Prognostic Value of an Activation State Marker for Epidermal Growth Factor Receptor in Tissue Microarrays of Head and Neck Cancer

Anthony Kong,1,2 Pierre Leboucher,4 Russell Leek,3 Véronique Calleja,1 Stuart Winter,3 Adrian Harris, Peter J. Parker,5 and Banafshe Larijani1

1 Cell Biophysics Lab, 2 Protein Phosphorylation Lab, London Research Institute, Cancer Research UK, London, United Kingdom; 3 Weatherall Institute of Molecular Medicine, Cancer Research UK, University of Oxford, John Radcliffe Hospital, Headley Way, Oxford, United Kingdom; and 4 Laboratoire de Physiologie de la Perception et de l’Action, College de France, Paris, France

Abstract

Overexpression and mutation of epidermal growth factor receptors (EGFR) have been shown to be important in the prognosis of several cancers, including head and neck cancers. However, our inability to define the activation status of these receptors limits our ability to assess the importance of these pathways and to exploit effectively new molecularly targeted treatments directed at their catalytic activities. Here we describe the use of automated, high-throughput fluorescence lifetime imaging microscopy to measure EGFR auto-phosphorylation status by fluorescence resonance energy transfer (FRET) in head and neck tumors. We have correlated FRET efficiency with the clinical and survival data. The results from head and neck arrays show that high FRET efficiency is correlated with worsening disease-free survival but not with overall survival. This powerful tool could be exploited as a new independent prognostic tool in clinical decisions and cancer management. (Cancer Res 2006; 66(5): 2834-43)

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world and represents 6% of all cancers (1, 2). It comprises a heterogeneous group of tumors in terms of its etiology and molecular mechanisms (3). Traditionally, the management of this cancer represents a combination of surgery, radiotherapy, and chemotherapy (4). However, molecular therapy is gaining importance in this group of cancers. Elevated levels of transforming growth factor α (TGF-α) and epidermal growth factor receptor (EGFR) mRNAs have been detected in the normal mucosa samples of head and neck cancer patients, and hence implicated in the pathogenesis of head and neck cancer (5), and have been shown to be significant predictors of disease-free survival in these patients (6). In addition, several studies have also shown correlation of overexpression of EGFR with overall survival, disease-free survival, increased locoregional recurrence, and decreased sensitivity to radiation treatment in these patients (7–10). To assess EGFR overexpression, its expression determined by immunohistochemistry has been the standard. However, not all studies have found a correlation of overexpression of the EGFR and prognosis (11, 12). Furthermore, the EGFR level does not necessarily predict the response to molecular therapy (13). Part of the variation may derive from the fact that immunohistochemistry does not provide a truly quantitative analysis of EGFR expression. The results tend to vary between the laboratories because of the lack of a standardized scoring system and the subjective process of the interpretation of stained samples (14). Moreover, fixatives used and the storage time of unstained tissue sections are the cause of variations (15). In a recent article on molecular classification of HNSCC using patterns of gene expression, it was found that the poorest outcome group was the group with tumors characterized by the high expression of TGF-α with evidence of activation of EGFR pathway (16). In addition, another study has found that it is the overexpression of multiple receptors, mainly EGFR1 (ErbB1), with other ErbB receptors (ErbB2-4) that correlates more with metastatic disease (17). Based on our understanding of the behavior of these receptors, these studies indicate that not only is the actual level of EGFR expression important, but more so the activation/phosphorylation state of these receptors.

The current methods of measuring EGFR levels, including immunohistochemistry, cannot be unequivocally endorsed as predictive of patient prognosis or response to treatment and they are also unable to detect the functional status of the EGFR pathway (18). Therefore, there is a need for a quantitative method to be set up to measure EGFR and its activation status. To achieve this, we have exploited fluorescence resonance energy transfer (FRET) monitored by fluorescence lifetime imaging microscopy (FLIM) as a molecular prognostic tool to identify HNSCC patients who show overexpression and/or phosphorylation of the EGFR. Using A431 cells as a test bed, we have evaluated the use of a suitable pair of donor (Cy3b) and acceptor fluorophores (Cy5) conjugated to F4 (EGFR cytoplasmic domain antibody) and FB2 (antiphosphotyrosine), respectively, and monitored FRET between the fluorophores detected by FLIM on EGFR stimulation. We have illustrated that the FRET associated with the coincident binding of the labeled monoclonals is specific. These validated reagents have been applied to head and neck tumor arrays using for the first time a high-throughput automated FLIM. Employing this highly selective FRET assay in tumor arrays, we show that increased FRET can be correlated with disease recurrence and prognosis of the patients. Moreover, we have investigated whether the EGFR status determined by immunohistochemistry or by average fluorescence intensity is also correlated with activation of EGFR.

