A Novel Somatic Point Mutation of the *RET* Proto-oncogene in Tumor Tissues of Small Cell Lung Cancer Patients

Hitoyasu Futami,[1, 3] Shin-ichi Egawa,[1] Toshihiko Tsukada,[1] Kouji Maruyama,[1] Shuji Bandoh,[1] Masayuki Noguchi[2] and Ken Yamaguchi[1]

[1]*Growth Factor Division and* [2]*Pathology Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104*

We examined whether the novel point mutation from GCC (Ala) to GAC (Asp) at codon 664 in exon 11 of *RET* proto-oncogene, which we had found in two small cell lung carcinoma (SCLC) cell lines, existed in genomic DNA of tumor tissues of the two SCLC patients from whom these SCLC cell lines were derived. Sequence analysis revealed that point mutation identical to that of the SCLC cell lines was present in amplified alleles of single-strand conformational variants in genomic DNA of the tumor tissues, whereas it was not detected in genomic DNA of non-tumor tissues of the patients. These results indicate that this mutation had initially occurred in the SCLC patients and was of somatic origin.

Key words: Small cell lung carcinoma — *RET* — Point mutation — Multiple endocrine neoplasia — Medullary thyroid carcinoma

The *RET* proto-oncogene, which encodes receptor tyrosine kinase for an unidentified ligand,[1, 2] has been reported to be associated with several neoplasms and inherited diseases.[3-13] Rearrangements and overexpression of the *RET* proto-oncogene have been found in human papillary thyroid carcinomas,[3-5] and neuroblastomas,[6, 7] respectively. Recently, germ-line point mutations of the *RET* proto-oncogene have been reported to be closely associated with several inherited diseases including multiple endocrine neoplasia type 2A (MEN2A),[8, 9] and 2B (MEN2B),[10] familial medullary thyroid carcinoma (FMTC),[8] and Hirschsprung's disease.[11, 12] Moreover, somatic mutations of this gene were also found in some sporadic cases of medullary thyroid carcinoma (MTC) and pheochromocytoma.[10, 13] Based on these lines of evidence, point mutations of the *RET* proto-oncogene have been thought to play an important role in the carcinogenesis of at least some neuroendocrine tumors.

Small cell lung carcinoma (SCLC) is a highly malignant neoplasm and follows an aggressive clinical course. This cancer is characterized by an ability to secrete a variety of hormonal neuropeptides, as do several neuroendocrine tumors including MTC.[14, 15] Since it was expected that some genetic changes of SCLC might be shared with these neuroendocrine tumors, we previously examined whether SCLC possessed genetic alteration of the *RET* proto-oncogene, using SCLC cell lines. We found a novel point mutation of the *RET* proto-oncogene

at codon 664 in exon 11 in Lu130 and Lu134A SCLC cell lines out of six SCLC cell lines examined.[16] However, it has not been determined whether this genetic alteration occurred in the patients or during the course of the establishment or the *in vitro* culture of these cell lines. In the present study, we examined whether this point mutation existed in genomic DNA of tumor tissues of the two SCLC patients from whom these two SCLC cell lines were derived.

We first performed polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis to detect the point mutation of the *RET* proto-oncogene of exon 11 in both the tumor and the normal tissue of each patient using the same primers as previously described.[16] One tumor tissue was obtained by biopsy of subcutaneous metastases of the 61-year-old male patient from whom Lu130 was derived. This patient exhibited features of ectopic ACTH syndrome. Laboratory examinations showed high plasma ACTH and cortisol levels, which were not suppressed by administration of high-dose dexamethasone. Histologically, small-sized tumor cells were diffusely infiltrating into subepithelial connective tissue. They showed hyperchromatic and finely granular nuclei with a thin nuclear membrane and inconspicuous nuclei. The histological diagnosis was small cell carcinoma, oat cell type. The diagnosis of ectopic ACTH syndrome was confirmed by the finding of a high concentration of ACTH (940 ng/g wet weight) in the tumor specimen. Other hormonal studies revealed that the tumor tissue contained a large amount of gastrin-releasing peptide (GRP) (270 ng/g wet weight).[17] A

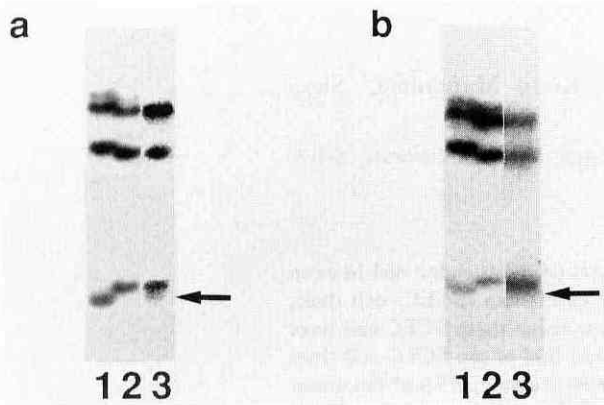[3] To whom requests for reprints should be addressed.

