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The Significance of Co-Expression of Epidermal Growth Factor Receptor (EGFR) and Ki67 on Clinical Outcome in Patients with Anal Cancer Treated with Chemoradiotherapy: An Analysis of NRG Oncology RTOG 9811

Corinne M. Doll, MD¹, Jennifer Moughan, MS², Alexander Klimowicz, PhD¹, Clement K. Ho, MD¹, Elizabeth N. Kornaga, MS¹, Susan P. Lees-Miller, PhD³, Jaffer A. Ajani, MD⁴, Christopher H. Crane, MD⁴, Lisa A. Kachnic, MD⁵, Gordon S. Okawara, MD⁶, Lawrence B. Berk, MD⁷, Kevin S. Roof, MD⁸, Mark J. Becker, MD⁹, David L. Grisell, DO¹⁰, Robert J. Ellis, MD¹¹, Paul Sperduto, MD¹², Gerald Marsa, MD¹³, Chandan Guha, MD¹⁴, and Anthony Magliocco, MD¹⁵

¹Tom Baker Cancer Centre, Calgary, AB, Canada

²NRG Oncology Statistics and Data Management Center, Philadelphia, PA

³University of Calgary, Calgary, AB, Canada

⁴University of Texas MD Anderson Cancer Center, Houston, TX

⁵Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN

⁶McMaster University Juravinski Cancer Center, Hamilton Health Sciences, Hamilton, ON, Canada

⁷Mount Sinai Comprehensive Cancer Center CCOP, Miami Beach, FL

⁸Southeast Cancer Control Consortium, Inc., CCOP, Winston-Salem, NC

⁹Columbus Community Clinical Oncology Program, Columbus, OH

¹⁰Greenville CCOP, Greenville, SC

¹¹Cancer Research of the Ozarks, Springfield, MO

¹²Metro-MN CCOP, St Louis Park, MN

¹³Toledo Community Hospital Oncology Program CCOP, Toledo, OH

Corresponding author: Corinne M. Doll, MD, Tom Baker Cancer Centre, Department of Oncology, University of Calgary, 1331 29th St NW Calgary, AB, T2N 4N2, Canada, Phone: 1-403-521-3095, Corinne.Doll@albertahealthservices.ca.

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¹⁴Montefiore Medical Center, Bronx, NY

¹⁵H. Lee Moffitt Cancer Center, Tampa, FL

Abstract

Purpose—To measure co-expression of EGFR and Ki67 proteins in pre-treatment tumor biopsies of anal cancer patients enrolled on NRG Oncology RTOG 9811, a phase III trial comparing 5FU/MMC/RT (Arm A) vs. 5FU/cisplatin/RT (Arm B), and to correlate expression with clinical outcome.

Materials/Methods—EGFR and Ki67 co-expression was measured after constructing a tissue microarray using fluorescence immunohistochemistry and automated quantitative image analysis (AQUA). Ki67 score within EGFR high vs. low areas (Ki67ratio in EGFR_{high:low}) in each tumor core was analyzed at the median, quartiles and as a continuous variable. Associations between the tumor markers and clinical endpoints [overall and disease-free survival (OS, DFS), locoregional and colostomy failure (LRF, CF), and distant metastases (DM)] were explored.

Results—282 pre-treatment tumors were analyzed from NRG Oncology RTOG 9811. Of evaluated specimens, 183 (65%, n=89, Arm A; n=94, Arm B) were eligible and analyzable. There were no significant differences in baseline characteristics or outcomes between analyzable and unanalyzable patient cases. Median follow-up was 6.0 years. On multivariate analysis, after adjusting for gender, patients with Ki67ratio in EGFR_{high:low} median had worse OS (HR=2.41, 95% C.I. = [1.38, 4.19], p=0.0019). After adjusting for N stage and largest tumor dimension, patients with Ki67ratio in EGFR_{high:low} median had a higher risk of a disease-free failure (HR=1.85, 95% C.I. = [1.18, 2.92], p=0.0078). Technical validation with an independent anal cancer patient cohort was performed and shows a very similar biomarker score distribution.

Conclusions—High Ki67ratio in EGFR_{high:low} is associated with worse clinical outcome in this subset of patients with anal cancer treated with chemoradiation on NRG Oncology RTOG 9811. Evaluation within a clinical trial will be required to determine whether patients with these tumor characteristics may specifically benefit from an EGFR targeted therapeutic agent.