Materials and Methods

Patients. The work was carried out with the approval of the Oxford Ethics Committee. One hundred thirty consecutive cases of HNSCC from the Ear Nose Throat Department, Radcliffe Infirmary (Oxford, United...
FRET Efficiency as an EGFR Activation Reporter in HNSCC

**Materials and cell lines.** A431 cells were obtained from cell services at Cancer Research UK London Research Institute. F4 immunoglobulin G1 (IgG1) mouse antibody, residues 985-996 (monoclonal against EGFR cytoplasmic domain), and FB2 IgG3 (monoclonal against phosphotyrosine) antibodies were obtained from the Monoclonal Antibody Lab, London Research Institute, Cancer Research UK. The secondary sheep anti-mouse IgG was purchased from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, United Kingdom). AG 1478, a selective inhibitor of the EGFR tyrosine kinase (IC$_{50}$ = 3 nmol/L), was from Calbiochem (Nottingham, United Kingdom). The monoclonal fluorophores Cy3B and Cy5 were from Amersham Biosciences. Protein tyrosine phosphatase from *Verrisia enterocolitica* (recombinant, *E. coli*) was purchased from Calbiochem.

**Western blotting.** A431 cells were grown to 80% confluency in six 10-cm cell culture plates. Four of the six plates of cells were treated with different doses of AG 1478 (0.3, 0.6, 1.5, and 3 mmol/L) for 2 hours. All six plates apart from one were then stimulated with EGF (50 ng/mL) for 10 minutes after being serum starved for 16 hours. The cells from all the plates were then lysed in lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 100 mmol/L NaF, 10 mmol/L Na$_3$P$_2$O$_7$, 10 mmol/L EDTA with 1% Triton and protease inhibitor cocktail; Roche) and centrifuged at 4°C with the removal of the cell pellets. PAGE was carried out employing 10 μg of protein in each lane. Western blots were done using F4 and FB2 antibodies. A dilution of 1:15,000 of F4 (stock concentration 1.7 mg/mL) and a dilution of 1:500 of FB2 (stock concentration of 1.3 mg/mL) were used to probe the cell lysates. Antibodies were incubated overnight at 4°C. They were detected using horseradish peroxidase–linked secondary antibody (a dilution of 1:5000 sheep anti-mouse IgG) and visualized with an enhanced chemiluminescent system (Amersham).

**Fluorescence resonance energy transfer by fluorescence lifetime imaging microscopy.** FRET involves the transfer of energy from an excited donor molecule to a nearby (<7 nm) spectrally overlapping fluorophore. 100 μL of 0.2% (v/v) Triton X-100 were added in the well for 5 minutes to make the cell membrane permeable followed by 1 mg/mL fresh sodium borohydrate/PBS for 10 minutes to quench background fluorescence. The cells were then blocked with 1% w/v bovine serum albumin (BSA)/PBS for 30 minutes. To label the cells with conjugated F4-Cy3b, we incubated the cells with 10 μg/mL F4-Cy3b/1% BSA/PBS for 1 hour. For cells that required detection with the acceptor fluorophore, we incubated them further with conjugated phospho-EGFR antibody, FB2-Cy5, for 1 hour. The coverslips were mounted on the slide with Mowiol mounting medium containing 2.5% (w/v) 1,4-diazabicyclo (2.2.2) octane as an antifade. The slides were left at 37°C for 1 hour and then at room temperature overnight before image acquisition.

For FRET experiments, all images were taken using a Zeiss Plan-Apochromat ×100/1.4 NA phase 3-oil objective with images recorded at a modulation frequency of 80.218 MHz. The donor (F4-Cy3b) was excited using 514-nm line of an argon/krypton laser and the resultant fluorescence was separated from free dyes by column chromatography. The dye/protein ratios were maintained constant per experiment. The dye/protein ratios were 1.6 for F4-Cy3b and 2.3 for FB2-Cy5. The dye/protein ratios were measured by UV/Visible spectroscopy at 280 nm to determine F4 and FB2 concentrations. The concentrations of F4-Cy3b and FB2-Cy5 were detected at 516, 552, and 650 nm, respectively. The dye/protein (D/P) ratios were calculated using the protocol provided by Amersham Biosciences for Cy3b monoreactive dye:

\[
D/P = \frac{[\text{absorption } A_{\text{max}}] \times (\text{antibody extinction coefficient})}{[(A_{\text{dyn}} - \text{correction factor } \times A_{\text{max}}) \times \text{(Cy dye extinction coefficient})].}
\]

