Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

Tumor and Stem Cell Biology

Cancer Research

A Single Nucleotide Change in the Mouse Genome Accelerates Breast Cancer Progression

Nina Seitzer, Thomas Mayr, Sylvia Streit, and Axel Ullrich

Abstract

In the growth factor receptor gene *FGFR4* the presence of the common single nucleotide polymorphism Arg388 has been associated with progression of various types of cancer including breast cancer. However, a causative relationship is not readily assigned due to genetic heterogeneity in different patient cohorts. To address this issue, we compared the effects of this allele on malignant progression in the *WAP-TGFα* transgenic mouse model of breast cancer. A knock-in strain was generated to introduce an analogous Arg385 allele into the murine *FGFR4* gene. Mouse embryonic fibroblasts derived from this strain displayed accelerated cell transformation, with transformed cells exhibiting greater motility and invasive behavior. In the *in vivo* context of TGFα-induced mammary carcinogenesis, tumor development and progression was significantly advanced in tumor mass, size, and onset of pulmonary metastases. Our findings definitively identify the *FGFR4 Arg388* allele as a functional prognostic marker for breast cancer progression. *Cancer Res; 70(2); 802–12. ©2010 AACR.*

Introduction

In recent years, it has become increasingly clear that in addition to somatic mutations, germ-line alterations such as single nucleotide polymorphisms (SNP) have clinical significance for the development and progression of diseases such as cancer as well as for the definition of a patient's individual response to therapeutic agents (1, 2).

In the human *FGFR4*, a polymorphic nucleotide change in codon 388 converts glycine to arginine in the transmembrane region of the receptor (3). This SNP was shown to be implicated in progression and poor prognosis of various types of human cancer (3–8). Discovered by Bange and colleagues (3), the *FGFR4Arg388* allele could be associated with tumor progression in breast and colon cancer patients. Similarly, soft tissue sarcoma patients, who carried the *FGFR4Arg388* allele, had a poor clinical outcome (9). In melanoma, the *FGFR4Arg388* allele is associated with increased tumor thickness, whereas in head and neck squamous cell carcinoma the glycine-arginine substitution correlates with reduced overall patient survival and advanced tumor stage (6, 7). Furthermore, a recent study on prostate cancer patients associated the *FGFR4Arg388* allele not only with tumor progression but also with initiation (8). Breast cancer studies correlate the *FGFR4Arg388* allele with both accelerated disease progression and higher resistance to adjuvant systemic or chemotherapy, leading to a significantly shorter disease-free and overall survival (3, 10).

The main conclusion of these studies was that the presence of one or two *FGFR4Arg388* alleles in the genome does not initiate cancer development but predisposes the carrier to a more aggressive form. Unfortunately, due to the highly complex and heterogeneous genetic background of the patients, statistical analysis yielded at times marginal results, and because of differences in patient stratification and statistical evaluation, diverging results led to controversies (11). Because of that and in spite of the strong association of the *FGFR4Arg388* allele with disease progression, this genetic configuration is not yet established as progression marker for clinical outcome or as basis for individual patient treatment decisions.

Here, we show in a genetically "clean" system the effect of a single nucleotide difference in codon 385 of the mouse *FGFR4* gene (corresponding to codon 388 in the human) on breast cancer progression. We generated an *FGFR4Arg385* knock-in (KI) mouse model to investigate the effect of the *FGFR4* isotypes on the physiology of mouse embryonic fibroblasts (MEF) and on mammary cancer progression *in vivo*. For this purpose, we crossed the *FGFR4Arg385* KI mice to *WAP-TGFα* and *MMTV-PymT* transgenic mice. In the *WAP-TGFα* model, transforming growth factor α (TGFα) overexpression is controlled by the whey acidic protein (*WAP*) promoter, which specifically activates the transgene in mammary epithelial cells in mid-pregnancy, thereby promoting mammary carcinogenesis (12, 13). In the *MMTV-PymT* model, overexpression of PymT leads to the constitutive activation of the proto-oncogene src, which in turn causes the development of breast cancer (14).

Here, we report that the mouse *FGFR4Arg385* allele enhances the transformation rate of isolated MEFs; promotes

Authors' Affiliation: Department of Molecular Biology, Max Planck Institute of Biochemistry, Martinsried, Germany

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for S. Streit: Department of Surgery, Technische Universität München, Munich, Germany.

Corresponding Author: Axel Ullrich, Department of Molecular Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 85152 Martinsried, Germany. Phone: 49-89-8578-2512; Fax: 49-89-8578-2454; E-mail: ullrich@biochem.mpg.de.

doi: 10.1158/0008-5472.CAN-09-3239

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

their migration, anchorage independence, and invasion after stable transformation; and, above all, enhances the progression of breast cancer in the *WAP-TGFα* mouse mammary carcinoma model. Furthermore, the *FGFR4Arg385* allele accelerates lung metastatic lesions *in vivo*.

These results support previous clinical correlation studies on cancer patients with diverse genetic profiles and highlight the importance of the *FGFR4Arg388* allele in cancer progression. This SNP may therefore serve as a prognostic marker of clinical outcome for breast cancer patients.

Materials and Methods

Mouse embryonic fibroblasts. MEFs were isolated from 13.5-day postcoitum embryos and maintained following the 3T3 protocol (15). To stably overexpress epidermal growth factor receptor (EGFR), v-src, and empty pLXSN, MEFs were selected with G418 24 h after infection. Transmigration of MEFs was analyzed in Boyden chambers (Schubert & Weiss). Cells (1.5×10^4) were seeded in DMEM containing 0% FCS. Migration was performed to DMEM containing 4% FCS for 16 h. Afterward, cells were stained with crystal violet and migrated cells were analyzed microscopically. For quantification, Boyden chamber membranes were destained in 5% acetic acid and analyzed in an ELISA reader. For the soft agar assay, cells (1×10^5) were added to 3 mL DMEM supplemented with 10% fetal bovine serum and 0.3% agar and layered onto 6 mL of 0.5% agar beds in 60-mm dishes. After 24 to 96 h, anchorage independence of cells was calculated and quantified microscopically. To perform a Matrigel assay, 5×10^3 cells were seeded on Matrigel-coated (BD Biosciences) 96-well plates. After 24 to 96 h, Matrigel outgrowth was calculated and quantified microscopically.

RNA and reverse transcription-PCR. Total RNA of minced murine tissues was isolated using the RNeasy kit (Qiagen) according to the manufacturer's recommendation. cDNA was produced with the first-strand cDNA kit (Boehringer Mannheim) according to the manufacturer's protocol. Raw data were quantified via ImageJ software, normalized on the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and plotted relatively to the control that was set on 100%.

Immunoprecipitation and Western blotting. Tumor samples were minced by an Ultratorax (Janke & Kunkel, IKA Labortechnik). Tumor samples and cultured cells were lysed in radioimmunoprecipitation assay lysis buffer containing phosphatase and proteinase inhibitors for 30 min and precleared by centrifugation. For immunoprecipitation, lysates (1,000 μg protein) were incubated with protein A–Sepharose beads (GE Healthcare) and the primary antibody at 4°C overnight and subjected to Western blot analysis. Raw data were quantified via ImageJ software, normalized on the expression levels of actin/tubulin, and plotted relatively to the control. The following primary antibodies were used: FGFR4 (Santa Cruz Biotechnology), 4G10 (Upstate), and α-actin/α-tubulin (Sigma). The following secondary antibodies were used: horseradish peroxidase (HRP)–conjugated α-rabbit (Bio-Rad) and HRP-conjugated α-mouse (Sigma).

Tumor analysis. Mice were sacrificed by cervical dislocation and opened ventrally. Mammary glands and tumors were excised for measurement. The area and mass of tumors or normal mammary glands were analyzed by metrical measurement and weighing of the tumor tissue and the mammary gland tissue independently. Raw data were normalized on body weight.

