[CANCER RESEARCH 55, 5545–5547, December 1, 1995]

Advances in Brief

A Transforming Growth Factor β Receptor Type II Gene Mutation Common in Colon and Gastric but Rare in Endometrial Cancers with Microsatellite Instability[1]

Lois L. Myeroff, Ramon Parsons, Seong-Jin Kim, Lora Hedrick, Kathleen R. Cho, Kim Orth, Michael Mathis, Kenneth W. Kinzler, James Lutterbaugh, Keunchil Park, Yung-Jue Bang, Hwa Young Lee, Jae-Gahb Park, Henry T. Lynch, Anita B. Roberts, Bert Vogelstein, and Sanford D. Markowitz[2]

Department of Medicine, Case Western Reserve University and Ireland Cancer Center, University Hospitals of Cleveland, Cleveland, Ohio 44106 [L. L. M., J. L., S. D. M.]; Department of Pathology [L. H., K. R. C.] and Johns Hopkins University Oncology Center [R. P., L. H., K. R. C., K. W. K., B. V.] Johns Hopkins University, Baltimore, Maryland 21231; Howard Hughes Medical Institute, Chevy Chase, Maryland 20815 [B. V.]; Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892 [S-J. K., K. P., H. Y. L., A. B. R.]; Division of Molecular Medicine and Genetics, University of Michigan, Ann Arbor, Michigan 48109 [K. O.]; Simmons Cancer Center, University of Texas Southwestern Medical School, Dallas, Texas 75235 [M. M.]; Cancer Research Center, Seoul National University College of Medicine, Seoul, Korea [Y-J. B., J-G. P.]; and Department of Preventive Medicine and Public Health, Creighton University School of Medicine, Omaha, Nebraska 68178 [H. T. L.]

Abstract

We have recently demonstrated that mutation of the transforming growth factor-β (TGF-β) receptor type II (*RII*) gene is characteristic of colon cancers exhibiting microsatellite instability or replication errors (RER+). Moreover, we have shown that RII mutations in these RER+ colon cancers are characteristically frameshift mutations within a 10-bp polyadenine repeat present in the *RII*-coding region. We now show that *RII* gene mutations in this polyadenine repeat are also commonly present in RER+ gastric cancers (71%). In contrast, we find these same *RII* gene mutations are distinctly uncommon in RER+ endometrial cancers (17%, $P < 0.02$). These results suggest that *RII* gene mutations confer a growth advantage and are selected for in RER+ cancers of both the upper and lower gastrointestinal tract. The genesis of RER+ endometrial tumors must, however, be by a different route.

Introduction

TGF-β[3] inhibits the growth of many epithelial cell types (1–4) and, for example, abolishes growth of and induces apoptosis in nontransformed colon epithelial cells (5). In contrast, colon and many other cancer cell lines are resistant to suppression of growth by TGF-β (1–4, 6, 7). We have recently shown that 8 of 11 cases of RER+ (8) colon cancer were resistant to TGF-β due to mutations inactivating the RII component of the TGF-β receptor (9). We have now additionally confirmed the presence of RII mutation in 100 of 111 subsequent cases of RER+ colon cancers (10). Consistent with our original report, in each of these cases, a frameshift mutation was detected within a small adenine mononucleotide repeat at nucleotides 709–718 of the RII cDNA (9, 10). Insertions or deletions of adenines within this repeat produce −1, −2, or +1 frameshift mutations, resulting in predicted synthesis of truncated RII receptors of 161, 129, or 130 amino acids, respectively. The RER+ phenotype is characteristic of cancers arising in individuals with HNPCC, a familial cancer syndrome, characterized by a high incidence of colon, endometrial, and gastric cancers (8, 11). The RER+ phenotype also is present in many sporadic colon, endometrial, and gastric cancers found in individuals not belonging to cancer families (8). We show here that *RII* gene mutations are also commonly present in RER+ gastric cancers. In contrast, we find that *RII* gene mutations are distinctly uncommon in RER+ endometrial cancers. These results suggest that *RII* gene mutations confer a growth advantage and are selected for in RER+ gastrointestinal cancers but not in RER+ endometrial tumors.

Materials and Methods

Tumors and Cell Lines. Gastric carcinoma cell lines and the Ishikawa cell line have been described previously (12, 13); other endometrial carcinoma cell lines are from the American Type Culture Collection. Genomic DNA was isolated by a standard SDS and proteinase K protocol (14). Genomic DNA was extracted from paraffin-embedded and frozen normal or tumor tissues using standard methods (15).

