Gender-Related Survival Differences Associated with EGFR Polymorphisms in Metastatic Colon Cancer

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Abstract

Evidence is accumulating supporting gender-related differences in the development of colonic carcinomas. Sex steroid hormone receptors are expressed in the colon and interact with epidermal growth factor receptor (EGFR), a gene widely expressed in colonic tissue. Increased EGFR expression is linked with poor prognosis in colon cancer. Within the EGFR gene there are two functional polymorphisms of interest: a polymorphism located at codon 497 (HER-1 R497K) and a dinucleotide (CA)n repeat polymorphism located within intron 1. These germ-line polymorphisms of EGFR were analyzed in genomic DNA from 318 metastatic colon cancer patients, 177 males and 141 females, collected from 1992 to 2003. Gender-related survival differences were associated with the HER-1 R497K polymorphism (PInteraction = 0.003). Females with the HER-1 497 Arg/Arg variant had better overall survival (OS) when compared with the Lys/Lys and/or Lys/Arg variants. In males the opposite was true. The EGFR dinucleotide (CA)n repeat also trended with a gender-related OS difference (PInteraction = 0.11). Females with both short <20 (CA)n repeat alleles had better OS than those with any long ≥20 (CA)n repeats. In males the opposite was true. Combination analysis of the two polymorphisms taken together also revealed the same gender-related survival difference (PInteraction = 0.002). These associations were observed using multivariable analysis. The two polymorphisms were not in linkage disequilibrium and are independent of one another. This study supports the role of functional EGFR polymorphisms as independent prognostic markers in metastatic colon cancer. As a prognostic factor, these variants had opposite prognostic implications based on gender. [Cancer Res 2008;68(8):3037–42]

Introduction

Colorectal cancer is the second most common cause of cancer-related death in the United States. In 2007, an estimated 153,760 new cases will be diagnosed and 52,180 deaths will occur (1). Epidermal growth factor receptor (EGFR), also known as HER-1 or erbB-1, is a transmembrane protein, a member of a human epithelial receptor tyrosine kinase family, and is widely expressed in colonic tissues (2). Activation of EGFR initiates signal transduction cascades that affect gene expression, cellular proliferation, inhibition of apoptosis, and angiogenesis (3). EGFR has shown prognostic value and is associated with poor survival, more aggressive behavior, and an increased risk of invasion/metastasis in colorectal cancers (4). Additionally, blocking EGFR ligand binding through interaction with therapeutic monoclonal antibody such as cetuximab and panitumumab has been shown to be an effective treatment for advanced colon cancer (5).

Two functional polymorphic variants of EGFR are of particular interest for this study. The first polymorphism is a single nucleotide change (G to A) leading to an arginine (Arg) to lysine (Lys) substitution in codon 497 (HER-1 R497K) in the extracellular domain within subdomain IV of the EGFR gene. An in vitro study has shown that the variant HER-1 497K has attenuated functions in ligand binding, growth stimulation, tyrosine kinase activation, and induction of proto-oncogenes (myc, fos, and jun) compared with the more prevalent “wild-type” HER-1 497R variant (6).

Another functional polymorphism is located within intron 1 of the EGFR gene. This polymorphism is associated with altering levels of EGFR transcription both in vitro and in vivo (7, 8). The length of this (CA)n dinucleotide polymorphism inversely correlates with transcriptional activity of the gene. In vitro models have shown more transcriptional activity in cell lines expressing the shorter polymorphic variant (16 CA repeats) compared with the longer polymorphic variant (>20 CA repeats; ref. 8). In vivo human breast tumors seem to select the shorter number of CA repeats ensuring higher expression levels of EGFR, which in turn propagates tumor growth and development (9).

