Published in final edited form as:
Int J Radiat Oncol Biol Phys. 2007 ; 69(2 Suppl): S102–S105.

GENE EXPRESSION PROFILES AS MARKERS OF AGGRESSIVE DISEASE—EGFR AS A FACTOR

Christine H. CHung, M.D.[*,†], **Joel Parker, M.S.**[‡], **Shawn Levy, PH.D.**[§], **Robbert J. Slebos, PH.D.**[†,‖], **Adam P. Dicker, M.D.,PH.D.**[¶], and **Ulrich RObeck, M.D.,PH.D.**[#]

*Department of Medicine, Division of Hematology/Oncology, Vanderbilt University School of Medicine, Nashville, TN

†Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN

§Department of Biomedical Informatics, Vanderbilt University School of Medicine, Nashville, TN

‖Department of Otolaryngology, Vanderbilt University School of Medicine, Nashville, TN

‡Constella Health Sciences, Thomas Jefferson University, Philadelphia, PA

¶Department of Radiation Oncology, Division of Experimental Radiation Oncology, Thomas Jefferson University, Philadelphia, PA

#Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, PA

Abstract

We previously reported that 43 (58%) of 75 head and neck squamous cell carcinoma (HNSCC) tumors harbor increased epidermal growth factor receptor (EGFR) gene copy numbers as determined by fluorescent *in situ* hybridization. In this study, an increased EGFR copy number was associated with decreased progression-free survival and overall survival of HNSCC patients. However, activated EGFR protein levels are difficult to quantify by immunohistochemistry and are subject to dynamic regulation, specifically receptor downregulation on ligand binding. Therefore, we generated an activated EGFR gene expression signature in an *in vitro* HaCaT keratinocyte model system to further study genes involved in the EGFR signaling pathway in HNSCC. The results from this model system have suggested that the activated EGFR signature might reflect the activated state of the EGFR pathway in human HNSCC tumors and that it is associated with the increased EGFR gene copy number by fluorescent *in situ* hybridization. Furthermore, the activated EGFR signature has provided additional leads, because they are related to co-regulated molecular pathways and associated gene products on activation of EGFR. These could be exploited to refine and optimize combination therapies to be used in conjunction with available EGFR inhibitors in individual HNSCC patients.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) remains one of the most devastating cancers in the United States. The sites affected by HNSCC (*i.e.*, oral cavity, oropharynx, hypopharynx, and larynx) are critical to the complex and vital functions of speech and swallowing. Despite the advances of primary treatments, a large group of patients (30–40%) develop recurrence, with very little improvement in overall survival (OS). Recently, the dearth of therapeutic

Reprint requests to: Christine H. Chung, M.D., Division of Hematology/Oncology, Departments of Medicine and Cancer Biology, Vanderbilt University School of Medicine, 2220 Pierce Ave., 777 Preston Research Bldg., Nashville, TN 37232−6307. Tel: (615) 322−4967; Fax: (615) 343−7602; E-mail: Christine.Chung@Vanderbilt.edu.

Conflict of interest: C. H. Chung received research funding from AstraZeneca and Ely Lilly, and received honoraria from Bristol-Myers Squibb and Array BioPharma. The other authors have no conflicts of interest.

options for HNSCC has motivated the search for targeted therapies for HNSCC. These efforts have largely focused on the epidermal growth factor receptor (EGFR) because of epidemiologic and mechanistic data implicating aberrant activation of this pathway in HNSCC. EGFR is a type 1 membrane tyrosine kinase that plays important roles in the differentiation, proliferation, and metastatic potential of a variety of human cancer cells, mostly those of epithelial origin (1). Once the EGFR is activated, it triggers, in a cell type-specific manner, multiple signaling events lead to phosphorylation of downstream effectors, including mitogen activated protein kinase, Akt, and signal transducer and activator of transcription 3 (2,3). The EGFR is highly expressed in >95% of HNSCCs, and increased protein expression of EGFR and its ligand, transforming growth factor-α (TGF-α), by immunohistochemical staining is associated with a poor prognosis in HNSCC patients (4,5).