Amplification of RII. A 73-bp region of the *RII* gene (nucleotides 665–737) was amplified from 50–200 ng of genomic DNA with approximately 10 ng of ^{32}P end-labeled TA10-F1 primer (5′-CTTTATTCTGGAAGATGCTGC-3′) and 150 ng of reverse primer TA10-R1 (5′-GAAGAAAGTCTCACCAGG-3′) using 30 cycles of 95°C for 30 s, 55°C for 1 min, and 70°C for 1 min. The PCR products were separated by electrophoresis at 52°C on a 6% LongRanger (FMC Bioproducts) polyacrylamide/7M urea gel and visualized by autoradiography. The standard in each assay was established by amplifying wild-type RII from plasmid H2-3FF (16). All assays were repeated in triplicate. Amplification, cloning, and sequencing of RII cDNA were performed as described previously (9).

RER Assays. Reconfirmation of microsatellite instability in endometrial cancers and determination of microsatellite instability in gastric cancers was performed using primer sets BAT26 and BAT40, which amplify adenine-mononucleotide repeats and are greater than 98% sensitive for detecting RER+ (10). RER+ samples displayed microsatellite lengths divergent from matched normal tissue or, in the case of some cell lines, divergent from 10 unmatched normal samples.

Statistical Analysis. Statistical comparisons were performed using the χ^2 test with the Yates continuity correction.

Results and Discussion

To detect RII mutations in RER+ tumor types, we developed a PCR-based assay for the mutational hotspot comprising the RII polyadenine repeat. PCR amplification from genomic DNA of a 73-bp sequence encompassing this repeat is followed by PAGE to register a shift of 1 or more basepairs in the size of the amplified fragment. Fig. 1*A* shows the expected 73-bp *RII* fragment amplified from the *RII*

Received 10/6/95; accepted 10/25/95.

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.

[1] Supported by NIH Grants CA57208 and P01 CA51183 (S. D. M.); P30 CA4370301 to the Case Western Reserve University Cancer Center; CA09320 (R. P.), CA68769 (K. O.), CA66720 (L. H.), CA62924 (K. W. K.), and CA35494 (B. V.); American Cancer Society Grants FRA-451 (S. M.), RD-381 (H. L.), and EDT-84 (H. L.); Council for Tobacco Research Grant 1297ER1 (H. L.); Korea Science and Engineering Foundation Grant KOSEF-SRC-56-CRC-94K3-0401-01-03-3 (Y-J. B., J-G. P.); and by grants from the Ohio Cancer Research Associates (S. M.) and the Clayton Fund (B. V.). B. V. is an investigator of the Howard Hughes Medical Institute.

[2] To whom request for reprints should be addressed, at Ireland Cancer Center, University Circle Research Center 2, Room 200, 11001 Cedar Road, Cleveland, OH 44106.

[3] The abbreviations used are: TGF-β, transforming growth factor β; RII, TGF-β receptor type II; RER, replication error phenotype; HNPCC, hereditary nonpolyposis colorectal cancer; RT-PCR, reverse transcription-PCR.

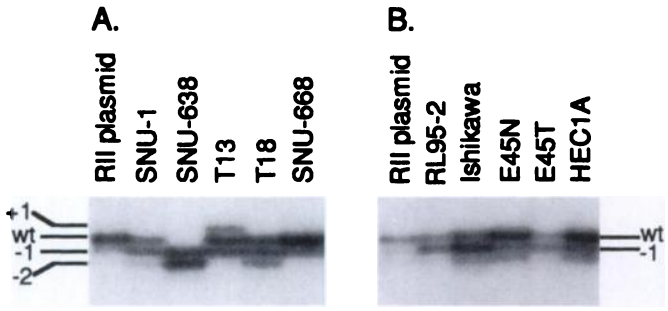

Fig. 1. PCR detection of RII mutations. Size of wild type (*wt*) PCR product is indicated, as are +1-, −1-, and −2-bp frameshifts. A, gastric carcinomas. Control RII cDNA plasmid, RII mutant cell lines SNU-1 and SNU-638, RII mutant tumors T13 and T18, and wild-type cell line SNU-668. B, endometrial carcinomas. Control RII cDNA plasmid, RII mutant cell lines RL95-2 and Ishikawa, RII mutant tumor E45T with matched normal tissue E45N, and wild-type RII cell line HEC1A.

