A Novel Type of RET Rearrangement (PTC8) in Childhood Papillary Thyroid Carcinomas and Characterization of the Involved Gene (RFG8)

Sabine Klugbauer, Anna Jauch, Edmund Lengfelder, Evgenij Demidchik, and Hartmut M. Rabes

Institute of Pathology [S. K., H. M. R.], and Radiation Biology [E. L.], Ludwig Maximilians University of Munich, D-80337 Munich, Germany; Institute of Human Genetics, University of Heidelberg, Heidelberg D-69120, Germany [A. J.]; and Thyroid Cancer Center, 220600 Minsk, Belarus [E. D.]

ABSTRACT

As part of ongoing studies on the RET rearrangement frequency in children with papillary thyroid carcinoma (PTC) after their exposure to radioactive iodine after the Chernobyl reactor accident, new methods for the detection of novel types of RET rearrangements are being developed. In this study, an improved reverse transcription-PCR strategy is used successfully to identify a new type of RET rearrangement. This rearrangement is designated PTC8 and the involved RET-fused gene (RFG) as RFG8. The identification of two reciprocal transcripts coding for the RFG8/RET and RET/RFG8 fusions suggests that the PTC8 rearrangement results from a balanced chromosomal translocation. With a view to clarify its role in tumor induction, we compared the fusion products with those of previously described RET rearrangements. We therefore sequenced and characterized the RFG8 cDNA, which showed no significant similarity to any functional protein described as yet. RFG8 is located on chromosome 18q21–22 and is expressed ubiquitously. Bioinformatic analysis predicts with a high probability that the corresponding rfg8 protein is located in the cytoplasm and is involved putatively in intracellular transport processes. Furthermore, we identified coiled-coil structures upstream of the breakpoint with one of the coiled-coils showing dimerization capability. Thus the rfg8/ret fusion protein exhibits structures for oncopgenic activation that are similar to those observed in previously described RET fusions.

INTRODUCTION

Proto RET codes for a membrane-associated receptor TK3 that is expressed in a developmental stage-specific manner in subsets of neural crest-derived cells (1). Oncogenic RET rearrangements are detected frequently in PTCs of children from Belarus who had been exposed to radioactive iodine after the Chernobyl power plant accident (2–4). These RET activations are caused by balanced chromosomal translocations that lead to the expression of at least one aberrant fusion product. Typically, these fusion proteins have a deletion of the RET NH2-terminal and fusion of its remaining TK domain to the expressed 5′end of RFGs. The described RFGs are expressed ubiquitously and contain dimerization domains. Each RET rearrangement produces chimeric mRNAs and proteins in the thyroid epithelial cells, which is a cell type that does not originate from the neural crest (for review, Refs. 5, 6). Moreover, the fusion proteins show an intrinsic and constitutive TK activity that is sufficient to induce PTC in transgenic mice (7, 8).

Previously, we have published a RT PCR-based strategy to rapidly identify hitherto unknown RET rearrangements in a large series of tumor samples (2, 9). Using RT PCR-based strategies, we have investigated thus far 191 PTCs of children and adolescents who were up to 18.3 years of age at the time of the reactor accident (10). As a result, we have identified three new types of RET rearrangement (PTC5, PTC6, and PTC7; Refs. 6, 11) and two variants of the PTC3 rearrangement (12). Thus, six different types of RET rearrangements have been reported until now for childhood thyroid carcinomas. The involved RFGs are H4 (PTC1), Rla (PTC2), LE1/ARA70 (PTC3 and PTC4; for review, Ref. 13), RFG5/GOLGIN84 (PTC5), HTIF1 (PTC6), and RFG7 (PTC7). We have now improved the identification of novel RET rearrangements by the establishment of the MPI PCR. In this study, we report the discovery of the PTC8 rearrangement in two tumor samples of our series and discuss its role in tumor induction.

MATERIALS AND METHODS

Thyroid Tissues. Tumor material was obtained from patients who underwent thyroidectomy at the Department of Surgery, Medical High School of Minsk, Belarus. The tumor specimens of interest were from a female (M119) and a male (M163) patient exposed to radioactive fallout at the age of 2 years 3 months and 2 years 10 months, respectively. Both tumors were follicular variants of PTC (14) with Tumor-Node-Metastasis classifications T2a N1a M0 (M119) and T3a N2a M0 (M163).

RT PCR, 5′ RACE, Marathon RACE, and Sequence Analysis. Poly(A)+ mRNA isolation and RT PCR were performed as described in detail elsewhere (2) with the following modification. The identification PCR (2) was replaced by the MPI PCR that was carried out using specific primer pairs for all of the known types of RET rearrangements in one PCR reaction under the conditions described for the identification PCR. Unknown fusion partners of RET were identified by the 5′ RACE system (Roche Diagnostics, Boehringer Mannheim, Germany). To obtain the cDNA sequence of the RET fusion partner, the Thyroid Marathon Ready cDNA Kit (Clontech, Palo Alto, CA; marathon RACE) was used. The marathon RACE products were cloned using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, NM). Cycle sequencing of vector DNA and direct sequencing of the RT PCR fragments were performed according to protocols for the ABI PRISM 310 genetic analyzer (Applied Biosystems-Perkin Elmer, Weiterstadt, Germany). The strategy used in this study, including data of primers tm1 and retc5, has been described in detail (2, 6, 9). Additional details concerning primer sequences and positions are shown in Figs. 1 and 3 or are available on request.

