Tissue-specific Carcinogenesis in Transgenic Mice Expressing the RET Proto-Oncogene with a Multiple Endocrine Neoplasia Type 2A Mutation

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ABSTRACT

Germ line mutations of the RET proto-oncogene are responsible for the development of multiple endocrine neoplasia type 2A (MEN 2A), an inherited cancer syndrome characterized by medullary thyroid carcinoma, pheochromocytoma, and parathyroid hyperplasia. To study the mechanism of tissue-specific tumor development by RET with a MEN2A (cysteine 634→arginine) mutation, we generated transgenic mice by introducing the RET-MEN2A gene fused to Moloney murine leukemia virus long terminal repeat. Expression of the transgene and its product was detected at variable levels in a variety of tissues including thyroid, heart, liver, colon, parotid gland, and brain. All of 29 mice analyzed developed thyroid C-cell hyperplasia or medullary carcinoma, accompanying high levels of serum calcitonin. In addition, development of mammary or parotid gland adenocarcinoma was observed in one-half of the transgenic mice. RET dimerization and its complex formation with Shc and Grb2 adaptor proteins were detected in tumor tissues. Unexpectedly, no tumor formation was found in other tissues despite RET-MEN2A expression where RET dimerization was undetectable. Because these tissues but not tumors expressed glial cell line-derived neurotrophic factor family receptor α (GFRα) at high levels, this suggested that GFRα expression may interfere in the dimerization of the RET-MEN2A mutant proteins, leading to tissue-specific tumor development in vivo.

INTRODUCTION

The RET proto-oncogene encodes a receptor tyrosine kinase the ligands of which are members of the GDNF protein family (1, 2). It has been demonstrated that the intracellular signaling through RET is triggered by its interaction with GDNF family members and glycosylphosphatidylinositol (GPI)-anchored coreceptor, GFRα (3–11). On the basis of analysis of Ret+/−, Gdnf−/−, and Grfαl−/− mice, it turned out that the GDNF/GFRα/RET signaling complexes play a crucial role in the development of the enteric nervous system and kidney (12–17).

Germ line mutations in RET result in human hereditary diseases including MEN 2A and MEN 2B, FMTC and Hirschsprung’s disease (18–23). MEN 2A and MEN 2B are autosomal dominant cancer syndromes characterized by medullary thyroid carcinoma and pheochromocytoma. In addition to these tumors, ~20–30% of MEN 2A patients develop parathyroid hyperplasia, whereas MEN 2B includes mucosal neuroma, intestinal ganglioneuromatosis, and skeletal abnormalities. Most MEN2A mutations were identified in one of six cysteine residues in the extracellular domain of the RET gene (24).

Biochemical and biological analyses revealed that these cysteine mutations induced disulfide-linked dimerization of the RET protein, leading to constitutive activation of its intrinsic tyrosine kinase (25, 26). On the other hand, mutations in the kinase domain of RET that result in substitution of threonine for methionine at codon 918 or of phenylalanine for alanine at codon 883 are responsible for MEN 2B (20, 22, 27, 28). The MEN2B mutations appear to activate RET without dimerization (26, 29, 30). Despite different molecular mechanisms of RET activation by MEN2A and MEN2B mutations, however, the binding of Shc adaptor proteins to tyrosine 1062 of RET was important for the transforming activity of both RET-MEN2A and RET-MEN2B mutant proteins in vivo (31, 32).

Recently, transgenic mice expressing RET-MEN2A or RET-MEN2B mutant proteins were reported by two groups (33, 34). In these cases, the RET-MEN2A and RET-MEN2B genes were linked to the calcitonin gene-related peptide/calcitonin promoter and dopamine β-hydroxylase promoter to induce their expression specifically in calcitonin-secreting parafollicular C cells and in developing sympathetic and enteric nervous systems, respectively. As expected, transgenic mice carrying the RET-MEN2A gene developed thyroid CCH and medullary carcinoma (33). Transgenic mice that carried the RET-MEN2B gene developed ganglioneuromas in the sympathetic nervous system and adrenal glands as well as renal malformation, although the enteric nervous system was not affected (34).

