Novel Prognostic Value of Nuclear Epidermal Growth Factor Receptor in Breast Cancer

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Abstract

Epidermal growth factor receptor (EGFR) has been detected in the nucleus of cancer cells and primary tumors for decades. While localized in the nucleus, EGFR functions as a transcriptional regulator resulting in the activation of the cyclin D1 gene. Despite nuclear accumulation of EGFR, it is linked to increased DNA synthesis and proliferative potential, the pathological significance of nuclear EGFR, however, remains uninvestigated. Furthermore, expression of EGFR has not provided a consistent predictive value for survival of breast cancer patients. Here, we analyzed 130 breast carcinomas via immunohistochemical analyses for the levels of nuclear and non-nuclear EGFR. We found 37.7% of the cohort immunostained positively for nuclear EGFR and 6.9% with high levels of expression. Importantly, Kaplan-Meier survival analysis and log-rank test revealed a significant inverse correlation between high nuclear EGFR and overall survival (P = 0.009). Expression of nuclear EGFR correlated positively with increased levels of cyclin D1 and Ki-67, both are indicators for cell proliferation. In contrast, expression of non-nuclear EGFR did not significantly correlate with those of cyclin D1 and Ki-67 or the overall survival rate. In addition, we analyzed 37 oral squamous carcinomas for EGFR expression and found 24.3% of the cases to contain moderate/high levels of nuclear EGFR. Taken together, our findings indicate pathological significance of nuclear EGFR and may have important clinical implication. (Cancer Res 2005; 65(1): 338–48)

Introduction

Nuclear detection of epidermal growth factor (EGF) receptor (EGFR) has been reported in highly proliferative tissues, including those of cancer cells, primary tumor specimens, pregnant uterus, and regenerating liver (1–7). In addition to EGFR, many receptor tyrosine kinases, such as rat erbB1, ErbB2/HER-2, rat p185/neu, ErbB3/HER-3, ErbB4/HER-4, TrkA/nerve growth factor receptor, transforming growth factor-β type I receptor, and fibroblast growth factor receptor, have also been found in the nucleus (8–18). Receptors to inflammatory cytokines, such as interleukin-1, interleukin-5, and IFN-γ, also exist in the nuclear compartment (19–25). Ligands to most of these receptors, including EGF, nerve growth factor, fibroblast growth factor, interleukin-1, interleukin-5, transforming growth factor-α and -β, and IFN-γ, were also found in the nucleus (19, 25, 26).

Despite a large body of evidence showing the nuclear existence of cell-surface receptors, the physiological function and pathological consequences of which remain largely unknown. Nevertheless, EGFR nuclear translocation occurs following EGF stimulation as shown in tumor cells (3). In the nucleus, EGFR complex associates with the cyclin D1 promoter leading to its transcriptional activation (3). More recently, HER-2 was found to translocate into the cell nucleus, binds to the promoter of cyclooxygenase, and activates its expression (27). Nuclear EGFR and HER-2 interact with specific DNA sequences designated as AT-rich sequence (ATRS) and HER-2-associated sequence (HAS), respectively (3, 18). TrkA/nerve growth factor receptor and the ligand nerve growth factor associate with chromatin (13). In addition, nuclear HER-4 and fibroblast growth factor receptor also involve in promoting gene transcription (12, 16). Shown in the GAL4 reporter system, EGFR, HER-2, rat p185/neu, and HER-4 were found to display transactivational ability (3, 10, 12, 18).

Together, these emerging evidences suggest a novel mode of growth factor signaling in which extracellular signals can be transmitted, via receptor nuclear transport, directly from the cytoplasmic membrane to their transcriptional targets in the nucleus (28, 29). This direct route of cell signaling is distinct from the classic pathway where extracellular signals are transduced into the nucleus following activation of multiple signaling cascades (30–33).

Breast cancer remains as one of the top malignancies that affect women in the United States despite the mortality rate has steadily declined since 1990 (34). It is estimated that ~1 in 8 (~13.3%) women in the United States will develop breast cancer during her lifetime. Eradication of breast cancer will require prevention, early diagnosis, and effective treatment that are developed based on a good understanding of the biology of breast tumors. As EGFR deregulation occurs frequently in human breast tumors, extensive research efforts have been focused on finding a correlation between EGFR expression and clinical outcome. However, inconsistent findings have been reported suggesting that the biological complexity of the EGFR pathway might have been underestimated (35).

A consistent inverse correlation exists between EGFR expression and patient survival in several human cancers, such as those of head and neck, ovary, cervix, and bladder (35, 36). Such correlation, however, has not been consistently reported in breast cancer patients (35, 37–42). In patients with non–small cell lung cancer (NSCLC), EGFR expression rarely correlates with survival (35, 43, 44). Interestingly, recent reports indicate that activating mutations occurring in the kinase region of EGFR serve as a hallmark for NSCLC patients who respond to Iressa, an anti-EGFR agent (45, 46). Unlike the wild-type EGFR, the mutant form was found to selectively
activate the downstream antiapoptotic pathways (i.e., those of the Akt and signal transducer and activator of transcription-5 but not the extracellular signal-regulated kinase–mediated proliferative pathway; ref. 47). In NSCLC patients, phosphorylated EGFR has been shown to be a better prognostic indicator than total EGFR (44). Additionally, in tumors of stomach, uterus, and colon, EGFR is considered as a weak prognostic indicator (35). Collectively, our current understanding of EGFR pathway in its association with the malignant biology and therapeutic response, particularly in breast cancer and NSCLC, seems to be incomplete. In particular, the role of EGFR in the prediction of clinical outcome in breast cancer patients remains elusive.

