Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 264, No. 18, Issue of June 25, pp. 10331-10334, 1989

Deregulation of Alternative Splicing of Fibronectin Pre-mRNA in Malignant Human Liver Tumors*

(Received for publication, January 17, 1989)

Fumitaka Oyama, Setsuo Hirohashi‡, Yukio Shimosato‡, Koiti Titani, and Kiyotoshi Sekiguchi¶

From the Institute for Comprehensive Medical Science, Fujita-Gakuen Health University, Toyoake, Aichi 470-11 and the ‡Pathology Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan

Alternative splicing of fibronectin pre-mRNA at the ED-A region has been shown to be regulated in a tissue- and developmental stage-specific manner. We investigated the splicing pattern at this region in malignant and nonmalignant human liver tissues and found that the relative population of the fibronectin mRNA containing the ED-A sequence is markedly increased in malignant liver tumors. Nontumorous liver tissues including those with chronic hepatitis and cirrhosis did not show any significant change in the alternative splicing at the ED-A region. It was also found that the increased expression of the ED-A-containing fibronectin mRNA closely correlates with the occurrence of portal vein tumor thrombus and intrahepatic metastasis, which are two characteristic features of invasive liver tumors. These results indicate that tissue-specific alternative splicing of fibronectin mRNA is modified in human liver cancer and raise a possibility that the putative molecular machinery governing alternative RNA splicing of not only fibronectin but also other cellular proteins is deregulated in malignant human tumors.

Fibronectin (FN),[1] a high molecular weight glycoprotein present in the extracellular matrix and various body fluids, plays an important role in many aspects of cell-substrate interactions including cell adhesion, migration, differentiation, and malignant transformation (1–5). FN is made up of three types of internally homologous repeats referred to as type I, type II, and type III (6), each encoded by a single, or at most a pair of exon(s) (4, 7, 8). Recent studies on the cloning and sequencing of the FN gene and cDNAs have revealed that up to 20 different FN isoforms can be generated from a single gene by alternative pre-mRNA splicing at three distinct regions termed ED-A, ED-B, and IIICS (8–12). Alternative splicing at these regions appears to be regulated in a tissue- or cell type-specific manner; for example, more ED-A$^+$ mRNAs than ED-A$^-$ mRNA are expressed in the lung and cultured lung fibroblasts whereas the liver expresses almost exclusively the ED-A$^-$ mRNA (13–15). Tissue- or cell type-specific expression of the ED-A region has been also demonstrated at the protein level using site-specific antibodies (16–20).

Recently, we have found that human fetal liver expresses a significant amount of the ED-A$^+$ mRNA, as contrasted to the almost exclusive expression of the ED-A$^-$ mRNA in the adult liver (15). These results suggest that the alternative splicing of FN pre-mRNA is developmentally regulated in the liver. Since many fetal antigens and isozymes are expressed in malignant tumors, these results raised a possibility that the ED-A$^+$ mRNA is retrodifferentially expressed in liver cancer. In this report, we present evidence that the ED-A$^+$ mRNA is indeed expressed in human liver cancer and that the increase of the ED-A$^+$ mRNA is closely associated with the invasiveness of the tumor.

MATERIALS AND METHODS

Tissues—Human hepatocellular carcinoma (HCC) tissues as well as the surrounding nontumorous tissues were obtained from the surgically resected specimens for pathological examination. The macroscopic grading of HCCs was determined as described by Kanai *et al.* (21). The tissue specimens were immediately frozen at −85 °C after surgical resection. Tissue specimens with chronic hepatitis and cirrhosis were obtained from the nontumorous area distal from the tumor loci. The numbers of specimens examined for each tissue type were as follows; normal adult lung, 3; normal adult liver, 4; fetal liver, 5; liver with chronic hepatitis, 6; liver cirrhosis, 10; hepatocellular adenoma, 1; type I HCC, 7; type II HCC, 10; HCC transplanted in nude mouse, 2.