Statistical analysis. All data are shown as mean ± SD; all *P* values were calculated using the Student's *t* test and values ≤0.03 were considered statistically significant.

Histology and immunohistochemistry. Murine tissues were fixed in 70% ethanol at 4°C overnight. Samples were embedded in paraffin and sectioned (4–8 μm) on a microtome (HM355S, Microm). Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was achieved by cooking in citrate buffer (pH 6) in a microwave. Immunohistochemistry was done with the Vectastain staining kit (Vector Laboratories) following the manufacturer's protocol. After blocking with 10% horse serum in PBS/3% Triton X-100 for 1 h, the sections were incubated with the primary antibody at 4°C overnight. The secondary antibody (α-rabbit; Vector Laboratories) was incubated for 1 h in PBS/3% Triton X-100. Mayer's hematoxylin (Fluka) was used as a counterstain.

For analysis of metastases, lungs were sectioned and analyzed at 800- to 1,000-μm intervals. Sections were stained with H&E (Fluka). Metastatic burden was calculated microscopically.

Results

The* FGFR4Arg385 *allele facilitates cell transformation, migration, anchorage-independent growth, and branching of EGFR-transformed MEFs. To ultimately prove the influence of the *FGFR4Arg388* allele on tumor progression *in vivo*, we generated an *FGFR4Arg385* KI model in the genetic background of SV/129 mice, which represents the first directly targeted KI mouse model to investigate the effect of a SNP on the progression of cancer (Supplementary Fig. S1).

Similar to the human situation (3), the *FGFR4Arg385* KI mouse model displays no obvious phenotype that distinguishes it from *FGFR4Gly385*-carrying mice, and FGFR4 expression and localization does not differ between the different *FGFR4* isotypes (Supplementary Fig. S2).

MEFs represent a useful *in vitro* system to investigate the effect of altered genetic loci. Thus, we investigated the effect of the *FGFR4Arg385* allele on biological functions of isolated MEFs. Western blot analysis displays neither an overexpression nor a hyperactivation of the FGFR4Arg385 in MEFs (Supplementary Fig. S3*A*). We further analyzed the effect of the *FGFR4Arg385* allele on several processes, including migration, cell proliferation, and cellular life span; however, *FGFR4Arg385*-carrying MEFs do not show an increased proliferative capacity, life span, or migration compared with *FGFR4Gly385* MEFs (Supplementary Fig. S3*B* and *C*). Previous reports of clinical studies do not implicate the *FGFR4Arg388* allele in tumor initiation but rather associate it with enhanced disease progression (3, 6). For that reason, we

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

performed focus formation assays with HER2, EGFR, and v-kit to determine if a certain *FGFR4* genotype could enhance the transformation and the progression of MEFs together with different receptor tyrosine kinases acting as the transformation-initiating oncogenes. Infection with the oncogene v-src or empty pLXSN vector served as a positive or negative control in all experiments. *FGFR4Gly/Arg385* MEFs display a significantly increased number of foci in cooperation

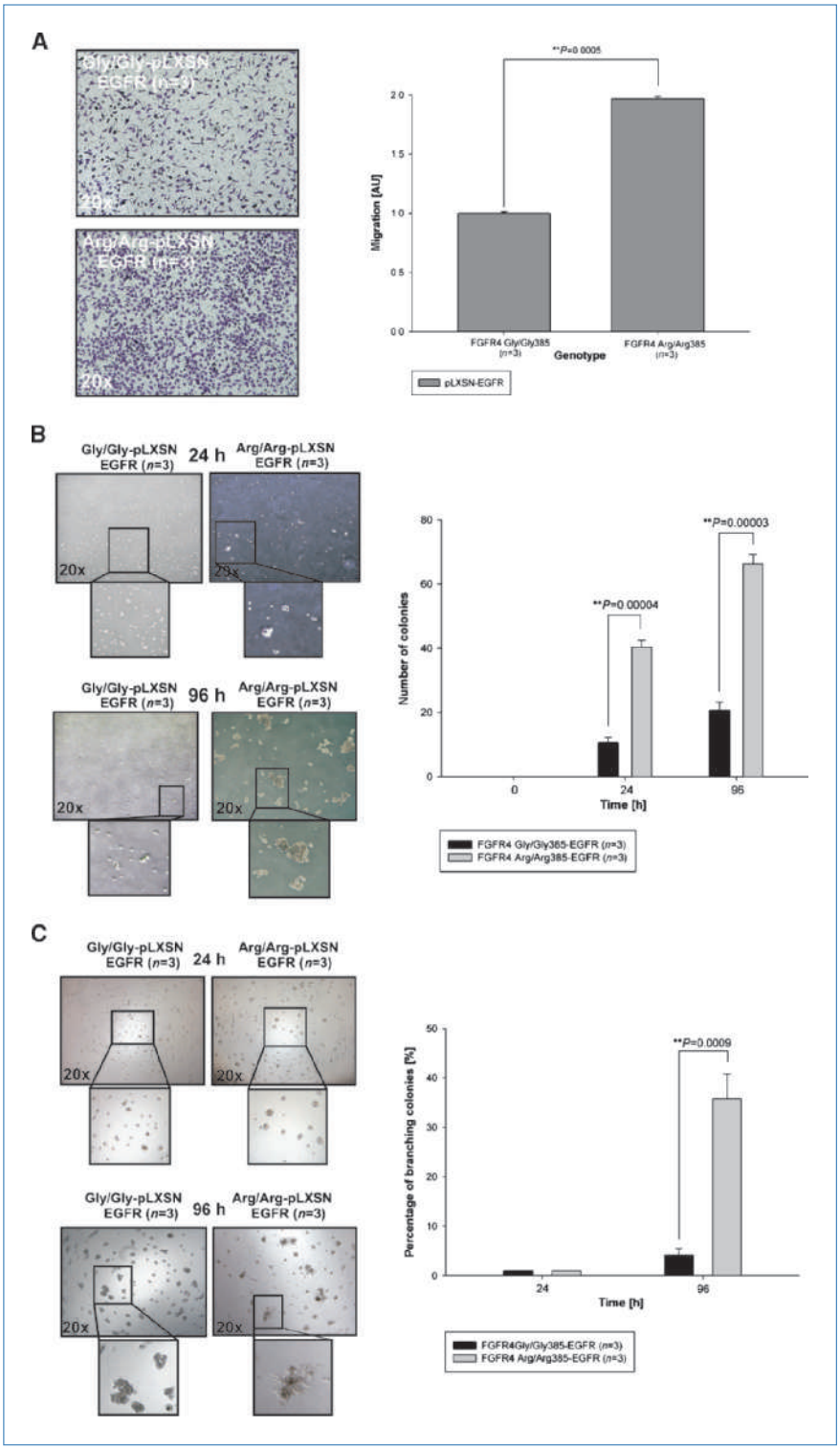

Figure 1. The *FGFR4Arg385* allele facilitates cellular transformation, migration, anchorage-independent growth, and branching in EGFR-transformed MEFs. *A,* migration assay of EGFR-transformed *FGFR4Gly/Gly385* and *Arg/Arg385* MEFs. Migration was analyzed after 16 h microscopically and quantified. *B,* soft agar colony formation assay of EGFR-transformed *FGFR4Gly/Gly385* and *Arg/Arg385* MEFs. Anchorage independence was analyzed after 24 to 96 h and quantified microscopically. *C,* Matrigel outgrowth of EGFR-transformed *FGFR4Gly/Gly385* and *Arg/Arg385* MEFs. Matrigel outgrowth was analyzed after 24 to 96 h and quantified microscopically.