Given the potential involvement of nuclear EGFR in promoting tumor growth (3–7), we rationalized that nuclear EGFR may play a negative role in patient survival. Emerging evidences also suggest that the subcellular localization of cellular proteins plays a critical pathological role in predicting clinical outcome, such as p21WAF1, p27Kip1, FoxO3a, and nuclear factor-κB (48–52). The findings in this study describe, for the first time, a significant inverse correlation between nuclear levels of EGFR in tumors and overall survival rate in patients with breast cancer. Our correlative data suggest that the observed inverse relationship may, in part, be attributed to increased proliferative potential in tumors with high EGFR in their nucleus. Together, the current study describes a novel prognostic value for nuclear EGFR and sheds light into the clinical behavior of breast cancer patients who express increased EGFR in tumor nuclei.

Materials and Methods

Cell Lines, Cell Culture, and Cell Fractionation. MDA-MB-468 human breast carcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and in DMEM with 10% FCS. Cellular fractionation was performed as described previously (33). Briefly, cells treated with 100 ng/ml EGF (Sigma, St. Louis, MO) were collected, washed with PBS, and swelled in hypotonic buffer [25 mmol/l Tris-HCl (pH 7.5), 5 mmol/l KCl, 0.5 mmol/l DTT, 1 mmol/l phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin] for 20 minutes on ice. Following homogenization using a Dounce homogenizer, nuclei were pelleted and washed. The supernatants were also collected, debris was removed by centrifugation, sonicated, and the resulting supernatants collected as cytosolic lysates. Nuclear proteins were then extracted from the isolated nuclei by an extraction buffer containing 50 mmol/l Tris-HCl (pH 7.5), 1 mmol/l DTT, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.15 units/ml aprotinin. The supernatant was centrifuged at 15,000 g for 15 minutes at 4°C. The resulting supernatant was collected as the nuclear extract.

Antibodies used in the immunohistochemical studies included EGFR antibodies from Santa Cruz Biotechnology (sc-03, rabbit polyclonal antibody, 1:150, COOH terminus, Santa Cruz, CA), Zymed (S157G, ready-to-use, mouse monoclonal antibody, NH2 terminus, South San Francisco, CA), Novocastra (RTU-EGFR-384, ready-to-use, mouse monoclonal antibody, COOH terminus, Newcastle upon Tyne, UK), DAKO Corp. (H11, ready-to-use, mouse monoclonal antibody, NH2 terminus, Carpinteria, CA), and Neomarkers (Ab-3, mouse monoclonal antibody, 1:200, NH2 terminus, Fremont, CA), CD44 and proliferating cell nuclear antigen antibodies were purchased from Neomarkers (Ab-3, 1:100) and Novoceastra (1:100), respectively. In the peptide competition studies, EGF-specific and nonspecific antibodies (Santa Cruz Biotechnology) were used before the incubation with EGFR antibody as described previously (3).

Patients and Tumor Specimens. The cohort of primary breast carcinoma specimens, stained previously for cyclin D1, was consisted of 130 cases (55). These archived blocks containing formalin-fixed, paraffin-embedded infiltrating breast carcinomas were obtained from the Department of Pathology, Shanghai East Breast Disease Hospital (Shanghai, People's Republic of China). Patients were women with nonmetastatic disease following mastectomy and axillary lymph node dissection between 1988 and 1994. Stage assessment followed the tumor-node-metastasis clinical staging system of the American Joint Committee on Cancer. Patient follow-up was done for 4 to 72 months with a median follow-up of 48 months. The pathological characteristics of this cohort were described previously (49). The second cohort consisted of 37 primary oral squamous carcinoma specimens was described previously (36).

Immunohistochemical Analyses. The immunoperoxidase staining method used in these studies was a modification of the avidin-biotin complex technique as described previously (36, 55, 56). The modifications from the standard method were incorporated to ensure high sensitivity and specificity. Tissue sections (5 μm) were deparaffinized, dehydrated, and subjected to antigen retrieval using microwave oven (2 minutes at 1,000 W and 6 minutes at 200 W) followed by incubation with 0.05% trypsin in PBS for 15 minutes at room temperature. The endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide and the slides were then treated with 10% normal goat or horse serum for 30 minutes. Incubation with primary antibodies was performed at 4°C overnight. Following washes with PBS, the slides were incubated with biotinylated secondary antibodies and incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Detection was performed with the 0.125% aminoethylcarbazole chromogen substrate solution (Sigma). After counterstaining with Mayer's hematoxylin (Sigma), the slides were mounted. In the study for the correlation of nuclear EGFR and Ki-67 expression, a monoclonal antibody (Zymed) was used to detect the level of Ki-67 in a total of 27 cases consisted of three groups of tumor specimens. To ensure absolute objectivity of these immunohistochemical studies, these experiments were conducted in a double-blind manner in which two experienced pathologists (W.X. and Y.W.) stained and evaluated primary tumor sections independently. The slides were independently viewed and scored by two pathologists. Slides in which there was a scoring discrepancy >10% were re-evaluated and reconciled on a two-headed microscope.

