2.44$ in 58 benign breast diseases (Table I). Especially in intraductal papilloma of the breast, the IRS was significantly reduced to $1.34 \pm 1.70$, compared with that of fibrocystic change (IRS, $3.88 \pm 2.45$) or of fibroadenoma (IRS, $4.11 \pm 2.16$) (Table II).

**Breast cancer**

In breast cancer, the expression of c-kit proto-oncogene product was often deleted. Even in the tumour showing positive staining, the expression was observed in the cytoplasm and/or on the cell membrane of the limited number of cancer cells (Figure 2). The expression was found

![Figure 1 Immunohistochemical expression of c-kit proto-oncogene product in human normal breast tissues and benign diseases. (a) Normal mammary epithelia. Bar = 25 μm. All of the epithelial cells expressed c-kit proto-oncogene product on plasma membrane or in the cytoplasm. (b) Fibrocystic change. The expression of c-kit proto-oncogene product was observed in the ductal epithelial hyperplasia in the lesion of fibrocystic change. Bar = 25 μm. (c) Intraductal papilloma. Bar = 50 μm. (d) Fibroadenoma. Bar = 50 μm. b–d represent the heterogeneous expression of c-kit proto-oncogene product in benign breast diseases.](image)
to be restricted in cancer cells showing acinar differentiation (Figure 2c). The average IRS of breast cancer was only 0.43 ± 1.27 (Table I). The complete deletion was found in 40

diseases
Number of tissues
IRS of c-kit expression (mean ± s.d.)
Fibrocystic change 23 3.88 ± 2.45*
Fibroadenoma 20 4.11 ± 2.16**
Intraductal papilloma 15 1.34 ± 1.70*
* \( P = 0.0013; \) ** \( P = 0.0003. \)

Figure 2 Immunohistochemical expression of c-kit proto-oncogene product in human breast cancer. (a) The staining of immunoreactive c-kit proto-oncogene protein was distributed focally in this breast cancer tissue. (b) Complete deletion of the expression of c-kit proto-oncogene product was observed in cancer cells. In contrast, diffuse staining was observed on the alveolar mammary epithelia in this specimen. (c) The expression of c-kit proto-oncogene product was detected on cancer cells showing acinar differentiation. Bar = 50 μm.

Table II Expression of c-kit proto-oncogene product in benign diseases of the breast

| Benign diseases | Number of tissues | IRS of c-kit expression (mean ± s.d.) |
|-----------------|-------------------|---------------------------------------|
| Fibrocystic change | 23                | 3.88 ± 2.45*                          |
| Fibroadenoma     | 20                | 4.11 ± 2.16**                         |
| Intraductal papilloma | 15              | 1.34 ± 1.70*                          |

Table III Changes in the expression of c-kit proto-oncogene product in human breast diseases

| Tissues                 | Number of tissues | IRS of c-kit expression (mean ± s.d.) |
|-------------------------|-------------------|---------------------------------------|
| Normal duct             | 16                | 7.13 ± 1.82                           |
| Intraductal papilloma   | 15                | 1.34 ± 1.70                           |
| Non-invasive carcinoma  | 4                 | 0.90 ± 1.55                           |
| Invasive carcinoma      | 50                | 0.41 ± 1.02                           |

Table IV Correlation between the expression of the c-kit proto-oncogene protein and the clinicopathological parameters of breast cancer

