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

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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 FGFR4Arg385 allele is associated with increased tumor thickness, whereas in head and neck squamous cell carcinoma the glycine-arginine substitution correlates with reduced 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
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 FGFR4Arg385 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 ST3 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). To ultimately prove the influence of the FGFR4Arg385 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).

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 microscope (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 FGFR4Arg385 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. S3A). 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. S3B 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
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.
with HER2 and EGFR, whereas FGFR4Arg385 MEFs show a significantly enhanced focus formation with all three investigated oncogenes (Supplementary Fig. S3D). Furthermore, a time course analysis showed that FGFR4Arg385-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. S4A). 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. S4B). In contrast, FGFR4Arg385 MEFs transformed with EGFR display a significantly increased migratory capacity compared with Gly385 MEFs (Fig. 1A). Next, we analyzed anchorage independence of EGFR-transformed MEFs in soft

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 mean of values from 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.
agar colony formation assays. FGFR4Arg/Arg385 EGFR-transformed MEFs display a significantly accelerated anchorage-independent growth after 24 and 96 hours (Fig. 1F). Subsequently, we analyzed the effect of the FGFR4Arg385 allele on invasiveness in a Matrigel assay (Fig. 1C). 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 physiological 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. S6A 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/Gly385, 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. 2A, 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. 2A). Moreover, the tumor area and the percentage of tumor area are significantly accelerated in WAP-TGFα;FGFR4Arg/Arg385 mice (Fig. 2B). 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. S6C 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. 2C). 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. S7A 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. S8A). Contrarily, the FGFR4Arg/Arg385 displays a significantly enhanced activation compared with the FGFR4Gly/Gly385 (Fig. 3A, 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. S8B). 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. 3B), indicating that the FGFR4Arg/Arg385 allele potentially affects the onset of malignant 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. S8C).

In addition, we wanted to investigate the mRNA expression of genes associated with an aggressive tumor phenotype, such as motility, invasiveness, and angiogenesis, in 6-month-old tumors from WAP-TGFα;FGFR4Gly/Gly385 and WAP-TGFα;FGFR4Arg/Arg385 mice (Fig. 3C). The FGFR4 and the EGFR
display no overexpression in the presence of the \textit{FGFR4Arg385} allele. Among analyzed tumor suppressors, p21 was found to be significantly downregulated in \textit{WAP-TGF\alpha;FGFR4Arg/Arg385}–induced tumors. About proliferation markers, cyclin-dependent kinase 1 (CDK1) was significantly upregulated in \textit{WAP-TGF\alpha;FGFR4Arg/Arg385}–induced tumors. As CDK1 is strongly associated with migration, this significant overexpression might promote a more aggressive phenotype of \textit{FGFR4Arg385}-carrying tumors (18). In the group of invasion and angiogenic markers, CD44 and flk-1 were significantly overexpressed in \textit{FGFR4Arg385} tumors, which both might contribute to a more aggressive phenotype of \textit{FGFR4Arg385} tumors (19). In the cluster of matrix metalloproteinases (MMP), MMP13 as well as MMP14 were overexpressed in \textit{FGFR4Arg385} tumors, possibly contributing to a higher metastatic potential (20).

These data strongly suggest that \textit{WAP-TGF\alpha;FGFR4Arg/Arg385}–induced tumors display a more aggressive behavior resulting in an accelerated tumor progression.

\textit{The FGFR4Arg385 allele decreases the time point of tumor incidence and promotes tumor progression over time in the WAP-TGF\alpha transgenic model}. To further analyze the
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. 4A, 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. S7C).

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. 4B, 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. 4C). 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. 4D).

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

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**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;FGFR4Arg/Arg385 mice show a significantly earlier time point of visible tumor incidence compared with WAP-TGFα;FGFR4Gly/Gly385 mice. B, WAP-TGFα;FGFR4Arg/Arg385 mice partly establish a significantly higher number of tumors over time compared with WAP-TGFα;FGFR4Gly/Gly385 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;FGFR4Gly/Gly385 mice. D, WAP-TGFα;FGFR4Arg/Arg385 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.
distant metastases in the lungs of WAP-TGFα;FGFR4Arg385 mice show a significantly decreased time point of metastasis onset compared with WAP-TGFα;FGFR4Gly385 mice. Strikingly, FGFR4Arg385-carrying mice display a significantly earlier incidence of lung metastases compared with WAP-TGFα;FGFR4Gly385 after 8 mo of tumor progression (Fig. 5A). However, as seen in Fig. 5B, 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;FGFR4Gly385, WAP-TGFα;FGFR4Arg385, 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. 5C).

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 FGFR4Arg388 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
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 heterogenic 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 FGFR4Gly388. 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.\footnote{In preparation.}

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.
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.

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