Histologic Scoring. The immunoreactivity of EGFR in the nucleus was ranked into four groups according to the percentage of the positive tumor cells: high (+++, >35%), medium (+++, 18-35%), low (+, 1-17%), and negative (−, 0%). For the non-nuclear EGFR, tumors were grouped into four groups: high (+++, >30%), medium (+++, 26-50%), low (+, 1-25%), and negative (−, 0%). In addition, the immunoreactivity for non-nuclear EGFR was semiquantitatively scored using a well-established immunoreactivity score system in which immunoreactivity score was generated by incorporating both the percentage of positive tumor cells and the intensity of staining (57, 58). We found the results derived from both systems (percentage of positive tumor cells and the immunoreactivity score) were comparable and, therefore, the results derived from only one scoring system, the percentage of positive tumor cells, were shown.

Survival and Statistical Analyses. The correlation between the expression levels of cyclin D1 and EGFR in the immunostained tumor specimens was analyzed using the Pearson χ2 test. To correlate the levels of nuclear and non-nuclear EGFR with overall patient survival, Kaplan-Meier survival analysis and log-rank test were performed. T-Test was used in the analysis for the correlation of nuclear EGFR and Ki-67 expression/overall patient survival. All statistical analyses were done using Statistica 6.0 software (StatSoft, Inc., Tulsa, OK).

Immunofluorescent Microscopy and Confocal Analyses. Following serum starvation for 24 hours, MDA-MB-468 cells were treated without and with EGF (100 ng/ml) for 30 minutes, washed twice with ice-cold PBS, fixed in 4% paragormaldehyde for 15 minutes, and permeabilized using 0.2% Triton X-100. Following treatment with 0.1% normal goat serum for 30 minutes, cells were incubated with indicated primary antibodies (i.e., monoclonal EGFR antibodies 1:300, NH2 terminus, Zymed and 1:300, COOH terminus, Novoceastra) for 1 hour. Following washes, cells were further incubated with goat anti-mouse secondary antibody (Vector Laboratories) tagged with Texas red diluted at 1:300. To delineate the nuclear morphology, nuclear marker ToPro3 was used. Immunostained cells were examined under a Zeiss Axiosplan2 (Germany) equipped with Carl Zeiss with CSM510.
Western Blot Analyses. Nuclear fractions extracted from serum-starved MDA-MB-468 cells treated without and with 100 ng/mL EGF for 0 and 30 minutes were subjected to SDS-PAGE and Western blot analysis as described previously (27). The antibodies used for Western blot analyses included polyclonal EGFR antibody from Santa Cruz Biotechnology, monoclonal β-actin antibody from Sigma, and polyclonal histone H3A antibody from Cell Signaling (Beverly, MA).

Results

Differential Ability of Different EGFR Antibodies in Detecting Nuclear EGFR via Immunohistochemistry. As immunohistochemical analysis is a primary approach used to analyze protein levels and subcellular localization in tumor specimens, we aimed to produce a standardized immunohistochemical protocol to detect nuclear EGFR in paraffin-embedded tumor sections. To approach this, we evaluated five commercially available EGFR antibodies for their ability to detect nuclear and non-nuclear EGFR immunohistochemically. Each antibody was used to immunostain two groups of tumors, nuclear EGFR positive and negative, with three tumor specimens in each group. As summarized in Fig. 1A to C and Table 1, we found two of five tested antibodies to be effective in recognizing both non-nuclear and nuclear EGFR.

Figure 1. Differential ability of different EGFR antibodies in detecting nuclear EGFR via immunohistochemistry. A, representative breast tumor stained positive for nuclear EGFR. An example of nuclear EGFR-positive tumor was examined via immunohistochemical for EGFR using a monoclonal antibody from Novocastra (a), CD44 (b), and proliferating cell nuclear antigen (PCNA; c). Adjacent sections from the same tumor were immunostained for EGFR using a rabbit polyclonal antibody from Santa Cruz Biotechnology. d, tumor sections preincubated with a specific (e) and nonspecific (f) neutralizing peptides before incubation with the primary antibody used for d. Solid arrows, EGFR-positive nuclei; dashed arrows, EGFR-negative nuclei; clear arrow, membrane EGFR; striped arrows, cell membrane stained negative for EGFR. B, representative breast tumor stained negative for nuclear EGFR. Experiments in B were performed in a tumor example containing only non-nuclear EGFR but no nuclear EGFR. C, immunohistochemical staining using three EGFR antibodies. Two representative tumors, nuclear EGFR-positive and EGFR-negative previously shown in A and B, were immunostained for EGFR using three commercially available antibodies as indicated.
in paraffin-embedded tumor sections. Specifically, a monoclonal antibody from Novocastra (RTU-EGFR-384; Fig. 1A and B) and a polyclonal antibody from Santa Cruz Biotechnology were effective in detecting nuclear EGFR, whereas the other four antibodies from DAKO (H11), Zymed (31G7), and Neomarkers (Ab-3) only recognized the non-nuclear but not the nuclear EGFR (Fig. 1C; Table 1). The ability of the polyclonal antibody from Santa Cruz Biotechnology to detect nuclear EGFR and the inability with the DAKO (H11) antibody were also observed when we analyzed other tumor types, including oral squamous carcinomas and lung tumors (data not shown).

