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Am J Surg. Author manuscript; available in PMC 2011 September 1.

Published in final edited form as:
Am J Surg. 2010 September ; 200(3): 398–405. doi:10.1016/j.amjsurg.2009.10.014.

Epidermal growth factor receptor (*EGFR*) intron 1 polymorphism and clinical outcome in pancreatic adenocarcinoma

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Abstract

Background—*EGFR* intron1 has a polymorphic region of CA-repeats which is believed to be associated with increased EGFR expression, tumor aggressiveness, and worse survival in cancer patients.

Methods—We investigated a large population of pancreatic adenocarcinoma patients to evaluate this polymorphism as a potential prognostic marker of clinical outcome. We included DNA obtained from 50 resected pancreatic adenocarcinomas and from 85 diagnostic EUS-FNA corresponding to patients with unresectable tumors. The correlation between CA-repeats length and *EGFR* mRNA levels was also examined.

Results—Analysis of the 135 patients revealed no correlation between *EGFR* intron 1 CA repeats length and tumor stage. There was no difference in overall patient survival when stratified by allele length. A correlation between *EGFR* intron 1 length and EGFR transcript and protein levels could not be established.

Conclusions—The length of the *EGFR* intron1 CA repeats does not correlate with levels of EGFR expression and can not be employed as marker of clinical prognosis in pancreatic cancer patients.

Keywords

EGFR; pancreatic cancer; intron 1 polymorphism

Introduction

Pancreatic adenocarcinoma remains the fourth deadliest cancer in the United States with 42,470 new cases and 35,240 deaths estimated in 2009(1). The five year survival rate remains dismal

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at roughly 5% despite aggressive therapy(1). There is a critical need to identify molecular markers of prognosis that could improve patient selection for surgical treatment and which may also be employed in the identification of candidates for targeted systemic therapeutic strategies. The epidermal growth factor receptor (EGFR) is one of four members of the ErbB receptor family and is believed to play an integral part in tumorigenesis of multiple epithelial cancers including pancreatic cancer (2). In addition, EGFR is overexpressed in pancreatic cancer (3) and overexpression has been shown to correlate with aggressive tumor behavior (4) and decreased overall survival (5). The *EGFR* gene intron 1 has a polymorphic region of CA dinucleotide repeats, ranging from 9 to 26 repeats, which is believed by some researchers to affect *EGFR* transcription efficiency, influence clinical prognosis and modulate anti-EGFR drug sensitivity in colorectal (6), head and neck (7), and breast cancers (8).

We have previously demonstrated that short length of the *EGFR* intron 1 CA repeats is associated with decreased overall survival among a small number of patients undergoing pancreatic cancer resection (9). Recent reports, however, have challenged the role of *EGFR* CA repeat length in the regulation of EGFR transcription and its potential role as a predictive indicator of cancer patient survival, tumor aggressiveness, and response to anti-EGFR therapy in colorectal cancer and osteosarcoma (10,11).

In the present study, we sought to expand the analysis of *EGFR* intron 1 length in pancreatic cancer by significantly increasing our patient population size and the duration of its clinical follow-up. In this analysis, we have included tissue from patients with locally advanced and/or metastatic pancreatic cancer collected at the time of diagnostic endoscopic ultrasoundguided fine needle apsiration (EUS-FNA). We thus performed an analysis of the relationship between *EGFR* intron 1 length and clinical outcome in the entire spectrum of pancreatic adenocarcinoma clinical presentations including pancreatic cancer patients with unresectable tumors which constitute the majority. The objectives of our study were to correlate the length of the *EGFR* intron 1 CA repeats with EGFR mRNA and protein expression levels, tumor characteristics, patient demographics and overall survival in a large cohort of pancreatic cancer patients while attempting to validate the role of *EGFR* intron 1 length as predictor of clinical outcome.