Nuclease S1 Protection Analysis—Total RNA was prepared from 0.5–2.0 g of frozen tissues as described previously (22). Nuclease S1 protection analysis was performed as described by Berk and Sharp (23). The probe DNA for the ED-A region was prepared from the FN cDNA clone pFH111, which was kindly provided by Drs. A. R. Kornblihtt and F. E. Baralle, as described previously (15). The 3′ end-labeled single-stranded probe DNA was hybridized with 5–10 μg of RNAs extracted from various liver tissues in 30 μl of 40 mM PIPES buffer, pH 6.4, containing 1 mM EDTA, 0.4 M NaCl, and 80% formamide at 53 °C for 18 h. The DNA/RNA hybrids were digested with 800 units of nuclease S1 (Boehringer Mannheim Yamanouchi, Tokyo, Japan) at 37 °C for 30 min as described (15). The nuclease-resistant fragments were analyzed on 6% polyacrylamide sequencing gels containing 7 M urea and subsequent autoradiography. The relative radioactivities of the nuclease-resistant fragments were determined with a Fujix Bio-Image Analyzer BA100 (Fuji Photo Film Co., Ltd., Kanagawa, Japan) using phosphor imaging plates (24).

RESULTS AND DISCUSSION

Alternative splicing of FN pre-mRNA at the ED-A region was analyzed by nuclease S1 protection analysis using as a probe the antisense strand cDNA covering the entire ED-A sequence (Fig. 1). The probe DNA is designed to yield, upon nuclease S1 digestion, either a 481- or 105-base fragment (referred to as "ED-A$^+$" and "ED-A$^-$" fragment, respectively) when protected by the FN mRNA species containing or lacking the ED-A sequence (Fig. 1*A*). Hybridization of the probe DNA with total RNA extracted from the adult liver gave almost exclusively the ED-A$^-$ fragment which was actually

* This work was partly supported by grants-in-aid from Fujita-Gakuen Health University, the Ministry of Science, Education, and Culture of Japan, Uehara Memorial Foundation, and Naito Science Foundation. 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.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: FN, fibronectin; ED-A$^+$ mRNA, FN mRNA containing the ED-A sequence; ED-A$^-$ mRNA, FN mRNA lacking the ED-A sequence; HCC, hepatocellular carcinoma; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