All five antibodies have been used and proven effective by other researchers (Table 1) and, in our immunohistochemical study, successfully recognized both cell-surface and cytoplasmic EGFR (Fig. 1A–C). In addition, adjacent tumor sections were immunostained for CD44 to mark the cell-surface and proliferating cell nuclear antigen, a nuclear protein to indicate the nucleus, and together to show the structural integrity of our tumor samples and to clearly confirm the subcellular localization of EGFR (Fig. 1A, b and c, and B, b and c). We further showed the staining specificity of our immunohistochemical procedures using a neutralizing peptide to compete for staining signals and the results are shown in Fig. 1A, d to f and B, d to f). Importantly, EGFR immunoreactivity (Fig. 1A, d, and B, d) was successfully out-competed by a specific EGFR peptide (Fig. 1A, e, and B, e) but not by a nonspecific peptide (Fig. 1A, f, and B, f).

The reason why only two of five antibodies can detect nuclear EGFR in primary tumor specimens is not yet clear. It is possible that different conformation exists between the cell-surface and nuclear EGFR, as the EGFR may exist in a non-membrane-bound form in the nucleus (3, 59). As all of the EGFR antibodies that did not detect nuclear EGFR in tumor specimens were monoclonal antibodies that recognize the NH2 terminus (DAKO, Zymed, and Neomarkers), another possibility is that these antibodies have been pre-selected during screening process to primarily recognize the membrane form of EGFR. Also possible is that, in the nucleus, EGFR forms complex with other proteins resulting in a blockage for efficient recognition by some antibodies. Additionally, both antibodies that are capable of detecting nuclear form of EGFR were raised against the COOH terminus of EGFR (Table 1). Thus, it is possible that nuclear EGFR exists in a truncated form in primary tumors that we examined despite an intact receptor was detected in cultured cells (Fig. 4) (2, 3). Such possibility will need to be exploited by extensive research.

### Table 1. Comparison of the ability of five EGFR antibodies to recognize EGFR in immunohistochemical studies

<table>
<thead>
<tr>
<th>EGFR antibody Species</th>
<th>Terminus*</th>
<th>mEGFR</th>
<th>nEGFR</th>
<th>Clinical Trials</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomarkers Mouse monoclonal antibody</td>
<td>NH2</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>J Pathol 2003;200:290</td>
</tr>
</tbody>
</table>

*Epitope that each EGFR antibody raised against.
†Ability of each EGFR antibody to recognize membrane EGFR.
‡Ability of each EGFR antibody to recognize nuclear EGFR.
§Use of EGFR antibody in staining samples from clinical trials.
||Studies used the indicated antibody for immunohistochemical analyses.
Figure 2. Correlation of high nuclear EGFR with poor patient survival in primary breast tumors. A cohort of 130 cases of human breast carcinomas was immunostained for EGFR and analyzed for the correlation between EGFR expression and overall survival. A, examples of tumors immunostained negative (0%, −), low (1-17%, +), medium (18-35%, ++), and high (>35%, ++++) levels of nuclear EGFR. Dashed arrows, EGFR-negative nuclei (blue); solid arrows, EGFR-positive nuclei (brown). Levels of EGFR were detected using the polyclonal antibody recognizing the cytoplasmic domain of EGFR. B, high nuclear EGFR correlates with poor patient survival. Kaplan-Meier survival analysis and log-rank test were performed to correlate the levels of nuclear EGFR with overall patient survival. C, lack of correlation between non-nuclear EGFR and patient survival. D, positive correlation between high nuclear EGFR and high proliferating activity. Top, representative tumor stained negative for nuclear EGFR and weakly for Ki-67. Middle, tumor stained strongly for both nuclear EGFR and Ki-67. Solid arrows, EGFR-positive and Ki-67-positive nuclei (brown); dashed arrows; negatively stained nuclei (blue). Bottom, analysis of three groups of tumors with a total of 27 tumor sections for the levels of EGFR and Ki-67. Grouping criteria are summarized in Table 3.
of nuclear EGFR in a cohort of 130 breast carcinoma specimens using immunohistochemical analyses. These primary breast carcinomas were stained previously for cyclin D1 (55) and the pathological characteristics were also described previously (49). In these studies, levels of EGFR were determined using a polyclonal antibody (Santa Cruz Biotechnology) that was determined to recognize both non-nuclear and nuclear EGFR (Fig. 1). Immunostained tumor sections were scored according to non-nuclear and nuclear EGFR by two pathologists (W.X. and Y.W.). In the analysis for nuclear EGFR, tumors were divided into four groups: negative (−, 0%), low (+, 1−17%), medium (+++, 18−35%), and high (+++++, >35%).

Figure 2A shows representative tumors for each of the four groups in which dashed arrows mark EGFR-negative nuclei (blue) and solid arrows mark EGFR-positive nuclei (brown). As indicated in Table 2, nuclear EGFR staining was detected in 38% of the tumor tissues we examined. It is worthwhile to mention that nuclear EGFR was also detected in a similar percentage of other tumor tissues, such as cancers of bladder (31%) and cervix (37%; refs. 5, 7).