Materials and Methods

Study subjects

After IRB-approval and informed consent were obtained, tumor specimens were collected from 135 pancreatic cancer patients evaluated at the University of Alabama at Birmingham between 4/1999 and 5/2007. Patients were staged using helical computed tomography with triple phase intravenous contrast pancreatic protocol as well as endoscopic ultrasound. There were 50 patients who underwent laparotomy with curative intent. Tumor specimens were collected at the time of operation, snap-frozen, and stored in liquid nitrogen for later analysis. This group of 50 patients includes a subset (n=30) which has already been described in our previous report (9). In addition, 85 patients who underwent diagnostic EUS-FNA were determined to have unresectable disease by imaging studies. FNA-acquired tumor specimens were collected, snap-frozen, and stored in liquid nitrogen for later analysis. Clinical follow-up was obtained from hospital records.

Human pancreatic cancer cell lines

S2-013 and S2-VP10 cell lines, cloned sublines of SUIT-2 (12) (a gift from Dr. Michael Hollingsworth, University of Nebraska Medical Center), were cultured in DMEM supplemented with L-glutamine and 10% FBS, in a 37 °C incubator with 5% CO_2. ASPC-1, BxPC-3, CAPAN-1, HPAC, HPAF-II, MIA PaCa-2, and PANC-1 were obtained from the

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American Type Culture Collection (Rockville, MD) and propagated according to provider's recommendations.

Tumor and cell lines DNA isolation

We have previously demonstrated that *EGFR* intron 1 polymorphism can be reliably measured in any source of patient genomic DNA (9). Tumor DNA from resected pancreatic cancer was isolated using the AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA). Tumor genomic DNA from EUS-FNA material was isolated by incubating the entire FNA specimen with 50 μL of a DNA extraction solution at 56 C overnight. The DNA extraction solution consisted of 100 mM Tris-HCl, 2 mM EDTA, 1% Tween-20, and 0.42 mg/mL Proteinase K. The DNA was subsequently purified using Wizard Plus DNA purification system (Promega, Madison, WI). Genomic DNA from nine pancreatic cancer cell lines was isolated using the AquaPure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA).

Laser capture microdissection of resected tumor samples

Fresh-frozen samples were embedded in Optimal Cutting Temperature (OCT) compound and 8-μm sections were stained with hematoxyllin and eosin. Sections were reviewed by a pancreatic pathologist (N.C.J.) to localize and verify the presence of cancer cells on each slide. Tumor cells were collected using a PixCell II Laser Capture Microdissection (LCM) System (Arcturus Molecular Devices, Sunnydale, CA). Approximately 3,000 tumor cells were captured on each LCM cap for RNA extraction.

RNA isolation and real time RT-PCR analysis

Total RNA from the microdissected sections and cell lines was isolated with the RNAqueous Micro Kit (Ambion, Austin, TX) using the manufacturer protocol. A gross yield of 18 μL of RNA solution per sample was subsequently stored at −80 C. cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Using patient cDNA, real-time quantitative PCR was performed using TaqMan Gene Expression *EGFR* Assay-on-Demand (Applied Biosystems, Foster, CA) in an ABI Prism 7700 Detection System. *RPLPO* was used as an endogenous reference gene.

Immunoblotting

Cell line protein lysates were prepared, and standard SDS-PAGE was performed as previously described (13). Anti-EGFR (Sigma-Aldrich) was diluted 1:1000.

Immunohistochemical (IHC) analysis of EGFR expression

Serial 5 μm sections were cut one day prior to immunostaining from the representative formalin-fixed and paraffin-embedded blocks and mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were then incubated overnight with anti-EGFR monoclonal antibodies (Santa Cruz biotechnology, Santa Cruz, CA). Secondary detection was performed using a multi-species detection system (Signet Lab Inc., Dedham, MA). Sections were incubated in biotinylated anti-mouse antibodies for 20 min, then incubated with peroxidase-labeled streptavidin for 20 min (Signet Lab Inc.). Antigen–antibody complexes were visualized by incubation with 3,3′-diaminobenzidine substrate (BioGenex, San Ramon, CA) and counterstained with diluted Harris hematoxylin. The stained slides were systematically evaluated by a pathologist for the staining intensity and sub-cellular localization. The intensity was scored from 0 (equals no staining) to 3 (strongest staining, equivalent to A431 cell line control). The H-score was calculated by multiplying staining intensity by percent of cells positive (0 to 100). All negative control slides (omitted primary antibodies) were negative for staining.