**FRET Experiments on A431 cells.** A431 cells were grown in 24-well plates with coverslips after seeding ~15,000 per well. The cells were then stimulated with EGF 50 ng/mL for 10 minutes following serum starvation for 16 hours. Following stimulation, the cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. Five hundred microliters of 0.2% (v/v) Triton X-100 were added in the well for 5 minutes to make the cell membrane permeable followed by 1 mg/mL fresh sodium borohydrate/PBS for 10 minutes to quench background fluorescence. The cells were then blocked with 1% w/v bovine serum albumin (BSA)/PBS for 30 minutes. To label the cells with conjugated F4-Cy3b, we incubated the cells with 10 μg/mL F4-Cy3b/1% BSA/PBS for 1 hour. For cells that required detection with the acceptor fluorophore, we incubated them further with conjugated phospho-EGFR antibody, FB2-Cy5, for 1 hour. The coverslips were mounted on the slide with Mowiol mounting medium containing 2.5% (w/v) 1,4-diazabicyclo (2.2.2) octane as an antifade. The slides were left at 37°C for 1 hour and then at room temperature overnight before image acquisition.

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**Tissue microarrays—lifetime and average intensity measurements.** Tissue arrays were prepared from formalin-fixed, paraffin-embedded tumor blocks derived by surgical resection from the Pathology Department, John Radcliffe Hospital (Oxford, United Kingdom). Quality control for tumor specimens has been undertaken. All slides were examined by a specialist consultant pathologist. It is not possible for each core to be uniformly involved by the tumor and naturally there is variation between the individual cores with the percentage of the tumor. However, it was not thought appropriate to correct for the percentage of the area occupied by the tumor because EGFR was only expressed in the tumor and not in stromal cells by our immunohistochemistry studies and the analysis undertaken here measures the intensity of activation of the receptor. Serial sections adjacent to those used in the FRET analysis were stained with H&E to confirm the presence of tumor in the cores. The t Toniol sections are provided as a reference point for slice orientation and contain heterogeneous tissues including squamous, lymphoid, and vascular tissues as well as supporting stroma. Sections which contained squamous tissue were analyzed in the study. Cores of 500 μm were taken and transferred to an
8 × 15 core recipient array block using a Beecher Instruments Manual Tissue Arrayer-1 (Beecher Instruments, Inc., Sun Prairie, WI). Five-micrometer sections were cut from each array block and dewaxed in xylene. The sections were then rehydrated through graded alcohols to water.

For antigen retrieval process, 0.01 mol/L citrate buffer (pH 6) was heated in an 800-W microwave oven at full power for 4 minutes. The sections were then placed in the buffer and heated in the microwave for a further 4 minutes at full power. The citrate buffer was then topped up and microwave heated again for 4 minutes. The citrate solution and sections were then allowed to cool for 10 minutes before handling.

To label the tumor arrays with F4-Cy3b, we immersed the arrays with 400 μL of 0.2% (v/v) Triton X-100 for 5 minutes to permeabilize the membrane, followed by 1 mg/mL fresh sodium borohydride/PBS for 10 minutes to quench background fluorescence. 1% (w/v) BSA in PBS was used for blocking. The conjugated EGFR antibody F4-Cy3b, stock solution 100 μg/mL, was diluted in 1% BSA/PBS to 10 μg/mL. We incubated the tumor arrays with 400 μL of 10 μg/mL F4-Cy3b/1% BSA/PBS on the slide for 1 hour and then fixed the arrays with 4% paraformaldehyde for 10 minutes. To label the tumor arrays with the acceptor, we incubated the tumor slide with FB2-Cy5 (diluted 2-fold) for 1 hour. The arrays were then mounted on a glass cover with Mowiol mounting medium containing 2.5% (w/v) 1,4-diazabicyclo (2,2,2) octane.

Using the automated FLIM system, we mapped each tumor core according to the position on the arrays. Images from each tumor core were acquired automatically from the arrays according to their positioning. The phase, modulation, and average lifetimes of each tumor core were calculated automatically and the average FRET efficiency for each tumor core was obtained. Average FRET efficiency for each tumor core was correlated with survival data. We first carried out a pilot study to assess the first set of arrays with the automated system. To validate the pilot study, we prepared a new set of arrays with new antibodies and fluorophores and processed the arrays with automated FLIM. In the validation study, the system was programmed to run two loops so that two lifetime measurements were taken from each tumor core (n = 2; i.e., 572 tumor cores in total). However, in any one array a patient would have a duplicate sample; thus, for each patient the final lifetime represents an average of four measurements. A total of 1,114 tumor core recordings were made and analyzed in the validation study.

To obtain the EGFR status by FLIM, we calculated the donor intensity. For each tumor core, images were acquired and an intensity distribution histogram was plotted using Matlab version 7 and the median of the intensity distribution was calculated by Matlab 7. This median was normalized to the background intensity. We used the average of two median intensity values from the two tumor cores of the same patient in each array. Thereafter, these values were used to correlate with immunohistochemistry stains and average FRET efficiency.