To investigate further the action of the RET-MEN2A gene in various tissues, we used MoMuLV LTR. All of the transgenic mice from the high copy line developed CCH or medullary thyroid carcinoma, and one-half of them developed mammary or parotid gland adenocarcinoma. Interestingly, although mutant RET proteins were expressed in liver, heart, and brain at high levels, no tumor formation was observed in them. Analysis of GFRα expression in various tissues suggested that high levels of its expression may interfere with RET dimerization, which results in suppression of tumor development. In addition, the present study may partly explain the differences of clinical phenotypes between MEN 2A and MEN 2B.

MATERIALS AND METHODS

Construction of the Transgene. A cDNA clone containing the entire coding sequence (for 1114 amino acids) of the human c-RET gene was inserted into the APtag-1 vector including MoMuLV LTR, kindly provided by Dr. P. Leder (Harvard Medical School, Boston, MA). A MEN2A mutation (Cys634→Arg) was introduced by PCR. The mutation and absence of polymerase errors were verified by sequencing.

Generation of Transgenic Mice. The MoMuLV/RET-MEN2A transgene was excised from the vector by Dru1 digestion and purified from agarose gels using Gene Clean II (BIO101 Inc.). The linearized DNA (7.5 kb) was injected into the pronuclei of fertilized oocytes from (C57BL/6 × BALB/c)A mice at a concentration of 2 μg/ml in TE buffer (5 mm Tris-HCl (pH 7.4)/0.2 mm EDTA). Eggs surviving microinjection were transferred into the oviducts of pseudopregnant ICR females. The offspring were weaned at 4 weeks of age, and genomic DNA was extracted from their tails. Integration of the transgene was screened by PCR analysis and Southern blotting. The forward primer used for polymerase chain reaction (PCR) amplification was 5'-CCGCTGCTGCTTCCGCTCGG-3' and the reverse primer was 5'-CTGGGTTGTCGGTGTCGGGG-3'. PCR products were size separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Southern blotting was used to determine the presence of the transgene. The isolated DNA was digested with appropriate restriction enzymes and transferred to nylon membranes for hybridization. The DNA probe used was a 600-bp fragment containing the calcitonin gene-related peptide/calcitonin promoter and dopamine β-hydroxylase promoter, which was subcloned into the APtag-1 vector.
for PCR (5'-TCCCTTTTTGTACATATCTACACCA-3') was derived from exon 16 of the human RET gene, and the reverse primer (5'-AATCCATGCTGAGGAAGGGGTTCTGA-3') from exon 19. Two transgenic mouse lines (lines 121 and 180) were established by crossing founder mice with BALB/cA or C57BL/6 mice.

**Analysis of Transgene Expression.** Mouse tissues were removed under general anesthesia and total cellular RNAs were isolated using RNeasy Mini Kit (QIAGEN). RNAs were reverse-transcribed with AMV reverse transcriptase XL (TaKaRa) for 30 min at 55°C, and the resulting cDNA was subjected to 30 cycles of PCR with a thermal cycler. (94°C for 30 s, 56°C for 30 s, and 72°C for 30 s). The forward primer (5'-CTCTGCAGGAGGGCCTCTGCA-3') annealed to exon 10 of the human RET gene, and the reverse primer (5'-TGAATCCGACCCTGGCTCC-3') hybridized to exons 15 and 16. The primer set was designed not to anneal to the mouse Ret gene at the 3' end of the primers. Primer sets for other genes are as follows: mouse Ret (forward primer, 5'-CTCTGGAAGGGCCTCTGCA-3'; reverse primer, 5'-GGATAATCCGGCCTGCTCTTT-3'); mouse GFRα1 (forward primer, 5'-TCATTGGGAAATCCTGAGT-3'; reverse primer, 5'-GCTCATGGT-GGTTACAGTCT-3'); mouse GFRα2 (forward primer, 5'-GCTG-CCCTGGCAGACACTAC-3'; reverse primer, 5'-GAGCTCTGTTGAAACA-CATGGC-3'); mouse GFRα3 (forward primer, 5'-CTTGTGCAATCTGAGCAGTCC-3'; reverse primer, 5'-CCACAGGGGCAATCTGAC-3'); and mouse β-actin (forward primer, 5'-AGCTGCTGACGCGCAGGCCATG-3'; reverse primer, 5'-GCTACGAGGAGGACATGTAC-3').