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

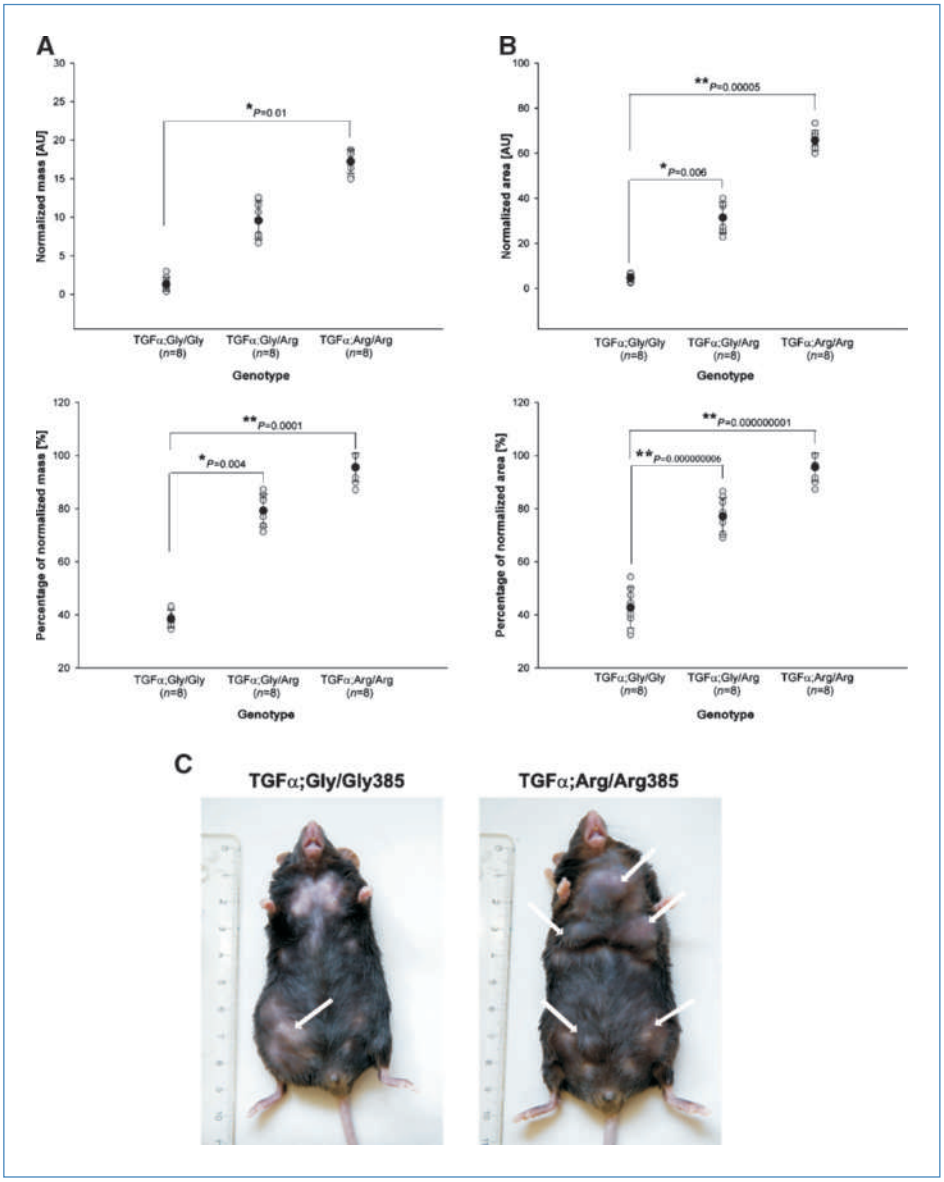

Figure 2. The *FGFR4Arg385* allele promotes breast tumor progression in the *WAP-TGFα* mouse mammary carcinoma model. In *A* and *B*, every data point represents the values of one female *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, or *WAP-TGFα;FGFR4Arg/Arg385* mouse. Mice were sacrificed after 6 mo of tumor progression. *A, WAP-TGFα;FGFR4Arg/Arg385* mice display a significantly increased tumor mass and percentage of tumor mass compared with *WAP-TGFα;FGFR4Gly/Gly385* mice. *B, WAP-TGFα;FGFR4Arg385* mice display a significantly increased tumor area and percentage of tumor area compared with *WAP-TGFα;FGFR4Gly/Gly385* mice. *C, white arrows, WAP-TGFα;FGFR4Arg/Arg385* mice display a visibly increased tumor progression after 8 mo compared with *WAP-TGFα;FGFR4Gly/Gly385* mice.

with HER2 and EGFR, whereas *FGFR4Arg/Arg385* MEFs show a significantly enhanced focus formation with all three investigated oncogenes (Supplementary Fig. S3*D*). Furthermore, a time course analysis showed that *FGFR4Arg/Arg385*-carrying MEFs not only transform considerably faster but also generate an increased number of foci over time. These results indicate that the *FGFR4Arg385* isotype clearly promotes the transformation that is initiated by different oncogenes and seems to facilitate the transformation of MEFs, resulting in a higher number of foci that occur at earlier time points.

Next to v-kit, the transformation of *FGFR4Arg385* MEFs with EGFR displays an unusually high activity in the focus formation assay. Therefore, we aimed to investigate the involvement of the *FGFR4Arg385* allele on several physiologic processes using stable EGFR-overexpressing *FGFR4Gly385* and *Arg385* MEFs. To ensure equal expression among the infected MEFs, overexpression of EGFR and v-src was analyzed via immunoblot analysis and quantification (Supplementary Fig. S4*A*). Interestingly, FGFR4 is clearly upregulated in EGFR-transformed cells compared with v-src–transformed MEFs and even more upregulated and hyperactivated in *FGFR4Arg385* relative to *Gly385* MEFs. We further investigated if this upregulation of the *FGFR4Arg385* allele compared with *FGFR4Gly385* in EGFR-transformed MEFs influences certain biological processes. About proliferation, EGFR-transformed MEFs display no differences between the *FGFR4* isotypes (Supplementary Fig. S4*B*). In contrast, *FGFR4Arg385* MEFs transformed with EGFR display a significantly increased migratory capacity compared with *Gly385* MEFs (Fig. 1*A*). Next, we analyzed anchorage independence of EGFR-transformed MEFs in soft

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

agar colony formation assays. *FGFR4Arg/Arg385* EGFR-transformed MEFs display a significantly accelerated anchorage-independent growth after 24 and 96 hours (Fig. 1*B*). Subsequently, we analyzed the effect of the *FGFR4Arg385* allele on invasivity in a Matrigel assay (Fig. 1*C*). EGFR-transformed MEFs display significantly accelerated branching in Matrigel after 24 and 96 hours in the presence of the *FGFR4Arg385* allele. In contrast, cell migration, soft agar colony formation, and branching in Matrigel were not promoted by the *FGFR4Arg385* allele in v- src–transformed MEFs (Supplementary Fig. S5).

These results show that the *FGFR4Arg385* allele accelerates physiologic processes in EGFR-transformed MEFs, including migration, invasion, and anchorage independence, which all contribute to tumor progression. Furthermore, the effect of the *FGFR4Arg385* allele is dependent on the genetic background that triggers malignant transformation.

The* FGFR4Arg385 *allele promotes breast cancer progression in the* WAP-TGFα *mouse mammary carcinoma model. The *in vitro* experiments in primary and transformed MEFs show the effect of the *FGFR4Arg385* allele on tumor progression on several cell biological processes. Furthermore, the effect of the *FGFR4Arg385* isotype seems to be dependent on the oncogenic background. To ultimately clarify the influence of the *FGFR4Arg385* allele on tumor progression and accelerated aggressiveness, we investigated the effect of the *FGFR4Arg385* allele on breast cancer progression *in vivo*. Similar to the experiments *in vitro*, we analyzed the involvement of the *FGFR4Arg385* allele on tumor progression in combination with the well-established *WAP-TGFα* and the *MMTV-PymT* transgenes (Supplementary Fig. S6*A* and *B*).