| Parameters             | Number of tissues | IRS of c-kit expression (mean ± s.d.) | P-value |
|------------------------|-------------------|---------------------------------------|---------|
| ER (-)                 | 31                | 0.51 ± 1.67                           | NS      |
| ER (+)                 | 26                | 0.36 ± 0.84                           | NS      |
| Menopausal status      |                   |                                       |         |
| Pre-                   | 26                | 0.44 ± 0.92                           | NS      |
| Post-                  | 31                | 0.42 ± 1.52                           | NS      |
| Tumour size            |                   |                                       |         |
| T1                     | 20                | 0.55 ± 1.87                           | NS      |
| T2                     | 25                | 0.40 ± 0.91                           | NS      |
| T3                     | 8                 | 0.43 ± 0.69                           | NS      |
| T4                     | 4                 | 0.00 ± 0.00                           | NS      |
| Histological           |                   |                                       |         |
| Solid tubular          | 29                | 0.46 ± 1.58                           | NS      |
| Papillotubular         | 10                | 0.50 ± 1.10                           | NS      |
| Scirrhus               | 11                | 0.22 ± 0.48                           | NS      |
| Non-invasive           | 4                 | 0.90 ± 1.55                           | NS      |
| Mucinous               | 3                 | 0.00 ± 0.00                           | NS      |
| Differentiation        |                   |                                       |         |
| I                      | 19                | 0.41 ± 0.38                           | NS      |
| II                     | 23                | 0.69 ± 0.18                           | NS      |
| III                    | 15                | 0.38 ± 0.84                           | NS      |
| Lymph node metastasis  |                   |                                       |         |
| N0                     | 15                | 0.56 ± 1.70                           | NS      |
| N1a                    | 8                 | 0.75 ± 1.69                           | NS      |
| N1β                    | 1                 | 0.09 ± 0.25                           | NS      |
| N2                     | 3                 | 0.00 ± 0.00                           | NS      |
| N3                     | 7                 | 0.67 ± 1.16                           | NS      |
| Distant metastasis     |                   |                                       |         |
| MO                     | 41                | 0.43 ± 1.40                           | NS      |
| M                      | 16                | 0.41 ± 0.91                           | NS      |
| TNM stage              |                   |                                       |         |
| I                      | 29                | 0.53 ± 1.64                           | NS      |
| II                     | 11                | 0.21 ± 0.47                           | NS      |
| III                    | 1                 | 0.00 ± 0.00                           | NS      |
| IV                     | 16                | 0.41 ± 0.91                           | NS      |

IRS, immunoreactive score; ER, oestrogen receptor; NS, not significant.
no significant difference was found between non-invasive and invasive carcinoma, the IRS of intraductal papilloma was higher than that in invasive carcinoma, and the intensity and the pattern of the staining were found to be similar to those seen in non-invasive carcinoma.

No significant relationship was found between the expression of the c-kit proto-oncogene product and the clinicopathological parameters of the breast cancer, such as grade of differentiation, tumour size, lymph node metastasis, distant metastasis, TNM stage, presence of ER, and menopausal status of the patient (Table IV). The patients were followed-up for three years in this study and no significant association was observed between the expression of this protein and the prognosis of patients with breast cancer.

Discussion

The immunoreactive expression of c-kit proto-oncogene product was not observed in human normal lung or seminal vesicles tissue (Natali et al., 1992a; Matsuda et al., 1993), whereas it was found in 56% of SCLC (Matsuda et al., 1993 and 80% of seminomas (Strohmeyer et al., 1991). On the other hand, the expression was diffusely observed in human melanocytes, but, in primary melanomas, the deletion of the c-kit proto-oncogene product was observed in more invasive lesions (Natali et al., 1992c). Previously a complete inverse pattern of the expression of c-kit proto-oncogene product has also been found in the normal tissues and the malignant tumours of the breast (Natali et al., 1992a; Matsuda et al., 1993).

In the present study the c-kit proto-oncogene product was uniformly expressed in normal breast tissues. In contrast, it was expressed heterogeneously in benign breast disease and the expression was observed in breast cancer specimens with high incidence suggesting that the reduced c-kit proto-oncogene product expression is a general phenomenon in breast cancer. Previous studies have shown that direct cell–cell interaction between c-kit and its ligand, the membrane-bound form of stem cell factor (SCF), plays an important role in signal transduction (Flanagan, et al., 1991; Reith et al., 1991). Therefore, the high expression of the c-kit proto-oncogene product in normal breast tissue indicated that this protein may be related to the regulation of the proliferation and/or the differentiation of human normal mammary epithelia through the c-kit signalling pathway. Although it is possible to consider that the absence of staining in neoplastic cells is due to the presence of mutant c-kit product which is not reactive with the antibody used, the deletion of c-kit proto-oncogene product in breast cancer may indicate the changes of the signal transduction during malignancy in human mammary epithelia.

Recently, clonal analysis by means of polymerase chain reaction revealed that the intraductal papilloma was monoclonal in origin consisting of breast carcinoma, indicating that certain genetic changes had already occurred in the intraductal papilloma (Noguchi et al., 1992, 1994). In the present study we found that the IRS of c-kit proto-oncogene product in intraductal papilloma was significantly reduced compared with that of other benign breast diseases. Since the loss of the expression of this protein could be considered to be related to the malignant transformation of human mammary epithelia (Natali et al., 1992b), the observations in this study may indicate that the intraductal papilloma possesses higher malignant potential in benign breast diseases.

