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Validation of new aromatase monoclonal antibodies for immunohistochemistry: progress report☆

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Abstract

Intratumoral aromatase is a potential therapeutic target for the treatment of postmenopausal estrogen-dependent breast cancers. Therefore, reliable methods should be developed for routine application for the detection of intratumoral aromatase. A multi-center collaborative group has been established to generate and validate new aromatase monoclonal antibodies (MAbs). A recombinant GST–aromatase fusion protein was expressed in baculovirus and the purified protein was used for immunization of mice either as a native or formalin-fixed antigen. Hybridomas were generated using standard techniques and screened biochemically prior to immunohistochemistry (IHC) evaluation in human placenta, ovary and breast cancer tissues. Twenty-three MAbs selected by biochemical assays were further evaluated by IHC of paraffin-embedded tissue sections including normal ovary, and placenta, and a small series of 10 breast carcinomas. Of the 23 MAbs, 2 (clones 677 and F2) were determined to specifically stain cell types known to express aromatase in normal tissues. In breast carcinomas staining of malignant epithelium, adipose tissue, normal/benign and stromal compartments was detected. IHC was performed and independently evaluated by three pathologists (HS, TJA and SGS), each using the same evaluation criteria for staining intensity and proportion of immunopositive cells. With these two MAbs, interpathologist and intralaboratory variations were minimal in comparison with differences which could be detected between tissue specimens and antibodies.

Keywords: Breast; Cancer; Aromatase; Immunohistochemistry; Antibody

1. Introduction

Estrogens are considered to play important roles in the development and progression of hormone-dependent human breast cancer. Human breast cancers express increased aromatase enzyme and activity compared with normal breast tissue. The overexpression of aromatase appears to play an important role in estrogen related development and progression of some human breast cancers [1–4]. Aromatase inhibitor therapy is one of the endocrine treatments available to breast cancer patients. It has therefore become very important to predict which patients will respond prior to initiation of therapy.

At present, aromatase inhibitors appear to exert their effects primarily through the reduction of aromatase activity in non-ovarian tissue in postmenopausal women. Some studies have demonstrated a positive correlation between intratumoral aromatase activity and response to treatment with various aromatase inhibitors [3,4]. If in situ estrogen biosynthesis and local concentrations are important in the cancer cases that respond to aromatase inhibitor treatment, the tumors must also express the aromatase enzyme as well as estrogen and progesterone receptors. Accordingly, measurement of aromatase activity might serve as a useful tool for prediction of responses to aromatase inhibitors. Test of this concept has been limited by the need to determine aromatase by biochemical methods in fresh tissue. What is needed is a precise, sensitive, and quantifiable method for

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detecting aromatase in archival materials or formalin-fixed and paraffin-embedded tissue. No anti-aromatase antibodies have been specifically designed for this purpose.

Existing antibodies directed against aromatase are available. However, they are in ever dwindling supplies and the results from studies using them have been controversial in terms of tumor aromatase localization [5–9]. Therefore, this study was undertaken as an international collaboration in order to develop aromatase antibodies that can be used to assess aromatase expression in fixed breast cancer tissue and test whether these measurements are predictive of responsiveness to aromatase inhibitors. This manuscript represents an interim progress report of this project.

2. Materials and methods

2.1. Materials

Tissues were fixed in 3.7% formalin and embedded in paraffin. Control tissues included normal placenta and cycling ovary. All human breast carcinoma (10 cases) used for initial screening were invasive ductal carcinoma and retrieved from surgical pathology files of Department of Pathology, Tohoku University Hospital.

2.2. Methods

2.2.1. Production of monoclonal antibodies (MAbs) against aromatase

Preparation of aromatase antigen, its fixation prior to injection, initial screening for antibody production, and development of hybridomas were carried out in a fashion similar to that described by Press et al. for formalin-fixed and native progesterone receptor [10–13]. Cell fusion and construction of B-cell hybridomas was performed as previously described [11–13]. Selected colonies were expanded further in culture and cryopreserved in liquid nitrogen. Subcloning and screening was repeated to assure that cell lines were clonal and stable antibody producers. Screening assays utilized previously described standard techniques [10–13].