To investigate the effect of the *FGFR4Arg385* allele on tumor progression in the *WAP-TGFα* model, we analyzed tumors of 6-month-old female *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, and *WAP-TGFα;FGFR4Arg/Arg385* mice. The analyzed criteria for tumor progression are the mass, area, and the percentage of mass and area compared with the whole mammary gland of the analyzed tumors. As shown in Fig. 2*A*, the tumor mass is significantly increased in *WAP-TGFα;FGFR4Arg/Arg385* mice when compared with *WAP-TGFα;FGFR4Gly/Gly385* controls. Furthermore, the percentage of tumor mass is significantly promoted in *WAP-TGFα;FGFR4Arg/Arg385* mice (Fig. 2*A*). Moreover, the tumor area and the percentage of tumor area are significantly accelerated in *WAP-TGFα;FGFR4Arg/Arg385* mice (Fig. 2*B*). These results indicate that the *FGFR4Arg385* allele is a potent enhancer of *WAP-TGFα*–induced mammary tumors. Additionally, the higher significance in the area of tumors suggests that the *FGFR4Arg385* allele is not an enhancer of cancer cell proliferation but seems to accelerate migration, resulting in an increased invaded area of the mammary gland. The analyzed control mammary glands of *FGFR4Gly/Gly385, Gly/Arg385*, and *Arg/Arg385* mice without an oncogenic background do not display any changes in their mass, size, or pathology (Supplementary Fig. S6*C* and *D*).

The potent tumor-enhancing effect of the *FGFR4Arg385* allele is apparent when comparing *WAP-TGFα;FGFR4Arg/Arg385* mice with *WAP-TGFα;FGFR4Gly/Gly385* controls sacrificed after 8 months of tumor progression (Fig. 2*C*). Mice transgenic for *WAP-TGFα* display more as well as larger tumors in the presence of the *FGFR4Arg385* allele (*white arrows*).

In addition to the *WAP-TGFα* mouse model, we also investigated the tumor-promoting effect of the *FGFR4Arg385* allele in the *MMTV-PymT* mouse mammary carcinoma model. Because of the *in vitro* results with v-src–transformed MEFs, we aimed to investigate if the tumor-promoting action of the *FGFR4Arg385* allele is likewise *in vitro* not apparent *in vivo*.

Therefore, we analyzed the tumors of 3-month-old female *MMTV-PymT;FGFR4Gly/Gly385, MMTV-PymT;FGFR4Gly/Arg385*, and *MMTV-PymT;FGFR4Arg/Arg385* mice. As seen in Supplementary Fig. S7*A* and *B*, there is a significant difference neither in tumor size nor in tumor mass between the *FGFR4* isotypes in mice transgenic for *MMTV-PymT*. Thus, the tumor-promoting effect of the *FGFR4Arg385* allele *in vivo* is likewise *in vitro* dependent on the genetic background, which triggers oncogenesis.

The* FGFR4Arg385 *allele is hyperactivated and promotes a more aggressive phenotype in the expression pattern of* WAP-TGFα*–derived tumors. To further investigate the underlying mechanism of the tumor-promoting effect of the *FGFR4Arg385* allele, we studied molecular differences of the *FGFR4* alleles. In many human cancers, overexpression of FGFR4 is a commonly observed feature of tumors (16, 17). Therefore, we examined FGFR4 expression in tumors derived from 6-month-old *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, and *WAP-TGFα;FGFR4Arg/Arg385* mice. Here, the FGFR4 protein is clearly overexpressed in tumors compared with normal mammary glands; however, there was no detectable difference in the presence of the *FGFR4Arg385* alleles (Supplementary Fig. S8*A*). Contrarily, the FGFR4Arg/Arg385 displays a significantly enhanced activation compared with the FGFR4Gly/Gly385 (Fig. 3*A*), suggesting that the tumor-promoting potential of the *FGFR4Arg385* allele is possibly due to an enhanced kinase activity. Because of the higher FGFR4Arg/Arg385 activity, we aimed to determine the expression and activation of phosphorylated extracellular signal-regulated kinase and phosphorylated Akt; however, we could not detect a difference in the activation of these molecules (Supplementary Fig. S8*B*). We further investigated the expression levels of FGFR4 in 3-month-old hyperplastic mammary glands and 6-month-old adenocarcinomas from *WAP-TGFα;FGFR4Gly/Gly385* and *WAP-TGFα;FGFR4Arg/Arg385* mice immunohistochemically. Interestingly, the expression of FGFR4 in *WAP-TGFα;FGFR4Arg/Arg385* hyperplasias is clearly increased compared with *WAP-TGFα;FGFR4Gly/Gly385* mice (Fig. 3*B*), indicating that the *FGFR4Arg/Arg385* allele potentially affects the onset of mammary tumor progression. Similarly to the Western blot analysis, the expression of FGFR4 in adenocarcinomas does not alter the presence of the *FGFR4Arg385* allele (Supplementary Fig. S8*C*).

In addition, we wanted to investigate the mRNA expression of genes associated with an aggressive tumor phenotype, such as motility, invasivity, and angiogenesis, in 6-month-old tumors from *WAP-TGFα;FGFR4Gly/Gly385* and *WAP-TGFα;FGFR4Arg/Arg385* mice (Fig. 3*C*). The FGFR4 and the EGFR