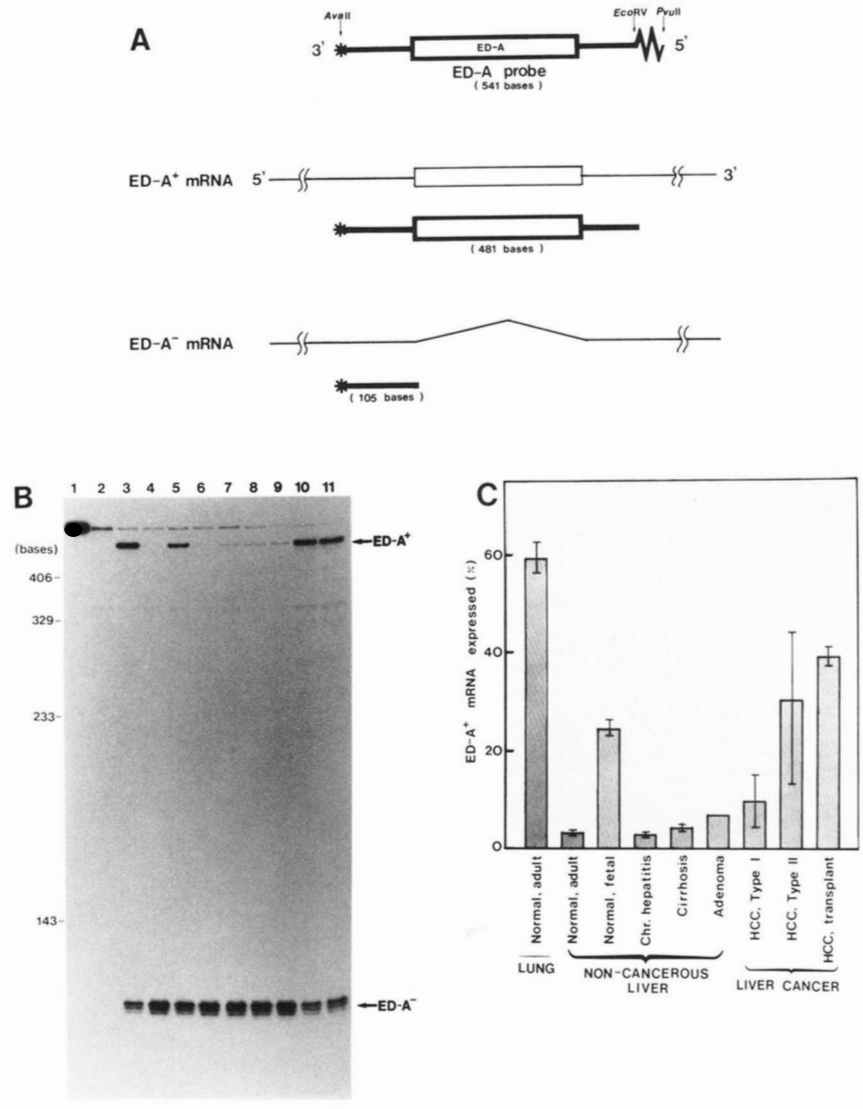

FIG. 1. **Patterns of the alternative splicing of FN pre-mRNA at the ED-A region studied by nuclease S1 protection analysis.** *A*, schematic representation of the probe DNA and its putative fragments protected by FN mRNA species containing or lacking the ED-A sequence. The probe DNA contains an extra nucleotide derived from a cloning vector at the 5' end (shown by *wavy line*), which serves as a control substrate for monitoring nuclease activity. The *open box* represents the ED-A sequence. *B*, autoradiogram of the nuclease-resistant fragments of the probe DNA protected by hybridization with RNAs extracted from rat pituitary (*lane 2*) and the following human tissues except *lane 1* (the probe DNA without nuclease digestion); *lane 3*, normal adult lung; *lane 4*, normal adult liver; *lane 5*, normal fetal liver; *lane 6*, liver with chronic hepatitis; *lane 7*, liver with cirrhosis; *lane 8*, benign hepatocellular adenoma; *lane 9*, HCC without extranodular tumor growth (type I HCC); *lane 10*, HCC with extranodular tumor growth (type II HCC); *lane 11*, transplanted HCC grown in a nude mouse. The positions of the expected fragments protected by the ED-A$^+$ and ED-A$^-$ FN mRNA species (*right*) and the molecular weight markers (the *Taq*I digest of ϕX174 DNA, *left*) are indicated in the *margin*. *C*, relative abundance of the ED-A$^+$ mRNA species in noncancerous and cancerous human liver tissues. Relative radioactivities of the ED-A$^+$ and ED-A$^-$ fragments were determined by a Fujix Bio-Image Analyzer BA100 and expressed as a percentage of the total radioactivities recovered in both fragments. The results are expressed with mean standard deviation.

resolved into three bands differing in size by one or two bases (Fig. 1*B*, *lane 4*; see also Fig. 1*C*). Heterogeneity of this fragment is probably due to the excessive attack of the termini of the DNA/RNA hybrid by the nuclease. In contrast, more ED-A$^+$ than ED-A$^-$ fragment was produced upon protection with the RNA extracted from adult lung (Fig. 1*B*, *lane 3*), confirming tissue specificity in alternative pre-mRNA splicing at the ED-A region (15). None of these fragments was generated when the probe DNA was hybridized with rat RNA (Fig. 1*B*, *lane 2*). Unlike the adult liver, a significant amount of the ED-A$^+$ mRNA was expressed in the fetal liver (Fig. 1*B*, *lane 5*; also see Fig. 1*C*), being consistent with our previous observations (15).

