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Advances in Brief

Deficient Transforming Growth Factor-β1 Activation and Excessive Insulin-like Growth Factor II (IGFII) Expression in IGFII Receptor-Mutant Tumors[1]

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Abstract

The insulin-like growth factor II receptor (*IGFIIR*) gene has been identified as a coding region target of microsatellite instability in human gastrointestinal (GI) tumors. IGFIIR normally has two growth-suppressive functions: it binds and stimulates the plasmin-mediated cleavage and activation of the latent transforming growth factor-β1 (LTGF-β1) complex, and it mediates the internalization and degradation of IGFII ligand, a mitogen. We used an immunohistochemical approach to determine whether *IGFIIR* mutation affected expression of these proteins in GI tumors. Four highly specific antibodies were used: LC(1-30), which recognizes the active form of TGF-β1; anti-LTGF-β1, which detects the LTGF-β1 precursor protein; anti-IGFIIR; and anti-IGFII ligand. Twenty GI tumors either with (6 of 20) or without (14 of 20) known *IGFIIR* mutation were examined, along with matching normal tissues. Results were statistically significant in the following categories: (*a*) decreased active TGF-β1 protein expression in *IGFIIR*-mutant tumor tissues *versus* matching normal tissues or *IGFIIR*-wild-type tumor tissues; (*b*) increased LTGF-β1 protein expression in *IGFIIR*-mutant tumor tissues *versus* matching normal tissues or *IGFIIR*-wild-type tumor tissues; and (*c*) increased IGFII ligand protein expression in *IGFIIR*-mutant tumor tissues *versus* matching normal tissues or *IGFIIR*-wild-type tumor tissues. These data suggest that in genetically unstable GI tumors, mutation of a microsatellite within the coding region of *IGFIIR* functionally inactivates this gene, causing both diminished growth suppression (via decreased activation of TGF-β1) and augmented growth stimulation (via decreased degradation of the IGFII ligand).

Introduction

MI[3] is a genome-wide phenomenon occurring as a result of mutations in DNA mismatch repair enzymes. Although most MI has been described in noncoding regions of the genome, several genes such as the *TGF-β1 type II receptor* (1), *hMSH3* (2), *hMSH6* (2), *hMLH1* (3), *β2-microglobulin* (4), and *BAX* (5) were demonstrated to contain coding region targets of MI. Recently, the *IGFIIR* gene was also identified as a coding region target of MI in human gastrointestinal tumors (6).

IGFIIR has several mechanistic and clinical links to human cancer. One physiological function of this receptor is to bind the latent complex of TGF-β1 (7, 8). In its active form, TGF-β1 primes a growth-suppressive cascade in epithelial cells. The binding of TGF-β1 latent complex to IGFIIR is essential for TGF-β1 activation. Another function of IGFIIR involves the binding of its own ligand, IGFII (9, 10). IGFII ligand can bind to both the IGFIR and the IGFIIR, but it binds with higher affinity to IGFIIR. Through its interaction with IGFIR, IGFII ligand is a potent growth stimulator. Nevertheless, although IGFII is itself a potent mitogen, IGFIIR actually limits IGFII-mediated growth stimulation by binding, internalizing, and allowing the degradation of IGFII ligand. These studies support the view that IGFIIR plays a critical role as a negative regulator of cell growth.

Further support for this view comes from reports of LOH and cytogenetic deletions at the human *IGFIIR* locus on chromosome 6q26–27 in breast cancer, melanoma, ovarian carcinoma, lymphoma, and renal cell carcinoma (11–14). In fact, De Souza *et al.* (15) identified loss-of-function mutations in the remaining *IGFIIR* allele in 3 of 12 hepatocellular carcinomas showing 6q-LOH, as well as one mutation in a case lacking 6q-LOH. For these mutations to affect tumor growth, they should compromise the known growth-suppressive functions of IGFIIR, such as TGF-β1 activation and IGFII ligand degradation. We, therefore, used an immunohistochemical approach to assess whether *IGFIIR* microsatellite mutations cause loss of function: specifically, whether these mutations alter the expression in primary tumors of relevant growth-governing proteins known to be regulated by IGFIIR.