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

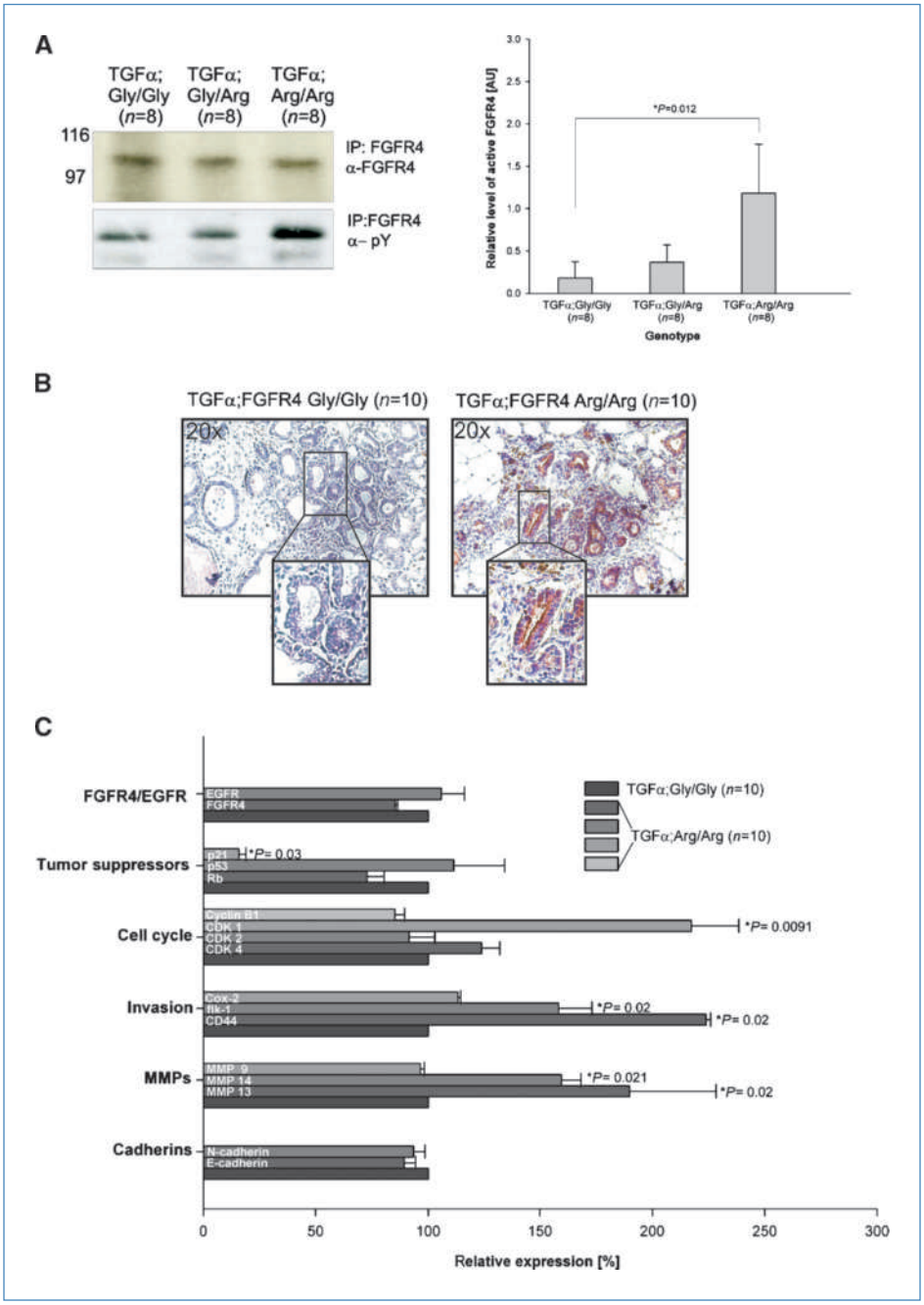

Figure 3. The *FGFR4Arg385* allele is hyperactivated and promotes a more aggressive phenotype in the expression pattern of *WAP-TGFα*–derived tumors. *A,* activation of FGFR4 in 6-mo-old *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, or *WAP-TGFα;FGFR4Arg/Arg385* tumors. FGFR4Arg/Arg385 display a significantly increased activation compared with FGFR4Gly385. Quantification was done relative to FGFR4 expression levels. *B,* increased FGFR4Arg/Arg385 expression in 3-mo-old *WAP-TGFα;FGFR4Arg/Arg385* hyperplasias compared with *WAP-TGFα;FGFR4Gly/Gly385*. FGFR4 expression was detected immunohistochemically and analyzed microscopically. *C,* reverse transcription-PCR analysis of 6-mo-old *WAP-TGFα; FGFR4Gly/Gly385* or *WAP-TGFα; FGFR4Arg/Arg385* tumors. GAPDH served as normalization value. Expression values of *WAP-TGFα;FGFR4Arg/Arg385* tumors are blotted relatively to the expression values of *WAP-TGFα; FGFR4Gly/Gly385* tumors and grouped according to their physiologic function. *WAP-TGFα; FGFR4Arg/Arg385* tumors significantly overexpress genes involved in migration, invasion, and vascularization.

display no overexpression in the presence of the *FGFR4Arg385* allele. Among analyzed tumor suppressors, p21 was found to be significantly downregulated in *WAP-TGFα;FGFR4Arg/Arg385*–induced tumors. About proliferation markers, cyclin-dependent kinase 1 (CDK1) was significantly upregulated in *WAP-TGFα;FGFR4Arg/Arg385*–induced tumors. As CDK1 is strongly associated with migration, this significant overexpression might promote a more aggressive phenotype of *FGFR4Arg/Arg385*-carrying tumors (18). In the group of invasion and angiogenic markers, CD44 and flk-1 were significantly overexpressed in *FGFR4Arg385* tumors, which both might contribute to a more aggressive phenotype of *FGFR4Arg385* tumors (19). In the cluster of matrix metalloproteinases (MMP), MMP13 as well as MMP14 were overexpressed in *FGFR4Arg385* tumors, possibly contributing to a higher metastatic potential (20).

These data strongly suggest that *WAP-TGFα;FGFR4Arg/Arg385*–induced tumors display a more aggressive behavior resulting in an accelerated tumor progression.

The* FGFR4Arg385 *allele decreases the time point of tumor incidence and promotes tumor progression over time in the* WAP-TGFα *transgenic model. To further analyze the

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

tumor-promoting effect of FGFR4Arg385 in the *WAP-TGFα* model, we followed tumor progression by sacrificing the female mice at defined periods.

As shown in Fig. 4*A*, the visible time point of tumor incidence is significantly decreased in *WAP-TGFα;FGFR4Arg385* mice, suggesting that the *FGFR4Arg385* allele facilitates neoplastic transformation and thereby decreases the time point of tumor incidence. To ensure that these data are independent of the genetic background, we backcrossed the *WAP-TGFα* and the *FGFR4Arg385* KI mice to the FVB background. Here, we also analyzed the visible tumor incidence in *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, and *WAP-TGFα;FGFR4Arg/Arg385* mice. Like in the C57BL/6 background, the visible tumor incidence is significantly decreased in mice carrying the *FGFR4Arg385* allele (Supplementary Fig. S7*C*).

In the C57BL/6 background, we further investigated tumor progression over time by analyzing the number of tumors, mass, area, and the percentage of mass and area of the dissected tumors of *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, and *WAP-TGFα;FGFR4Arg/Arg385* mice. As shown in Fig. 4*B*, *WAP-TGFα;FGFR4Arg/Arg385* mice just partly establish a significant larger amount of tumors at very late points of tumor progression. However, *FGFR4Arg385*-carrying mice seem to induce a larger amount of tumors but, importantly, increase their number of tumors over time faster than the *FGFR4Gly/Gly385* mice. In addition, *WAP-TGFα;FGFR4Arg/Arg385* mice establish only a significantly higher tumor mass at very early time points (Fig. 4*C*). Nevertheless, the *FGFR4Arg385* allele seems to clearly progress tumor mass over time. In contrast, the percentage of tumor mass is significantly increased in *WAP-TGFα;FGFR4Arg/Arg385* mice. The tumor area is mostly significantly increased in *WAP-TGFα;FGFR4Arg/Arg385* mice (Fig. 4*D*). The most significant difference is shown in the percentage of tumor area, where both *WAP-TGFα;FGFR4Gly/Arg385* mice and *WAP-TGFα;FGFR4Arg/Arg385* mice display a significant increase in the percentage of tumor area compared with *WAP-TGFα;FGFR4Gly/Gly385* mice (Fig. 4*D*).

In summary, the *FGFR4Arg385* allele promotes breast tumor progression over time and facilitates the initiation of oncogenesis and thereby shortens the time point of tumor onset.

The* FGFR4Arg385 *allele promotes cancer cell metastasis. As clinical outcome of cancer is strongly dependent on the invasive stage of the primary tumor, it is essential to investigate the effect of the *FGFR4Arg385* allele on metastases of *WAP-TGFα*–derived tumors. Importantly, the expression of genes involved in metastasis and invasion is significantly upregulated in *WAP-TGFα;FGFR4Arg/Arg385*–derived tumors. Therefore, we investigated the occurrence of