**Antibodies.** Anti-RET antibody was raised against the COOH-terminal 19 amino acids of the long isoform as described previously (10). Anti-Shc polyclonal antibody and anti-Grb2 monoclonal antibody were purchased from Transduction Laboratories. Anti-phosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology. Anti-phosphoErk polyclonal antibody and anti-Erk2 monoclonal antibody were purchased from New England Bio-labs and Santa Cruz Biotechnology, respectively.

**Histology and Immunohistochemistry.** Fresh tissues sampled from transgenic mice or normal littersmates were fixed in 10% neutral buffered formalin or C57BL/6 mice. (lines 121 and 180) were established by crossing founder mice with BALB/cA and anti-RET antibody overnight at 4°C. Endogenous peroxidase was blocked by incubating the tissues with TPBS three times. The resulting immunocomplex was eluted by boiling in SDS sample buffer in the presence of 80 mM DTT and was subjected to Western blotting.

**Expression of the Transgene.** Mice from the F1 to F3 generations were sacrificed at 3 months of age and transgene expression was analyzed by RT-PCR. Total RNAs were prepared from various tissues of transgenic mice as well as nontransgenic littersmates and were amplified using primers specific for the human RET proto-oncogene. In the case of transgenic mice from the high copy line, the transgene expression was detected in brain, parotid gland, heart, liver, and testis at high levels and in lung, kidney, colon, and thyroid at low levels (Fig. 1A), whereas no expression was observed in any organs of control nontransgenic littersmates (data not shown). Four transgenic mice examined showed the same tissue distribution of the transgene expression. Transcription of endogenous mouse Ret gene was detected in submandibular gland at high levels and in brain, heart, colon, and testis at low levels, although it was undetectable in thyroid gland under our experimental conditions (Fig. 1A). When amplification of actin mRNA was carried out as a control, approximately equal efficiency was observed in each tissue sample (Fig. 1A). The transgene expression in transgenic mice from the low copy line was relatively high in heart but very low or undetectable in other tissues (Fig. 1B).

**RESULTS**

**Generation of MoMuLV/RET-MEN2A Transgenic Mice.** A full-length human RET cDNA with a MEN2A (Cys634→Arg) mutation was fused to MoMuLV LTR, followed by an intron and SV40 poly(A) signal. By injecting a 7.5-kb linearized fragment into fertilized eggs from C57BL/6 × BALB/cA mice, we obtained two founder mice carrying the MoMuLV/RET-MEN2A gene. Transgenic mouse lines 121 and 180 were established by crossing these founder mice with C57BL/6 or BALB/cA mice. Southern blot analyses revealed that the number of transgene copies in line 121 (22 copies) was approximately seven times higher than that in line 180 (three copies; data not shown). Although the founder mouse of the high copy line was mosaic for the transgene (data not shown), F1 mice of the high copy line, and the founder of the low copy line, transmitted the transgene in a Mendelian fashion.

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The tissues used for RT-PCR were histologically normal, except the thyroid gland of transgenic mice from the high copy line in which CCH or medullary carcinoma already developed at 3 months of age as described below.

Pathological Findings in Transgenic Mice. Both of two transgenic mouse lines were examined for pathological changes in various tissues. Mice of line 180, characterized by low transgene copies, showed no pathological phenotype in any tissues when they were sacrificed at 6 months of age. In contrast, mice of line 121, with high transgene copies, developed thyroid tumor with a complete penetrance (Table 1). Gross appearance of thyroids was normal at 4 weeks of age but microscopic analysis revealed nodular CCH (Fig. 2A) or MTC in tissues. Mice of line 180, characterized by low transgene copies, showed no pathological phenotype in any tissues when they were sacrificed at 6 months of age. In contrast, mice of line 121, with high transgene copies, developed thyroid tumor with a complete penetrance (Table 1). Gross appearance of thyroids was normal at 4 weeks of age but microscopic analysis revealed nodular CCH (Fig. 2A) or MTC in