Fig. 1. PCR-SSCP analysis of the *RET* proto-oncogene in exon 11 in genomic DNA from SCLC cell lines and tumor tissues of SCLC patients. Lanes indicate samples prepared from the following: (a) 1, Lu130 cell line; 2, peripheral blood mononuclear cells from a normal volunteer; 3, tumor tissue of the SCLC patient from whom Lu130 was derived; (b) 1, Lu134A cell line; 2, peripheral blood mononuclear cells from a normal volunteer; 3, tumor tissue of the SCLC patient from whom Lu134A was derived. Arrows indicate a single-strand conformational variant.
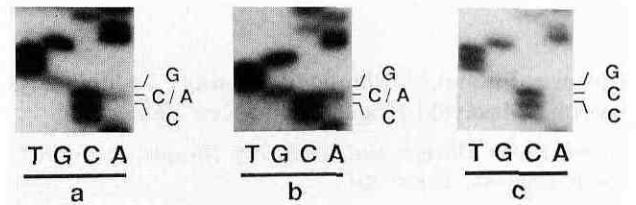

Fig. 2. Direct sequencing analysis of amplified mutated alleles of single-strand conformational variants of the *RET* proto-oncogene in the tumor tissues of the two SCLC patients. Nucleotide sequence showed the point mutation from GCC (Ala) to GAC (Asp) at codon 664, in addition to the normal sequence at this codon. Lanes from left to right are nucleotides T, G, C, A of the sense strand. a, tumor tissue of the SCLC patient from whom Lu130 was derived; b, tumor tissue of the SCLC patient from whom Lu134A was derived; c, peripheral blood mononuclear cells from a normal volunteer.

normal tissue sample for the preparation of the genomic DNA of this patient was obtained from the spleen at autopsy. Another tumor tissue was obtained by surgical operation on the primary tumor of the 80-year-old male patient from whom Lu134A was derived. This patient did not show particular symptoms or signs due to hormonal excess. Histologically, most of the tumor cells were small and showed hyperchromatic nuclei, compatible with oat cell or intermediate cell type small cell carcinoma. But there was a minor component of tumor cells which showed large and varied nuclei. The cytoplasm was abundant with an ill-defined cell border. The histological diagnosis was small cell carcinoma with a large cell component. Hormonal studies also revealed that tumor tissue contained a large amount of GRP (280 ng/g wet weight) and growth hormone-releasing hormone (1600 ng/g wet weight).[18] A normal tissue sample for the preparation of the genomic DNA of this patient was obtained from the normal tissue of the resected lung. Genomic DNA for PCR-SSCP analysis was extracted from formalin-fixed and paraffin-embedded samples,[19] and PCR-SSCP analysis was carried out as described previously.[16, 20] PCR-SSCP analysis for genomic DNA from both tumor tissues revealed a faint band of a single strand conformational variant (SSCV) at a position adjacent to the normal band (Fig. 1, arrows). The mobility shifts of these SSCVs in the gel were identical to those of Lu130 and Lu134A cell lines. Subsequently, these small

areas of SSCVs below the normal bands were cut out and subjected to DNA extraction for secondary PCR as described previously.[21] The secondary PCR was performed using the primers described previously.[16] The direct sequencing of the PCR products revealed the point mutation from GCC (Ala) to GAC (Asp) at codon 664 in addition to the normal sequence at this codon (Fig. 2). This nucleotide alteration was identical to those observed in the two cell lines Lu130 and Lu134A.[16] The direct sequencing of genomic DNA from normal tissues of these patients revealed only the wild-type codon 664 in exon 11 (data not shown).