Introduction

Anal cancer, although a relatively rare malignancy, has been steadily increasing in incidence, notably in females (1,2). Age-adjusted incidence rates have increased in all Western countries during the last decades, up to 2.2% per year (2,3). Chemoradiotherapy (CRT) is standard treatment for patients with this disease; however despite the investigation of alternate regimens and dose escalation, there has been little improvement in outcomes over the years (4–7). The results for treatment of recurrent or disseminated disease are very poor, thus investigation of better treatment options in the first-line setting are needed. As in other tumor sites, there is emerging interest in the determination of tumoral molecular factors that may predict for response or resistance to standard CRT and to guide the use of targeted therapies.

There has been much interest in the addition of epidermal growth factor receptor (EGFR) inhibition to standard cancer treatment, in an effort to improve clinical outcomes (8,9). To

date, the most commonly used such agent in clinical and investigative use is cetuximab, an IgG1 chimeric monoclonal antibody against EGFR. Overexpression of the EGFR signaling pathway may be responsible for malignant transformation and tumor progression. Activation of EGFR leads to downstream signaling, primarily through the KRAS/BRAF/ERK/MAPK pathway, ultimately resulting in abnormal proliferation of cells (10). EGFR activation can be mediated by gene mutation, amplification, and overexpression. Specifically in anal cancer, there is evidence that EGFR is overexpressed, and is independent of gene amplification (11). Moreover, KRAS and BRAF mutations appear rare in these tumors, supporting a role for EGFR inhibitor therapies, since these therapies have not shown benefit in patients with mutant tumors (12–18). These observations suggest that the addition of EGFR inhibition to CRT has potential for clinical benefit for patients with anal cancer. There is evidence of clinical benefit in treating metastatic anal cancer patients with cetuximab alone or in combination with irinotecan, even in patients who are heavily pre-treated (18). Unfortunately, recent clinical trials using cetuximab have shown significant toxicity when combined with conventional CRT for patients with anal cancer (19,20). Moreover, the addition of cetuximab to concurrent CRT in patients with esophageal cancer did not demonstrate benefit over CRT alone (21). However, these trials did not pre-select patients whose tumors had abnormal EGFR pathway activation. Thus further work is needed to better explore cancer molecular biology and to determine which patients have a greater chance of responding to this targeted therapy.

Although most anal cancers express EGFR, detection of pathway activation using IHC techniques has been challenging. A lung cancer study showed that cetuximab was only beneficial in patients with EGFR tumor expression level above a specific threshold (22); consequently, defining an appropriate cut point is important for clinical relevance. However, EGFR expression alone is not consistently associated with survival outcomes in cancer, possibly due to the fact that EGFR over-expression does not necessarily translate into downstream pathway activation and tumor proliferation. Ki67 is a nuclear protein that is tightly linked to the cell cycle, and its expression is intimately related to growth fraction (23, 24). It is considered to be a standard marker of tumor proliferation, and has been used as a prognostic marker in some tumor sites (25, 26). As a single marker, there have contradictory findings as to its role as a predictive marker, with many studies based on very small cohorts. Moreover, the relationship of Ki67-defined proliferative activity with its upstream biologic stimulus has not been well studied. The ability to positively correlate EGFR receptor activation with its corresponding downstream proliferation effect on the tumor cell could be an important molecular link to biologic behaviour. To date, detection of an activated EGFR pathway has been challenging using conventional immunohistochemistry techniques due to the inability to co-localizing these markers in tumor specimens.

The purpose of this study was to measure co-expression of EGFR and Ki67 proteins in pre-treatment tumor biopsies of anal cancer patients enrolled on NRG Oncology RTOG 9811, a phase III trial comparing 5FU/mitomycin-C (MMC)/RT vs. 5FU/cisplatin (CDDP)/RT, and to correlate expression with clinical outcome. It was hypothesized that the co-localization of the proliferation marker Ki67 with EGFR expression may be used as a surrogate for EGFR activity to identify tumors in which EGFR is driving the downstream signaling effect, reflecting the biologic behavior of the tumor.