We previously reported that 43 (58%) of 75 HNSCC tumors harbor an increased EGFR gene copy number as determined by fluorescent *in situ* hybridization (FISH) and that an increased EGFR copy number is associated with decreased progression-free survival and overall survival for HNSCC patients (6). Patients with FISH-positive tumors had a median time to progression of 18 months and to death of 20 months and those with FISH-negative tumors had a median time to progression of 25 months and to death of 29 months. This difference was statistically significant for progression-free survival ($p < 0.05$, log–rank test) and overall survival ($p < 0.01$, log–rank test), although we were limited by the heterogeneity of the patient population and treatments received. FISH status did not correlate with the EGFR gene or protein expression levels assessed either by quantitative polymerase chain reaction (PCR) or immunohistochemical staining using the DAKO EGFR PharmDx kit. This discrepancy was presumably due to the dilution effects of the DNA and RNA levels in quantitative PCR, reverse transcriptase (RT)-PCR, and microarray assays in which entire tumors are analyzed compared with the FISH analysis in which only 50–100 tumor cells within the area of FISH positivity are counted. Furthermore, the EGFR protein levels are difficult to quantify by immunohistochemical staining and are subject to dynamic regulation, specifically receptor downregulation on ligand binding. Therefore, we generated an EGFR signature using a HaCaT cell model system that reflected the overall gene expression pattern of the activated state of the EGFR pathway and correlated this EGFR signature with previously determined EGFR gene copy number data in HNSCC tumors.

METHODS AND MATERIALS

Cell preparation

The HaCaT cells overexpressing EGFR (HaCaT-EGFR) were maintained in culture media (W489) as described previously (7,8). After incubation in serum-free media, the cells were stimulated with TGF-α 10 ng/mL for 4 h and harvested for total RNA isolation in parallel with unstimulated control cells.

RNA preparation and DNA microarray analyses

Gene expression was determined from three HaCaT-EGFR cell cultures to increase our confidence in the statistical analyses. Total RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) and amplified and labeled using the NuGEN WT-Ovation RNA Amplification kit (NuGEN, San Carlos, CA), according to the manufacturer's recommendations. Next, 15 μg of biotin-labeled aRNA was fragmented and hybridized on to the Affymetrix Human Genome U133 plus 2.0 GeneChip. After hybridization, the GeneChip was washed, stained with strepavidin/phycoerythrin conjugate and biotinylated antibody, and scanned according to the manufacturer's recommendations. The raw microarray data were normalized and uploaded into the VMSR database for analysis.

Statistical analysis

All arrays were analyzed using the Robust Multichip Analysis approach. Differentially expressed genes between EGF stimulation and no stimulation in the HaCaT-EGFR cells were selected by statistical criteria of greater than twofold changes and $p < 0.05$ by Student's *t* test.

Determination of co-regulated genes/pathways with activation of EGFR on stimulation of EGF

The data were further mined using Ingenuity Pathways Analysis (IPA). The selected differential gene log ratios were submitted to the IPA application. The IPA mapped these genes to their corresponding gene objects in the Ingenuity Pathways Knowledge Base. A p value was generated for each mapped network by comparing the number of differential genes that participated in a given network relative to the total number of occurrences of those genes in all the possible network permutations for all the objects in the Ingenuity Pathways Knowledge Base. The network score was assessed for statistical significance, and the generated pathway maps were examined for biologic correlations.

Validation of array data using RT-PCR

The mRNA expression levels of EGFR and the EGFR ligands (EGF, TGF-α [TGFA], heparin-binding EGF-like growth factor [HB-EGF], and neuregulin 1 [NRG1]) were confirmed by RTPCR in HaCaT cells, first with and without EGFR overexpression (HaCaT-vector vs. HaCaT-EGFR) and, second, with and without EGF stimulation. The RT-PCR for each gene was performed in triplicate with control genes, including GUSB, 18S, and PPIA. The RT-PCR data were analyzed using the $2^{-\Delta\Delta}$Ct method, as described previously (9).

RESULTS

Examination of the EGFR mRNA levels using RT-PCR in the HaCaT-vector and HaCaT-EGFR cells with and without EGF stimulation after serum stimulation has shown that HaCaT-EGFR cells express greater levels of EGFR mRNA compared with HaCaT-vector and that ligand stimulation up-regulates the expression of the receptor itself (Fig. 1A). The activated EGFR signature determined by comparing the gene expression profile of the EGF-stimulated vs. EGF-unstimulated HaCaT-EGFR cells contained 234 probe sets or 207 unique genes. Examination of these genes in the context of biologic pathways using IPA revealed two key signaling pathways with increased expression levels on EGF stimulation. The two pathways included upregulation of Myc and vascular endothelial growth factor as their central genes in the network, as well as upregulation of EGFR ligands, NRG1, heparin-binding EGF-like growth factor, and TGFA. The differential expression levels of these ligands were confirmed using RT-PCR (Fig. 1B). Furthermore, downregulation of CDKN1B and CEPB4 was consistently observed in EGF-stimulated HaCaT-EGFR cells.