**Correlation of High Nuclear EGFR with Poor Patient Survival in Breast Cancer.** Importantly, tumors with high levels of nuclear EGFR staining, in the same cohort, were found to have worse overall patient survival compared with those without detectable EGFR in the nucleus (33.4 ± 14.6 versus 47.8 ± 15.3 months; Table 2). The difference in overall survival between these two groups is statistically significant following Kaplan-Meier survival analysis and log-rank test (P = 0.009; Fig. 2B; Table 2). In contrast, non-nuclear EGFR did not correlate significantly with patient survival rate (P >0.05; Fig. 2C; Table 2). These data suggest that expression of nuclear EGFR may be of prognostic value for predicting survival in patients with breast cancer.

**Correlation of High Nuclear EGFR with Increased Ki-67 and Cyclin D1 Expression in Primary Breast Tumors.** We then rationalized that high nuclear EGFR may correlate with high proliferating potential in tumors and thus in part leads to poor survival. We therefore examined the levels of Ki-67, a biomarker for cell proliferation, in three groups of tumors with 8 to 10 samples in each group. The grouping criteria are summarized in Table 3. Representative tumors stained for EGFR and Ki-67 are shown in Fig. 2D (top). The tumor (top) was stained negative for nuclear EGFR (right) and weak for Ki-67 (left). The tumor shown in the mid-panel was stained positive for nuclear EGFR (right) and strong for Ki-67 (left). Solid arrows indicate positively stained brown nuclei, whereas dashed arrows mark negatively stained blue nuclei. Consistently, tumors with high levels of nuclear EGFR (group C) contained increased expression of Ki-67 compared with those with no nuclear EGFR (groups A and B; P <0.001; Fig. 2D, bottom; Table 3). Patients with tumors containing high EGFR and Ki-67 expression (group C) survived the worst among the three groups (33.4 ± 14.6 versus 57.0 ± 8.3 and 61.5 ± 3.2 months; P <0.001). In contrast, in the nuclear EGFR-negative tumors, no significant differences in the Ki-67 immunoactivity and overall patient survival were found between non-nuclear EGFR-negative (group A) and EGFR-positive (group B) tumors (Table 3), indicating that non-nuclear EGFR was not an important predictor for tumor growth and patient prognosis in this cohort.

As cyclin D1 is a transcriptional target of nuclear EGFR (3), we next aimed to validate such correlation in primary tumor specimens. In these studies (Table 4), we performed the Pearson χ² test to correlate levels of EGFR with cyclin D1 in which cyclin D1 expression was determined previously (55). As summarized in Table 4, we found that nuclear EGFR staining correlated positively with cyclin D1 expression (P <0.00001). In contrast, no significant

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**Table 2. Nuclear EGFR levels positively correlate with poor survival in patients with breast carcinomas**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Survival (mean ± SD), mo</th>
<th>Survival (median), mo</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−, 0%</td>
<td>47.8 ± 15.3†</td>
<td>49</td>
<td>81</td>
</tr>
<tr>
<td>+, 1−17%</td>
<td>47.9 ± 16.0</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>++, 18−35%</td>
<td>45.3 ± 20.7</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>++++, &gt;35%</td>
<td>33.4 ± 14.6†</td>
<td>30</td>
<td>9</td>
</tr>
<tr>
<td>Non-nuclear EGFR</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>−, 0%</td>
<td>48.3 ± 13.4</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>+, 1−25%</td>
<td>40.1 ± 18.0</td>
<td>46</td>
<td>31</td>
</tr>
<tr>
<td>++, 26−50%</td>
<td>50.2 ± 16.1</td>
<td>55</td>
<td>32</td>
</tr>
<tr>
<td>++++, &gt;50%</td>
<td>48.1 ± 16.8</td>
<td>50</td>
<td>25</td>
</tr>
</tbody>
</table>

*The percentage of entire cohort.
†P = 0.009, statistically significant.

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**Table 3. Positive correlation between high nuclear EGFR levels and high Ki-67 in human breast carcinomas**

<table>
<thead>
<tr>
<th>Group</th>
<th>EGFR status</th>
<th>Total N</th>
<th>Ki-67 (mean ± SD), %</th>
<th>Survival (mean ± SD), mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Non-nuclear EGFR negative, nuclear EGFR negative</td>
<td>8</td>
<td>4.7 ± 4.3²,†</td>
<td>57.0 ± 8.3⁵</td>
</tr>
<tr>
<td>B</td>
<td>Non-nuclear EGFR positive, nuclear EGFR negative</td>
<td>10</td>
<td>7.4 ± 8.3¹,‡</td>
<td>61.5 ± 3.2¹</td>
</tr>
<tr>
<td>C</td>
<td>Non-nuclear EGFR positive, high nuclear EGFR</td>
<td>9</td>
<td>31.2 ± 13.5³,²</td>
<td>33.4 ± 14.6⁴,†</td>
</tr>
</tbody>
</table>

*P < 0.001, statistically significant.
†P = 0.42.
‡P < 0.001, statistically significant.
correlation ($P = 0.36$) was found between non-nuclear EGFR and cyclin D1 in this cohort. This is consistent with the lack of correlation between levels of non-nuclear EGFR and patient survival (Fig. 2C; Table 2). Together, these data indicate a positive correlation between expression of nuclear EGFR and two markers for cell proliferation, Ki-67 and cyclin D1, in primary breast carcinomas. Importantly, these results validate our in vitro observation that expression of cyclin D1 is up-regulated by the nuclear EGFR pathway (3).