In order to see whether alternative splicing of FN pre-mRNA is modified in various liver diseases, we examined the splicing pattern at the ED-A region in both nonmalignant and malignant liver tissues. Hybridization of the probe DNA with RNAs extracted from human liver tissues with chronic hepatitis, cirrhosis, and benign hepatocellular adenoma yielded predominantly the ED-A$^-$ fragment (Fig. 1*B*, *lanes 6–8*; see also Fig. 1*C*), indicating that tissue-specific pre-mRNA splicing at the ED-A region is not affected in these nonmalignant liver diseases.

However, a significant increase of the ED-A$^+$ mRNA was observed in some, but not all, of human HCC tissues. Interestingly, the HCC without extranodular tumor growth, macroscopically classified as type I (21), exhibited only a marginal increase, if any, in the ED-A$^+$ mRNA (Fig. 1*B*, *lane 9*), although the HCC showing extranodular tumor growth, macroscopically classified as type II, was found to express a large amount of the ED-A$^+$ mRNA (Fig. 1*B*, *lane 10*). Quantitation of the relative radioactivities of the ED-A$^+$ and ED-A$^-$ fragments generated by protection with RNAs from 17 HCC tissues indicated that the ED-A$^+$ mRNA comprises, on the average, 9.6 and 30.4% of the total FN mRNA in type I and type II HCCs, respectively (Fig. 1*C*). Thus, the increase in the ED-A$^+$ mRNA species appears to be associated with the malignancy of the tumor. Furthermore, the relative abundance of the ED-A$^+$ mRNA is increased slightly, but significantly, even in type I HCCs when compared with those of nonmalignant liver tissues except benign adenoma ($0.02 < t < 0.1$).

In support of this notion, the increase of the ED-A$^+$ mRNA correlates with the occurrence of portal vein tumor thrombus and intrahepatic metastasis, characteristic features of HCCs with higher grades of malignancy (Fig. 2). HCCs with portal vein tumor thrombus expressed three to four times more ED-A$^+$ mRNA than those without it. A similar, but less pronounced, difference in the expression of the ED-A$^+$ mRNA was observed between the HCCs with and without intrahepatic metastasis.

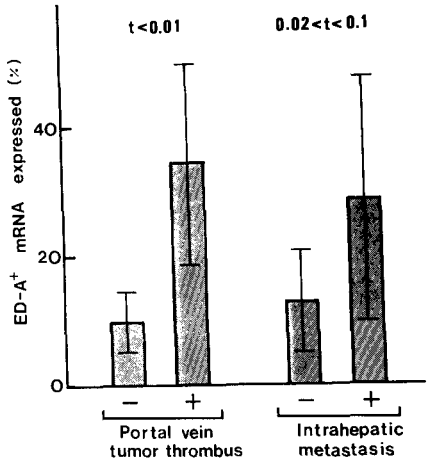

FIG. 2. **Expression of the ED-A$^+$ mRNA in noninvasive and invasive hepatocellular carcinomas.** Relative abundance of the ED-A$^+$ mRNA species was determined from the relative radioactivity of the ED-A$^+$ and ED-A$^-$ nuclease-resistant fragments as described in the legend for Fig. 1C. The *t* values are indicated above the *bars*.

Histological examination indicates that the increased expression of the ED-A$^+$ mRNA in malignant HCCs is not due to the outgrowth of fibroblasts or infiltration of macrophages but rather to the deregulation of the alternative splicing of FN pre-mRNA. Although elaboration of the "feeding artery" by the tumors may also contribute to the increase of the ED-A$^+$ mRNA, it is unlikely to be the major cause since the relative mass of the endothelial cells in the tumor tissues was far less than that of the tumor cells. Furthermore, sinusoidal vessels have been known to decrease in the tumors.