In previous work, we identified high expression of Myc and vascular endothelial growth factor in HNSCC patients with a poor prognosis (10,11). TGFA is an important EGFR ligand, and high expression of TGFA is also associated with a poor prognosis in HNSCC (5). NRG1 or heregulin is a ligand for Her3 that can heterodimerize with other Her family receptors and provide an escape mechanism to EGFR inhibitors (12,13). These key genes in the center of each pathway are important because manipulation of the central genes can regulate many genes linked within the network. These types of pathway analyses can provide information on novel targets and mechanisms of drug resistance and sensitivity. For instance, if a specific pathway is consistently upregulated in patients who were resistant to cetuximab or gefitinib, a combination regimen targeting that particular pathway could overcome the resistance. For example, anti-angiogenic agents to block vascular endothelial growth factor are already available in clinical settings and can be combined with EGFR inhibitors.

To determine the relevance of the activated EGFR signature as observed in HaCaT-EGFR cells to human HNSCC, the signature generated from HaCaT-EGFR cells was queried in a human data set generated from 44 HNSCC tumors, 3 normal tonsillar epithelia, and 3 normal mucosal epithelia adjacent to tumors and clustered to visualize the signature (Fig. 2). The tumors in Cluster 2 had a positive median correlation with the activated EGFR signature, indicating that the signature in the human data set resembled the EGF-stimulated cells. The tumors in Cluster 1 had a negative median correlation with the signature, resembling the unstimulated cells. Therefore, Cluster 2 with the positive correlation was determined to have the activated EGFR pathway. Of the 44 HNSCC tumors in Fig. 2, 33 had an increased EGFR gene copy number, as determined by FISH. Cluster 2 had more samples with FISH positivity than did Cluster 1 ($p < 0.0001$, pair-wise *t* test comparison).

DISCUSSION

These preliminary data suggest that the activated EGFR signature generated from HaCaT cells *in vitro* might reflect the activated state of the EGFR pathway in human HNSCC and that an increased EGFR gene copy number leads to increased EGFR activity. Therefore, the observed poor prognosis in patients with EGFR FISH positivity is most likely related to activation of the EGFR pathway. Furthermore, the activated EGFR signature provides additional leads, because they relate to co-regulated molecular pathways and associated gene products on activation of EGFR. However, the genes suggested as the activated EGFR signature are also frequently induced by other stimulants and are dependent on the *in vitro* experimental conditions, indicating that the diagnosis and/or prognosis using this signature could be misleading in general clinical applications. In addition, the HNSCC tumor data were generated from a small retrospective study of a heterogeneous patient population. Therefore, these preliminary data must be validated with a larger study of homogeneously treated patients with EGFR inhibitors before being exploited for refining and optimizing combination therapies in conjunction with available EGFR inhibitors in individual patients.

Acknowledgements

Funded by the Damon Runyon Clinical Investigator Award (CI-28-05) to C. H. Chung, Grants NIH CA081008 and DAMD 17-02-1-0216 to U. Rodeck, Grant RO1 CA106633-01 to A. P. Dicker.

REFERENCES

1. Arteaga CL. Targeting HER1/EGFR: A molecular approach to cancer therapy. Semin Oncol 2003;30:3–14.
2. Arteaga CL. ErbB-targeted therapeutic approaches in human cancer. Exp Cell Res 2003;284:122–130. [PubMed: 12648471]
3. Albanell J, Codony-Servat J, Rojo F, et al. Activated extracellular signal-regulated kinases: Association with epidermal growth factor receptor/transforming growth factor alpha expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. Cancer Res 2001;61:6500–6510. [PubMed: 11522647]
4. Grandis JR, Tweardy DJ. TGF-alpha and EGFR in head and neck cancer. J Cell Biochem Suppl 1993;17F:188–191. [PubMed: 8412192]
5. Grandis JR, Melhem M, Gooding W, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst 1998;90:824–832. [PubMed: 9625170]
6. Chung CH, Ely K, McGavran L, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol 2006;24:4170–4176. [PubMed: 16943533]

7. Boukamp P, Petrussevska RT, Breitkreutz D, et al. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761–771. [PubMed: 2450098]
8. Quadros MR, Peruzzi F, Kari C, et al. Complex regulation of signal transducers and activators of transcription 3 activation in normal and malignant keratinocytes. Cancer Res 2004;64:3934–3939. [PubMed: 15173005]
9. Heid CA, Stevens J, Livak KJ, et al. Real time quantitative PCR. Genome Res 1996;6:986–994. [PubMed: 8908518]
10. Chung CH, Parker JS, Karaca G, et al. Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression. Cancer Cell 2004;5:489–500. [PubMed: 15144956]
11. Chung CH, Parker JS, Ely K, et al. Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-{kappa}B signaling as characteristics of a high-risk head and neck squamous cell carcinoma. Cancer Res 2006;66:8210–8218. [PubMed: 16912200]
12. Sergina NV, Rausch M, Wang D, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 2007;445:437–441. [PubMed: 17206155]
13. Zhou BB, Peyton M, He B, et al. Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. Cancer Cell 2006;10:39–50. [PubMed: 16843264]