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**Table 1** Histopathology of MoMuLV/RET-MEN2A transgenic mice

<table>
<thead>
<tr>
<th>Age (transgenic mouse line no.)</th>
<th>Thyroid pathology</th>
<th>Tumors in other organs</th>
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<td></td>
<td>CCH only</td>
<td>MTC</td>
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<tr>
<td>4 wk (121)</td>
<td>2/4 (50%)</td>
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<td>3 mo (121)</td>
<td>1/6 (17%)</td>
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<td>5 mo (121)</td>
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<td>6–9 mo (121)</td>
<td>0/13 (0%)</td>
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<tr>
<td>6 mo (180)</td>
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*ca., carcinoma.

*One transgenic mouse developed both parotid gland and mammary adenocarcinoma.

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Fig. 2. Histopathology of tumors developed in MoMuLV/RET-MEN2A transgenic mice. A–C, CCH developed in the thyroid gland of a 4-week-old transgenic mouse from line 121; A, H&E staining. The section was stained with anticalcitonin (B) or anti-RET (C) antibody. D–F, medullary thyroid carcinoma developed in a 5-month-old transgenic mouse; (D) H&E staining; the section was stained with anticalcitonin (E) or anti-RET (F) antibody. G, parotid gland adenocarcinoma developed in a 5-month-old transgenic mouse. H, electron micrograph of a medullary thyroid carcinoma cell.
all of the four mice examined. Thyroids from older transgenic mice (≥3 months old) were bigger in size than normal thyroids and often developed bilateral MTC (9 of 25 mice examined). Although 17% of transgenic mice at 3–5 months of age (2 of 12 mice) still showed the development of only CCH, all of the mice at 6–9 months of age (13 mice) developed MTC (Table 1). MTC cells grew in a solid or papillary pattern (Fig. 2), and mitotic figures were infrequently seen. DM mice) developed MTC (Table 1). MTC cells grew in a solid or papillary pattern (Fig. 2), and mitotic figures were infrequently seen. Some MTCs were associated with amyloid-like material (data not shown). No evidence of metastasis of MTC to other organs and lymph-nodes was found.

Immunohistochemical analysis of thyroid tumors revealed positive staining for calcitonin and RET proteins (Fig. 2, B, C, E, and F). In addition, it was shown by transmission electron microscopy that tumor cells contain many electron-dense neurosecretory-type granules (Fig. 2H). Consistent with these findings, serum calcitonin levels were elevated in all of the transgenic mice from line 121 and appeared to correlate with tumor sizes. For example, serum calcitonin levels in transgenic mice with only CCH and with bilateral MTC were approximately, 2-fold and 8-fold, respectively, higher than those in normal transgenic mice of line 121 and normal littermates.

In addition to thyroid tumors, transgenic mice from the high copy line developed mammary adenocarcinoma or parotid gland adenocarcinoma (Fig. 2G). Nearly one-half of the mice developed one of the two types of carcinoma at 3–9 months of age (Table 1). Four transgenic mice exhibited lung metastasis of mammary carcinoma. On the other hand, no histological change was observed in adrenal glands in which the RET-MEN2A transgene was not expressed.

Detection of Disulfide-linked RET Homodimer and RET-Shc-Grb2 Complex Formation. We and others previously demonstrated that the MEN2A mutations that involve cysteine residues present in the RET extracellular domain activate RET by inducing its ligand-independent dimerization (25, 26). To examine the state of the RET-MEN2A mutant proteins in tumor tissues, lysates that were prepared from MTC and mammary and parotid gland adenocarcinomas that developed in transgenic mice were analyzed by Western blotting with anti-RET antibody under nonreducing conditions. A lysate from NIH 3T3 cells stably expressing the RET-MEN2A protein [designated NIH(C634R)] was used as a positive control. As shown in Fig. 3A, 350-kDa RET dimers, in addition to 155-kDa and 175-kDa monomers, were detected in lysates of these tumors from transgenic mice under nonreducing conditions.