Methods and Materials

Patient and tumor characteristics

NRG Oncology RTOG 9811 cohort—This has been well described in previous reports (27). Briefly, patients were eligible for inclusion on this study if they were ≥ 18 years of age, had histological diagnosis of squamous, basaloid, or cloacogenic carcinoma of the anal canal, had Karnofsky performance status ≥ 60, had T2–4 category tumors with any N category, and adequate organ function. Patients were randomly assigned to RT + FU/MMC or induction FU/CDDP followed by RT + FU/CDDP. Patients were stratified according to gender, clinical node status and size of primary tumor. Specific details of the treatment are as per the initial publication (28).

Calgary technical validation cohort—A second cohort was evaluated to technically validate the study assay. The patients in this subgroup were included based on the following criteria: ≥ 18 years of age, had histologic diagnosis of squamous, basaloid, or cloacogenic carcinoma of the anal canal, were treated with definitive CRT at Tom Baker Cancer Centre, Calgary, and had pre-treatment tumor biopsies adequate for evaluation. Treatment was delivered between 1997–2005; a similar time frame as the trial cohort. Full scientific and ethics approval for this study were granted by the University of Calgary's Office of Medical Bioethics.

Quantitative fluorescence immunohistochemistry—Pre-treatment biopsies were placed into tissue microarrays (TMAs). TMA sections (4 μm) were deparaffinized in xylene, rinsed in ethanol, and rehydrated. Heat induced epitope retrieval was performed by heating slides to 121°C in a citrate-based buffer (pH 6.0) Target Retrieval Solution (Dako) for 6 minutes in a decloaking chamber (Biocare Medical). Slides were then processed in a Dako Autostainer: endogenous peroxidase activity was quenched using Peroxidase Block from the Dako EnVision™ + System (Dako) for 10 minutes and non-specific staining was further reduced with a 15 minute incubation in Signal Stain® Antibody Diluent (Cell Signalling). Slides were stained for 60 minutes at room temperature in Signal Stain® Antibody Diluent with a 1:5000 dilution of Ki67 (mouse monoclonal anti-Ki67, Dako). After three washes in wash buffer (1×TBST, Dako), the secondary antibody was applied for 60 minutes at room temperature (goat anti-mouse antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone from the Dako EnVision™+ system, Dako). Slides were washed three times in wash buffer and incubated for 5 minutes with the TSA-Plus Cy3 tyramide signal amplification reagent (PerkinElmer). To stop horseradish peroxidase enzyme activity slides were removed from the autostainer and treated for 10 minutes in a 0.1% sodium azide solution. After two rinses with wash buffer, they were re-introduced into the Autostainer. Slides were incubated for 15 minutes in Signal Stain® Antibody Diluent, followed by a 60 minute room temperature incubation in Signal Stain® Antibody Diluent with a 1:1000 dilution of EGFR (rabbit monoclonal anti-EGFR, Eptomics) combined with a 1:100 dilution of guinea pig anti-pan-cytokeratin antibody (polyclonal, Acris) to identify tumor cells. After three washes in wash buffer, the secondary antibody was applied for 60 minutes at room temperature (goat anti-rabbit antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone from the Dako EnVision™+ system and a 1:200

dilution of Alexa488 conjugated anti-guinea pig secondary antibody, Invitrogen). Slides were washed three times in wash buffer and incubated for 5 minutes with the TSA-Plus Cy5 tyramide signal amplification reagent (PerkinElmer). After three washes in TBST wash buffer, the tissue microarray slides were mounted with ProLong Gold anti-fade mounting medium containing DAPI (Invitrogen), sealed with nail polish, and stored at 4°C until use.

Automated image acquisition and analysis—Automated image acquisition was performed using an Aperio ScanscopeFL and digital images were analyzed using AQUAnalysis® software version 2.3.4.1 (29). Briefly, high-resolution monochromatic 10-bit digital images were obtained for each TMA core using separate filters to define the nuclear (DAPI) and tumor (FITC) compartments as well as to capture the target biomarker staining for Ki67 (Cy3) and EGFR (Cy5). An analysis algorithm was constructed to identify compartments of interest, including: high EGFR expressing tumor area, low EGFR expressing tumor area, Ki67 positive tumor cell nuclei, high EGFR expressing Ki67 positive tumor cell nuclei, and low EGFR expressing Ki67 positive tumor cell nuclei. Compartment specific EGFR AQUA scores, defined as the average Cy5 (EGFR) pixel intensity within the defined compartment area for each TMA core, were calculated. An EGFR expression AQUA score threshold was developed to define high EGFR expression and low EGFR expression by measuring the AQUA EGFR expression scores using normal anal tissue; basal epithelial cell EGFR expression was considered to be EGFR high and stratified epithelial layers were considered to be EGFR low. EGFR and Ki67 expression data was forwarded to the RTOG central office where the samples were matched with clinical outcomes.