2.2.2. Biochemical screening assays

Enzyme-linked immunoabsorption assays (ELISAs) were performed as previously described with 96-well microtiter plates (Immulon 2B, Thermo Labsystems, Franklin, MA) coated at 2.5 μg/ml with purified GST–aromatase used as antigen or with free GST as previously described [10–13]. Western blots were performed by electrophoresis on SDS–8 or 10% polyacrylamide gels by an enhanced chemiluminescence procedure as previously described [10,13].

2.2.3. Immunohistochemistry

Immunohistochemical analyses were performed employing the streptavidin–biotin amplification method, and have been previously described in detail [5,6].With this methodology, non-specific control sections were processed according to standard methods and contained low background staining.

3. Results

3.1. Selection criteria for MAbs to aromatase and results of hybridoma screening

The strategy for screening hybridomas was to assay the initial fusion wells by ELISA against purified GST–aromatase used as antigen and with free GST. Only positives for GST–aromatase and negatives for the GST moiety of the antigen were selected. ELISA positives were further screened by Western blot against purified GST–aromatase, GST–aromatase in crude extracts of Sf9 cells and free GST. Hybridoma products that gave a specific reaction for GST–aromatase by Western blot with little or no cross-reaction with other proteins in crude cell extracts were submitted to screening by immunohistochemistry (IHC) of normal ovary and placenta. The criteria for specific IHC detection of aromatase in placenta was staining exclusively in the plasma membrane of syncytiotrophoblasts of chorionic villi and in the ovary by staining in granulosa cells of ovarian follicles. Hybridomas that passed this screening criteria were subcloned, isotyped and the MAb products were purified and used for subsequent characterizations.

From the cell fusions of mice injected with unfixed antigen, 60 hybridomas were positive by ELISA, 11 of these reacted specifically by Western blot with aromatase and 4 of the Western blot positives gave specific staining of placenta and ovary by IHC (clones 636, 677, 1157 and 1255). From the cell fusions with fixed antigen, there were 105 ELISA positives, 22 of these were positive by Western blot criteria and 5 of the Western blot positives gave specific staining of placenta and ovary by IHC. Purified MAbs were used for further characterization of all nine of the hybridomas selected from the two cell fusions by the above screening strategy. By IHC of 10 cases of human invasive ductal breast carcinoma, 4 of the 9 MAbs were determined to be optimal in terms of specific cytoplasmic staining of epithelial cancer cells and minimal background staining, i.e. no staining in nucleus or acellular areas. The four MAbs selected by this screening strategy are listed in Table 1, along with antibody subtypes for each.

Western blot screening results of the four selected MAbs are shown in Fig. 1. As a positive control, a previously

Table 1
Summary of monoclonal antibodies selected

Clone	Mouse isotype	Antigen
677/H7	IgG2a	Unfixed GST–aromatase
1255/H6	IgG1	Unfixed GST–aromatase
Grp10/F2	IgG1	Fixed GST–aromatase
Grp15/F11	IgG1	Fixed GST–aromatase

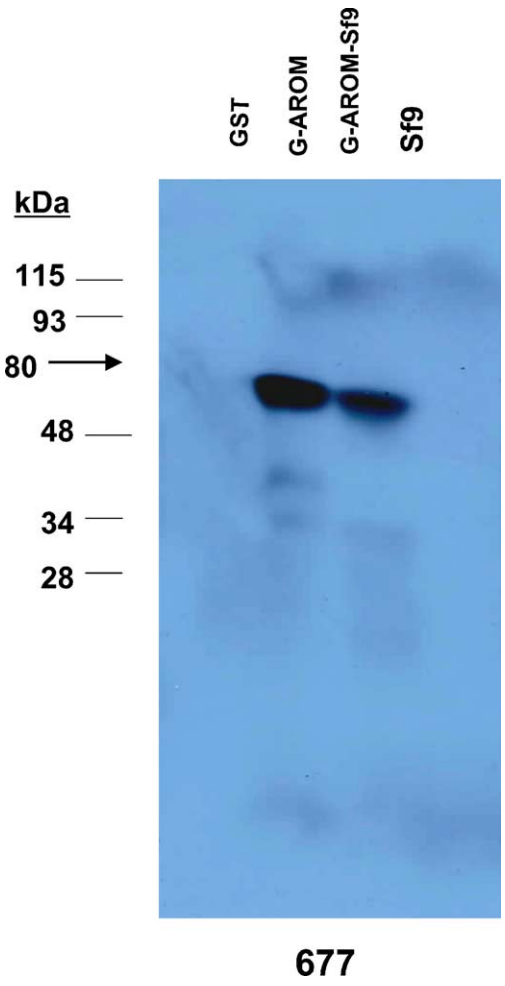

Fig. 1. Western blot screening of MAbs 677, 1255, F2 and F11. Each antibody was reacted by Western blot against purified GST–aromatase, GST–aromatase present in crude cell extracts of Sf9 cells and free GST. As a positive control, a previously described mouse MAb was used as an ascites fluid diluted 1:500 in PBS.