Materials and Methods

Antibodies. Anti-human IGFIIR was a rabbit polyclonal antibody obtained from Dr. Chris Gable at Pfizer Pharmaceuticals (New Haven, CT); anti-human IGFII ligand was a goat polyclonal antibody obtained from R&D Systems; anti-human TGF-β1 (active protein, LC1-30) was a rabbit polyclonal IgG fraction prepared against amino acids 1–30 of mature TGF-β1; and anti-human LTGF-β1 (precursor protein, LTGF) was a rabbit polyclonal antibody prepared against amino acids 266–278 of prepro-TGF-β1, as described previously (16, 17). On Western blot analysis of media conditioned by cells expressing either recombinant TGF-β1, -β2, or -β3, anti-LTGF reacted only with the TGF-β1 precursor. Anti-LC1–30 showed slight cross-reactivity with mature TGF-β3, but this cross-reactivity probably does not occur in immunohistochemistry, because in a number of instances where staining has been seen with anti-TGF-β3-specific antibody, no staining was seen with anti-LC. The LC antibody reacts with active, but not latent, TGF-β1 in formalin-fixed, paraffin-embedded tissue sections of xenografts of human tumor cells modified by transfection to overexpress either LTGF-β1 or constitutively active TGF-β1 (18). Because immunoreactivity may be affected by tissue composition, antigen abundance, and detection strategy, each antibody was individually titered on tissue sections. Titration dilution centered on the suggested dilution, using concentrations higher and lower than this value. The concentration resulting in

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[3] The abbreviations used are: MI, microsatellite instability; TGF, transforming growth factor; LTGF, latent TGF; IGFIR and IGFIIR, insulin-like growth factor types I and II receptor, respectively; LOH, loss of heterozygosity; WT, wild type.

Table 1 *Levels of IGFIIR, IGFII, TGF-β1, and LTGF-β1 expression in gastrointestinal tissues*

		IGFIIR		IGFII		active TGF-β1		LTGF-β1	
Case no.[a]	Tissue type	N	T	N	T	N	T	N	T
MUT 29	SP	++	+	−	−	+	−	+	++
MUT 31	SP	++	++	−	+	++	+	−	++
MUT 44	SP	+	−	−	++	+	−	−	++
MUT 613	G	+	+	−	++	+	+	−	++
MUT 813	G	++	−	++	++	++	−	−	++
MUT 933	G	+	+	+	++	+	−	−	++
WT 3	SP	++	++	−	+	−	−	−	−
WT 5	SP	+	+	−	−	+	+	−	−
WT 7	SP	++	++	−	−	+	+	−	−
WT 8	SP	++	++	+	+	+	+	−	−
WT 18	SP	++	++	−	+	++	++	+	+
WT 25	SP	++	++	−	−	++	++	+	+
WT 26	SP	+	+	−	+	++	++	−	−
WT 28	SP	++	++	+	+	++	++	+	+
WT 41	SP	+	+	−	+	+	+	+	++
WT 43	SP	ND	++	ND	++	ND	++	ND	−
WT 46	SP	++	+	−	−	+	+	−	−
WT 47	SP	+	+	−	−	++	++	−	−
WT 53	SP	++	+	+	+	+	++	−	−
WT 58	SP	+	+	+	++	++	++	−	−

[a] MUT, tumor with mutant-type IGFIIR; WT, tumor with wild-type IGFIIR; N, normal tissue; T, tumor; −, no staining; +, weak staining or less than 30% positive cells; ++, intense staining or more than 30% positive cells; ND, not detected; SP, sporadic colon cancer; G, gastric cancer.

highest staining intensity with lowest background became the working concentration.

Tissue Samples. Twenty paired normal and tumor tissues, either with (6 cases) or without (14 cases) known *IGFII* microsatellite mutation (6), were included in this study. Three cases were obtained from Japanese patients with gastric cancer. Seventeen patients with sporadic colorectal carcinoma were from Australia. Tissue samples were fixed in 10% buffered formalin, and whole-cut sections were prepared. The tissues were embedded in paraffin and sliced 4-μm thick for use.

Immunohistochemical Analysis. Sections were deparaffinized with xylene, rehydrated with graded alcohol, and washed in PBS. After blocking the endogenous peroxidase activity and nonspecific protein by H_2O_2 and normal serum, sections were incubated with primary antibody diluted to a working concentration in blocking buffer at 4°C. Antibody controls were incubated with nonimmunized animal serum at the same time. Sections were stained with avidin-biotin complex horseradish peroxidase (ABC kit; Vector Laboratories) and 3,3′-diaminobenzidine (Vector). Sections were then counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher). Slides were evaluated under the light microscope by three independent viewers. Expression of the four proteins was classified into three grades, according to intensity of staining and proportion of positive cells, as follows: −, no staining; +, weak staining or involving less than 30% of cells; and ++, intense staining or more than 30% immunopositive cells.

Results

The relationship between *IGFIIR* mutation and expression of related proteins is summarized in Tables 1 and 2; examples are illustrated in Fig. 1. We observed several statistically significant relationships between mutation and immunohistochemical findings.