polyadenine region in a control plasmid bearing the wild-type *RII* sequences (16). Amplification of the wild-type polyadenine tract is also associated with production of a minor PCR product sized 1 bp shorter (−1) than the wild-type fragment. Similar shortened minor reaction products are seen after PCR amplification of longer repetitive DNA sequences from genomic microsatellites and are commonly attributed to slippage by Taq polymerase (17). In comparison to the wild-type pattern, Fig. 1*B* shows the amplification of the *RII* polyadenine tract from endometrial tumor E45T, in which the signal associated with the −1-bp product is equal in intensity to that resulting from the full-length allele. This suggests that tumor E45T is heterozygous, bearing one RII allele with a −1 frameshift and one allele that is wild type within the polyadenine tract. To confirm that the RII-PCR assay is sufficiently sensitive to detect a heterozygous *RII* mutation, we amplified by RT-PCR the RII cDNA from tumor E45T and determined the RII sequence of individual cDNA clones. Consistent with the genomic RII PCR results, we detected wild-type RII sequences in 4 of 9 cDNA clones, and detected a 1-bp deletion within the polyadenine tract in the remaining 5 RII cDNA clones (not shown). We further confirmed the accuracy of the new assay by demonstrating that it detected the correct RII mutations in all 10 RER+ colon cancers examined previously by sequencing RII cDNA clones (not shown).

We next analyzed 29 cases of gastric cancer, including 21 gastric cancer tumors and 8 gastric cancer cell lines, to determine the presence of *RII* mutations and the association of such mutations with the RER+ phenotype. Seven of the 29 (24 ± 7.9%) gastric cancers were determined to be RER+ (Table 1). Five of 7 (71 ± 17%) of these RER+ gastric cancers were demonstrated to bear mutations within the *RII* polyadenine repeat (Table 1). In each of three cases (SNU-638, T13, and T18), two RII alleles were inactivated by two different mutations within the RII polyadenine tract (Table 1). No wild-type *RII* allele was detected in cell line SNU-638. The presence of residual wild-type sequence detected in T13 and T18 presumably arose from stromal cells still present in these tumor samples. Because two cases bearing *RII* mutations were cell lines (SNU-1 and SNU-638), DNAs from the paraffin-embedded tumors from which these lines were established were also studied. In both cases, the RII mutations detected in the cell lines were also present in the original tumor samples, demonstrating that the mutations did not arise during cell culture. In contrast to the RER+ gastric cancers, *RII* mutations within the polyadenine tract were detected in only 1 of 22 RER− cases (4.5 ± 4.4%). Thus, like colon cancers, gastric cancers also demonstrate mutations

Table 1 *TGF-β-RII mutation and RER status in gastric and endometrial cancers*

Gastric Carcinomas				Endometrial Carcinomas			
	(Line/tumor)[a]	TGF-β-RII[b]	RER[c]		(Line/tumor)[a]	TGF-β-RII[b]	RER[c]
SNU-1	L	−1/wt	+	Ishikawa	L	−1/wt	+
SNU-638	L	−1/−2	+	RL 95-2	L	−1/wt	+
SNU-5	L	wt	−	AN3CA	L	wt	+
SNU-16	L	wt	−	HEC1A	L	wt	+
SNU-484	L	wt	−	KLE	L	wt	−
SNU-601	L	wt	−	15T	T-F	−1/wt	+
SNU-668	L	wt	−	4T	T-F	wt	+
SNU-719	L	wt	−	5T	T-F	wt	+
T13	T-S	+1/−1	+	31T	T-F	wt	+
T18	T-S	−1/−2	+	T601	T-F	wt	+
T21	T-S	−1/wt	+	T603	T-F	wt	+
T24	T-S	−1/wt	−	E45T	T-S	−1/wt	+
T12	T-S	wt	+	E3T	T-S	wt	+
T15	T-S	wt	+	E5T	T-S	wt	+
T1	T-S	wt	−	E6T	T-S	wt	+
T2	T-S	wt	−	E8T	T-S	wt	+
T3	T-S	wt	−	E13T	T-S	wt	+
T5	T-S	wt	−	E14T	T-S	wt	+
T7	T-S	wt	−	E29T	T-S	wt	+
T8	T-S	wt	−	E30T	T-S	wt	+
T9	T-S	wt	−	E34T	T-S	wt	+
T10	T-S	wt	−	E39T	T-S	wt	+
T11	T-S	wt	−	E46T	T-S	wt	+
T14	T-S	wt	−	E57T	T-S	wt	+
T16	T-S	wt	−	E68T	T-S	wt	+
T17	T-S	wt	−	E1T	T-S	wt	−
T19	T-S	wt	−	E2T	T-S	−1/wt	−
T20	T-S	wt	−	E4T	T-S	wt	−
T25	T-S	wt	−	E7T	T-S	wt	−
				E10T	T-S	wt	−
				E12T	T-S	wt	−
				E15T	T-S	wt	−
				E16T	T-S	wt	−

[a] Indicates whether sample derived from cell line (L) or tumor (T), and whether tumor sample is familial (F) or sporadic (S).