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EGFR intron 1 polymorphism (CA repeat)

After extraction, 25 ng of DNA was amplified by PCR reaction using an unlabeled forward EGFR primer (5′-GGGCTCACAGCAAACTTCTC-3′) and a fluorescent HEX-labeled reverse EGFR primer (5′-AAGCCAGACTCGCTCATGTT-3′). Conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds, and final extension of 72°C for 10 min. Final concentrations in a 50 μL PCR reaction were 5 μL of 10x PCR Buffer II (Applied Biosystems, ABI), 2.5 μM $MgCl_2$, 200 μM each dNTP, 0.25 μM forward primer, 0.25 μM labeled reverse primer, 1.25 units AmpliTaq Gold Polymerase (ABI), and 1 μL DMSO. One to two μL of the amplified PCR products were diluted in 20 μL of water (high-performance liquid chromatography grade) containing 0.5 μL of 400 HD fluorescent size standard. Genotypes were resolved on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) to determine allele lengths and number of CA repeats. All analyses were performed at least in duplicates of independent PCRs.

Statistical analysis

Statistical analysis was performed with SPSS (SPSS, Chicago, IL). Patient overall survival was measured from the day of diagnosis by either EUS-FNA cytology or computed tomography. Kaplan-Meier analysis with log-rank test was employed to analyze patient survival and to test differences between patients who underwent pancreatic cancer resection and patients with unresectable tumors. Multivariate Cox regression analysis was applied to evaluate effect on survival of multiple factors simultaneously. Fisher exact test was used to estimate significance of associations between *EGFR* intron 1 length and various factors (age, race, stage, resection with curative intent, microscopic margins for surgical patients, perineural invasion for surgical patients, adjuvant chemotherapy for surgical patients, and radiation therapy for surgical patients). Spearman correlation coefficient was employed to estimate the association between *EGFR* intron 1 length and expression levels of *EGFR* transcript and EGFR protein. Statistical significance was defined at $p<0.05$.

Results

The median allele-specific length of the *EGFR* intron 1 CA repeats for all the analyzed patients was 18 (range 14-24), with 16 repeats being the most frequent allele-specific length (63% patients) followed by 20 repeats (41% patients). This corroborated the interethnic distribution seen by Liu et al. (14), who found that CA repeat length 16 was the most common allele-specific CA repeat length among Americans (42-43% of all alleles). When allele-specific CA repeats were added, the median sum of repeats (Asum) was 36 (range 29-44), with 43% patients having less than 36 repeats. As described in our previous report, CA repeat length shorter than 36 was classified as short *EGFR* intron 1 length and CA repeat length ≥ 36 was considered as long *EGFR* intron 1 length. There was no difference in the proportion of patients with short *EGFR* intron 1 length between the resectable and unresectable pancreatic cancer subgroups.

We then analyzed the correlation between *EGFR* intron 1 short (<36) and long (≥36) Asum and patient demographics as well as clinical outcome (Table 1). There was no association between the length of the *EGFR* intron 1 CA repeats and overall survival in the combined group of pancreatic cancer patients or in the subset of unresectable patients (Figure 1). Interestingly, the association of short length of the *EGFR* intron 1 CA repeats (Asum<36) with worse overall survival (19.7 vs. 23.1 months, p=0.114) did not reach statistical significance in the subset of resectable patients, and this expanded analysis failed to corroborate our previous findings (9).

Median patient follow-up ranged from 0.7 to 42.4 months. Median overall survival in the pancreatic cancer patient group who underwent surgical resection was 21 months (range 0.8 –

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42 months) which is consistent with described pancreatic cancer surgical outcomes (15). Median overall survival for patients with unresectable pancreatic cancer was 7.1 months (range 0.7-27.9 months) ($p<0.0001$).