**EGFR immunohistochemistry staining.** Five-micrometer sections were dewaxed in xylene and rehydrated through graded alcohols to water. High-temperature antigen retrieval was done in 0.01 mol/L citrate buffer (pH 6) heated for 1 minute in a microwaveable pressure cooker. The sections were then cooled and washed in water. Following peroxidase block, sections were protein blocked for 15 minutes in 2.5% normal horse serum. The primary monoclonal antibody F4 was incubated on the sections for 1 hour at room temperature at a concentration of 10 ng/mL and then washed twice in PBS. The primary antibody was visualized using a horseradish peroxidase Vector Labs ImmPRESS Mouse Kit (Vector Laboratories, Burlington, CA) and the sections were developed with diaminobenzidine. After washing in water, sections were mounted on coverslips in aqueous mounting medium.

![Figure 1](https://cancerres.aacrjournals.org/content/canres/66/3/2836.full)

**Figure 1.** Autophosphorylation of the EGFR is prevented by AG 1478 on EGF stimulation. Its activation is monitored by FRET. **A,** A431 cells were treated with 50 ng/mL of EGF for 10 minutes. Cells lysates were probed with F4 (antibody against the cytoplasmic domain of EGFR) and FB2 (EGFR auto-antiphosphorylation site monoclonal). The 170-kDa F4 immunoreactive band (EGF stimulation) was plotted using Matlab version 7 and the value of the intensity distribution was calculated by Matlab 7. This median was normalized to the background intensity. We used the average of two median intensity values from the two tumor cores of the same patient in each array. Thereafter, these values were used to correlate with immunohistochemistry stains and average FRET efficiency.

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Statistical analysis. Disease-free survival is defined as the length of time after treatment during which no cancer is found, and overall survival is a defined period of time that the subjects in a study have survived since diagnosis or treatment. All statistical analyses were done using GraphPad Prisms 3cx (Macintosh Version) except the univariate and multivariate analyses for prognostic factors which were done using “R” (see below). The Kaplan-Meier survival curves were used to compare between the groups and a log-rank test was used to assess the hazard ratios. For the univariate and multivariate analysis of the prognostic factors, we analyzed the data by fitting Cox proportional hazard models to the data with top 10% FRET efficiency as the group indicator with a common baseline hazard function, and including immunohistochemistry, Unio Internationale Contra Cancrum (UICC) tumor stage, grade, necrosis, age, and sex as covariates. To fit the models, we used the “coxph” function from the “survival” package within R. R is a language and environment for statistical computing and is available as Free Software under the terms of the Free Software Foundation’s GNU General Public License in source code form.

Results

Detection of EGFR phosphorylation in A431 cells by FRET. Traditionally immunochemistry has been used to measure the level of EGFR expression in tumors without informing on activation status. To develop an activation state readout with suitable specificity for the EGFR, we postulated that following EGFR activation, FRET would be detectable in fixed cell samples between F4-Cy3b (a monoclonal to the cytoplasmic domain of the receptor linked to a donor fluorophore, Cy3b) and FB2-Cy5 (an EGFR...
autophosphorylation site monoclonal linked to an acceptor fluorophor, Cy5). A431 was chosen as our test cell line to validate the assay because of its high EGF receptor expression and its extensive use for nearly 20 years in the analysis of EGFR function. Initially, the specificity of the antibodies F4 and FB2 was tested through Western blot of A431 cell lysates (Fig. 1A). The receptor is specifically detected by the F4 monoclonal whereas FB2 recognizes both the phosphorylated receptor and two pairs of faster migrating (phospho-) proteins. The degree of immunorecognition of the receptor by FB2 specifically increased in response to EGF as expected for this photophosphoryl site-directed monoclonal. Importantly, this immunoreactivity was reduced in a dose-dependent manner by the selective EGFR tyrosine kinase inhibitor AG 1478. It is notable that in these cells, there is a basal degree of EGFR phosphorylation consistent with prior data indicating a degree of autocrine receptor activation (21). It is also important to note that the additional recognition of faster migrating protein species by the FB2 monoclonal does not interfere with the two-site FRET assay reported here because the specificity of the analysis is determined by F4 (this is an important feature of the two-site immunohistochemistry assay; see further below).

Fixed A431 cells were employed to test whether, in an immunohistochemistry format, the coincident binding of F4-Cy3b and FB2-Cy5 to phosphorylated EGFR produced a specific FRET signal. Cells were treated with or without EGF, fixed, and processed as described in Materials and Methods. Employing this assay, it was found that following EGF stimulation, there is a marked increase in FRET as illustrated in Fig. 1B. This is a specific property of the coincident binding of the two fluorescently labeled antibodies because the lifetime change observed for the donor fluorophore following EGF treatment is not observed if the antibody-acceptor conjugate is omitted. In the absence of the EGF stimulus, there is a reduced degree of FRET but with lifetimes for the donor in the presence of the acceptor below those observed for donor alone (see Fig. 1B).