Considerable evidence is accumulating supporting gender-related differences in the development of colonic carcinomas, with women of all ages less likely to develop colon cancer (9–11). Large comprehensive studies such as the Women’s Health Initiative have conclusively shown that postmenopausal women treated with estrogen replacement therapy have a significant reduction in both risk and rate of developing colon cancer (12, 13). The molecular mechanisms behind this protective effect against colon cancer are not fully understood. However, the colon does express both estrogen receptor β (14) and androgen receptor (15). Important functional linkage and bidirectional signaling between epidermal growth factor receptor (EGFR) and estrogen receptor have been shown (16). Important signaling regulation between EGFR and androgen receptor has also been shown (17). Because EGFR is affected by both female estrogen receptors (16) and male androgen receptors (17), EGFR may be a potential mediator of gender-related differences in colon cancer.

Based on this information, we tested the hypothesis that functional EGFR polymorphisms could be associated with the
gender-specific overall survival (OS) differences in patients with metastatic colon cancer.

Materials and Methods

Eligible patients. A total of 318 patients with metastatic colon cancer treated at the University of Southern California/Norris Comprehensive Cancer Center (USC/NCCC) or the Los Angeles County/University of Southern California Medical Center (LAC/USCMC), between 1992 and 2003, were eligible for the present study. This population included only metastatic or recurrent colon cancer patients. All patients in this study signed informed consents and enrolled in protocols designed to study the molecular determinants of colon cancer. These protocols permitted blood collection (USC protocol 05-99-10) and/or tissue collection (USC protocol 05-00-15).

All patients were entered and followed in an institutional database. Patient information was collected through database review and retrospective chart review when additional patient information was necessary. A large number of the patients (69%) were initially treated at an outside institution until, because of failure to respond to prior treatment, they were referred to USC/NCCC or LAC/USCMC for subsequent treatments. The end point of this study, OS, was determined by calculating the difference between the date of first treatment at USC/NCCC or LAC/USC and the date of last follow-up appointment or date of death from disease.

All 318 patients were enrolled in at least one chemotherapy clinical trial while being treated at this institution (USC/NCCC or LAC/USCMC). All patients were treated with 5-fluorouracil (5-FU)–based chemotherapy regimens, and response to chemotherapy was not investigated as an end point for this study. This is a heavily pretreated cohort with 20 (6%) patients treated with one line of chemotherapy, 19 (6%) patients treated with two different chemotherapy regimens, 183 (58%) patients treated with three chemotherapy regimens, and 96 (30%) patients treated with four or more chemotherapy regimens. Although the treatment regimens varied among patients, the majority of the patients were exposed to similar chemotherapies. All 318 patients received treatment with 5-FU, 298 (94%) patients received treatment with 5-FU/irinotecan (CPT-11), and 279 (88%) patients received treatment with 5-FU/oxaliplatin.

DNA extraction. Peripheral blood and paraffin-embedded tissue samples were collected from each patient. Genomic DNA was extracted from WBC or paraffinized tissue using the QiaAmp kit (Qiagen). Genomic DNA was obtained from peripheral blood for 314 of the patients. There were four samples for which peripheral blood was not available, and therefore genomic DNA was obtained from paraffin-embedded tissue. These 318 genomic DNA samples were used to analyze both polymorphisms.

HER-1 R497K polymorphism. The HER-1 R497K polymorphism was analyzed by PCR-RFLP as previously described (18). Briefly, a forward primer 5′-TGCTGTGACCCACTCTGTCT-3′ and a reverse primer 5′-CACAAGGTTGCACTTTGTCC-3′ were used for PCR amplification. After initial denaturation at 95°C for 3 min, the reaction was carried out at 94°C denaturation for 1 min, 59°C annealing for 1 min, and 72°C extension for 1 min for 35 cycles. PCR product was digested with BstNI restriction enzyme (New England Biolabs) at 60°C overnight and alleles were separated on 4% NuSieve ethidium bromide–stained agarose gel. For quality assurance purposes, 55 (17%) blind duplicate controls were matched. Results for all 55 duplicate controls were identical.