**Nuclear EGFR Detected in Oral Cancer.** To further demonstrate that nuclear EGFR can also be detected in other primary tumors using our standardized immunohistochemical protocol, we analyzed 37 cases of human oral squamous carcinomas. A representative nuclear EGFR-positive tumor is shown in Fig. 3A and a nuclear EGFR-negative tumor is shown in Fig. 3B. Solid arrows marked the nuclei stained positive for EGFR, whereas dashed arrows marked those stained negative. We found 9 of 37 (24.3%) tumor specimens contained nuclear EGFR in >5% of the tumor cells. Patients with high nuclear EGFR expression in their tumor specimens contained nuclear EGFR in >5% of the tumors using our standardized immunohistochemical protocol, strongly suggesting that nuclear EGFR can also be detected in other primary tumors including HER-2, ErbB3, and fibroblast growth factor receptor 3, 11, 18, 61). These observations together suggest that, in paraffin-embedded tumors, nuclear EGFR may

<table>
<thead>
<tr>
<th>EGFR</th>
<th>Cyclin D1, n (%)</th>
<th>Total N</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Negative, low</td>
<td>Medium, high</td>
<td></td>
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<tr>
<td>Nuclear</td>
<td>90 (69.2)</td>
<td>21 (16.2)</td>
<td>111</td>
</tr>
<tr>
<td>Medium</td>
<td>6 (4.6)</td>
<td>13 (10)</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>34</td>
<td>130</td>
</tr>
<tr>
<td>Non-nuclear</td>
<td>61 (46.9)</td>
<td>12 (9.2)</td>
<td>73</td>
</tr>
<tr>
<td>Medium, high</td>
<td>44 (33.8)</td>
<td>13 (10)</td>
<td>57</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>18</td>
<td>130</td>
</tr>
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*The percentage of entire cohort  
†Statistically significant

Table 4. Nuclear EGFR levels positively correlate with cyclin D1 in human breast carcinomas: a $\chi^2$ analysis

immunofluorescent microscopy/confocal analyses (Fig. 4A). In these studies, we used two EGFR antibodies that recognize the NH$_2$ and COOH terminus of EGFR, Zymed, and Novocastra, respectively. As indicated in Fig. 4A, the Zymed antibody against the NH$_2$ terminus of EGFR immunostained both membrane-bound and nuclear EGFR. Nuclear EGFR is indicated by pink signals (solid white arrows) representing merge of EGFR (red) and DNA (blue), whereas the non-nuclear EGFR remains as red (dashed white arrows). Similarly, the Novocastra antibody against the COOH terminus also recognized nuclear EGFR as indicated in Fig. 4B. These results indicate that both NH$_2$ and COOH termini of EGFR can be detected in the nuclei of the cultured tumor cells, suggesting the nuclear existence of intact EGFR but not a truncated NH$_2$-terminal form. This notion is further supported by the results from Western blot analysis (Fig. 4C), indicating the molecular weight of both nuclear (left) and cytoplasmic (right) EGFR as 170 kDa, which corresponds to the full-length protein. The efficiency of cell fractionation was indicated by a lack of the cytoplasmic protein, $\beta$-actin, in nuclear fraction (left) as well as absence of the nuclear protein histone H3A in the cytosolic fraction (right; Fig. 4C).

Moreover, we found that all five antibodies recognized both non-nuclear and nuclear EGFR in EGF-treated human breast carcinoma MDA-MB-468 cells immunocytochemically (data not shown). Thus, EGFR has been consistently detected as an intact molecule in the nucleus of cultured cells and so have other membrane receptors including HER-2, ErbB3, and fibroblast growth factor receptor 3, 11, 18, 61). These observations together suggest that, in paraffin-embedded tumors, nuclear EGFR may

\[
\text{A. Nuclear EGFR-positive primary oral squamous carcinomas}
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\[
\text{B. Nuclear EGFR-negative primary oral squamous carcinomas}
\]
exist in a complex conformation that cannot be recognized by some antibodies that can effectively recognized nuclear EGFR in cultured tumor cells.

**Discussion**

The current study reports several key findings that describe a novel prognostic value for nuclear EGFR contributing to a better understanding of the pathological nature of tumors with increased nuclear EGFR. First, we established an immunohistochemical protocol that can be of future use to detect EGFR localized on the cell-surface and within the nucleus of paraffin-embedded tumor sections. Secondly, we provided evidences showing that expression of nuclear EGFR may serve as a prognostic indicator for poor survival in breast cancer patients. Thirdly, we found significant coexpression of nuclear counterpart of EGFR and two molecules indicative of active cell proliferation (i.e., Ki-67 and cyclin D1). Lastly, nuclear EGFR can be also detected in primary oral squamous carcinomas.