[b] TGF-β-RII mutation status at the polyadenine repeat is denoted by "wt" if sample is wild type on both alleles, or by number (*e.g.*, −1/−2), indicating the number of bases lost on each allele as indicated by the RII PCR assay.

[c] RER phenotype: +, microsatellite instability in a sample; −, no instability.

within the *RII* polyadenine tract, and these mutations are significantly associated with the presence of the RER+ phenotype ($P < 0.01$).

To study the role of the *RII* polyadenine tract mutations in a non-gastrointestinal malignancy, we next studied 24 cases of endometrial cancer that had previously been shown to be RER+ by virtue of instability of CA repeat microsatellites (15).[4] In each of these cases, polyadenine repeat sequences within genomic microsatellites were also confirmed as being unstable; yet, mutations in the *RII* polyadenine tract were detected in only 4 (17 ± 7.6%). *RII* mutations are thus significantly less common in RER+ endometrial than in RER+ colon ($P < 0.001$) or in RER+ gastric ($P < 0.02$) cancers. Notably, mutations in the *RII* polyadenine tract were absent in five of six RER+ endometrial tumors that arose in individuals belonging to HNPCC kindreds. *RII* mutations were also absent in two endometrial cancer cell lines (HEC1A and AN3CA), which, like HNPCC-derived cancers, have demonstrable defects in DNA mismatch repair (Table 1; Refs. 18 and 19). The paucity of *RII* mutations in RER+ endometrial cancers is, therefore, unlikely to be due to a difference in the genesis of the RER+ phenotype in endometrial compared with gastrointestinal malignancies. It was not surprising that mutations in the *RII* polyadenine tract were detected in only 1 of 9 RER− endometrial cancers (11 ± 10%) additionally studied.

Mutation in the *RII* polyadenine tract thus provides a common route for RER+ carcinogenesis in both the upper and lower gastrointestinal tract, whereas RER+ endometrial cancers must proceed by a different mechanism. Perhaps endometrial cancers escape TGF-β growth control by other means, or perhaps in endometrium steroid hormones supersede TGF-β as the dominant negative regulator of epithelial proliferation. TGF-β has certainly been reported to stimulate, as well as to inhibit, endometrial cell growth both in intact animals (20) and in different cell culture models (21–24). In contrast, TGF-β induces apoptosis of gastric epithelial cells (25). Gastric cancers have commonly acquired TGF-β resistance (12), and the *RII* mutations we detect in RER+ gastric cancers SNU-1 and SNU-638 account for the known TGF-β resistance of these cell lines (12). Moreover, RII inactivation is documented among RER− gastric cancers, caused in some cases by gene deletion (12) and, as illustrated by T24, in others by gene mutation. The relative absence of *RII* polyadenine tract mutations demonstrated in RER+ endometrial cancers provides added and independent support for the idea that in RER+ gastrointestinal malignancies, these mutations do not simply reflect the RER+ status of a tumor, but rather are selected for and contribute to cancer pathogenesis.

Acknowledgments

We thank Dr. Harvey Lodish for the gift of *RII* cDNA plasmid H2-3FF.