described mouse MAb to aromatase was used in Western blot screenings [14,15]. Each MAb reacted with the 82–85 kDa GST–aromatase fusion protein, but failed to react with free GST, indicating they detect an epitope in the aromatase portion of the antigen. The fusion protein is expected to be this size as the GST moiety is 27 kDa and the aromatase is 55 kDa. Little or no cross-reaction with other proteins was detected in crude Sf9 cell extracts (Fig. 1). To confirm that these MAbs recognize native aromatase without the GST moiety, Western blots were also performed with crude protein extracts of human MCF-7 breast cancer cell transfected to express aromatase as a non-fusion protein from a tetracycline-inducible promoter. As shown in Fig. 2, the 677 and F2 MAbs, reacted with a tetracycline-inducible protein of approximately 55 kDa with little or no cross-reaction with other proteins in the MCF-7 cell extract. The positive control antibody detected the same-sized tetracycline-inducible protein in MCF-7 cells (Fig. 2). Examples of IHC staining of term human placenta and normal cycling human ovary with one of the selected MAbs (677), are shown in Figs. 3 and 4, respectively. Immunostaining was detected in syncytiotrophoblasts of chorionic villi of placenta (Fig. 3) and predominantly in granulosa cells of ovarian follicles (Fig. 4); both are known cellular sites of aromatase expression.

3.2. Independent scoring and immunocytochemistry evaluation of breast carcinomas

Three co-authors (HS, TJA and SGS) independently performed and evaluated IHC of the same 10 cases of breast carcinomas with the four selected MAbs from Table 1. Immunostaining was evaluated based on the following criteria: (1) the proportion of the area of the cells which occupied the tissue sections; (2) the proportion of positively stained cells; and (3) the overall staining intensity of a tissue section. This criteria was developed through simultaneous evaluation using multi-headed light microscopy by three of the authors. All three co-authors agreed that immunohistochemistry using MAbs 677 or F2 yielded the most satisfactory results in terms of minimal background staining, specificity, reproducibility and interpretation of the results based on these staining criteria. With these two MAbs, immunoreactivity was detected in different compartments of breast carcinomas including parenchymal or carcinoma cells, stromal cells or fibroblasts, adipocytes, macrophages and normal duct epithelial cells (Fig. 5). The proportion of positively stained cells varied among the 10 cases. An *H*-score or semiquantitative scoring system for MAbs 677 and F2 was established based on staining of these 10 cases of breast carcinomas (Table 2) that can be applied to future clinical correlation studies between biochemical and immunohistochemical results.

4. Discussion

Due to marked improvements in immunostaining methods and the antibodies used, it is now possible to immunolocalize the increasing number of antigens in routinely processed specimens (i.e. 10% formalin-fixed and paraffin-embedded tissue). Immunohistochemistry can now be performed rapidly and without many technical difficulties. We reasoned that antibodies against aromatase and an immunohistochemical staining system would allow semiquantitation of aromatase immunoreactivity in tissue sections in situ [5–9]. In addition, resected breast cancer specimens, which are fixed in formalin and embedded in paraffin, are stored as archives in the greater majority of hospitals and institutions in many countries. This method has the enormous potential to provide information about breast cancer patients worldwide, allowing us to assess intratumoral aromatase, both prospectively and retrospectively.