Statistical analysis—A mathematical expression to define the localization of Ki67 expression to areas with high or low EGFR expression within each TMA core was developed: $Ki67_{ratio} \text{ in } EGFR_{high:low} = Ki67 \text{ AQUA score in high EGFR expressing tumor} / Ki67 \text{ AQUA score in low EGFR expressing tumor}$. A $Ki67_{ratio} \text{ in } EGFR_{high:low} > 1.0$ indicates Ki67 is more strongly associated with high levels of EGFR in a given TMA core, (i.e. a positive correlation between EGFR expression and Ki67). Statistical comparisons to assess potential associations between baseline characteristics and tumor marker categories were carried out using the Chi-square test. Overall (OS) and disease-free survival (DFS) were estimated univariately with the Kaplan-Meier method (30), and the tumor marker categories were compared using the log-rank test (31). Time to local-regional failure (LRF), distant metastases (DM), and colostomy failure (CF) were estimated by the cumulative incidence method (32) and the tumor marker categories were compared using Gray's test (33). LRF, DM, second primary, and death from any cause are considered failures for DFS. Each marker was analyzed as continuous and categorical using its median and upper quartile as cut points (< median vs. median or < upper quartile vs. upper quartile). Cox proportional hazards models (34) were used to determine if there are any associations between the tumor marker categories with OS and DFS. Fine-Gray regression models (35) were used to obtain HRs for LRF, DM, and CF. Models assessing the tumor markers are coded such that the reference level is < median (< upper quartile). A hazard ratio (HR) > 1 indicates an increased risk of failure for median (upper quartile) relative to the reference level. For the multivariate analyses, models were built using the backwards selection procedure. Variables with p-values < 0.05 were retained in the models. Treatment

arm, gender, N stage (N0/NX vs. N+), and tumor size (≤ 5 cm vs. >5 cm) were assessed in the models along with the tumor marker. In all models, tumor marker was forced to remain in the models regardless of the p-value. In the analyses, RT+5FU/CDDP, females, N0/NX, and ≤ 5 cm were the reference groups.

Results

Of 682 patients randomized to the NRG Oncology RTOG 9811 clinical trial, 282 (41%) patients had pre-treatment tumor available for analysis. Of these 282 patients, 13 were ineligible. Of the remaining 269 patients, 86 tumor cores had a final result of redacted or fail, resulting in 183 analyzable patients with evaluable specimens for this analysis. Loss of data was due to unavailability of pre-treatment biopsy specimens, loss of cores due to sectioning from the TMA, and eliminating cores where no tumor tissue was evident on pathology review. Median followup was 6.0 years (min–max: 0.2–11.1). There were no statistically significant differences in baseline characteristics between the tested and non-tested groups, as shown in Supplemental Table 1. In addition, there were no statistically significant differences in OS, DFS, LRF, DM, and CF between those who were analyzable and those who were not analyzable (data not shown).

Immunofluorescent staining for EGFR and Ki67 is shown in Figure 1. For Ki67ratio in EGFR_{high:low}, the median is 1.14 (min–max: 0.26–3.25) and the upper quartile is 1.45. Pretreatment characteristics by Ki67ratio in EGFR_{high:low} ($<$ median vs. median) are shown in Table 1. There are no statistically significant differences in patient, tumor or treatment characteristics between the two groups.