Further support for the deregulated alternative splicing of FN pre-mRNA at the ED-A region was obtained with HCC tumors grown in nude mice. The tumors were found to express a high level of the ED-A$^+$ mRNA (Fig. 1*B, lane 11*; also see Fig. 1*C*). Since the transplanted tumors should be free from human fibroblasts, macrophages, or endothelial cells, the increase of the ED-A$^+$ mRNA must be due to the deregulation of the alternative mRNA splicing *per se*. Mouse macrophages and endothelial cells infiltrating the tumors should not affect the determination of the relative abundance of the ED-A$^+$ mRNA, since the probe DNA used can only be protected by human FN mRNA. It should be noted, however, that we cannot exclude a possibility that the observed changes of the ED-A$^+$ mRNA level could be due to the differential turnover of the ED-A$^+$ and ED-A$^-$ mRNAs between normal and malignant liver tissues, since only the steady-state levels of these two mRNA species can be determined by nuclease protection analysis. There has been reported, however, no evidence that these two FN mRNA species differ in the stability in cultured cells or tissues.

Physiological significance of the increased expression of the ED-A containing FN isoforms has not yet been well understood but may well be involved in the manifestation of the invasive properties of the malignant tumor cells. It should be noted, however, that the increased expression of the ED-A-containing isoforms *per se* is not sufficient for the HCC cells to acquire invasive properties, because normal lung and endometrial tissues are shown to express a large amount of the ED-A-containing isoforms (15).

Tumor progression is considered to be a multistep process requiring not only altered cell-cell and cell-substrate adhesion but also alterations in cytoskeltal organization, signal transduction, and secretion of degradative enzymes (25, 26). A possible scenario would be, therefore, that the molecular machinery governing alternative pre-mRNA splicing is deregulated in malignant HCC cells, triggering pleiotropic alteration of the isoform diversity of not only FN but also many other proteins whose RNA transcripts are alternatively processed. An increasing number of genes, including those of cytoskeltal components such as tropomyosin (27, 28), troponin (29), myosin light chain (30, 31), and vinculin (32), as well as that of protein kinase C (33), have now been shown to be processed by alternative RNA splicing.

Transformation-associated changes of the alternative splicing at the ED-A region have been reported for virally transformed cultured cells at both the protein and mRNA levels (8, 14, 18). Similarly, the isoform diversity of tropomyosin was shown to be significantly altered in virally transformed fibroblasts (34, 35). Despite these *in vitro* observations, it has not been clear whether the alternative pre-mRNA splicing of these proteins is also altered in naturally occurring human cancer. Our results provide, as far as we know, the first direct evidence for the aberrant regulation of the alternative RNA splicing in human cancer. Further studies on the tissue-specific and oncodevelopmental regulation of differential RNA processing should provide an insight for better understanding the molecular basis of tumor progression.

Acknowledgments—We thank Drs. Nobuhiko Suganuma and Yoshiharu Murata for a generous gift of the RNAs from human fetal liver and rat pituitary and Drs. F. E. Baralle and A. R. Kornblihtt for kindly providing the FN cDNA clone, pFH111.