EGFR Intron 1 (CA)n repeat polymorphism. Genotyping of the EGFR CA Microsatellite polymorphisms was analyzed by PCR in combination with fluorescently labeled oligonucleotide primers. Briefly, the region of interest is amplified using a pair of oligonucleotide primers located in the unique flanking region on either side of the microsatellite repeat using primers GC023for (5′-TGCTGTGACCCACTCTGTCT-3′) and a reverse primer 5′-CACAAGGTTGCACTTTGTCC-3′. PCR reaction mix was prepared with HotStart Taq Polymerase (Qiagen) according to the manufacturer’s instructions using 20 ng of genomic DNA, 2 mmol/L MgCl2, and 300 μmol/L of each primer. PCR amplification was done in a thermal cycler (MWG Biotech) using a touchdown protocol with an initial step of 95°C for 15 min, finishing with 35 cycles of 95°C/25 s, 57°C/1 min, and 72°C/1 min. One of the oligonucleotides (GC023rev) was labeled with 6-FAM and the size of the PCR product, which was directly proportional to the number of

<table>
<thead>
<tr>
<th>Table 1. Demographic and baseline clinical information by sex</th>
<th>Total, n (%)</th>
<th>Female, n (%)</th>
<th>Male, n (%)</th>
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<tbody>
<tr>
<td>n</td>
<td>318</td>
<td>141</td>
<td>177</td>
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<td>Age, median (range), y</td>
<td>58 (25–86)</td>
<td>57 (25–82)</td>
<td>59 (25–86)</td>
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<td>≤39</td>
<td>28 (9)</td>
<td>16 (11)</td>
<td>12 (7)</td>
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<td>66 (47)</td>
<td>78 (44)</td>
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<td>59 (42)</td>
<td>87 (49)</td>
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<td>Ethnic</td>
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<td>Asian</td>
<td>43 (14)</td>
<td>24 (17)</td>
<td>19 (11)</td>
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<td>6 (4)</td>
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<td>7 (5)</td>
<td>17 (10)</td>
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<td>1 (1)</td>
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<td>Left</td>
<td>144 (46)</td>
<td>62 (30)</td>
<td>82 (57)</td>
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</tr>
<tr>
<td>Right</td>
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<td>65 (46)</td>
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<td>1</td>
<td>2</td>
<td></td>
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</table>

*Based on Fisher’s exact test.
The associations of cative product terms and nested Cox proportional hazards models that included the multiplicative terms were examined using stratified models and were tested by comparing corresponding likelihood ratio statistics between the baseline and nested models.

Results for all 38 duplicate controls were identical. In addition, 24 negative results files were automatically, modified manually if required, and output by the GeneMapper software in a tab-delimited format. The size of each allele was then converted into the number of CA repeats using Microsoft Excel. For quality assurance purposes, 92 (29%) blind duplicate controls were matched.

The allelic distribution of this polymorphism did not vary among genomic DNA was either consumed or degraded. Fifty percent of 316) of the patients had the Arg/Lys or the Lys/Lys variant. The location of the primary tumor within the colon was as follows: 144 (54%) left-sided tumors, 124 (46%) right-sided tumors, and 51 with the side unknown. The location of the first metastatic site was 156 (49%) liver, 56 (18%) intra-abdominal, and 46 (15%) other (lung, bone, stomach, spleen, pancreas, or gallbladder); there were 57 (18%) patients that presented with two or more metastatic sites at the onset of metastatic disease (Table 1).

OS differences in this population were not associated with gender differences, racial/ethnic distribution, location of primary tumor, or location of first metastatic lesion (data not shown). The EGFR polymorphisms were not associated with the age of onset of metastatic disease, location of primary tumor, or location of first metastatic lesion (data not shown).