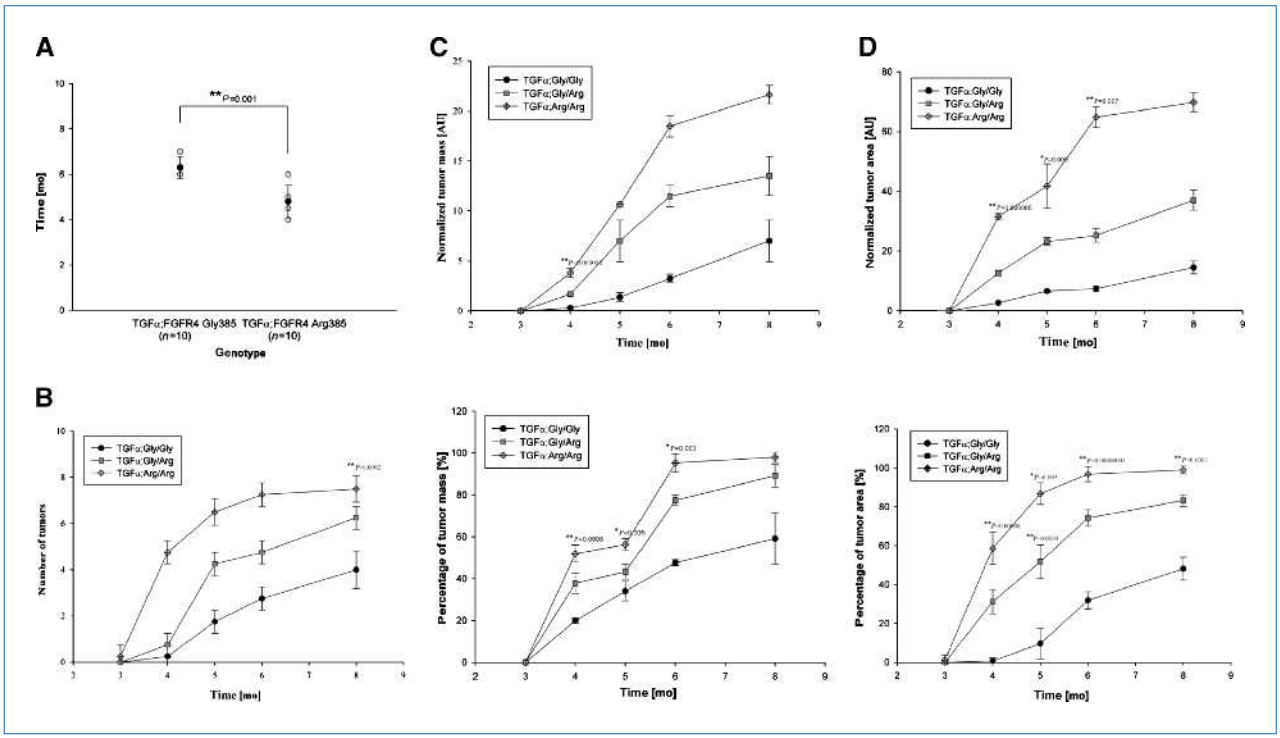

Figure 4. The *FGFR4Arg385* allele decreases time point of tumor incidence and promotes tumor progression over time in the *WAP-TGFα* transgenic model. In *B* to *D*, every data point represents the values of three or more female *WAP-TGFα;FGFR4Gly/Gly385*, *WAP-TGFα;FGFR4Gly/Arg385*, or *WAP-TGFα;FGFR4Arg/Arg385* mice. *A, WAP-TGFα;FGFR4Arg385* mice show a significantly earlier time point of visible tumor incidence compared with *WAP-TGFα;FGFR4Gly385* mice. *B, WAP-TGFα;FGFR4Arg/Arg385* mice partly establish a significantly higher number of tumors over time compared with *WAP-TGFα;FGFR4Gly385* mice. *C, WAP-TGFα;FGFR4Arg/Arg385* mice partly display a significant increase of tumor mass and the percentage of tumor mass over time compared with *WAP-TGFα;FGFR4Gly385* mice. *D, WAP-TGFα;FGFR4Arg385* mice partly display a significant increase in the tumor area and the percentage of tumor area over time compared with *WAP-TGFα;FGFR4Gly/Gly385* mice.

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

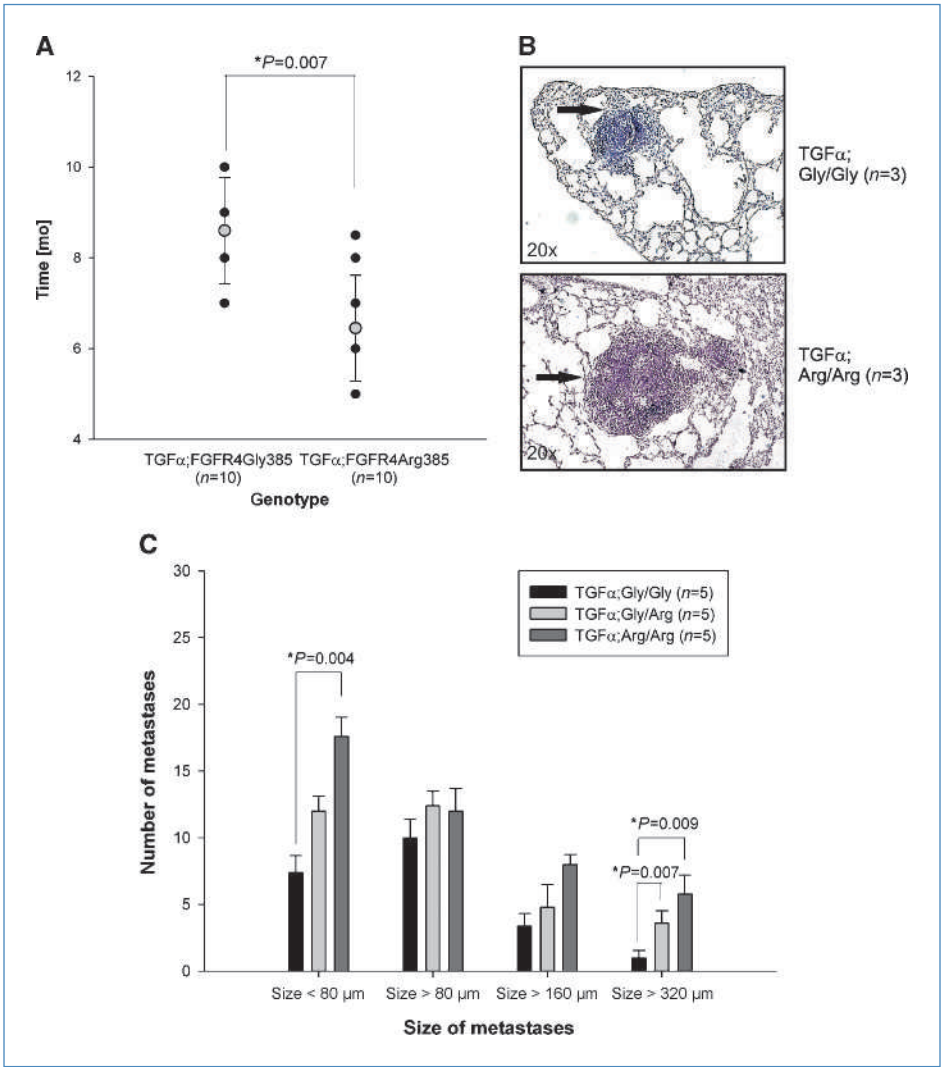

Figure 5. The *FGFR4Arg385* allele promotes cancer cell metastasis in the *WAP-TGFα* mouse mammary tumor model. *A, WAP-TGFα; FGFR4Arg385* mice show a significantly decreased time point of metastasis onset compared with *WAP-TGFα;FGFR4Gly385* mice. *B, black arrows,* metastases display no obvious pathohistologic differences about the different *FGFR4* genotypes after 6 mo of tumor progression (H&E staining). *C, WAP-TGFα;FGFR4Arg385* mice partly display a significantly accelerated number of metastases compared with *WAP-TGFα; FGFR4Gly/Gly385* after 8 mo of tumor progression. Size is plotted against number of metastases.

distant metastases in the lungs of *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, and *WAP-TGFα;FGFR4Arg/Arg385* mice. Strikingly, *FGFR4Arg385*-carrying mice display a significantly earlier incidence of lung metastases when compared with *WAP-TGFα;FGFR4Gly/Gly385* mice (Fig. 5*A*). However, as seen in Fig. 5*B*, the mice display no pathohistologic alterations of lung metastases in the presence of the *FGFR4Arg385* allele (*black arrows*). Furthermore, we investigated the number and size of metastases in the invaded lungs of *WAP-TGFα;FGFR4Gly/Gly385, WAP-TGFα;FGFR4Gly/Arg385*, and *WAP-TGFα;FGFR4Arg/Arg385* mice after 8 months of tumor progression. Here, *WAP-TGFα;FGFR4Gly/Arg385* mice show significantly more metastases that are bigger than 320 μm, whereas *WAP-TGFα;FGFR4Arg/Arg385* mice show significantly more metastases that are smaller than 80 μm or bigger than 320 μm (Fig. 5*C*).

