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Extra View

Fhit Expression Protects Against HER2-Driven Breast Tumor Development

Unraveling the Molecular Interconnections

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KEY WORDS

Fhit, EGFR, HER2, breast tumors, signaling

ABBREVIATIONS

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
MMTV	mouse mammary tumor virus

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ABSTRACT

The tumor suppressor gene *FHIT* is inactivated by genetic and epigenetic changes, i.e., loss of heterozygosity or promoter hypermethylation, in common human cancers. We recently showed that Fhit protein levels can be regulated by Fhit proteasome degradation mediated by EGF-dependent activation of EGFR family members, including HER2, whose overexpression is linked to poor prognosis in breast cancer. Analysis of a series of 384 human primary breast carcinomas revealed low/absent Fhit protein levels more frequently in HER2-overexpressing tumors. To test for a possible complementation of the *FHIT* and HER2 genes, tumor incidence was assessed in mice carrying one inactivated *Fhit* allele (*Fhit*$^{+/-}$) crossed with FVB/N mice carrying the rat HER2/*neu* proto-oncogene driven by the mouse mammary tumor virus promoter. All *Fhit* heterozygous mice developed mammary tumors, whereas when both *Fhit* alleles (*Fhit*$^{+/+}$) were present, tumor incidence was reduced in 27% of the mice, which remained tumor-free at twenty months. These findings suggest a protective role for *FHIT* in HER2-driven mammary tumors. Together, these data argue for the cooperation between Fhit and HER2 in breast carcinogenesis.

THE *FHIT* TUMOR SUPPRESSOR GENE

The development and progression of breast cancer involves a number of genetic and epigenetic alterations in tumor suppressor and tumor-related genes. *FHIT* (fragile histidine triad) is a newcomer to breast cancer research, but some of its features, such as its tumor suppressor action and the highest frequency of gene alteration in epithelial tumors (reviewed in Ref. 1), has brought attention to the role of *FHIT* in cancer. The *FHIT* gene maps to the short arm of chromosome 3 (3p14.2), encompasses the common *FRA3B* fragile region, and encodes a small protein of 16.8 kDa.[2] Fhit is an enigmatic protein, exerting an AP_3A-hydrolase activity that is not required for its tumor suppression function.[3] The tumor suppressor role of *FHIT* has been widely demonstrated in mouse models (reviewed in ref. 4). Mice with inactivation of one or both *Fhit* alleles are more susceptible than wild-type mice to the development of spontaneous or carcinogen-induced tumors.[5,6] Moreover, restoration of Fhit protein expression in *Fhit*-null models prevents tumor formation.[7] To date, the exact mechanism by which Fhit exerts its anti-tumor activity remains obscure. A considerable body of evidence points to the role of Fhit in apoptosis induction and cell cycle regulation, including its role as modulator of DNA damage checkpoint response,[8] but few data are available regarding the molecular pathways of Fhit anti-tumor activity. Some authors have investigated a possible cooperation in carcinogenesis between *FHIT* and Vhl, another oncosuppressor gene near the *FHIT* locus.[9] To date, no further data are available on the possible interplay of Fhit with oncogenes or tumor suppressor genes in exerting a key role in development and progression of cancers.

FHIT EXPRESSION IS FREQUENTLY LOST IN HUMAN CANCERS

Fhit mRNA expression is detectable in most normal human tissues, with the highest levels expressed in epithelial cells and tissues.[10] Originally, the absence of Fhit protein in tumors was investigated as a loss of heterozygosity, mainly due to allelic deletions of the gene leading to absent and/or aberrantly coding Fhit transcripts.[10] Interestingly, when the first studies on Fhit protein expression appeared, a discrepancy emerged between the prevalence of aberrant transcripts versus absence of Fhit protein. However, such discrepancies do not extend to all tumor histotypes (Table 1). For example, differences between the percentage of Fhit protein absence and percentage of Fhit aberrant transcripts are only slight in non small cell lung cancers (NSCLC), with aberrant transcripts observed

Table1 **Fhit protein expression in breast tumors***

Parameters	Strong	Weak	Negative	P	P for Trend
No. of cases	127	159	98		
High mitosis	37% (43/116)	53% (78/146)	64% (60/94)	0.0004	0.0001
T2	45% (57/127)	61% (96/157)	64% (62/97)	0.0051	0.0029
Grade III	21% (24/117)	36% (52/146)	36% (34/94)	0.013	
PgR-neg	27% (34/127)	47% (74/157)	44% (43/98)	0.001	
HER2-pos	19% (24/127)	26% (42/159)	30% (29/98)	0.149	0.058

*Immunohistochemical staining of tumors was scored as strong, weak or negative relative to staining intensity in matched normal breast epithelium.