OS and DFS by Ki67ratio in EGFR_{high:low} status are shown in Figure 2, illustrating median and quartile cut points. On Cox univariate analysis, patients with Ki67ratio in EGFR_{high:low} median (HR=2.24, 95% C.I. = [1.29, 3.87], $p=0.0032$) or upper quartile (HR=2.05, 95% C.I. = [1.21, 3.47], $p=0.0065$) are associated with a statistically significantly greater chance of dying than patients with Ki67ratio in EGFR_{high:low} $<$ median or $<$ upper quartile. In addition, patients with Ki67ratio in EGFR_{high:low} median have a greater chance of having a disease-free failure than patients with Ki67ratio in EGFR_{high:low} $<$ median (HR=1.72, 95% C.I. = [1.10, 2.69], $p=0.02$). Similarly, patients with Ki67ratio in EGFR_{high:low} upper quartile have a greater chance of having a disease-free failure than patients with Ki67ratio in EGFR_{high:low} $<$ upper quartile (HR=1.74, 95% C.I. = [1.10, 2.76], $p=0.02$). Likewise, an incremental increase in Ki67ratio in EGFR_{high:low} corresponds to an increase in the risk of failing local-regionally (HR=2.19, 95% C.I. = [1.13, 4.25], $p=0.02$) and patients with Ki67ratio in EGFR_{high:low} upper quartile have a two times greater chance of having a LRF than patients with Ki67ratio in EGFR_{high:low} $<$ upper quartile (HR=2.04, 95% C.I. = [1.12, 3.69], $p=0.02$).

On multivariate analysis, after adjusting for gender, patients with Ki67ratio in EGFR_{high:low} median have over double the chance of dying versus patients with values $<$ median (HR=2.41, 95% C.I. = [1.38, 4.19], $p=0.0019$, Table 2). Considering quartile analysis, Ki67ratio in EGFR_{high:low} is the only variable statistically significantly associated with OS. After adjusting for N stage and largest tumor dimension, patients with Ki67ratio in EGFR

median have a higher risk of a disease-free failure than patients with values < median (HR=1.85, 95% C.I. = [1.18, 2.92], p=0.0078, Table 2). After adjusting for N stage, an incremental increase in Ki67ratio in EGFR_{high:low} corresponds to an increase in the risk of failing local-regionally (HR=2.13, 95% C.I. = [1.09, 4.13], p=0.026) and patients with Ki67ratio in EGFR_{high:low} upper quartile have a two times greater chance of having a local-regional failure than patients with < upper quartile (HR=1.94, 95% C.I. = [1.06, 3.55], p=0.032). Considering tumor Ki67 and EGFR as single markers, there were no statistically significant differences seen in any of the clinical endpoints in multivariate Cox proportional hazards models (data not shown).

Clinical characteristics between the NRG Oncology RTOG 9811 (n=183) cohort and the Calgary technical validation cohort (n=38) were similar, except for a higher proportion of node positive patients in the NRG Oncology RTOG 9811 group (Table 3). The Ki67ratio in EGFR_{high:low} distribution and median value for the Calgary cohort were very similar to the NRG Oncology RTOG 9811 cohort (median Ki67ratio in EGFR_{high:low} 1.14 versus 1.13), Figure 3. There were no significant association between Ki67ratio in EGFR_{high:low} cut points and clinical variables in the Calgary cohort (data not shown).

Discussion

In this study, the ratio of Ki67 expression between high versus low EGFR regions within each tumor core (Ki67ratio in EGFR_{high:low}) was evaluated to assess the relationship between proliferation and EGFR expression. It was hypothesized that this biomarker may be a measure of EGFR activation, reflecting the biologic behavior of the tumor. It was determined that a high Ki67ratio in EGFR_{high:low} (above median, upper quartile) is independently associated with worse outcome in this population of anal cancer patients treated with definitive CRT on the NRG Oncology RTOG 9811 clinical trial. This novel assay defines a subgroup of patients whose tumors are not only defined by EGFR expression, but by its downstream signaling effect leading to proliferation and cell cycle activation. To the co-authors knowledge, this is the first study to show that co-expression of EGFR and Ki67 is significantly and independently associated with clinical outcome.