We demonstrated in this study that the novel point mutation of the *RET* proto-oncogene was also present in the tumor tissues of the two SCLC patients. This result indicates that the genetic alteration of the mutated *RET* proto-oncogene observed in Lu130 and Lu134A cells had initially occurred in these patients. In addition, the fact that only the wild-type codon 664 in exon 11 was observed in the genomic DNA from normal tissues of these patients indicates that this mutation was of somatic origin. Moreover, in order to determine the frequency of this point mutation of exon 11 in SCLC, we further examined whether this mutation was present in tumor specimens from another 5 SCLC patients using PCR-SSCP analysis as described above. However, SSCVs corresponding to those detected in the two SCLC cell lines were not detected in these specimens (data not shown). Thus, so far, this point mutation has been found in 2 of 7 SCLC patients (29%). Nevertheless, the finding that two SCLC cases independent of each other had the same novel point mutation of the *RET* proto-oncogene *in vivo* strongly suggests that this point mutation is closely

associated with a part of SCLC. PCR-SSCP analysis demonstrated that the bands of SSCVs in these patients were much fainter than the normal bands in the same lanes, indicating that the population of the mutated allele in the tumor specimen was small. One possible explanation for this is that the mutated allele existed only in a limited subpopulation of the cancer cells, although it remains to be determined whether the population of normal cells in the tumor tissues was relatively large.

As previously described, only the mutated allele of *RET* proto-oncogene was detected in both of the cell lines.[16] These cell lines were established through several passages of xenograft transplantation followed by *in vitro* culture.[22] Therefore, it is possible that the cancer cells carrying the point mutation of codon 664 might have been selected and become dominant in the transplanted tumor in nude mice or in cell culture. Recently, the mutated *RET* alleles reported in MEN2A, MEN2B and FMTC were shown to be transforming genes in NIH 3T3

cells as a consequence of constitutive activation of the *RET* kinases.[23] Thus, if the altered *RET* proto-oncogene carrying this novel point mutation also acted as a transforming gene, it might have led to transplantability for these tumors in nude mice or might have offered some growth advantage to the tumor cells in the culture medium. Currently, studies to examine whether *RET* protein with this point mutation has transforming activity are under way in our laboratory.

REFERENCES

1) Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene,* **3,** 571–578 (1988).

2) Tahira, T., Ishizaka, Y., Itoh, F., Sugimura, T. and Nagao, M. Characterization of *ret* proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. *Oncogene,* **5,** 97–102 (1990).

3) Ishizaka, Y., Itoh, F., Tahira, T., Ikeda, I., Ogura, T., Sugimura, T. and Nagao, M. Presence of aberrant transcripts of *ret* proto-oncogene in a human papillary thyroid carcinoma cell line. *Jpn. J. Cancer Res.,* **80,** 1149–1152 (1989).

4) Grieco, M., Santoro, M., Berlingieri, M. T., Melillo, R. M., Donghi, R., Bongarzone, I., Pierotti, M. A., Della, P. G., Fusco, A. and Vecchio, G. PTC is a novel rearranged form of the *ret* proto-oncogene and is frequently detected *in vivo* in human thyroid papillary carcinomas. *Cell,* **60,** 557–563 (1990).

5) Bongarzone, I., Monzini, N., Borrello, M. G., Carcano, C., Ferraresi, G., Arighi, E., Mondellini, P., Della, P. G. and Pierotti, M. A. Molecular characterization of a thyroid tumor-specific transforming sequence formed by the fusion of *ret* tyrosine kinase and the regulatory subunit RI alpha of cyclic AMP-dependent protein kinase A. *Mol. Cell. Biol.,* **13,** 358–366 (1993).

6) Nagao, M., Ishizaka, Y., Nakagawara, A., Kohno, K., Kuwano, M., Tahira, T., Itoh, F., Ikeda, I. and Sugimura, T. Expression of *ret* proto-oncogene in human neuroblastomas. *Jpn. J. Cancer Res.,* **81,** 309–312 (1990).

7) Ikeda, I., Ishizaka, Y., Tahira, T., Suzuki, T., Onda, M.,

Sugimura, T. and Nagao, M. Specific expression of the *ret* proto-oncogene in human neuroblastoma cell lines. *Oncogene,* **5,** 1291–1296 (1990).

8) Donis-Keller, H., Dow, S., Chi, D., Carlson, K. M., Toshima, K., Lairmore, T. C., Howe, J. R., Moley, J. F., Goodfellow, P. and Wells, S. A. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum. Mol. Genet.,* **2,** 851–856 (1993).