To determine whether the EGF-induced increase in FRET reflected an increase in immunorecognition of tyrosine-phosphorylated receptor by the FB2 antibody-acceptor conjugate, cells were pretreated with AG 1478. At effective doses of this EGFR inhibitor (1.5 and 3.0 μmol/L; see Fig. 1A), FRET was reduced to the basal unstimulated level (Fig. 1B). AG 1478 treatment of the MDAMB-231 cell line produced the same pattern of results as A431 cells (Supplementary Fig. S1A; Supplementary Table S1A). Dose dependency studies in A431 cells showed that at lower concentrations (0.3 and 0.6 μmol/L), FRET was not reversed (Supplementary Table S1B). Pretreating the cells with AG 1478 did not reverse the basal degree of immunoreactivity.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** Kaplan-Meier curves using average FRET efficiency as a prognostic marker illustrate that FRET efficiency is correlated with disease-free survival. **A,** pilot study: disease-free survival between patients in upper tertile versus the lower two tertiles of average FRET efficiency. Comparing the patients in the lower two tertiles with those in the upper tertile, the log-rank test revealed \( P = 0.05 \) and a hazard ratio of 0.57 (95% CI, 0.29-0.99). **B,** pilot study: disease-free survival between patients in upper 10% versus lower 90% of average FRET efficiency. Patients in the lower 90% range of average FRET efficiency had a hazard ratio of 0.43 (95% CI, 0.09-0.89; \( P = 0.03 \)) compared with the patients in the upper 10% range. **C,** validation study: disease-free survival curves comparing the upper 10% range with the lower 90% of average FRET efficiency (\( P = 0.04 \); hazard ratio, 0.48; 95% CI, 0.14-0.95). **D,** the linear regression shows no correlation between donor intensity Cy3b and average FRET efficiency \( r^2 = 0 \). In these studies, expression of EGFR is not correlated with its phosphorylation state.
degree of FRET. To verify that the signal was indeed due to phosphotyrosine recognition, we employed the protein tyrosine phosphatase from Y. enterocolitica (YOP). Pretreatment with YOP abolished both the basal activity and EGF-induced FRET (Supplementary Fig. S1B; Supplementary Table S1C). This suggests that FRET between F4-Cy3b and FB2-Cy5 in the basa state is not due to the direct autoactivation of the ErbB1 receptor (AG 1478 sensitive) but is probably mediated by heterodimerization with other activated EGFs or by other receptor-associated proteins, which are not inhibited by AG 1478.

An example of donor Cy3b intensity images and their corresponding average lifetime maps from the same FRET experiment are illustrated in Fig. 1C. The average lifetime of F4-Cy3b in these studies decreased from 1.63 to 1.50 ns when the acceptor FB2-Cy5 was present and decreased further to 1.38 ns on EGF stimulation. The changes in lifetime induced by EGF are localized mainly at the plasma membrane as seen from the pseudocolor changes of the lifetime map (Fig. 1C). This is entirely consistent with the expected increase in tyrosine phosphorylated receptor at the cell surface following a 10-minute stimulation with EGF. Concentrations of 1.5 and 3 μmol/L AG 1478 reversed the donor lifetime to 1.49 ns.

The observed ligand-induced FRET, its inhibitor-dependent reduction, and the defined plasma membrane response, coupled with the specificity of the F4 monoclonal antibody, validate the use of the F4-FB2 antibody pair to monitor EGFR expression (F4 immunoreaction) and EGFR activation (F4-FB2 coincident immunoreognition) in an immunohistochemistry format.

The activation state of EGFR in head and neck tumors. We used this validated two-site FRET assay to determine the pattern of EGFR phosphorylation in a series of HNSCC. An archive of head and neck tumor samples embedded in paraffin blocks was converted into three arrays. The arrays contained 286 tumor cores (a duplicate of a tumor core from each patient) and several cores comprised normal tonsil tissue as negative controls (Fig. 2A). These arrays were prepared for FRET experiments using the variables established in A431 cells.

A duplicate of each array was also prepared so that one array was labeled with donor alone (F4-Cy3b) and the other with donor and acceptor (F4-Cy3b + FB2-Cy5). For each tumor core, we obtained a pair of average lifetimes, one from the array with donor and acceptor (Fig. 2B). Comparing the two arrays, we were able to calculate average FRET efficiency for each tumor core (see Materials and Methods).