Neither EGFR nor Ki67 were found to be independently associated with clinical outcome in the analyses when considered separately. Single marker evaluation of EGFR in a secondary analysis of NRG Oncology RTOG 0324, a phase II trial of cetuximab plus CRT for stage III NSCLC, did not detect a difference in outcomes by protein expression level (36). Specifically in anal cancer, previous studies evaluating single-marker EGFR expression treated with CRT show inconsistent but generally negative results. Ajani et al previously evaluated a selected small cohort of patients with anal cancer treated with CRT. EGFR staining via conventional IHC technique was not associated with clinical outcomes, and on multivariate analysis, Ki67 was associated with DFS (37). Gilbert et al evaluated EGFR expression and KRAS mutational status in 153 anal cancer patients treated with radical CRT (38). Although EGFR status was not associated with clinical outcome, they found no KRAS mutations in primary or recurrent tumors. The lack of KRAS mutations in this tumor group was similarly reported by Paliga et al, who also did not identify an association between EGFR expression via conventional IHC and clinical outcome (39). Mistrangelo et al

measured expression of EGFR and Ki67 using conventional IHC techniques in a small cohort of CRT-treated patients, and did not find an association of expression with patient survival (40). Fraunholz et al investigated the prognostic value of EGFR in anal cancer patients treated with CRT using conventional IHC methods, and found EGFR expression was not independently associated with clinical outcomes on multivariate analysis (41). It is possible that these previous studies have failed to identify a population at increased risk of adverse clinical outcome, due to limitations of the assay (conventional IHC) or the inability to measure signaling activation of the EGFR pathway. In our technique, a mathematical expression to define the localization of Ki67 expression to areas with high or low EGFR expression within each TMA core was used. A Ki67ratio in EGFR_{high:low} of greater than 1.0 indicates Ki67 is more strongly associated with high levels of EGFR in a given TMA core. Although the full EGFR signaling pathway was not explored using this assay, it was felt that measuring Ki67 in conjunction with EGFR would link EGFR signaling with its downstream proliferation biologic effect. Given the novelty of this assay, a technical validation of the Ki67ratio in EGFR_{high:low} in an independent patient cohort was performed. A similar distribution of the biomarker was seen, and the median Ki67ratio in EGFR_{high:low} was virtually identical to the test NRG Oncology RTOG 9811 cohort. The fact that the median cut point for two distinct patient cohorts was almost identical provides additional evidence to the potential of this biomarker as a clinical assay. A similar association of the tested biomarker with clinical outcome was not seen in the validation cohort, however this was not unexpected, due to the small sample size (n=38). Given the significant clinical results found in the NRG Oncology RTOG 9811 cohort, and the consistency of the Ki67ratio in EGFR_{high:low} across the two cohorts, further exploration of this biomarker is warranted. This is of particular interest as data emerge on the benefit of EGFR inhibitors with EGFR activation - for example, the survival benefit seen for patients whose lung cancers had high EGFR expression status (42).

Although recent clinical trials have shown toxicity with the addition of cetuximab to standard CRT in locally advanced anal cancer patients, companion biomarker studies have not been published to evaluate whether there is a subset of patients who benefit from the addition of this targeted agent. These data do exist in the metastatic colorectal cancer (mCRC) literature. EGFR gain is associated with better outcome, and PTEN expression associated with longer time to progression in patients with KRAS wild-type mCRC tumours treated with cetuximab (43). Cetuximab efficacy was greater with intact/activated EGFR signaling, without KRAS/BRAF mutations and in the presence of PTEN inhibitory activation. In addition, in a retrospective analysis of RTOG 0617, a randomized, two-by-two factorial phase 3 study comparing standard- dose versus high-dose conformal RT with concurrent chemotherapy and the addition of cetuximab to concurrent CRT in patients with inoperable stage III NSCLC, high EGFR expression (quantified by H-score) status suggested benefit to cetuximab, whereas low scores showed a trend to worse median OS (44).

In conclusion, this study demonstrated that high tumor Ki67ratio in EGFR_{high:low} identified a subset of anal cancer patients enrolled on the NRG Oncology RTOG 9811 trial at risk of worse clinical outcome. Alternate therapeutic strategies, such as the addition of cetuximab or panitumumab, should be considered to improve the outcomes for patients whose tumors harbor these adverse features. Further investigation of this novel diagnostic tool for use as a

potential predictive biomarker of EGFR inhibitors with CRT is indicated to determine whether these patients may derive clinical benefit from this form of targeted intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Co-expression of EGFR and Ki67 was measured in pre-treatment tumor biopsies of anal cancer patients enrolled on NRG Oncology RTOG 9811. It was determined those patients whose tumors had Ki67ratio in EGFR_{high:low} median had worse overall survival and disease-free survival. This suggests that proliferative dysregulation in EGFR high expressing tumor cells is a potential marker of aggressiveness.

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Figure 1.