REFERENCES

1. Yamada, K. M. (1983) *Annu. Rev. Biochem.* **51,** 761–799
2. Mosher, D. M. (1984) *Annu. Rev. Med.* **35,** 561–575
3. Hakomori, S., Fukuda, M., Sekiguchi, K., and Carter, W. G. (1984) in *Extracellular Matrix Biochemistry* (Piez, K., and Reddi, A. H., eds) pp. 229–275, Elsevier Science Publishing Co., Inc., New York
4. Hynes, R. O. (1985) *Annu. Rev. Cell Biol.* **1,** 67–90
5. Ruoslahti, E. (1988) *Annu. Rev. Biochem.* **57,** 375–413
6. Petersen, T. E., Thøgersen, H. C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L., and Magnusson, S. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 137–141
7. Patel, R. S., Odermatt, E., Schwarzbauer, J. E., and Hynes, R. O. (1987) *EMBO J.* **6,** 2565–2572
8. Schwarzbauer, J. E., Patel, R. S., Fonda, D., and Hynes, R. O. (1987) *EMBO J.* **6,** 2573–2580
9. Sekiguchi, K., Klos, A. M., Kurachi, K., Yoshitake, S., and Hakomori, S. (1986) *Biochemistry* **25,** 4936–4941
10. Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F. E. (1985) *EMBO J.* **4,** 1755–1759
11. Zardi, L., Carnemolla, B., Siri, A., Petersen, T. E., Paolella, G., Sebastio, G., and Baralle, F. E. (1987) *EMBO J.* **6,** 2337–2342
12. Gutman, A., and Kornblihtt, A. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7179–7182
13. Kornblihtt, A. R., Vibe-Pedersen, K., and Baralle, F. E. (1984) *EMBO J.* **3,** 221–226
14. Norton, P. A., and Hynes, R. O. (1987) *Mol. Cell. Biol.* **7,** 4297–4307
15. Oyama, F., Murata, Y., Suganuma, N., Kimura, T., Titani, K., and Sekiguchi, K. (1989) *Biochemistry* **28,** 1428–1434
16. Paul, J. I., Schwarzbauer, J. E., Tamkun, J. W., and Hynes, R. O. (1986) *J. Biol. Chem.* **261,** 12258–12265
17. Peters, J. H., Ginsburg, M. H., Bohl, B. P., Sklar, L. A., and Cochrane, C. G. (1986) *J. Clin. Invest.* **78,** 1596–1603
18. Borsi, L., Carnemolla, B., Castellani, P., Rosellini, C., Vecchio, D., Allemanni, G., Chang, S. E., Taylor-Papadimitriou, J., Pande, H., and Zardi, L. (1987) *J. Cell Biol.* **104,** 595–600
19. Vartio, T., Laitinen, L., Narvanen, O., Cutolo, M., Thornell, L.-E., Zardi, L., and Virtanen, I. (1987) *J. Cell Sci.* **88,** 419–430
20. Sekiguchi, K., and Titani, K. (1989) *Biochemistry* **28,** 3293–3298
21. Kanai, T., Hirohashi, S., Upton, M. P., Noguchi, M., Kishi, K., Makuuchi, M., Yamasaki, S., Hasegawa, H., Takayasu, K., Moriyama, N., and Shimosato, Y. (1987) *Cancer* **60,** 810–819

22. Chomczynski, P., and Sacchi, N. (1987) *Annal. Biochem.* **162,** 156–159
23. Berk, A., and Sharp, P. A. (1977) *Cell* **12,** 721–732
24. Amemiya, Y., and Miyahara, J. (1988) *Nature* **336,** 89–90
25. Liotta, L. A. (1986) *Cancer Res.* **46,** 1–7
26. Nicolson, G. L. (1988) *Biochim. Biophys. Acta* **948,** 175–224
27. Ruiz-Opazo, N., Weinberger, J., and Nadal-Ginard, B. (1985) *Nature* **315,** 67–70
28. MacLeod, A. R., Houlker, C., Reinach, F. C., Smillie, L. B., Talbot, K., Modi, G., and Walsh, F. S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7835–7839
29. Breitbart, R. E., Nguyen, H. T., Medford, R. M., Destree, A. T., Maldavi, V., and Nadal-Ginard, B. (1985) *Cell* **41,** 67–82
30. Nabeshima, Y., Fujii-Kuriyama, Y., Muramatsu, M., and Ogata, K. (1984) *Nature* **308,** 333–338
31. Periasamy, M., Strehler, E. E., Garfinkel, L. I., Gubits, R. M., Ruiz-Opazo, N., and Nadal-Ginard, B. (1984) *J. Biol. Chem.* **259,** 13595–13604
32. Gimona, M., Small, J. V., Moeremans, M., Van Damme, J., Puype, M., and Vandekerckhove, J. (1988) *EMBO J.* **7,** 2329–2334
33. Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., and Hidaka, H. (1987) *Nature* **325,** 161–166
34. Matsumura, F., Lin, J. J.-C., Yamashiro-Matsumura, S., Thomas, G. P., and Topp, W. C. (1983) *J. Biol. Chem.* **258,** 13954–13964
35. Leavitt, J., Latter, G., Lutomski, L., Goldstein, D., and Burbeck, S. (1986) *Mol. Cell. Biol.* **6,** 2721–2726