Northern Blot Hybridization. Northern blot analysis was carried out as described earlier (11). We used the RT PCR fragment rfg8.1V/R (780 bp, Fig. 3) as a hybridization probe, which is specific for RFG8 and a commercial β-actin probe (Clontech).

FISH. We used the services of the ZRP2 to obtain a hybridization probe for the FISH analysis. The PCR fragment PTC8-M119 (primer pair: rfg8.1V/rfg8int1R, GCCCAATCAAAGGATAGTGGGCT) of −2.3 kb in length was sent to ZRP2 as a probe to screen the human PAC library No. 704. Potentially positive PAC clones were handled as advised by the ZRP2 and rescreened by PCR using the primer pair: rfg8.1V/rfg8int1R (see above). We isolated at least 2 μg of PAC DNA using the QAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany) from those clones that had been positive after rescreening. One μg of DNA from PACs RPCIP704B0676Q2 and RPCIP704H1121Q25 was biotin labeled (La Roche Diagnostics, Mannheim, Germany) by a standard nick-translation reaction. Before hybridization, metaphase spreads prepared from phytohemagglutinin-stimulated blood lymphocytes were treated with

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To whom requests for reprints should be addressed, at Institute of Pathology, Ludwig Maximilians University of Munich, Thalkirchner Str. 36, D-80337 Munich, Germany. Phone: 49-89-5160-4081; Fax: 49-89-5160-4083; E-mail: hm.rabes@lrz.uni-muenchen.de.

The abbreviations used are: TK, tyrosine kinase; MPI PCR, multiple primer identification PCR; RACE, rapid amplification of cDNA ends; 5′ RACE, rapid amplification of 5′cDNA ends; FISH, fluorescence in situ hybridization; PTC, papillary thyroid carcinoma; RT PCR, reverse transcription-PCR; RFG, RET-fused gene; ZRP2, Resource Center of the German Human Genome Project.
RNase and pepsin (15). Hybridization was performed as described by Lichter and Cremer (16) with the following modifications: 100 ng of biotinylated PAC DNA was precipitated together with 30 μg of Cot-1 DNA and 1 μg salmon sperm DNA. The DNA was resuspended in 10 ml of hybridization mixture, containing 50% deionized formamide, 1× SSC, and 10% dextran sulfate, and hybridized to metaphase spreads of a healthy male donor for at least 12 h. The hybridized metaphase slides were incubated with FITC-conjugated avidin DCS (Vector Laboratories, Burlingam, CA), and signals were amplified once using a biotinylated anti-avidin D affinity purified antibody from goat (Vector Laboratories), followed by a second layer of fluorescein avidin DCS (17). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole and mounted using Vectashield mounting medium (Vector Laboratories). Visualization of FISH signals was accomplished using an epifluorescence microscope (Axiophot, Zeiss) coupled to a cooled charge device camera (Photometrics, Kodak KAF 1400 chip). Image processing and pseudocoloring was performed using the Adobe Photoshop software package.

Homology Search and Computer-assisted Sequence Analysis. Programs provided on the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics were used for sequence analysis (18).

RESULTS AND DISCUSSION

Detection of PTC8 as a Novel Type of RET Rearrangement. A large series of tumor samples are being investigated using a RT PCR-based strategy to identify hitherto unknown RET rearrangements (2, 9). We have improved the identification method to screen the samples more efficiently. The resulting combination of multiplex PCR, the new MPI PCR, 5′ RACE, and direct sequencing was used successfully to identify a novel RET rearrangement in the thyroid tumor samples M119 and M163. In these two samples, the result of multiplex PCR strongly indicated the presence of a RET rearrangement. However, the six published fusion genes H4/RET, Rla/RET, ELE1/RET, RFG5/GOLG5A/RET, HTIF1a/RET, or RFG7/RET had not been detected by MPI PCR. 5′-RACE was carried out, and a PCR product was obtained that included a non-RET sequence of ~200 bp upstream of the ret TK domain. The novel type of RET rearrangement in tumor samples M119 and M163 was designated PTC8 and the respective gene RFG8.

In keeping with our previous experiences with RET rearrangements (2, 6, 11, 12), the PTC8 rearrangement also produces two transcripts, namely RFG8/RET and RET/RFG8. We conclude, therefore, that this new type of RET rearrangement is, like all of the other RET rearrangements, a balanced chromosomal translocation. In Fig. 1, we show the RT PCR results using the original thyroid cDNA of the two tumor samples and primer pairs made using the RFG8 and RET cDNA sequences. The PCR products were sequenced directly using the corresponding PCR primers specific for the RET cDNA (Fig. 1).