Consistently, previous studies showed a casual correlation of nuclear accumulation of EGFR/rat erb1 and their ligands, EGF and transforming growth factor-α, with liver regeneration, cell proliferation, and DNA synthesis (1–3, 8, 26). While our previous work (3) reported *in vivo* and *in vitro* association of nuclear EGFR and *cyclin D1* promoter, the current study further shows the importance of nuclear EGFR in *cyclin D1* gene expression. In support of these observations, our correlative data showed coexpression of EGFR and Ki-67 in the tumor nuclei. Interestingly,

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**Figure 4.** Nuclear accumulation of intact EGFR in MDA-MB-468 cells following EGF stimulation. MDA-MB-468 cells were serum starved for 24 hours, treated without and with EGF (100 ng/mL) for 30 minutes, and subjected to immunofluorescent/confocal and Western blot analyses. A and B, intact EGFR accumulates in cell nucleus following EGF treatment. Tumors cells without (-EGF) and with EGF (+EGF) were washed, fixed, permeabilized, and blocked with 0.1% normal goat serum for 30 minutes. Cells were incubated with primary antibodies: monoclonal EGFR antibodies from Zymed (NH2 terminus) in A and Novocastra (COOH terminus) in B. Immunostained cells were examined under a Zeiss Axioplan2 equipped with Carl Zeiss with CSM510. Red, EGFR; blue, nuclei are stained by ToPro3. Arrows, nuclear EGFR that is indicated by *pink signals* resulted from the merge of EGFR (red) and ToPro3 (blue). Dashed arrows, cell-surface EGFR. C, full-length 170-kDa EGFR detected in the cell nucleus. Nuclear (left) and cytosolic (right) lysates extracted from MDA-MB-468 cells treated with EGF for 0 and 30 minutes were subjected to SDS-PAGE and Western blot analysis. Expression levels and approximate molecular weight of EGFR, following comparison with size markers, were determined. As loading controls for nuclear and cytosolic lysates, expression of histone H3A and β-actin was simultaneously determined.
nuclear accumulation of fibroblast growth factor receptor is also associated with increased cell proliferation (17, 61) despite that the underlying molecular and cellular mechanisms remain unclear. Consistent with the lack of correlation between levels of non-nuclear EGFR and cyclin D1, no significant correlation was found between non-nuclear EGFR and patient survival. Taken together, increased expression of cyclin D1, a transcriptional target of nuclear EGFR, may in part contribute to the poor survival rate observed in patients with high nuclear EGFR in their breast tumors.

We detected nuclear EGFR staining in 38% and 24% of the breast and oral cancer tissues, respectively. Nuclear EGFR was also detected in a similar percentage of other tumor tissues, such as cancers of bladder (31%) and cervix (37%), although no significant clinical correlation was reported yet (5, 7). In comparison of the findings from our and other reports, we report 67% of the 130 breast carcinomas with EGFR expression and only 23% with high levels (+++). These percentages are consistent with other reports. For example, the review article by Klijn et al. (42) summarized the findings from a total of 57 studies with 5,232 patients and found EGFR positivity in 15% to 91% of breast tumors investigated. Overexpression of EGFR has been reported in ~15% to 40% of breast cancers (37, 41, 62). In the current study, we observed 23% of the cohort expressing high levels of EGFR, which falls within the reported range.

The immunohistochemical method that we established and used to detect nuclear EGFR in paraffin-embedded tumor sections contained significant modifications from the standard method (56) to ensure high sensitivity and specificity as described in Materials and Methods. For example, the antigen retrieval protocol using heat/microwave was established following extensive optimization for both power and duration time. Trypsin, a mild protease, was used for digestion rather than proteinase K, a more potent protease, to ensure that the cellular proteins were not over-digested. Moreover, the incubation with the primary antibody was carried out overnight at 4°C rather than the conventional 1 to 3 hours at room temperature to facilitate sufficient epitope-antibody interaction. Importantly, this method has been found to be effective and proven to be reliable (3, 36, 51, 55). To ensure absolute objectivity of these immunohistochemical studies, we conducted double-blind experiments in which two experienced pathologists (W.X. and Y.W.) stained and evaluated primary tumor sections independently. To note, all five antibodies have been used extensively by other groups and, in many cases, used for digestion rather than proteinase K, a more potent protease, to ensure that the cellular proteins were not over-digested.

Moreover, the immunohistochemical studies that we established and used to detect nuclear EGFR in paraffin-embedded tumor sections contained significant modifications from the standard method (56) to ensure high sensitivity and specificity as described in Materials and Methods. For example, the antigen retrieval protocol using heat/microwave was established following extensive optimization for both power and duration time. Trypsin, a mild protease, was used for digestion rather than proteinase K, a more potent protease, to ensure that the cellular proteins were not over-digested. Moreover, the incubation with the primary antibody was carried out overnight at 4°C rather than the conventional 1 to 3 hours at room temperature to facilitate sufficient epitope-antibody interaction. Importantly, this method has been found to be effective and proven to be reliable (3, 36, 51, 55). To ensure absolute objectivity of these immunohistochemical studies, we conducted double-blind experiments in which two experienced pathologists (W.X. and Y.W.) stained and evaluated primary tumor sections independently. To note, all five antibodies have been used extensively by other groups and, in many cases, used for immunostaining clinical specimens from clinical trials (Table 1).