**HER-1 R497K polymorphism.** The extracted genomic DNA was evaluated for HER-1 R497K polymorphism and the assay was successful in 316 of the 318 cases. There were two cases where the genomic DNA was either consumed or degraded. Fifty percent (157 of 316) of the patients had the Arg/Arg variant and 50% (159 of 316) of the patients had the Arg/Lys or the Lys/Lys variant. The allelic distribution of this polymorphism did not vary among the gender groups (Table 2). This distribution is consistent with other published findings (19). Asians were more likely to carry the Lys allele compared with other racial/ethnic groups (Table 2).
The HER-1 R497K genotypes were analyzed with regard to OS. When the population was not separated by gender, the genotypes were not associated with OS (data not shown). However, when the patient population was separated by gender, the polymorphic variants of HER-1 R497K were associated with OS ($P_{interaction} = 0.003$, likelihood ratio test). Male patients with the Arg/Arg variant ($n = 90$) had shorter OS (median OS, 103 months) than male patients with the Arg/Lys or the Lys/Lys variant ($n = 85$; median OS, 13.7 months; Table 2). In female patients the opposite OS difference was found. Female patients with the Arg/Arg variant ($n = 67$) had longer OS (median OS, 16.0 months) than female patients with the Arg/Lys or the Lys/Lys variant ($n = 74$; median OS, 14.0 months; Table 2). Therefore, as a prognostic factor, these HER-1 R497K polymorphic variants had opposite implications based on gender.

Patients with the Arg/Arg variant had a large shift in OS based on whether the patients were male (median OS, 10.3 months; $n = 78$) or female (median OS, 17.9 months; $n = 65$; Table 2; Fig. 1). However, patients with any long $\geq 20$ (CA)$_n$ repeat allele had their median OS remaining nearly constant: males, 13.1 months ($n = 96$); females, 14.1 months ($n = 72$; Table 2).

**Combination analysis.** The two EGFR polymorphisms were analyzed together using combination analysis. Again, the polymorphic variants were associated with opposite implications for survival based on gender ($P_{interaction} = 0.002$, likelihood ratio test; Table 2). Males with any long $\geq 20$ (CA)$_n$ repeat allele and the HER-1 Lys/Lys or Arg/Lys variant had statistically significant better survival (OS of 13.6 months) than the males with two short $<20$ (CA)$_n$ repeat alleles and the HER-1 Arg/Arg variant (OS of 8.9 months; adjusted $P = 0.094$; Fig. 3). In the female population the opposite was found. Females with any long $\geq 20$ (CA)$_n$ repeat allele and the HER-1 Lys/Lys or Arg/Lys variant had statistically significant shorter OS (12.2 months) than the females with two short $<20$ (CA)$_n$ repeat alleles and the HER-1 Arg/Arg variant (15.7 months; adjusted $P = 0.008$; Fig. 4). As a prognostic factor in combination these two EGFR polymorphic variants had opposite implications based on gender.

**Linkage disequilibrium.** The HER-1 R497K and the EGFR (CA)$_n$ repeat polymorphisms showed no statistically significant evidence of linkage disequilibrium in this patient population (data not shown).
Discussion

The HER-1 R497K polymorphic variant was associated with longer survival in females but with shorter survival in males. A previous in vitro study has shown that this HER-1 R497K polymorphism is related to attenuated ligand binding, reduced growth stimulation, diminished tyrosine kinase activation, and limited induction of proto-oncogenes. The "wild-type" Arg/Arg genotype does not cause this loss of EGFR ligand binding (6). Activation of EGFR in colon cancer is associated with a worse prognosis (4). Therefore, the Arg/Arg genotype would be expected to be associated with a worse prognosis. The males in our study did show this association [i.e., the Arg/Arg genotype (OS, 10.3 months) had a worse prognosis]. In contrast, the opposite was observed for females with the Arg/Arg genotype who had a better prognosis (OS, 16.0 months). This gender-related survival difference in the HER-1 R497K polymorphism in colon cancer is novel. The exact mechanistic interactions that contribute to this observed association are currently unclear.