Prior reports concerning immunohistochemistry of aromatase in breast tissue revealed difficulties with this method. Some groups reported aromatase in stromal cells, including

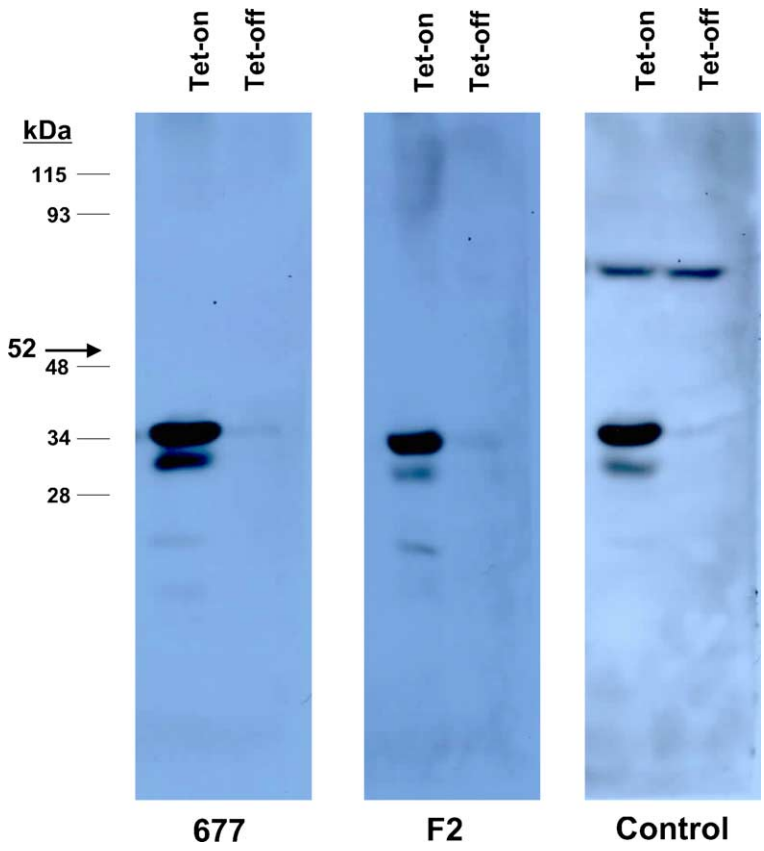

Fig. 2. Detection of a non-fusion human aromatase protein by Western blot. MCF-7 breast cancer cells were transfected with human aromatase from a vector regulated by a tetracycline-off promoter. Crude cell extracts from induced (Tet-off) and non-induced (Tet-on) cells were analyzed by Western blot with MAbs 677, F2 and the positive control mouse ascites MAb.

adipocytes [5,6], while others reported immunoreactivity in carcinoma cells [8,9]. Accordingly, the objective of this study was to produce specific monoclonal antibodies against aromatase, to fully characterize these antibodies, and to validate their use by comparison with a biochemical method for aromatase. A potential limitation of applying immunohistochemistry in evaluation of intratumoral aromatase or any antigen in low amounts is that results can be influenced by the quality of specimen preparations. Delayed fixation usually result in the degradation of immunoreactivity, leading to misinterpretation of data as false negative findings.

The immunization and screening strategy in this project was designed to produce MAbs that optimally detect aromatase through immunohistochemistry of routine archival formalin-fixed and paraffin-embedded sections of breast carcinomas. Whether the MAbs generated from formalin-fixed

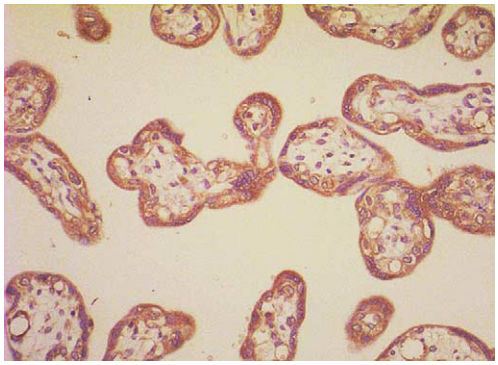

Fig. 3. Immunohistochemistry of aromatase in full-term human placenta using monoclonal antibody 677. Aromatase immunoreactivity was detected in syncytiotrophoblasts of chorionic villi.

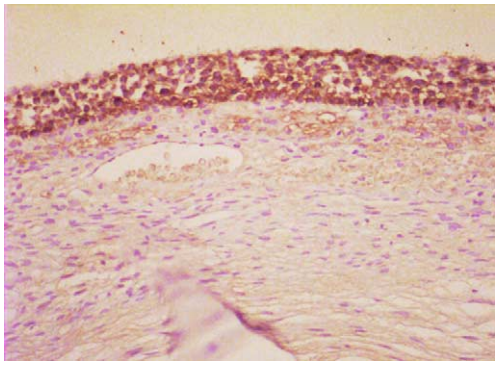

Fig. 4. Immunohistochemistry of aromatase in normal cycling human ovaries using monoclonal antibody 677, Aromatase immunoreactivity was predominantly detected in membrane granulosa.