To validate the consistency of our pancreatic cancer population as a representative sample, we analyzed the correlation between overall survival and pancreatic cancer patient demographics (Table 1). As expected, earlier tumor stage (I-II), Caucasian race, and ability to undergo resection with curative intent were factors associated with significantly improved overall survival in our cohort. In surgical patients, microscopic margins, peri-neural invasion, adjuvant chemotherapy and radiation therapy were not associated with either improved overall survival or length of *EGFR* intron 1 CA repeats.

We also performed multivariate Cox regression analysis to test the simultaneous influence of Asum, age, race, sex, tumor stage and surgical treatment on overall patient survival. Our analysis confirmed that stage, race and ability to undergo surgical treatment were the only significant factors correlated with clinical outcome ($p<0.0003$, $p<0.002$ and $p<10^{-7}$, respectively). We did find that age becomes a significant prognostic factor ($p<0.0003$) when other factors are taken into account.

Lastly, we wanted to test if shorter *EGFR* intron 1 length is indeed associated with increased *EGFR* transcript and protein expression levels. To test this hypothesis, we microdissected cancer cells in tumor specimens from a subset of 23 patients who had undergone pancreatic cancer resection. This procedure enabled us to separate carcinoma cells from surrounding stromal elements and to exclusively isolate tumor-specific RNA. Real time RT-PCR analysis of *EGFR* expression levels was then performed and correlated to the *EGFR* intron 1 length in the same group of patients. The calculated Spearman coefficient found no significant correlation (rho=0.099, p=0.57) between *EGFR* intron 1 length and *EGFR* mRNA levels in pancreatic adenocarcinoma patients. We then obtained paraffin blocks for the same 23 patients and performed IHC staining for EGFR protein (Figure 2). As predicted, there was a correlation between transcript and protein expression (rho=0.55, p=0.03), however, there was still no significant correlation between *EGFR* intron 1 length and EGFR protein expression (rho=0.15, p=0.56). To further prove the absence of correlation between *EGFR* intron 1 length and EGFR protein expression, we tested the intron 1 length and EGFR expression (both transcript and protein) in a panel of nine representative pancreatic cancer cell lines (Figure 3). The nine pancreatic cancer cell lines displayed the expected spectrum of CA repeat lengths (allele-specific range 15-22). There was a strong correlation between EGFR protein level and *EGFR* transcript (rho=0.821, p= 0.0067). However, average expression of EGFR protein did not vary significantly according to *EGFR* intron 1 length.

Discussion

Increased EGFR protein expression is associated with decreased survival in several epithelial cancers but the mechanisms regulating levels of EGFR expression in cancer are not fully understood. *EGFR* gene intron 1 length has been proposed as a determinant of EGFR overexpression by improving *EGFR* transcription efficiency. The molecular implications of this *EGFR* intron 1 polymorphism have not been completely characterized, but it has been suggested that this area of the intron acts like a joint, bringing the promoter in proximity to a putative repressor protein bound downstream of the CA repeat sequence (16). (6-8). In that regard, *EGFR* intron 1 length has been shown to affect gene transcription in skin, mammary, head and neck, and gastric cell lines, and this evidence resulted in enough momentum to justify testing its significance *in vivo* (6,17).