The other EGFR polymorphism, the dinucleotide (CA)n repeat, also had opposite OS associations based on gender. This variant was associated with longer survival in females and with shorter survival in males. Previous in vitro and in vivo studies have shown this EGFR dinucleotide (CA)n repeat polymorphism to effect the expression of EGFR. These studies have shown that shorter (CA)n repeat lengths, <20 base-pairs long, have higher expression of EGFR (7, 8). High expression of EGFR in the colon is a known poor prognostic factor (4). Therefore, the patients with the shorter (CA)n repeat variants are expected to have a worse prognosis. The males in our study population did show a clinical outcome that was consistent with these previous findings; males with two short <20 repeat alleles had a worse prognosis (OS, 10.4 months). However, the opposite was observed in females. Females with the two short <20 repeat alleles had a better prognosis (OS, 17.6 months). This gender-related survival difference for this (CA)n repeat polymorphism in colon cancer is novel. The exact mechanistic interactions that contribute to this observed association are unclear.

The HER-1 R497K and the EGFR dinucleotide (CA)n repeat polymorphisms had no evidence of linkage disequilibrium in this patient population. Despite their genetic independence, the two EGFR polymorphisms displayed similar gender-related inverse OS associations using combination statistical analysis. Again, the polymorphic variants were associated with longer survival in the females but were associated with shorter survival in the males. Therefore, they have opposite prognostic value based on gender and are independent of one another.

In this study, the male population had findings consistent with the literature (4, 6–8). However, the female population in this study had exactly the opposite findings from those in the male population and from the prior literature (4, 6–8). In the female population, both variant EGFR polymorphisms, previously described as poor prognostic markers and previously associated with high expression and/or ligand binding of EGFR (6–8), showed an unexpected survival benefit.

This study has shown for the first time that functional polymorphisms of EGFR are inversely related to gender-specific OS in patients with metastatic colon cancer. Gender differences in this population are important. When the study population was not separated by gender, the EGFR polymorphisms were not associated with survival; when the population was separated by gender, associations with survival were observed. This shows the potential importance of analyzing colon cancer data both with and without gender as a stratifying factor.

EGFR activation of its signaling pathways may occur through intermediaries that result in different activation in males and females. Because the colon expresses both estrogen receptor β (14) and androgen receptor (15), and EGFR interacts with both steroid hormone receptor pathways (16, 17), EGFR may have molecular intermediates that interact in a gender-specific way to effect EGFR pathway activation.

The design of this study is such that information about menopausal status, use of estrogen replacement therapy, and the relative amount of sex hormones present in each patient at the time of treatment was not known. Therefore, it is not possible to address the potential importance of sex steroid hormones in this patient population. Additional studies are warranted to determine the molecular reasons for the observations made in this study.

This study may have implications for treatment of males and females with metastatic colon cancer. A phase III clinical trial (European Organization for Research and Treatment of Cancer 05963) comparing two different chemotherapy regimens for optimal dynamic scheduling of oxaliplatin, 5-FU, and leucovorin...
in metastatic colorectal cancer patients showed no survival differences until the population was separated by gender (22). In this study, gender was associated with a treatment-specific inversion of OS benefit among males and females. Another study indicated that females with colorectal cancer are more likely to respond to 5-FU–based chemotherapy compared with males (23). Therefore, gender-related, treatment-specific differences in the colon have been shown. These differences may be due to different chemosensitivities resulting from hormonal variations among genders. Our study has shown that EGFR polymorphic variations are associated with gender-related OS differences.

In summary, this study supports the role of functional polymorphisms of EGFR as independent prognostic markers in metastatic colon cancer with opposite prognostic implications in males and females. To our knowledge, this is the first study that shows a relationship between EGFR gene polymorphisms and gender-related survival. Future studies are needed to confirm our study.

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