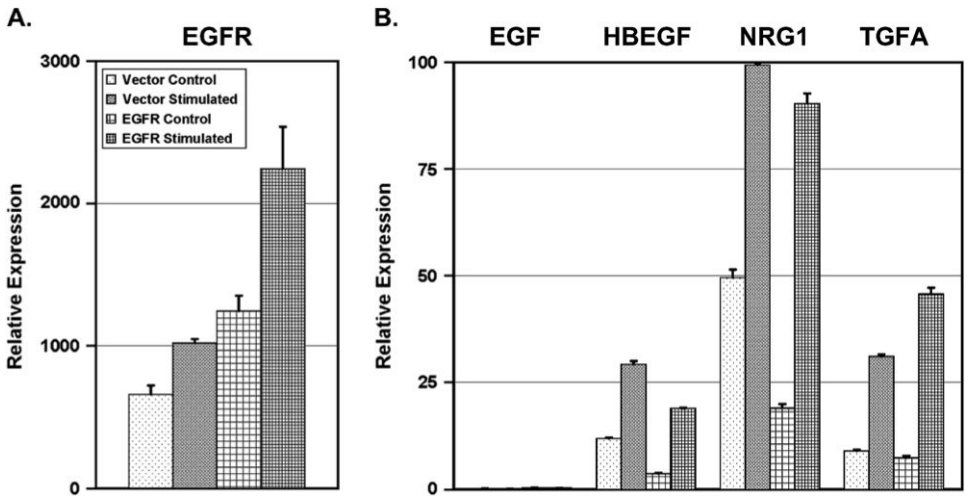

Fig. 1.

mRNA expression levels in HaCaT cells with empty vector without epidermal growth factor (EGF) stimulation (vector control) and with EGF stimulation (vector stimulated), and HaCaT cells with EGF receptor (EGFR) overexpression without EGF stimulation (EGFR control) and with EGF stimulation (EGFR stimulated) of (A) EGFR and (B) EGFR ligands, EGF, heparin-binding EGF-like growth factor (HBEGF), neuregulin 1 (NRG1), and transforming growth factor-TGF-α (TGFA).

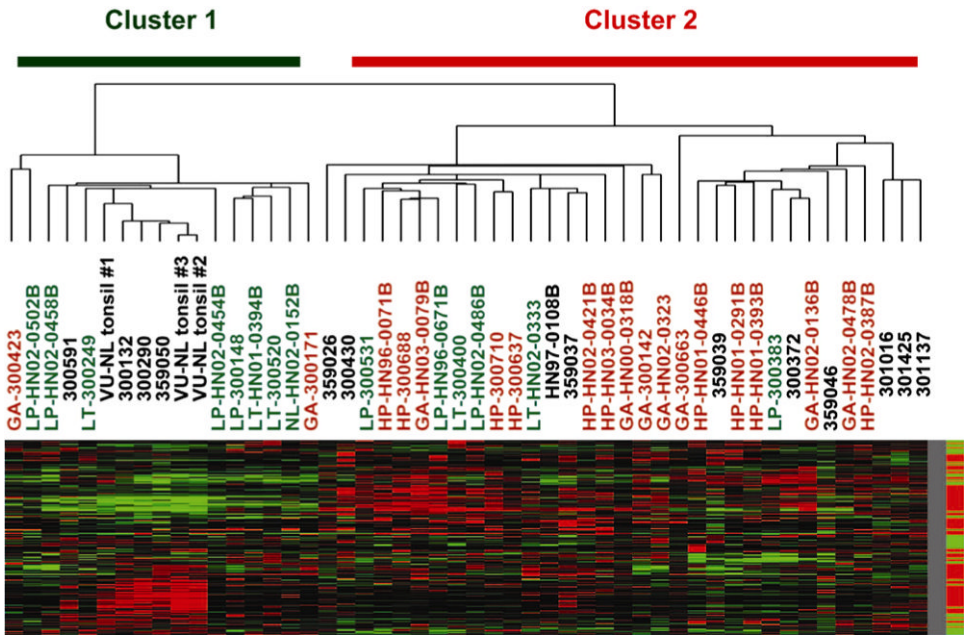

Fig. 2.

Hierarchical clustering of 50 frozen samples (44 head-and-neck squamous cell carcinoma [HNSCC] tumors, 3 normal mucosal epithelia adjacent to tumors, and 3 normal tonsillar epithelia) using activated epidermal growth factor receptor (EGFR) signature generated from EGFR overexpressing HaCaT cell with and without EGF stimulation. For 33 of 44 HNSCC tumors, EGFR fluorescent *in situ* hybridization (FISH) data were available. FISH data labeled as follows: HP = high polysomy; GA = gene amplification; LP = low polysomy; LT = low trisomy; and NL = normal disomy. Suffix “-AN” indicates mucosal epithelia adjacent to tumors.