Expression of LTGF-β1 was detected in 6 of 6 (100%) of tumors with *IGFIIR* mutation but in only 4 of 14 (29%) WT tumors ($P = 0.005$, Fisher's exact test). Moreover, LTGF-β1 expression level was greater in tumor than matching normal tissue in 6 of 6 *IGFIIR*-mutant cases (100%) but in only 1 of 13 (8%) *IGFIIR*-WT cases ($P = 0.0003$, Fisher's exact test). There was no difference in LTGF-β1 overexpression between *IGFIIR*-mutant gastric tumors (3/3) and *IGFIIR*-mutant sporadic colorectal tumors (3/3). In contrast, 18 of 19 total available normal tissues and 13 of 14 WT tumor tissues showed expression of TGF-β1 active protein, whereas only 2 of 6 tumors with *IGFIIR* mutation (33%) displayed positive staining with anti-TGF-β1 antibody ($P = 0.014$, Fisher's exact test). In addition, active TGF-β1 protein expression levels were lower in tumor than matching normal tissue in 5 of 6 *IGFIIR*-mutant cases (83%) but in 0 of 13 *IGFIIR*-WT cases ($P = 0.0005$, Fisher's exact test).

IGFII ligand expression was detected in 5 of 6 *IGFIIR*-mutant tumors (83%) and in 9 of 14 (64%) *IGFIIR*-WT tumors; this difference did not approach statistical significance ($P = 0.387$, Fisher's exact test). Similarly, IGFII ligand expression was higher in tumor than matching normal tissue in 4 of 6 *IGFIIR*-mutant cases (67%) and in 5 of 13 *IGFIIR*-WT cases (39%); this difference also failed to reach statistical significance ($P = 0.259$, Fisher's exact test). However, when analyzed as a group, the 9 *IGFIIR*-WT cancers that were IGFII-immunopositive stained significantly more weakly than did the 5 IGFII-immunopositive *IGFIIR*-mutant tumors ($P \leq 0.05$, rank sum test). In addition, staining was more intense in poorly differentiated cells. In fact, in both *IGFIIR*-WT and mutant cases, IGFII ligand was expressed more frequently in tumors (14 of 20, 70%) than in matching normal tissues (6 of 19, 32%; $P = 0.018$, Fisher's exact test). Moreover, when analyzed by degree of staining intensity, IGFII ligand was expressed at significantly higher levels in both *IGFIIR*-WT and *IGFIIR*-mutant tumors than in matching normal tissues ($P < 0.01$, rank sum test).

IGFIIR expression was detected in 4 of 6 tumors (66%) with *IGFIIR* mutation, whereas 14 of 14 WT tumors (100%) expressed this protein; this difference approached but did not achieve statistical significance ($P = 0.079$, Fisher's exact test). Similarly, diminished

Table 2 *Immunoreactivity of IGFIIR, IGFII, TGF-β1, and LTGF-β1 in gastrointestinal tumor tissues*

	IGFIIR			IGFII			TGF-β1			LTGF-β1		
Case no.[a]	−	+	P	−	+	P	−	+	P	−	+	P
MUT 6	2	4	0.079	1	5	0.387	4	2	0.014	0	6	0.005
WT 14	0	14		5	9		1	13		10	4	

[a] MUT, tumor with mutant-type IGFIIR.

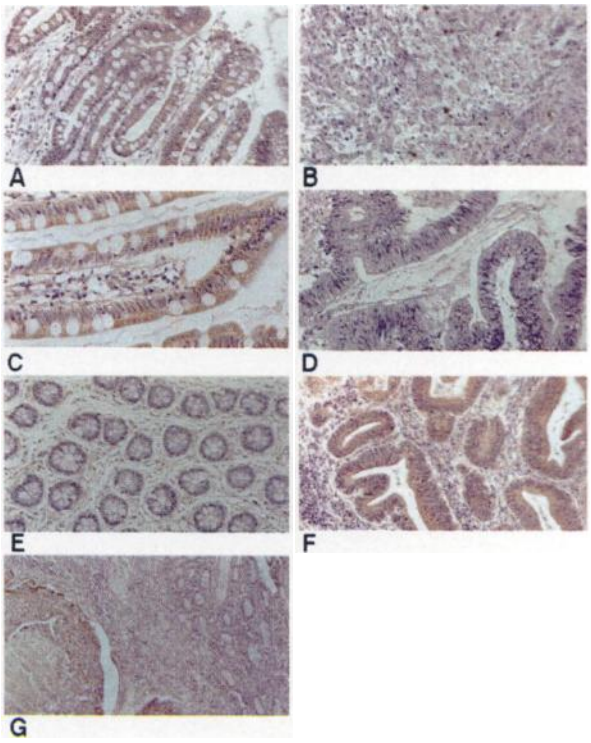

Fig. 1. Active TGF-β1, LTGF-β1, IGFII, and IGFIIR protein in tumors with *IGFIIR* mutation. *A*, active TGF-β1 in normal mucosa. Abundant protein (*brown* pigment) is visible. *B*, active TGF-β1 in gastric tumor. No protein is seen in cancer cells. *C*, IGFIIR in normal mucosa: expression is high. *D*, IGFIIR in tumor: no receptor protein is observed, consistent with inactivation of the gene at the DNA level. *E*, IGFII ligand in normal mucosa: no protein is seen. *F*, IGFII ligand in tumor: elevated expression is visible. *G*, LTGF-β1 precursor expression. Elevated protein levels are seen in tumor (*left, brown*) relative to normal epithelium (*right, purple*).