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Analysis of *EGFR* intron 1 length is a test that can be easily performed in peripheral blood DNA, making it inexpensive and practical as a predictive assay. Because of the described ease of measurement, *EGFR* intron 1 length has been postulated as an extremely attractive marker not only of EGFR expression but also as a potential predictor of response to anti-EGFR therapy (6). Via its role as a putative regulator of *EGFR* transcription and EGFR protein expression levels, *EGFR* intron 1 length has been described as a predictive marker of clinical outcome for a number of carcinomas. Buerger *et al*. described *EGFR* intron 1 length in breast cancer (8). Their report showed decreased *EGFR* transcription activity with increasing number of CA repeats. Since then, a growing number of publications have critically examined the functional meaning of *EGFR* intron 1 CA repeat length with conflicting results. Etienne-Grimaldi *et al* noticed that normal tissue EGFR concentrations were not significantly influenced by the length of the CA repeats while such correlation did exist in tumor samples (7). McKay *et al* showed no association between the distribution of *EGFR* intron 1 length in colorectal tumors and EGFR protein levels. More intriguingly, intron 1 genotype identified in normal colon samples was not predictive of *EGFR* mRNA expression levels (18). An additional observation by Buisine *et al* later confirmed these findings (10). Recent studies in osteosarcoma patients have failed to verify the functional importance of *EGFR* intron 1 length in EGFR expression (11). Possible reasons for this discordance include lack of tumor-specific EGFR expression regulation and variable significance of EGFR signaling in carcinogenesis and tumor progression depending on cancer type. While the scope of this manuscript was not to demonstrate the functional correlation between *EGFR* intron 1 length and EGFR expression, our results provide further evidence that CA repeats in intron 1 are unlikely regulators of *EGFR* gene expression. While this fact was originally postulated based on *in vitro* experiments, a growing body of literature indicates that there is no functional evidence supporting an association between intron 1 length and *EGFR* gene expression levels. The lack of relationship with clinical outcome demonstrated in our results further supports this claim, s suggesting the existence of alternative transcription control mechanisms which might account for the purported role of EGFR expression as clinical prognostic marker (11,19).

We have previously reported that *EGFR* intron 1 length could be potentially employed to predict pancreatic cancer aggressiveness in a group of 30 surgically resected patients (9). In the present study, we expanded our population size and increased the statistical power of our analysis by by providing extended clinical patient follow-up, and by including patients with unresectable pancreatic cancer. The inclusion of the latter patient subpopulation is of particular significance since over 70% of pancreatic cancer patients present with advanced disease and therefore are not candidates for surgical resection. This majority of patients are often excluded from pancreatic cancer studies due to the lack of available tumor tissue for analysis.

To our knowledge, this is the first comprehensive evaluation of *EGFR* intron 1 length as a potential surrogate marker of pancreatic cancer clinical outcome in a large population of patients. It is also the first attempt to correlate *EGFR* intron 1 length with *EGFR* mRNA and protein levels among patients with both resectable and unresectable pancreatic adenocarcinoma.

Our findings further challenge the putative role of *EGFR* intron 1 length as a modulator of EGFR transcription efficiency that can influence clinical outcome. The described analysis indicates that *EGFR* intron 1 length does not reliably discriminate patients with resectable pancreatic cancer from those with unresectable disease as there was no significant difference between the respective groups' CA repeat lengths. Longer *EGFR* intron 1 length could not be associated with prolonged patient survival. Furthermore, and at a functional level, *EGFR* intron 1 length does not seem to influence *EGFR* transcription efficiency. In the subset of pancreatic cancer patients in whom we analyzed *EGFR* transcript levels, we found no association between *EGFR* intron 1 length and either *EGFR* mRNA or protein expression.

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The discrepancy between our current findings and what we have previously reported can be explained by the increase in the sample size and the extension of clinical follow-up. The previously reported improved patient survival with longer *EGFR* intron 1 length did not hold statistical significance in our updated analysis when 20 patients were added to the resected group and also when a large number of patients with unresectable pancreatic cancer were included. These findings suggest that the length of the CA repeat has no influence on *EGFR* transcription and pancreatic cancer tumor progression. There are several possible explanations for the discrepancies among studies of *EGFR* intron 1 length, including the complexity of pancreatic cancer tumorigenesis with multiple signaling pathways that play a critical role in its inception and that may be relatively independent from the EGFR cascade. There is also a wide array of highly variable tumor-host interactions that probably influence tumor progression and that cannot be fully characterized or predicted by analysis of a single gene polymorphism.