Immunofluorescent staining for EGFR and Ki67. (a) Control staining with and without primary antibody on cell line and tissue controls. (b) Examples of EGFR and Ki67 staining in normal tissue (top panels), anal cancer NRG Oncology RTOG 9811 core with a below median Ki67ratio in EGFR_{high:low} expression (middle panels), and anal cancer with an above median Ki67ratio in EGFR_{high:low} (lower panels). The 'merge' panels are pseudo-coloured blue for DAPI, green for pan-cytokeratin, white for Ki67, and red for EGFR staining. Photos are exposure adjusted for visual illustration of signal localization.

Figure 2. Disease-free survival (a) and overall survival (b) by Ki67ratio in EGFR_{high:low} by (i) median and (ii) quartiles.

Figure 3.

Boxplot showing distribution of scores of Ki67ratio in EGFR_{high:low}, comparing the NRG Oncology RTOG 9811 cohort and the Calgary technical validation cohort. The box represents the range in which 75% of the data lies, with the middle line indicated the 50th percentile, or median, the top of the box indicating the 75th percentile (third quartile), and the bottom indicating the 25th percentile (first quartile). The whiskers indicate the range of maximum and minimum, accordingly, with extreme outliers indicated by dots.

Table 1

Characteristics of Patients by Ki67 ratio in EGFR_{high:low} [cut point=median], (n=183)

Age	< Median (n=91)				Median (n=92)				p-value [‡]
	Median	55		54	Median	36-83		36-83	
Min-Max		n	%	n	n	%	n	%	
RX									0.16
RT+5FU/MMC		49	53.8	40	43.5				
RT+5FU/CDDP		42	46.2	52	56.5				
Age									0.71
< 55		43	47.3	46	50.0				
55		48	52.7	46	50.0				
Gender									0.19
Male		34	37.4	26	28.3				
Female		57	62.6	66	71.7				
KPS									0.098
60-80		13	14.3	22	23.9				
90-100		78	85.7	70	76.1				
T-stage									0.70
T2		66	72.5	69	75.0				
T3/T4		25	27.5	23	25.0				
N stage									0.29
N0/NX		65	71.4	72	78.3				
N+		26	28.6	20	21.7				
Largest tumor dimension									0.83
5cm		69	75.8	71	77.2				
>5cm		22	24.2	21	22.8				

[‡] p-value from Chi-square Test

Multivariate Cox Proportional Hazards Model of OS and DFS: Ki67ratio in EGFR_{high:low} [cut point=median], (n=183)

Table 2

Outcome	Adjustment Variables	Comparison	Hazard Ratio	95% C.I. LL	95% C.I. UL	p-value [†]
OS	Ki67ratio in EGFR _{high:low}	< median vs. median	2.41	1.38	4.19	0.0019
	Gender	Female vs. Male	1.88	1.11	3.20	0.02
DFS	Ki67ratio in EGFR _{high:low}	< median vs. median	1.85	1.18	2.92	0.0078
	N stage	N0/NX vs. N+	1.92	1.18	3.10	0.0082
	Largest tumor dimension	5cm vs. > 5 cm	1.78	1.09	2.91	0.021

* Hazard Ratio: a hazard ratio of 1 indicates no difference between the subgroups. The variables were coded such that a HR > 1 indicates an increased risk of failure for the second level of the variables listed.

[†] p-value from the Cox proportional hazards model

Table 3

Characteristics of Patients in Test (NRG Oncology RTOG 9811) versus Technical Validation Group (Calgary Cohort)

		RTOG 9811 (n=183)		Calgary Cohort (n=38)				
Age		55		60				
Median		33-83		34-83				
Min-Max		n	%	n	%	n	%	p-value [†]
Age								0.18
< 55		89	48.6	14	36.8			
55		94	51.4	24	63.2			
Gender								0.15
Male		60	32.8	8	21.1			
Female		123	67.2	30	78.9			
T-stage								0.99
T1/T2		135	73.8	28	73.7			
T3/T4		48	26.2	10	26.3			
N stage								0.02
N0/NX		137	74.9	35	92.1			
N+		46	25.1	3	7.9			
Largest tumor dimension								0.21
5cm		140	76.5	26	86.7			
>5cm		43	23.5	4	13.3			
Missing info				8				

[†] p-value from Chi-square Test