From the cell model, we determined that for each experimental condition the average FRET efficiency was reproducible. Therefore, average FRET efficiency was used as the main variable to correlate with the clinical data (Fig. 3). The survival data of 130 head and neck cancer patients were compared with average FRET efficiency. The average FRET efficiencies of the normal tonsil tissue samples were used as controls. The values of the negative controls ranged from 0% to 8.90% with a median average FRET efficiency of 4.86%. For the tumors, the average FRET efficiencies ranged from 0% to 14.70% and the median was 4.13%. The patients were ranked according to their average FRET efficiency and the groups split for comparison into (a) upper median versus lower median, (b) upper tertile versus lower two tertiles, and (c) upper 10% versus lower 90% range of average FRET efficiency. The Kaplan-Meier method was used to compare the survival curves between the groups of patients and the log-rank test was used to calculate the hazard ratios (see Materials and Methods). We found that higher FRET efficiency was correlated with worse disease-free survival and this was statistically significant in the groups split by upper tertile and upper 10% but not the median (Fig. 3A and B). Although high FRET efficiency was correlated with disease-free survival, it was not correlated with overall survival in any of the groups (Supplementary Fig. S2A and B).

To ensure inter assay validity, the above experiments were repeated in a new set of six tumor arrays, which contained tumor cores from the same patients. New preparations of conjugated antibodies were used and average FRET efficiencies were again calculated from each tumor core using automated high-throughput FLIM. The automated system was programmed to do multiple loops and we did two loops for each array. Thus, we acquired two measurements for each process (572 measurements were acquired). The Kaplan-Meier survival curves (Fig. 3C) were used to compare between the patients in the upper 10% FRET efficiency and those in the lower 90% efficiency and the log-rank test was used to compare the hazard ratio. The results were similar to the initial study in that the patients in the lower 90% range of average FRET efficiency had a better disease-free survival compared with the upper 10% range (Fig. 3C). However, there was no statistical significance between the upper tertile and lower two tertile groups in this validation study [hazard ratio, 0.77; 95% confidence interval (95% CI), 0.76-1.9; P = 0.35]. From these FRET efficiency studies, we deduce that phosphorylation of EGFR assessed by high-throughput FLIM correlates with disease-free survival.

Using Mann-Whitney tests, it was found that there was no evidence to suggest that average FRET efficiency was associated with grade or UICC tumor stage (Supplementary Fig. S3A and B). To address whether EGFR expression itself was correlated with its activation, we used average donor fluorescence intensity measurements (i.e., F4 immunoreactivity) and compared them with average FRET efficiency. It is important to note that average fluorescence intensity measurements of F4-Cy3b are related to the amount of receptor in each tumor core (see Materials and Methods). To test the correlation between EGFR expression and its activity, we calculated the linear regression between the average fluorescence intensity and average FRET efficiency. The analysis showed that there was no linear relationship between the two variables \( r^2 = 0.39 \) (Fig. 3D). These studies illustrated that there was minimal correlation between EGFR concentration and its activation.

In summary, we have illustrated that there is a prominent correlation between EGFR activation and disease-free survival and a lack of correlation between EGFR expression and its activation status in HNSCC cores.

Conventional immunohistochemistry does not reveal a correlation of EGFR overexpression with disease-free survival and overall survival. Previously, immunohistochemistry analysis of EGFR expression has been shown to correlate with survival, particularly disease-free survival, and hence was considered useful in predicting disease recurrence (8, 9). To assess whether in this patient cohort conventional immunohistochemistry revealed a correlation between levels of EGFR expression and survival data, we marked the same tumors from the head and neck cancer patients with the F4 antibody using conventional methods although only 122 patient immunohistochemistry scores were obtainable due to inadequate tumor sample or loss of tumor sample from the array slide.
Cancer Research

Table 1. Characteristics of patients according to subsites of head and neck tumors

<table>
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Table 1 shows the characteristics of patients according to subsites of head and neck tumors and Table 2 shows the tumor characteristics according to immunohistochemistry EGFR scores. The majority of the tumors were of UICC tumor stage III and IV and moderate to poor grade. Fifty-seven of the 122 patients (47%) have overexpression of EGFR scored either 2+ or 3+ with only 17 of the 122 patients (14%) scored at 3+ overexpression.

As previously described, the same statistical tests were used to determine the correlation between disease-free survival, overall survival, and EGFR expression. Figure 4A and B shows that EGFR overexpression (scores 2+ and 3+) by immunohistochemistry was not correlated with disease-free survival or overall survival. Even a further division of the subgroups into EGFR 0 to 2+ versus EGFR 3+ did not show a statistically significant difference in disease-free survival (data not shown). The statistical results show that conventional immunohistochemistry does not reveal a correlation with disease-free survival and, therefore, does not seem to be informative as a single variable to predict disease recurrence in this retrospective study.