To note, the two EGFR antibodies from DAKO and Zymed failed to detect nuclear EGFR in tumors known to expression high levels of nuclear EGFR despite their frequent use in immunostaining EGFR in clinical samples. This may, in part, provide an explanation for the fact that only a limited number of immunohistochemical studies reported the detection of nuclear EGFR in primary tumor specimens. However, it remains unclear as why only two of five antibodies can detect nuclear EGFR in primary tumor specimens. Possibly, different conformation exists between membrane-bound cell-surface EGFR and nuclear EGFR (3). Also possible is that, in the nucleus, EGFR may be in complex with other proteins resulting in epitope blockade, which prevents efficient antibody recognition. Additionally, both antibodies that are capable of detecting nuclear form of EGFR were raised against the COOH terminus of EGFR (Table 1). Thus, it is not impossible that nuclear EGFR exists in a truncated form in primary tumors that we examined despite an intact receptor was detected in cultured cells and EGFR can be detected by both NH2- and COOH-terminal antibodies (Fig. 4; refs. 2, 3). All these possibilities will require extensive research to be exploited.

Moreover, the EGFR antibody used in the previous studies by Kamio et al. (4) and Lipponen and Eskelinen (5), both reported nuclear EGFR in primary tumors, was a monoclonal antibody obtained from Cambridge Research Biochemicals (Valleystream, NY). This antibody, however, is no longer commercially available. Another study by Marti et al. (6), also reported nuclear detection of EGFR in primary tumor specimens, used a mixture of monoclonal antibodies (291-3a and 291-4a), which are not available commercially. Therefore, the specific information provided by this study regarding the selection of EGFR antibodies for nuclear staining of primary tumors will be of particular importance to researchers who are interested in correlating nuclear EGFR with therapeutic outcome.

According to a review article by Klijn et al. (42), summarizing the findings from 57 studies with a total of 5,232 patients, only 11 of 57 studies performed correlation analysis between EGFR levels and relapse-free survival in which only 55% (6 of 11) of these studies found an inverse correlation. The numbers of samples among these studies ranged from 55 to 376 per study. Our sample size, 130 cases, seems to fall within the reasonable range. Interestingly, in patients with cancers of breast, stomach, uterus, and colon, EGFR expression is a moderate predictive factor for survival, as only 52% (13 of 25) of studies showed a significant correlation (35). Specifically, in patients with breast cancer, the focus of our study, a lack of correlation has been reported by several recent studies, including those by Ferrero et al. (63), Tolgay Ocal et al. (39), Seshadri et al. (41), and Bieche et al. (40).

To further examine whether nuclear EGFR also exist in other tumor specimens, we examined 37 cases, a small cohort, of oral squamous carcinoma samples and surprisingly found a correlation between high EGFR and poor survival. Future investigation using a larger number of samples will be necessary to draw a firm conclusion. Nevertheless, the value of nuclear EGFR in survival prediction may be important not only for malignancies of breast but also for head and neck, NSCLC, and tumors of stomach, uterus, and colon, as EGFR is considered as a weak prognostic indicator for these cancers (35). In patients with NSCLC, total EGFR expression rarely correlates with survival (35, 43, 44), whereas the phosphorylated EGFR has been shown to be a better prognostic indicator (44). Expression of the activating kinase mutants serves as an indicator for NSCLC responsiveness to EGFR kinase inhibitor Iressa but not to other agents that target other molecules/pathways (45–47).

The findings reported in this study raised a critical issue to pay attention to subcellular localization of EGFR and its contribution to clinical significance. Emerging evidences have suggested a critical pathological and clinical importance to examine cellular proteins for not only their expression level but also the subcellular localization (i.e., cytoplasm versus nucleoplasm). For example, p21<sup>Cip1/WAF1</sup>, a negative cell cycle regulator localized primarily in the cell nucleus, can exist in the cytoplasm following phosphorylation by Akt (64). The nuclear p21<sup>Cip1/WAF1</sup> elicits growth-inhibitory effect whereas the cytoplasmic counterpart plays a role in apoptotic protection (65–67). As p21<sup>Cip1/WAF1</sup> expression level has not provided a consistent predictive role in survival of breast cancer patients, several recent reports showed that the cytoplasmic Akt-phosphorylated
p21Cip1/WAF1 correlates with poor clinical outcome (48, 49). Similarly, another Akt substrate and a cell cycle inhibitor p27Kip1 was found to retain in the cytosol following Akt phosphorylation and such cytoplasmic localization correlates with poor survival in breast cancer patients (50, 68–70).

The forkhead transcription factor FOXO3a is another example showing the pathological significance for protein subcellular localization (51). FOXO3a, a transcription factor that can induce cancer patients (50, 68–70).

This current study raised a critical issue, namely, the pathological significance of nuclear EGFR. Because many other cell-surface receptors, including receptor tyrosine kinases and cytokine receptors, have also been reported to be detected in the nucleus of tumor cells (8–17, 20, 22, 24, 25, 27, 73), our findings here establish an alert to evaluate the pathological significance for nuclear accumulation of these receptors.

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References


Prognostic Value of Nuclear EGFR

In the article on the prognostic value of nuclear EGFR in the January 1, 2005, issue of Cancer Research (1), the affiliation for Dr. Shiu-Feng Huang is incorrect. The affiliation should have read Division of Molecular and Genomic Medicine, National Health Research Institutes, 128 Yen-Chiu-Yuan Road, sec. 2, Taipei, 115, Taiwan.

Novel Prognostic Value of Nuclear Epidermal Growth Factor Receptor in Breast Cancer

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