We conclude that pancreatic adenocarcinoma *EGFR* intron 1 does not contribute to the regulation of *EGFR* transcription. We also conclude that the length of the *EGFR* intron 1 CA repeats cannot be employed as marker for clinical prognosis in patients with pancreatic cancer.

Summary

Analysis of the 135 pancreatic cancer patieints revealed no creelation between the length of polymorphic region of CA-repeats in EGFR intron 1 tumor stage. The length of the EGFR intron1 CA repeats also does not correlate with levels of EGFR expression and can not be employed as marker of clinical prognosis in pancreatic cancer patients.

Acknowledgments

This study was supported by Pancreatic SPORE grant P20 CA10195-01 and by the Robert E. Reed Gastrointestinal Oncology Research Foundation.

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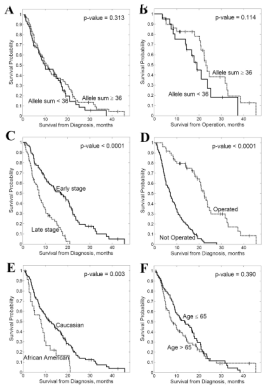

Figure 1.

Kaplan-Meier analysis of overall survival for (**A**) all pancreatic cancer patients stratified by *EGFR* intron 1 total CA repeat length; (**B**) patients who underwent pancreatic cancer resection stratified by *EGFR* intron 1 total CA repeat length.

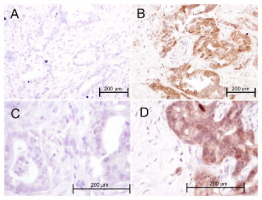

Figure 2.

The photographs show representative samples of pancreatic adenocarcinoma from 4 patients, stained using immunohistochemistry for EGFR. Each sections shows malignant glands composed of plump epithelial cells with nuclear enlargement and pleomorphism, and the intervening desmoplastic reaction of small fibroblasts. Panels A and C (magnification 200 and 400X, respectively) were scored as 0 (cytoplasmic), 0 (membranous). Panels B and D represent EGFR expressing cancers and were scored as 140 (cytoplasmic) and 60 (membranous). The stromal elements were negative in all samples.

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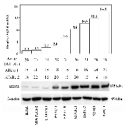

Figure 3.
Expression of EGFR protein, EGFR transcript and *EGFR* intron 1 total CA repeat length in a panel of nine pancreatic cancer cell lines. Allele-specific and total CA repeats are displayed for each cell line.

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Table I

Factor	Split	N	Non-censored N	Median Survival (months)	Log-rank p-value	Hazard Ratio	95% CI	N Allele Sum <36	N Allele Sum ≥36	Fisher p-value
Allele Sum (Surgical Patients)	≥36	29	19	23.1	0.114	1.86	0.86-3.99			
	<36	21	14	19.1						
Allele Sum (Combined Patients)	≥36	77	65	10.6	0.313	1.22	0.83-1.78			
	<36	58	50	9.9						
Min Allele	≥18	35	29	12.2	0.253	1.27	0.84-1.90			
	<18	100	86	10.1						
Max Allele	≥20	71	61	10.6	0.525	1.13	0.78-1.64			
	<20	64	54	9.9						
Age	≤65	64	57	13.6	0.390	1.18	0.81-1.70	24	40	0.296
	>65	71	58	7.9				34	37	
Ract	Caucasian	111	93	11.8	***0.003***	2.46	1.35-4.47	49	62	0.818
	African Am	23	22	7.9				9	14	
Sex	Female	71	61	12.2	0.456	1.12	0.78-1.62	35	36	0.163
	Male	64	54	9.1				23	41	
Stage	I - II	76	61	16.7	***< 0.0001***	3.47	2.230-5.39	33	43	1.000
	III - IV	59	54	7.1				25	34	
Resection with Curative Intent	Yes	50	33	22.3	***< 0.0001***	4.76	3.18-7.14	21	29	1.000
	No	85	82	7.1				37	48	