Table 2
Summary of scoring of aromatase immunoreactivity in human breast invasive ductal carcinoma

(1) Obtain the approximate percentage of the parenchymal or carcinoma cells in the foci of carcinoma. Both cellularity and areas of carcinoma should be considered at the time of evaluation (0: 0%; 1: 1–25%; 2: 26–50%; 3: 51–75%; 4: 76–100%)
(2) Obtain the approximate percentage of aromatase positive carcinoma (0: 0%; 1: 1–25%; 2: 26–50%; 3: 51–75%; 4: 76–100%)
(3) Choose the most representative areas of aromatase positivity in carcinoma cells and grade relative immunointensity (0: no immunoreactivity; 1: weak; 2: moderate; 3: intense)
(4) Evaluate relative immunointensity and proportion of stromal cells, macrophages and other inflammatory cells and adipocytes in and/or adjacent to carcinomatous foci as follows: proportion of adipocytes (0: 0%; 1: 1–25%; 2: 26–50%; 3: 51–75%; 4: 76–100%); proportion of normal ducts and stromal cells (0: 0%; 1: 1–25%; 3: 26–50%; 4: >50%); intensity (0: no immunoreactivity; 1: weak; 2: moderate; 3: intense)
(5) Describe the presence of absence of focal immunoreactivity in the tissue sections

antigen are less sensitive to formalin fixation and paraffin embedding than the MAbs generated to native antigen remains to be determined. The dependency of these MAbs on an antigen retrieval step also is not known as yet. However, prompt and brief fixation is likely to be ideal for the accurate assessment of intratumoral aromatase by immunohistochemistry.

Whether in parenchymal or stromal cells, aromatase immunoreactivity is located in the cytoplasm of cells, making it difficult to obtain the ratio or labeling index of aromatase, in immunostained slides as compared to a nuclear antigen such as ER. Computer-based image analysis can contribute in this aspect but this approach requires relatively expensive image analyzers and supporting computer programs. An alternative approach is to determine the percentage of stromal cells with aromatase immunoreactivity using routine light microscopy. We evaluated immunoreactivity based on the following histological scores: (1) the proportion of the area of the cells which occupied the tissue sections; (2) the proportion of aromatase positive cells; and (3) the overall immunointensity of aromatase in tissue sections. This approach is relatively straightforward and easily applicable and is considered more promising for widespread application, as it requires no special instruments or equipment. Using this approach in this investigation, three co-authors were able to agree on interpretation of staining results with the 677 and F2 MAbs on a limited number of breast carcinoma cases. However, as in any morphological or histological classification or criteria, a future goal with these two MAbs is to establish standards for intra- and inter-observer variations. This will be crucial to make results as subjective and reproducible as possible.

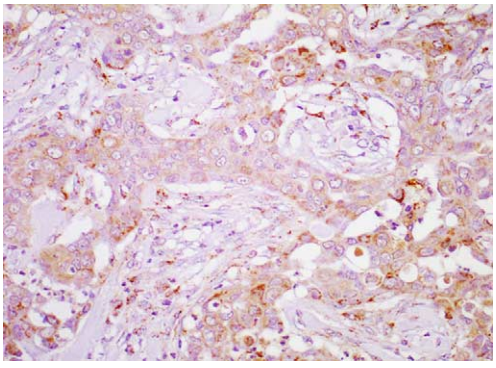

Fig. 5. Immunohistochemistry of aromatase in human breast invasive ductal carcinoma. Aromatase immunoreactivity was detected in both carcinoma (parenchymal) cells and stromal cells.

Of the MAbs produced in this project, two (677 and F2) were determined to be the most capable of detecting aromatase by immunohistochemical staining in formalin-fixed and paraffin-embedded tissue sections. To determine the reliability of these MAbs to detect aromatase by IHC in routine formalin-fixed paraffin sections of breast carcinomas will require correlating the results of immunohistochemistry and biochemical assays in the same specimen with a large series of breast tumors that have known biochemical values for aromatase. The *H*-score system (Table 2) developed with 677 and F2 MAbs in this investigation should be a useful starting point for these correlation studies and for refinement of an optimal immunohistochemistry method and semiquantitative scoring system for detection of intratumoral aromatase in the patients with breast carcinoma.

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