in 40% of NSCLC versus 57% of protein absence or reduction,[11-17] or in gastric tumors, where percentage of aberrant transcripts reaches 50–55% versus 60–65% of Fhit protein absence.[18-22] Similar findings have been reported for cervical cancer,[23-28] and oral carcinomas.[29] By contrast, the gap between the percentages of Fhit protein loss versus Fhit aberrant transcripts is wider in breast and ovarian cancers. Indeed, we and others observed aberrant transcripts expression in 30–35% of breast cancer cases[30-36] but Fhit protein absence or marked reduction compared to normal epithelium of the same patient in up to 60% of cases.[32,34] A wide discrepancy has also been reported in renal carcinomas, where transcript alterations were described in 51% of clear-cells and 10% of papillary sporadic renal carcinoma cells, while absent or Fhit protein reduction occurred in 88% and 26%, respectively.[37-39] Further studies have reported loss of Fhit not due to altered transcript but to promoter hypermethylation of the *FHIT* gene in breast, gallbladder and NSCL cancers.[36,40-42] On the other hand, we recently showed that downregulation of Fhit protein levels in the presence of a normal mRNA can occur through Fhit protein posttranslational modification.[43] Our work began based on the observation that Fhit can be phosphorylated by Src on tyrosine 114.[44] Src is a downstream molecule of several activated receptors, including members of the EGFR family (EGFR, HER2, HER3 and HER4). In breast and ovarian cancer cell lines expressing Fhit and overexpressing EGFR and HER2, we observed down-modulation of Fhit protein steady-state levels after mitogenic stimulation that activated EGFR, HER2 and Src. Fhit protein downmodulation results from proteasome degradation, and Fhit degradation depends on Fhit phosphorylation of tyrosine 114, the docking site of activated Src. Thus, during cancer cell proliferation promoted by activated EGFR family members that recruit Src, the phosphorylation of Fhit induces its degradation and the subsequent reduction in Fhit protein levels allows transmission of the mitogenic signal. Moreover, we observed a tight temporal regulation of Fhit protein levels during the mitogen-stimulated cell proliferation mediated by EGF-activated tyrosine kinase receptors. These data demonstrated for the first time a cross-regulation between EGFR family members and Fhit, suggesting a potential complementation between EGFR family members and the Fhit pathway.

IMPACT OF FHIT ON HER2-DRIVEN BREAST TUMORS

Several lines of evidence indicate that two members of the EGFR family, EGFR and HER2, are overexpressed individually or together in breast carcinomas.[45-47] These two receptors stand at the origin of the major signaling pathway involved in the growth of breast cancer. EGFR amplification and activating mutations are rare events in breast cancer and differences in EGFR expression appear to be controlled largely at the transcriptional level.[45,48] By contrast, it is well-established that about 20% of breast carcinomas are characterized by HER2 gene amplification, leading to an excess of HER2 protein in cancer cells and a high risk of recurrences (reviewed in ref. 49). The role of HER2 in breast carcinomas is so prominent that classification of breast tumors by microarray analysis has identified HER2-positive tumors as a distinct subset among five defined. The powerful role of the EGFR family in oncogenesis resides in its exquisitely mechanism of signaling within the cell. This receptor family has evolved a complex network in which the basic functional unit is a receptor dimer, to which each partner contributes a unique feature. HER2 is the preferred heterodimeric partner of the other three members of the family, due to its peculiar structure which primes it for interaction with ligand-bound receptors of the family (reviewed in ref. 50). Moreover, HER2 binds to a much larger subset of phosphotyrosine-binding proteins than the other ligand-binding receptors of the family.[51] Heterodimers that contain HER2, in particular EGFR-HER2 and HER2-HER3, confer superiority to these heterodimers that lead to more potent mitogenic signals.[52] After twenty years since the cloning of HER2 and almost 8000 publications regarding its activation mechanism, degradation and recycling pathways, clinical importance, and development of successful HER2-targeted therapies, the puzzle of HER2's powerful role in carcinogenesis needs to be completed. One piece of the puzzle may be represented by the oncosuppressor gene *FHIT*. In an effort to analyze the impact of Fhit downregulation due to EGFR family activation in human breast tumors development and progression, we focused on Fhit protein levels with respect to HER2 overexpression, whose assessment in breast carcinomas is well-standardized by the FDA-approved Herceptest test[53] in immunohistochemistry.

We previously found that Fhit protein was low or absent due to noncoding aberrant *FHIT* transcripts in 30% of the cases; however an additional 40% of the tumors showed loss of Fhit protein (evaluated as reduction or absence of Fhit protein) in the presence of normal *FHIT* mRNA. In either case, loss of Fhit protein resulted in more aggressive, highly proliferative and poorly differentiated tumors.[32] We performed the analysis of a larger series of primary breast carcinomas (384 samples) confirming that reduced Fhit levels were associated with a more aggressive phenotype (Table 1). Based on comparison with the strong immunohistochemical staining observed in normal breast epithelium, Fhit reactivity in the tumor was found to be strong in 33%, weaker than normal in 41% and negative in 26% of the 384 tumors. The reduction/loss of Fhit expression was directly associated with increased proliferation, evaluated as percent of mitotic cells ($p = 0.0004$) and tumor size ($p = 0.0051$), and with tumor differentiation, evaluated as absence of progesterone receptor (PgR) expression ($p = 0.001$) and high tumor grade ($p = 0.0013$). Moreover, analysis in this larger series demonstrated the higher frequency of HER2 overexpression in tumors with low levels of Fhit (*p for trend* = 0.058). The subset of HER2-positive/Fhit-negative tumors showed the highest proliferation rate and the largest size in the entire series.