Next, we addressed whether overall EGFR expression and EGFR phosphorylation calculated from the average fluorescence intensity of F4-Cy3b and the FRET efficiency, respectively, were correlated with EGFR expression by immunohistochemistry. To do this statistical study, the Mann-Whitney test was used. Figure 4C and D illustrates that there was no correlation between immunohistochemistry EGFR scoring and average F4 fluorescence intensity (EGFR expression) and between immunohistochemistry scoring and average FRET efficiency (P = 0.39 and P = 0.24, respectively).

Studies of EGFR expression (immunohistochemistry and average donor fluorescence intensity) when compared with activation reveal the absence of correlation between EGFR status and its activation.

Determination of the prognostic value of average FRET efficiency. Finally, we determined the prognostic value of the top 10% FRET efficiency by analysis of the disease-free survival and overall survival using univariate and multivariate analysis (see Materials and Methods). Table 3 shows the prognostic value of top 10% FRET efficiency in comparison with immunohistochemistry using univariate analysis. Top 10% FRET efficiency was a significant variable (P = 0.04) for disease-free survival but not for overall survival (P = 0.35), and overexpression of EGFR (2-3+) by immunohistochemistry was not a significant variable for either disease-free survival or overall survival. Table 4 shows the prognostic value of the top 10% FRET efficiency for disease-free survival and overall survival with UICC tumor stage and grade as covariates. The most significant variable for disease-free survival was top 10% FRET efficiency (P = 0.03) and none of the other variables were significant for overall survival by multivariate analysis. Further analysis including age and sex as covariates, in addition to UICC tumor stage and grade, showed again that top 10% FRET efficiency was the only significant variable for disease-free survival (P = 0.013; Supplementary Table S2).

Discussion

In this study, we have tested and validated an automated assay compatible with a microarray format that will inform on the phosphorylation status of the EGFR. The assay is designed to provide a two-site assay for phosphorylation of the EGFR using coincidence detection of protein and phospho-site selective monoclonal antibodies. This in-built specificity circumvents the requirement for monospecific modification site reagents because the specificity is determined by the protein-directed monoclonal. This is of particular importance in the context of tissue microarrays where there is no opportunity to test the validity of the immunoreactions observed. This assay has been developed for automated data capture in a format appropriate for tissue microarray analysis. We have exploited this assay with microarrays of head and neck cancers to determine the relationship between EGFR phosphorylation and prognosis. The study reveals that 10% of patients with a high degree of EGFR autophosphorylation (as evidenced by those with the highest FRET efficiency) have a poor disease-free survival. Importantly, this activation status does not correlate with EGFR concentration per se and this latter variable itself does not correlate with disease-free survival. This indicates that as might be predicted, the activation status of this receptor signaling pathway best defines its role in relation to the pattern of disease progression; as shown here, this seems to be a poor prognosis determinant with respect to disease recurrence.

Currently, the most accurate predictor of disease recurrence for head and neck cancer is the tumor-node-metastasis stage, particularly the lymph node status. There have been several studies seeking to find prognostic markers that can complement clinicopathologic information to predict survival and recurrence in HNSCC [e.g., EGFR overexpression level (8), TGF-α/EGFR mRNA (9), and p53 status (22, 23)]. At present, an elevated level of TGF-α seems to be as important or even more important in classifying prognostic groups than EGFR expression level (6, 12, 16, 24, 25). It is implicit that ligand is engaging and activating its receptor and, hence, the assessment of activation status should be informative; indeed, our data support this contention. The activation of the receptor measured by the method described here does give

| Table 2. Characteristics of tumors in relation to immunohistochemistry stains |
|----------------------------------|-----|-----|-----|-----|-----|
| EGFR Score                      | 0+  | 1+  | 2+  | 3+  | Total = 122 |
| Tumor stage                     |     |     |     |     |             |
| I                                | 3   | 0   | 5   | 1   | 9 (7%)     |
| II                               | 6   | 5   | 5   | 2   | 18 (15%)   |
| III                              | 11  | 6   | 9   | 0   | 26 (21%)   |
| IV                               | 16  | 18  | 21  | 14  | 69 (57%)   |
| Grade                            |     |     |     |     |             |
| Well                             | 2   | 4   | 2   | 0   | 8 (7%)     |
| Moderate                         | 17  | 10  | 19  | 8   | 54 (44%)   |
| Poor                             | 17  | 15  | 19  | 9   | 60 (49%)   |

prognostic information that is not available from analyzing EGFR in the conventional way or even with phospho-antibodies as indicated by Chung et al. (16).