References

1. Roberts, A., and Sporn, M. *In:* A. Roberts and M. Sporn (eds.), Peptide Growth Factors and Their Receptors. Handbook of Experimental Pharmacology, pp. 419–472. Heidelberg: Springer-Verlag, 1990.
2. Massague, J. The transforming growth factor-β family. Annu. Rev. Cell Biol., *6:* 597–641, 1990.
3. Moses, H., Yang, E., and Pietenpol, J. TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. Cell, *63:* 245–247, 1990.
4. Brattain, M., Howell, G., Sun, L., and Willson, J. K. V. Growth factor balance and tumor progression. Curr. Opin. Oncol., *7:* 77–81, 1994.
5. Wang, C. Y., Eshleman, J. R., Willson, J. K. V., and Markowitz, S. TGF-β and substrate release are both inducers of apoptosis in a human colon adenoma cell line. Cancer Res., *55:* 5101–5105, 1995.
6. Markowitz, S. D., Myeroff, L., Cooper, M., Traicoff, J., Kochera, M., Lutterbaugh, J., Swiriduk, M., and Willson, J. K. V. A benign cultured colon adenoma bears three genetically altered colon cancer oncogenes, but progresses to tumorigenicity and transforming growth factor-β independence without inactivating the *p53* tumor suppressor gene. J. Clin. Invest., *93:* 1005–1013, 1994.
7. Filmus, J., and Kerbel, R. Development of resistance mechanisms to the growth-inhibitory effects of transforming growth factor-β during tumor progression. Curr. Opin. Oncol., *5:* 123–129, 1993.
8. Eshleman, J. R., and Markowitz, S. D. Microsatellite instability in inherited and sporadic neoplasms. Curr. Opin. Oncol., *7:* 83–89, 1995.
9. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M., and Willson, J. K. V. Inactivation of the type II TGF-β receptor in colon cancer cells with microsatellite instability. Science (Washington DC), *268:* 1336–1338, 1995.
10. Parsons, R., Myeroff, L., Liu, B., Wilson, J. K. V., Markowitz, S., Kinzler, K. W., and Vogelstein, B. Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. Cancer Res., *55:* 5548–5550, 1995.
11. Marra, G., and Boland, C. R. Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. J. Natl. Cancer Inst., *87:* 1114–1125, 1995.
12. Park, K., Kim, S-J., Bang, Y-J., Park, J-G., Kim, N. K., Roberts, A. B., and Sporn, M. B. Genetic changes in the transforming growth factor β (TGF-β) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-β. Proc. Natl. Acad. Sci. USA *91:* 8772–8776, 1994.
13. Nishida, M., Kasahara, K., Kaneko, M., Iwasaki, H., and Hayashi, K. Establishment of a new human endometrial adenocarcinoma cell line, containing estrogen and progesterone receptors. Acta Obstet. Gynaecol. Jpn., *37:* 1103–1111, 1985.
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbor Laboratory, NY: Cold Spring Harbor Laboratory, 1989.
15. Burks, R. T., Kessis, T. D., Cho, K. R., and Hedrick, L. Microsatellite instability in endometrial carcinoma. Oncogene, *9:* 1163–1166, 1994.
16. Lin, H. Y., Wang, X-F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell, *68:* 775–785, 1992.
17. Hauge, X. Y., and Litt, L. A study of the origin of "shadow bands" seen when typing dinucleotide repeat polymorphisms by the PCR. Hum. Mol. Genet., *2:* 411–415, 1993.
18. Umar, A., Boyer, J. C., Thomas, D. C., Nguyen, D., Risinger, J., Boyd, J., Ionov, Y., Perucho, M., and Kunkel, T. Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. J. Biol. Chem., *269:* 14367–14370, 1994.
19. Risinger, J. I., Umar, A., Barrett, J. C., and Kunkel, T. A. A *hPMS2* mutant cell line is defective in strand-specific mismatch repair. J. Biol. Chem., *270:* 18183–18186, 1995.
20. Takahashi, T., Nelson, K., Goods, L., and McLachlan, J. A. Transforming growth factor-β promotes the growth of mouse uterus and vagina *in vivo*. J. Cell. Biochem. Suppl., *13B:* 201, 1989.
21. Rotello, R. J., Lieberman, R. C., Purchio, A. F., and Gerschenson, L. E. Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β1 in cultured uterine epithelial cells. Proc. Natl. Acad. Sci. USA, *88:* 3412–3415, 1991.
22. Croxtall, J. D., Ayub, M., Colletta, A. A., and White, J. O. TGF-β stimulation of endometrial and breast-cancer cell growth. Int. J. Cancer, *50:* 822–827, 1992.
23. Korc, M., Haussler, C. A., and Trookman, N. S. Divergent effects of epidermal growth factor and transforming growth factors on a human endometrial carcinoma cell line. Cancer Res., *47:* 4909–4914, 1987.
24. Boyd, J. A., and Kaufman, D. G. Expression of transforming growth factor β1 by human endometrial carcinoma cell lines: inverse correlation with effects on growth rate and morphology. Cancer Res., *50:* 3394–3399, 1990.
25. Yanagihara, K., and Tsumuraya, M. Transforming growth factor β1 induces apoptotic cell death in cultured human gastric carcinoma cells. Cancer Res., *52:* 4042–4045, 1992.

[4] L. Hendrick, K. R. Cho, K. Orth, and M. Mathis, unpublished data.