The trend between HER2 overexpression and loss of Fhit protein in human breast carcinomas raised the possibility of interplay in the Fhit and HER2 pathways. To examine the potential complemen-

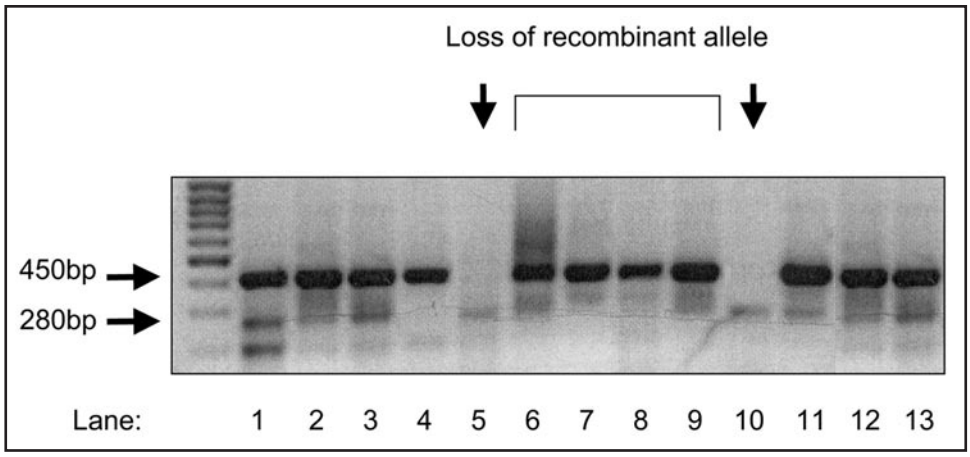

Figure 1. PCR analysis of *Fhit* alleles. DNA from mammary tumors (lanes 1–12) of 12 female *Fhit*$^{+/-}$ mice was genotyped by PCR to examine the loss of the second *FHIT* allele using a primer pair that amplifies the wild-type *Fhit* fragment of 450 bp (containing exon 5) or primers producing a 280 bp fragment spanning the inactivated *Fhit* allele. Lane 13: positive control.

tation between the two genes, we used murine models in which mice (C57Bl-6/129Sv-J/C_3H) carrying one inactivated *Fhit* allele (*Fhit*$^{+/-}$) were crossed with FVB/N mice carrying the rat HER2/*neu* proto-oncogene driven by the MMTV promoter. Due to the transgenic HER2 proto-oncogene, FVB/N virgin females develop atypical hyperplasia at about five months of age which progresses to in situ carcinoma and to invasive carcinoma within one year of age.[54] Of 96 female F1 mice obtained and screened for *Fhit* allele inactivation by a polymerase chain reaction (PCR) analysis of tail genomic DNA that enabled discrimination of the heterozygote from the homozygote form, a clear imbalance of *Fhit*$^{+/-}$ versus *Fhit*$^{+/+}$ frequency emerged (15% were *Fhit*$^{+/-}$ and 85% were *Fhit*$^{+/+}$) compared to the expected 1:1 ratio. Mice began to develop mammary tumors at seven months of age, with no differences between the *Fhit* heterozygous and homozygous mice in latency. Analysis of tumor incidence showed that all *Fhit* heterozygous mice (14/14) developed mammary tumors, whereas tumor incidence reduction in mice carrying both *Fhit* alleles (*Fhit*$^{+/+}$) was observed in 27% of the cases (22 mice) and tumor-free mice remained so at twenty months ($p = 0.02$). The F_1 *Fhit*$^{+/+}$ mice without tumors were distributed in each litter. PCR analysis performed in twelve of the fourteen tumors that arose in *Fhit* heterozygous mice revealed loss of the second *Fhit* allele in two of the tumors (Fig. 1). Immunohistochemical analysis of sections of these two tumors was scored negative for Fhit expression (data not shown), consistent with the PCR results. Together, these data point to a protective role for *Fhit* in HER2-driven mammary tumors.

CONCLUSIONS

Current data argue for the cooperation between Fhit and HER2 in breast carcinogenesis. Based on our data, we can argue that tumors HER2-positive and Fhit negative due to FHIT gene inactivation will do have a more aggressive prognosis. In the remaining HER2-positive tumors with normal Fhit transcript we can hypothesize that Fhit protein loss will be only in tumors with higher-level activation of EGFR/HER2, leading to Src activation and the ensuing Fhit degradation. Thus, the Fhit protein downregulation can be a parameter reflecting the EGFR-family activation state in the tumor.

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