9) Mulligan, L. M., Kwok, J. B. J., Healey, C. S., Elsdon, M. J., Eng, C., Gardner, E., Love, D. R., Mole, S. E., Moore, J. K., Papi, L., Ponder, M. A., Telenius, H., Tunnacliffe, A. and Ponder, B. A. J. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature,* **363,** 458–460 (1993).

10) Hofstra, R. M. W., Landsvater, R. M., Caccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W. M., van Amstel, H. K. P., Romeo, G., Lips, C. J. M. and Buys, C. H. C. M. A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature,* **367,** 375–376 (1994).

11) Edery, P., Lyonnet, S., Mulligan, L. M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul, F. C., Ponder, B. A. and Munnich, A. Mutations of the *RET* proto-oncogene in Hirschsprung's disease. *Nature,* **367,** 378–380 (1994).

12) Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. and Martucciello, G. Point mutations affecting the tyrosine kinase domain of the *RET* proto-oncogene in Hirschsprung's disease. *Nature,* **367,** 377–378 (1994).

13) Eng, C., Smith, D. P., Mulligan, L. M., Nagai, M. A.,

Healey, C. S., Ponder, M. A., Gardner, E., Scheumann, G. F. W., Jackson, C. E., Tunnacliffe, A. and Ponder, B. A. J. Point mutation within the tyrosine kinase domain of the *RET* proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. *Hum. Mol. Genet.*, **3**, 237–241 (1994).

14) Yamaguchi, K., Abe, K., Adachi, I., Suzuki, M., Kimura, S., Kameya, T. and Yanaihara, N. Concomitant production of immunoreactive gastrin-releasing peptide and calcitonin in medullary carcinoma of the thyroid. *Metabolism*, **33**, 724–727 (1984).

15) Yamaguchi, K., Abe, K., Adachi, I., Otsubo, K., Nagasaki, K., Suzuki, M., Maruno, K., Asanuma, F., Tsuchihashi, T., Miyake, Y., Yoshinaga, K., Matsubara, H., Imanishi, K., Kimura, S., Kodama, T., Kameya, T. and Shimosato, Y. Peptide hormone production in small cell lung carcinomas with particular reference to gastrin-releasing peptide. *Jpn. J. Clin. Oncol.*, **16**, 235–241 (1986).

16) Futami, H., Egawa, S. and Yamaguchi, K. A novel point mutation of the *RET* proto-oncogene in small cell lung carcinoma cell lines. *Proc. Jpn. Acad.*, **70B**, 210–214 (1994).

17) Yamaguchi, K., Abe, K., Kameya, T., Adachi, I., Taguchi, S., Otsubo, K. and Yanaihara, N. Production and molecular size heterogeneity of immunoreactive gastrin-releasing peptide in fetal and adult lungs and primary lung tumors. *Cancer Res.*, **43**, 3932–3939 (1983).

18) Yamaguchi, K., Abe, K., Suzuki, M., Adachi, I., Kimura, S., Shimada, A., Ohno, H., Kanai, A. and Kameya, T. Production of immunoreactive pancreatic growth hormone-releasing factor in small cell carcinoma of the lung. *Jpn. J. Cancer Res.*, **74**, 814–817 (1989).

19) Noguchi, M., Maezawa, N., Nakanishi, Y., Matsuno, Y., Shimosato, Y. and Hirohashi, S. Application of the p53 gene mutation pattern for differential diagnosis of primary versus metastatic lung carcinoma. *Diagn. Mol. Pathol.*, **2**, 29–35 (1993).

20) Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. Rapid and sensitive detection of point mutations and DNA polymorphism using the polymerase chain reaction. *Genomics*, **5**, 874–879 (1989).

21) Suzuki, Y., Sekiya, T. and Hayashi, K. Allele-specific polymerase chain reaction: a method for amplification and sequence determination of a single component among a mixture of sequence variants. *Anal. Biochem.*, **192**, 82–84 (1991).

22) Terasaki, T., Kameya, T., Nakajima, T., Tsumuraya, M., Shimosato, Y., Kato, K., Ichinose, H., Nagatsu, T. and Hasegawa, T. Interconversion of biological characteristics of small cell lung cancer depending on culture conditions. *Gann*, **75**, 1089–1099 (1984).

23) Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Grieco, M., Fusco, A., Vecchio, G., Matoskova, B., Kraus, M. H. and Fiore, P. P. D. Activation of *RET* as a dominant transforming gene by germline mutations of MEN2A and MEN2B. *Science*, **267**, 381–383 (1995).