These results suggest that the *FGFR4Arg385* allele contributes to accelerated tumor cell invasion as well as an earlier incidence and faster growth of metastases. In the MMTV-PymT breast cancer model, pulmonary metastasis was visible after 3 months of tumor development (data not shown); however, no acceleration of tumor progression was observed (Supplementary Fig. S7). We therefore did not further investigate the effect of the *FGFR4Arg385* allele on metastasis to the lung.

Discussion

In this study, we investigated for the first time the effect of the change of a single nucleotide in the mouse genome in the gene encoding the receptor tyrosine kinase *FGFR4* on breast cancer progression in the *WAP-TGFα* model. Here, we first analyzed the differential effect of the *FGFR4* alleles in MEFs. First, we investigated the effect of the *FGFR4Arg385* allele on MEF transformation by oncogenes such as HER2, EGFR, and v-kit, as the most prominent effect of the *FGFR4Arg388* allele is the disease progression rate once the cancer has been initiated (3, 6, 7, 9). Consistently, MEFs expressing the *Arg385* allele showed a significantly higher transformation rate than control fibroblasts. We then investigated if the *FGFR4Arg385* allele contributes to EGFR-driven transformation. To this end, we

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

stably transformed the MEF FGFR4 genotype variants by EGFR overexpression. Interestingly, FGFR4 was upregulated in EGFR-transformed MEFs and the FGFR4Arg385 was found to be hyperactivated in EGFR-transformed MEFs. These results indicated the possibility of cross-talk between these two receptors as it has been shown for HER2 and FGFR4 (21). In EGFR-transformed MEFs, the *FGFR4Arg385* isotype was significantly associated with accelerated cell motility, soft agar colony formation, as well as invasivity and branching in Matrigel. Furthermore, as a migratory effect is not detectable in any FGFR4 genotype MEFs not transformed by EGFR overexpression, these data clearly indicate that the *FGFR4Arg385* allele is not an oncogene per se, but rather support oncogenes by the enhancement of transformation-characteristic biological processes. Moreover, no effect of the *FGFR4Arg385* allele could be detected when MEFs were transformed with v-src. These results suggest that the effect of FGFR4Arg385 is clearly dependent on the specific oncogenic background that triggers the neoplastic transformation, and indicate a supportive rather than autonomous action of the *FGFR4Arg385* isotype.

After this clear implication of the FGFR4 and its Arg385 variant in biological processes that are involved in definition of tumor progression and aggressiveness, we investigated the effect of FGFR4 on tumor progression *in vivo*. We reasoned that the *FGFR4Arg385* KI mouse would overcome the problem of heterogenetic patient cohorts to clarify the possible effect of the *FGFR4Arg385* allele on tumor progression (11). As FGFR4 is upregulated in diverse cancers including that of the breast and, furthermore, the *FGFR4Arg388* allele was shown to promote mammary carcinoma in humans, we determined the effect of this allele on mammary cancer progression in the mouse (3, 17). Similar to the experiments *in vitro*, we analyzed the involvement of *FGFR4Arg385* in tumor progression in combination with the well-established *WAP-TGFα* and the *MMTV-PymT* transgenic cancer models. We showed that the *FGFR4Arg385* allele promotes *WAP-TGFα*–induced mammary tumors in mass and area. In addition, these tumors displayed faster progression with a significant increase of tumor size and metastases over time depending on the different *FGFR4* genotypes. Furthermore, FGFR4Arg385 decreased the time point of visible tumor incidence and therefore seems to facilitate tumor initiation. Moreover, the analysis of the criteria of tumor progression displayed a more significant difference in the tumor area rather than tumor mass, suggesting that the effect of the FGFR4Arg385 is rather on cell motility than proliferation. This is in line with the results obtained with EGFR-transformed *FGFR4Arg385* MEFs.

We further analyzed the molecular consequences of *FGFR4Arg385* isotype expression in tumors to investigate the underlying mechanism of accelerated tumor progression. Although FGFR4Arg385 is not overexpressed in primary tumors relative to FGFR4Gly385, its activity is enhanced. As the SNP in FGFR4 results in the conversion of a neutral to a hydrophilic amino acid, the structure of the FGFR4Arg385 allele possibly alters receptor regulation. Furthermore, Wang and colleagues (22) showed increased stability of the FGFR4Arg388 receptor in prostate cancer cell lines, which could result in a relatively higher phosphorylation status.

In this study, we further analyzed differences in the expression of several genes involved in tumor progression between *WAP-TGFα;FGFR4Arg/Arg385* and *WAP-TGFα;FGFR4Gly/Gly385* tumors. Here, *FGFR4Arg385*-carrying *WAP-TGFα*–derived tumors display a more "aggressive" gene expression pattern. The significant downregulation of the tumor suppressor p21 is known to predict the poorest prognosis together with high EGFR expression (23), and the upregulation of CDK1 involves FGFR4 in an enhanced migratory capacity of cancer cells (18). The unchanged expression of the other cell cycle proteins confirms the lack of involvement of FGFR4Arg385 in cell proliferation. Moreover, genes associated with cell invasivity were upregulated in FGFR4Arg385-expressing *WAP-TGFα*–derived tumors, such as flk-1, CD44, and MMPs, contributing to a higher metastatic potential (20). Previous studies also identified changes in the cellular gene expression profile in the presence of FGFR4Arg388. Here, *FGFR4Arg388* promoted the upregulation of the metastasis-associated gene Ehm2 in prostate cancer and the promigratory gene LPA receptor EDG-2 in MDA-MB-231 cells that is suppressed by FGFR4Gly388 (24, 25).

Besides changes in gene expression, *FGFR4* isotypes could differ in their affinity toward other functionally relevant proteins. To address this possibility, we performed a SILAC-based mass spectrometry analysis of immunoprecipitates of FGFR4Gly388 and Arg388 in MDA-MB-231 breast cancer cells (3). Here, we identified the EGFR as a strong interaction partner of the FGFR4. Subsequent experiments interestingly showed a significantly higher affinity of the EGFR to FGFR4Arg388 variant, resulting in enhanced downstream signaling. This interaction may likely be the key mechanism of the tumor progression–promoting effect of the FGFR4Arg388, which is supported by our results in the *FGFR4Arg385* KI *WAP-TGFα* mouse model in which a hyperactive EGFR drives mammary carcinogenesis.[1]

Consistent with the gene expression differences and our preliminary EGFR interaction hypothesis, mouse cancer cells expressing the *FGFR4Arg385* allele display an enhanced potential in invading the lung to form distant metastases *in vivo*. These data strongly associate the *FGFR4Arg388* allele with poor prognosis and thereby highlight this receptor as a marker of breast cancer progression. Our *in vivo* results are consistent with several clinical reports, which were published since the discovery of the *FGFR4Arg388* allele by our laboratory (3), which associate the *FGFR4Arg388* allele with poor clinical outcome in various cancers, including head and neck, breast, and melanoma (6–8).

In contrast, FGFR4Arg385 was not able to promote mammary cancer progression in mice transgenic for *MMTV-PymT* neither in tumor mass nor in area. This is well in line with the results obtained with v-src–transformed MEFs. In this case, FGFR4Arg385 could not enhance any of the analyzed biological properties. These findings underline the dependency of the *FGFR4Arg385* isotype on a specific oncogenic background.

[1] In preparation.