We next investigated the presence of a signal transducing complex consisting of RET, Shc, and Grb2 proteins in tumors. Lysates from MTC and mammary and parotid gland adenocarcinomas as well as from NIH 3T3 and NIH(C634R) cells were immunoprecipitated with anti-Shc antibody and then immunoblotted with anti-phosphotyrosine (pTyr), anti-RET, anti-Shc, or anti-Grb2 antibody. As shown in Fig. 3B, Shc was highly phosphorylated on tyrosine, and the mutant RET-MEN2A and Grb2 proteins were coprecipitated with Shc, which showed the complex formation of these signaling molecules.

When phosphorylation levels of Erk in tumors were analyzed by Western blotting with anti-phosphoErk (pErk) antibody, they were variable, depending on tumors (Fig. 3C). Among six tumors examined (one MTC, four parotid gland carcinomas, and one mammary carcinoma), the phosphorylation was almost undetectable in one parotid gland adenocarcinoma (Fig. 3C), although increased tyrosine phosphorylation of Shc was observed in the same tumor (Fig. 3B). In addition, the phosphorylation levels of Erk in two parotid gland carcinomas were similar to those in normal parotid glands (Fig. 3C).
Inverse Correlation between GFRα Expression and Dimerization of RET-MEN2A Mutant Proteins. Because no tumor development was observed in several tissues despite high levels of expression of the RET-MEN2A transgene, we further investigated the expression of the RET-MEN2A mutant protein in them. The expression of the mutant protein was detected in liver, heart, and brain at high levels (Fig. 4A). The expression level was also high in colon from a few transgenic mice (Fig. 4A). RET-MEN2A was expressed as 155-kDa and 175-kDa proteins in liver and heart as observed in MTC and parotid gland carcinoma, but the molecular mass was a bit different in colon and brain (Fig. 4A), probably because of the difference in glycosylation form of the mutant proteins. Although a 155-kDa band was present in liver from a normal littermate, it could be a nonspecific band because the expression of mouse Ret gene in liver was not detected by RT-PCR (Fig. 1).

We next examined dimerization and phosphorylation state of the mutant protein in these tissues. Its dimerization was almost undetectable in liver, heart (Fig. 4B), and brain (data not shown). Consistent with this result, the level of tyrosine-phosphorylation of the mutant protein was not increased (Fig. 4C), which confirms that dimerization was required for its activation.

Recently, Trupp et al. (11) reported that the spontaneous dimer formation of RET expressed in COS cells could be inhibited in a dose-dependent manner by coexpression with GFRα receptors (GFRα1, GFRα2 or GFRα3). It was also shown that GFRαs were expressed in heart, liver, and brain at high levels (35). Thus, we analyzed the expression of GFRαs in tumors developed in transgenic mice to investigate whether it influences the RET dimerization. RT-PCR analysis revealed that the expression of GFRα1, GFRα2, or GFRα3 mRNA was undetectable or was very low in tumors (Fig. 5). Heart, liver, brain, and testis in which no tumor formation was observed expressed at least one of the GFRαs at high levels, whereas their expression in mammary and parotid glands was undetectable or was very low (Fig. 5). These results suggested the possibility that high levels of expression of GFRα receptors at the cell surface may interfere with dimerization of the RET-MEN2A mutant proteins.

DISCUSSION

By transfection of NIH 3T3 cells, we, and previously others, elucidated the mechanism of RET activation by MEN2A mutations identified at cysteine residues in the RET extracellular domain. Biological and biochemical analyses demonstrated that all of the cysteine mutations activated RET by inducing its disulfide-linked dimerization, which resulted in the complex formation of RET with Shc and Grb2 adaptor proteins (25, 26, 31, 36–39). In the present study, to investigate the mechanisms of tissue-specific tumor development observed in MEN2A, we have established a transgenic mouse model with high transgene copies (~20), using MoMuLV LTR as a regulatory unit. In this line, the RET-MEN2A mutant gene was expressed at high levels in brain, parotid gland, heart, liver, and testis and at low levels in lung, kidney, colon, and thyroid. Although MoMuLV LTR contains promoter and enhancer elements known to be active in hematopoietic cells in vitro, expression of the transgene was undetectable in the spleen and thymus of our transgenic mice. It is possible that the unique combination of the sequences present in the MoMuLV/RET-MEN2A transgene and/or its integration site may affect the transcription pattern of the transgene.