IGFIIR expression in tumors *versus* matching normals was more common in mutant (3 of 6, 50%) than WT cases (2 of 13, 15%), but this trend did not reach statistical significance ($P = 0.151$, Fisher's exact test). However, when analyzed as a group, the WT tumors expressed significantly more IGFIIR protein than did the mutant tumors ($P \leq 0.05$, rank sum test).

Discussion

Our results show that there is a decrease in active TGF-β1 but an increase in LTGF-β1 and IGFII ligand protein expression in tumors possessing *IGFIIR* mutations, relative to *IGFIIR* WT tumors and/or matching normal tissues. Although the rate of detectable IGFII ligand expression did not differ significantly between tumors with and without *IGFIIR* mutation, the staining intensity of IGFII ligand in *IGFIIR*-mutant tumors was significantly higher than in *IGFIIR*-WT tumors. Interestingly, when we compared the level of IGFII ligand expression in all 19 paired normal and tumor tissues (both mutant and WT), expression of IGFII ligand protein was significantly more frequent and more intense in the tumor group than in the normal group. Thus, up-regulation of IGFII may be a more generalized tumorigenic mechanism than *IGFIIR* mutation *per se*; *IGFIIR* mutation probably results in IGFII overexpression, but there may be other means by which IGFII becomes up-regulated in tumors. In fact, Ellis *et al.* (9) suggested that in malignant cells expressing high levels of IGFII, there may be no requirement for loss-of function mutations in *IGFIIR*, because the capacity of IGFIIR to suppress extracellular IGFII levels may have been exceeded. Ellis' hypothesis is consistent with our findings regarding expression levels of the IGFIIR protein itself, which did not differ significantly between WT and mutant tumors.

These data suggest that mutation of a microsatellite tract within *IGFIIR* alters the tumor-suppressive function of IGFIIR in at least two ways:

(*a*) Mutation is associated with increased IGFII ligand expression. Although the IGFII interacts with two receptors (IGFIR and IGFIIR), IGFIIR plays a central role in these interactions. When IGFII binds to IGFIR, a growth-stimulatory signal is generated (19, 20). However, when IGFII binds to WT IGFIIR, the ligand is internalized and degraded, making it unavailable to bind to IGFIR. Mutation of *IGFIIR* may interfere with its ability to perform this internalization/degradative function. Thus, through this mechanism, we suspect that IGFIIR acts as an IGFII antagonist and a tumor suppressor gene.

(*b*) *IGFIIR* microsatellite mutation is associated with diminished TGF-β1 activation. IGFIIR normally binds LTGF-β1, permitting cleavage into its active form (7, 8). Inactivation of IGFIIR may paralyze TGF-β1 in its latent, inactive form, removing this normally growth-suppressive growth factor from the epithelial cell milieu. The role of IGFIIR in regulating the activity of other TGF-β isoforms is unknown. The *IGFIIR* mutations that we observed could interfere with LTGF-β1 binding; however, it should be noted that the *IGFIIR* poly(G) microsatellite is actually downstream of this binding site. Nevertheless, frameshifts and predicted truncation of the IGFIIR protein do occur as a consequence of our poly(G) mutations. This truncation could interfere with the stability of IGFIIR; alternatively, it could affect anchorage of IGFIIR, and consequently LTGF-β1, to the cell surface, *i.e.*, if LTGF-β1 is not properly fixed to the cell surface, cleavage by plasmin may not occur.

Finally, diminished but detectable IGFIIR protein expression in four of six *IGFIIR*-mutant tumors does not necessarily imply that IGFIIR function was normal in these tumors: for example, witness the excessive LTGF-β1 and absent or diminished active TGF-β1 expression in these same four tumors. Specifically, we did not rule out more subtle abnormalities in the remaining *IGFIIR* allele; only the poly(G) microsatellite tract was studied. It is possible that missense mutations occurred in the remaining allele, or that postranscriptional or post-translational modifications of *IGFIIR* prevented its normal function.

In summary, these findings are consistent with the hypothesis that mutation of the *IGFIIR* gene within a coding microsatellite interferes with its function as a tumor suppressor gene in at least a subset of genetically unstable gastrointestinal tumors.

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