Our data support the conclusion that the *FGFR4Arg388* allele is a potent enhancer of human breast tumor development, progression, and metastasis formation. As recent publications correlate the *FGFR4Arg388* allele with various types of human cancer, our mouse KI model strongly supports an exclusive effect of the *FGFR4Arg388* allele on a broad spectrum of cancers with respect to the rate of disease progression and outcome. The strong effect of FGFR4 on disease outcome is further underlined by Roidl and colleagues (26), who could show that upregulation of FGFR4 results in chemoresistance in breast cancer cell lines, and by the work of Meijer and colleagues (27), which shows that FGFR4 predicts failure in tamoxifen treatment of breast cancer patients. This notion is strongly supported by our previous findings—that the time of mammary cancer relapse after different drug treatments is associated with the two *FGFR4-388* alleles (10). In summary, we have characterized FGFR4 as an allele-specific "oncogene enhancer" that significantly accelerates neoplastic progression driven by classic oncogenes such as EGFR or kit. This novel scenario of oncogenesis is of high clinical relevance as the FGFR4-388 genotype of a patient may provide a diagnostic marker for the individual determination of therapy decisions. These data present opportunities for the further use of the *FGFR4Arg385* KI model for the investigation of *FGFR4* genotype-selective cancer treatment strategies and mechanisms of resistance in targeted therapies.

Our findings strongly support a role of the *FGFR4Arg388/385* allele as a marker for poor clinical outcome in breast cancer progression and metastasis. On this account, these data further validate the FGFR4 and its isotypes as a target for the development of prototypical drugs and emphasize the validity and importance of individualized therapy regimens for cancer patients.

Above all, our findings highlight the effect of germ-line alterations, including the great variety of SNPs in tyrosine kinase genes (28), on the clinical progression of cancer and thereby emphasize the individual nature of this deadly disease. Future cancer therapy decisions will have to include individual genetic characteristics, such as the *FGFR4Arg388* allele, in addition to histologic and molecular pathologic data of every individual tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Lothar Henninghausen (Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD) for the *WAP-TGFα*, Christian Bader (Department of Nutritional Medicine, Else Kröner-Fresenius-Center for Nutritional Medicine, Technische Universität München, Freising Weihenstephan, Germany) for the *MMTV-PymT* transgenic mice, Cord Brakebusch (Biomedical Institute, BRIC, University of Copenhagen, Copenhagen, Denmark) for supporting us in the generation of the *FGFR4Arg385* KI mice, and the animal facility of the Max Planck Institute of Biochemistry for animal care and a lot of support.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 9/2/09; revised 10/16/09; accepted 11/2/09; published OnlineFirst 1/12/10.

References

1. Ameyaw MM, Tayeb M, Thornton N, et al. Ethnic variation in the HER-2 codon 655 genetic polymorphism previously associated with breast cancer. J Hum Genet 2002;47:172–5.
2. Przybylowska K, Smolarczyk K, Blasiak J, et al. A C/T polymorphism in the urokinase-type plasminogen activator gene in colorectal cancer. J Exp Clin Cancer Res 2001;20:569–72.
3. Bange J, Prechtl D, Cheburkin Y, et al. Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. Cancer Res 2002;62:840–7.
4. Falvella FS, Frullanti E, Galvan A, et al. FGFR4 Gly388Arg polymorphism may affect the clinical stage of patients with lung cancer by modulating the transcriptional profile of normal lung. Int J Cancer 2009;124:2880–5.
5. Spinola M, Leoni V, Pignatiello C, et al. Functional FGFR4 Gly388Arg polymorphism predicts prognosis in lung adenocarcinoma patients. J Clin Oncol 2005;23:7307–11.
6. Streit S, Bange J, Fichtner A, Ihrler S, Issing W, Ullrich A. Involvement of the FGFR4 Arg388 allele in head and neck squamous cell carcinoma. Int J Cancer 2004;111:213–7.
7. Streit S, Mestel DS, Schmidt M, Ullrich A, Berking C. FGFR4 Arg388 allele correlates with tumour thickness and FGFR4 protein expression with survival of melanoma patients. Br J Cancer 2006;94:1879–86.
8. Wang J, Stockton DW, Ittmann M. The fibroblast growth factor receptor-4 Arg388 allele is associated with prostate cancer initiation and progression. Clin Cancer Res 2004:6169–78.
9. Morimoto Y, Ozaki T, Ouchida M, et al. Single nucleotide polymorphism in fibroblast growth factor receptor 4 at codon 388 is associated with prognosis in high-grade soft tissue sarcoma. Cancer 2003;98:2245–50.
10. Thussbas C, Nahrig J, Streit S, et al. FGFR4 Arg388 allele is associated with resistance to adjuvant therapy in primary breast cancer. J Clin Oncol 2006;24:3747–55.
11. Jezequel P, Campion L, Joalland MP, et al. G388R mutation of the FGFR4 gene is not relevant to breast cancer prognosis. Br J Cancer 2004;90:189–93.
12. Pittius CW, Hennighausen L, Lee E, et al. A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. Proc Natl Acad Sci U S A 1988;85:5874–8.
13. Sandgren EP, Schroeder JA, Qui TH, Palmiter RD, Brinster RL, Lee DC. Inhibition of mammary gland involution is associated with transforming growth factor α but not c-myc-induced tumorigenesis in transgenic mice. Cancer Res 1995;55:3915–27.
14. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12:954–61.
15. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 1963:299–313.
16. Gowardhan B, Douglas DA, Mathers ME, et al. Evaluation of the fibroblast growth factor system as a potential target for therapy in human prostate cancer. Br J Cancer 2005;92:320–7.

Published OnlineFirst January 12, 2010; DOI: 10.1158/0008-5472.CAN-09-3239

17. Jeffers M, LaRochelle WJ, Lichenstein HS. Fibroblast growth factors in cancer: therapeutic possibilities. Expert Opin Ther Targets 2002;6:469–82.
18. Manes T, Zheng DQ, Tognin S, Woodard AS, Marchisio PC, Languino LR. α(v)β3 integrin expression up-regulates cdc2, which modulates cell migration. J Cell Biol 2003;161:817–26.
19. Sheridan C, Kishimoto H, Fuchs RK, et al. $CD44^+/CD24^-$ breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Res 2006;8:R59.
20. Jiang WG, Davies G, Martin TA, et al. Expression of membrane type-1 matrix metalloproteinase, MT1-MMP in human breast cancer and its impact on invasiveness of breast cancer cells. Int J Mol Med 2006;17:583–90.
21. Koziczak M, Hynes NE. Cooperation between fibroblast growth factor receptor-4 and ErbB2 in regulation of cyclin D1 translation. J Biol Chem 2004;279:50004–11.
22. Wang J, Yu W, Cai Y, Ren C, Ittmann MM. Altered fibroblast growth factor receptor 4 stability promotes prostate cancer progression. Neoplasia 2008;10:847–56.
23. Somlo G, Chu P, Frankel P, et al. Molecular profiling including epidermal growth factor receptor and p21 expression in high-risk breast cancer patients as indicators of outcome. Ann Oncol 2008;19:1853–9.
24. Stadler CR, Knyazev P, Bange J, Ullrich A. FGFR4 GLY388 isotype suppresses motility of MDA-MB-231 breast cancer cells by EDG-2 gene repression. Cell Signal 2006;18:783–94.
25. Wang J, Cai Y, Penland R, Chauhan S, Miesfeld RL, Ittmann M. Increased expression of the metastasis-associated gene Ehm2 in prostate cancer. Prostate 2006;66:1641–52.
26. Roidl A, Berger HJ, Kumar S, Bange J, Knyazev P, Ullrich A. Resistance to chemotherapy is associated with fibroblast growth factor receptor 4 up-regulation. Clin Cancer Res 2009;15:2058–66.
27. Meijer D, Sieuwerts AM, Look MP, van Agthoven T, Foekens JA, Dorssers LC. Fibroblast growth factor receptor 4 predicts failure on tamoxifen therapy in patients with recurrent breast cancer. Endocr Relat Cancer 2008;15:101–11.
28. Ruhe JE, Streit S, Hart S, et al. Genetic alterations in the tyrosine kinase transcriptome of human cancer cell lines. Cancer Res 2007;67:11368–76.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

A Single Nucleotide Change in the Mouse Genome Accelerates Breast Cancer Progression

Nina Seitzer, Thomas Mayr, Sylvia Streit, et al.

Cancer Res 2010;70:802-812. Published OnlineFirst January 12, 2010.