Despite the widespread transgene expression, however, transgenic
mice displayed a very peculiar tissue-restricted phenotype. That is, transgenic mice developed calcitonin-secreting CCH and/or medullary thyroid carcinoma at as early as 4 weeks of age with a complete penetrance, and about one-half of them developed mammary and/or parotid gland adenocarcinoma at 5–9 months of age. In these tumors, disulfide-linked dimerization of the mutant RET protein was clearly detected as expected. In contrast, no tumor development was observed in liver, heart, and brain in which the RET-MEN2A mutant proteins were highly expressed. Unexpectedly, the levels of RET dimerization and tyrosine phosphorylation were very low in these tissues. Trupp et al. (11) reported recently that constitutive tyrosine autophosphorylation of RET overexpressed in COS cells was inhibited in a dose-dependent manner by coexpression with GFrα receptors (GFrα1, GFrα2, or GFrα3), which suggests that GFrαs could inhibit the spontaneous formation of RET homodimers in COS cells. In addition, the same group showed that RET and GFrα1 preassemble at the cell surface in the absence of GDNF (40). Thus, to investigate the relation between GFrαs expression and tumor development, we analyzed the expression of the receptors in tumors. RT-PCR analysis demonstrated that the expression of GFrα1, GFrα2, or GFrα3 mRNA was undetectable or very low in the tumors. In contrast, consistent with the previous report (35), GFrα1, GFrα2, and/or GFrα3 mRNAs were expressed in heart, liver, and brain at high levels. Moreover, it has recently been demonstrated that GFrα1 expression was undetectable in thyroid C cells (41). Taken together, these findings supported the view that high levels of GFrα expression may interfere in the dimer formation of the RET-MEN2A mutant proteins expressed in liver, heart, and brain, which results in suppression of tumor development in those organs, although it is possible that other cell surface proteins could also influence the RET-MEN2A dimerization.

In this respect, it is interesting to note that MEN 2A does not develop the mucosal neuroma and ganglioneuromatosis of the enteric nervous system that are the characteristic features of MEN 2B. These neuronal cells affected in MEN 2B are known to express GFrα1 at high levels (4). Because the MEN2B mutations activate RET in a monomeric form (26, 29, 30), this fact implied that expression of GFrα1 may not impair activation of the RET-MEN2B mutant protein in neuronal cells. Our results, thus, suggested the possibility that the expression pattern of GFrαs could be one of factors that determine the clinical features of MEN 2A and MEN 2B, in addition to possible differences of intracellular signaling activated by RET-MEN2A and RET-MEN2B proteins (26, 42, 43).

As a result of RET-MEN2A dimerization, its complex formation with Shc and Grb2 adaptor proteins was induced in tumors. In vitro experiments demonstrated that Shc binds to tyrosine 1062 of RET and is phosphorylated on tyrosine (31, 36, 38, 39). Then phosphorylated Shc binds to Grb2, leading to activation of Ras-MAPK signaling pathway. When tyrosine 1062 in RET was replaced with phenylalanine, the transfection activity of both RET-MEN2A and RET-MEN2B mutant proteins markedly decreased (30–32), which confirmed that Shc binding to RET is crucial for their activity. Despite tyrosine phosphorylation of Shc, however, the levels of Erk phosphorylation were variable, depending on tumors developed in transgenic mice. This implied that additional genetic events could be more important to acquire tumorigenic properties in these tissues.

In conclusion, the fact that CCH and/or MTC develop in the transgenic mice with a complete penetrance indicates that they constitute a useful animal model for developing therapeutic strategies for hereditary MTC caused by RET mutations. In addition, our results suggest that the tissue distributions of GFrαs may partly explain the differences in clinical phenotypes between MEN 2A and MEN 2B.

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