Overexpression and high levels of EGFR do not necessarily reflect the functional state of this pathway. In recent studies, immunohistochemistry reporting on EGFR expression levels has been shown to be inconsistent in its predictions of disease recurrence and notably response to treatment (14, 18). This emphasizes the need to understand at a higher level of detail the functional status of candidate markers employed in diagnostic, prognostic, and therapeutic settings. The approach exemplified here clearly can be employed in this manner and indeed can be exploited for other pathways where fixation-insensitive changes occur. Initial studies on protein kinase C (26) had provided data on a small patient group, indicating a subset of patients exist with activated protein kinase C (26). Although coincidence detection of EGFR activity can be employed in tumor cell lines using FRET efficiency, it is the automation of this method for significant patient numbers that is crucial for outcome analysis (27). Moreover, in these studies there were insufficient numbers to derive statistically significant predictions about disease progression. The developments described here provide a substantial advance that permit significant numbers of patient samples to be analyzed.

The two principal classes of EGFR inhibitors are monoclonal antibodies that bind to the extracellular domain (of which the most promising drug is the chimeric human:murine monoclonal antibody cetuximab) and quinazoline inhibitors of the intracellular kinase domain (of which the ones that are most commonly used are gefitinib and erlotinib; ref. 28). Gefitinib has been tested in a phase II trial in HNSCC and the results show a disease stabilization

![Figure 4.](image-url)

**Table 3.** Prognostic value of the top 10% FRET efficiency by univariate analysis as compared with conventional immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th></th>
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<tbody>
<tr>
<td>Disease-free survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR (2-3+) by immunohistochemistry (n = 122)</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Top 10% FRET efficiency (n = 130)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR (2-3+) by immunohistochemistry (n = 122)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Top 10% FRET efficiency (n = 130)</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Top 10% FRET is statistically significant variable for disease-free survival but not for overall survival and EGFR overexpression (2-3+) by immunohistochemistry is not statistically significant for both disease-free survival and overall survival.
of 29% (29). Preliminary results from a phase III trial, which randomly assigned 424 head and neck cancer patients to receive either radiation therapy alone or radiation with concurrent cetuximab, have shown that cetuximab nearly doubled the median survival in these patients (30). However, the two phase II head and neck cancer trials on cetuximab revealed a relatively low response rate (around 10%) despite the fact that the majority of the patients have a 2 to 3+ staining for EGFR (31, 32). And in colon cancer two trials found no relation of EGFR expression and response rate to cetuximab (33, 34). In fact, one trial has reported objective responses to cetuximab in chemotherapy-refractory colorectal cancer even when EGFR expression was not detected by immunohistochemistry (35). Therefore, these trials on cetuximab indicate poor predictive value of EGFR expression in response to cetuximab. It would seem important in future studies to determine how EGFR activation status, rather than concentration, track with responses to these and related EGFR-directed treatments.

Monitoring response to treatment is of increasing importance with respect to trials of new molecular-directed therapeutics, as well as in seeking to optimize the use of those reaching the clinic. It is well accepted that the use of biomarkers in the development of new agents is important and it is implicit that these markers reflect the action of the target (whether direct or through a validated surrogate) and not simply its presence. The logic applied to the targeting and monitoring of inhibition for these targets is no less relevant to the question of prognostic indicators in relation to disease. Indeed, the studies reported here clearly show the distinctive value of the information acquired in comparing the prognostically uninformative data set relating to two different means of assessing the expression pattern of EGFR levels in these HNSCC samples and the apparently informative data set obtained from determining the phosphorylation status of the receptor.

The need to be able to determine the functional status of potential therapeutic targets or prognostic indicators is irresistible. For the EGFR studied here, the ligand-induced autophosphorylation of tyrosine residues within the cytoplasmic domain serves as a useful marker of receptor activation that is stable with fixation and sample storage. Many such covalent modifications are well characterized functionally in this and other pathways. Hence, there is a significant opportunity to develop and exploit pairs of reagents to monitor these pathways in tissue microarrays using the type of highly selective, two-site, automated system described here.

For HNSCC, it is anticipated that FRET efficiency measurements by automated high-throughput FLIM will complement the traditional EGFR immunohistochemistry methods and the clinicopathologic information in aiding clinical decision and management of head and neck cancer patients. We recognize that our study is an exploratory assessment of disease-free survival. A prospective protocol-driven assessment for validation and determination of performance characteristics needs to be done using this potentially powerful technique as described by Hayes et al. (36, 37). Using this strategy, we would propose using this assay for prospective stratification of patients in randomized trials of EGFR inhibition; retrospective analysis of trials of EGFR inhibitors would also be possible. In the longer term, the identification of these patients at presentation should provide guidance on more aggressive treatment or, more specifically, on appropriate molecular therapy.

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**References**

Prognostic Value of an Activation State Marker for Epidermal Growth Factor Receptor in Tissue Microarrays of Head and Neck Cancer


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