In this study no significant relationship was found between the expression of c-kit proto-oncogene product and the clinicopathological factors of breast cancer, although it is well known that the c-kit proto-oncogene product displays pleiotropic functions, such as the migration of the pigment stem cells (Keshet et al., 1991) and the proliferation of the mast cells during normal development (Wershil et al., 1992).

The reduction of c-kit proto-oncogene product expression observed in intraductal papilloma as well as breast cancer cells suggested that the deletion of c-kit proto-oncogene product would occur in the early phase of malignant transformation of human mammary epithelia. Further investigations will be required to clarify the active mechanism of c-kit proto-oncogene product in human breast.

References

FLANAGAN JG, CHAN DC AND LEDER P. (1991). Transmembrane form of the kid ligand growth factor is determined by alternative splicing and is missing in the S12' mutant. Cell, 64, 1025–1035.

JAPANESE BREAST CANCER SOCIETY. (1992). General Rules for Clinical and Pathological Recording of Breast Cancer. 11th ed. Kanehara: Tokyo.

KESHT E, LYMAN SD, WILLIAMS DE, ANDERSON DM, JENKINS NA, COPELAND NG AND PARADA LF. (1991). Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. EMBO J., 10, 2425–2435.

MATSUDA R, TAKAHASHI T, NAKAMURA S, SEKIDO Y, NISHIDA K, SETO M, SEITO T, SUGIURA T, AIYOSHI Y, TALAHASHI T AND UEDA R. (1993). Expression of the c-kit protein in human solid tumors and in corresponding fetal and adult normal tissues. Am. J. Pathol., 142, 339–346.

NATALI PG, NICOTRA MR, SURES I, SANTORO E, BIGOTTI A AND ULLRICH A. (1992a). Expression of c-kit receptor in normal and transformed human nonlymphoid tissues. Cancer Res., 52, 6139–6143.

NATALI PG, NICOTRA MR, SURES I, MOTTOLESE M, BOTTI C AND ULLRICH A. (1992b). Breast cancer is associated with loss of the c-kit oncogene product. Int. J. Cancer, 52, 713–717.

NATALI PG, NICOTRA MR, WINKLER AB, CAVALIERE R, BIGOTTI A AND ULLRICH A. (1992c). Progression of human cutaneous melanoma is associated with loss of expression of c-kit proto-oncogene receptor. Int. J. Cancer, 52, 197–201.

NOGUCHI S, MOTOMURA K, INAJI H, IMAOKA S AND KOYAMA H. (1994). Clonal analysis of predominantly intraductal carcinoma and precancerous lesions of the breast by means of polymerase chain reaction. Cancer Res., 54, 1849–1853.

QUI F, RAY P, BROWN K, BARKER PE, JHANWAR S, RUDGE FF AND BESMER P. (1988). Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family-oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. EMBO J., 7, 1003–1011.

REITH AD, ELLIS C, LYMAN S, ANDERSON DM, WILLIAMS DE, BERNSTEIN A AND PAWSON T. (1991). Signal transduction by normal isoforms and W mutant variants of the kit receptor tyrosine kinase. EMBO J., 10, 2451–2459.

REMMELE W AND STEGNER HE. (1986). Immunohistochemischer Nachweis von Östrogenezeptoren (ERICA) in Mammakarzinomgewebe: Vorschlag zur Einheitlichen Formulierung des Untersuchungsbefundes. Dtsch. Arztebl., 83, 3362–3364.

STROHMeyer T, PETER S, HARTMANN M, MUNEMITSU S, ACKERMANN R, ULLRICH A AND SLAMON DJ. (1991). Expression of the hst-1 and c-kit protooncogenes in human testicular germ cell tumors. Cancer Res., 51, 1811–1816.

WERSHIL BK, TSAI M, GEISSLER W, ZSEBO KM AND GALLI SJ. (1992). The rat c-kit ligand, stem cell factor, induces c-kit receptor-dependent mouse mast cell activation in vivo. Evidence that signaling through the c-kit receptor can induce expression of cellular function. J. Exp. Med., 175, 245–255.