Consequences of the RFG8 Gene Expression for the PTC8 Rearrangement and for Identifying the RFG8 cDNA Sequence. To further characterize RFG8, we investigated its expression pattern. Northern blot analysis of mRNA from various human tissues was performed. A RFG8 specific probe detected two transcripts of about 6.0 and 4.4 kb in 6 of 7 tissues tested, including the thyroid gland (Fig. 2). These results demonstrate that the RFG8 gene is expressed ubiquitously, which is a feature shared with all of the RFGs identified thus far (5, 6, 11). It is assumed that the rearrangement process places the ret TK domain under the control of the ubiquitously expressed RFG8 promoter. For the reciprocal translocation, the 3′ end of the RFG8 cDNA was used for sequence analysis (37).
gene comes under the control of the RET promoter. The latter clearly occurs in the tumor cells, generating the reciprocal transcript RET/RFG8 (Fig. 1). The role of this transcript in thyroid carcinogenesis remains unknown.

A BLAST search with the RFG8 part of the 5' RACE sequence using all of the available databases revealed no considerable sequence homology to any other sequences. Therefore, we performed the marathon RACE technique to complete the RFG8 cDNA sequence. This technique is designed to identify unknown 5' and 3' cDNA ends, and very little sequence information is required for primer design. We successfully amplified the 3' end of the RFG8 message, including a putative stop codon. However, we could not identify a start codon in the 5' end of the RFG8 cDNA sequence, although the two detected RFG8 messages were of 4.4 and 6.0 kb in length (Fig. 2). Several 5'-RACE experiments using different primers showed shorter fragments without potential start site or transcripts of incorrectly spliced mRNA including Alu sequences. This is comparable with our recent published results (6) on the RFG7 cDNA sequence. RFG7 was expressed by two messages of 8.2 and 5.2 kb in length, and it was also not possible to obtain the 5' cDNA end with the start codon using the marathon RACE technique. Therefore, we conclude that only shorter messages can be amplified as complete cDNA sequences by the marathon RACE method. The entire RFG7 sequence has since been published and is designated HTIF1 (19). Our partial RFG8 cDNA sequence, which results from the marathon RACE technique, is shown in Fig. 3.

Chromosomal Location of the RFG8 Gene and the Frequency of the PTC8 Rearrangement in Our Tumor Series. We have detected the PTC8 rearrangement in two samples of 191 PTCs obtained from children from Belarus. This low frequency is in contrast to the high prevalence of PTC1 and PTC3 rearrangements, but comparable with that of the PTC5, PTC6 and PTC7 rearrangements. The PTC2 rearrangement was absent in our tumor series. However, in PTC2 a chromosomal translocation, t(10;17)(q11.2;q23) juxtaposes the TK domain of the RET proto-oncogene, which is located on chromosome 10, to the 5' region of the Rfa gene on chromosome 17 (20). In contrast, a paracentric inversion of chromosome 10 is responsible for the generation of PTC1 and PTC3 rearrangements (21, 22). This implies that RET rearrangements involving genes on chromosome 10 are created more often than those involving genes on different chromosomes. We tested this hypothesis by determining the chromosomal localization of RFG8 by FISH analysis. Approximately 20 metaphase chromosome spreads from a lymphocyte culture of a healthy male were hybridized with the two PAC probes described in
this study. The PAC probes harbored a considerable portion of the RFG8 genomic sequence or even the complete sequence (this was not investigated in detail). Both PACs revealed specific hybridization signals on chromosome 18q21–22 (Fig. 4) showing that the RFG8 gene is located on chromosome 18 most proximal to the q22 band.

### Computational Characterization of RFG8 and Consequences for the PTC8 Rearrangement

Thus far, the partial RFG8 cDNA sequence consists of 3673 bp and the corresponding amino acid sequence of 1081 amino acids (Fig. 3). Database searches using both cDNA and protein sequences revealed the following significant similarities: (a) the human bacterial artificial chromosome clone RGG00C03 (human bacterial artificial chromosome library CITBHS-A) mapped to chromosome 7q31.2 was detected showing several interrupted sequence similarities between 51 and 78%. We conclude that a RFG8-related gene on chromosome 7 may exist; (b) several EST clone sequences from mice, rats, and humans have been identified showing very high similarities to the RFG8 sequence (up to 99%), indicating that the same or a highly related gene is expressed in these species. Most of the human EST sequences cover 3′ RFG8 sequence regions, and some of them belong to clones that are mapped to chromosome 18 (e.g., cDNA clones NHTBCae15h12 and IMAGE: 36907). Therefore, it cannot be excluded that they have sequences identical to the RFG8 sequence, but because of sequencing ambiguities the identity is <100%. According to the nucleic and amino acid sequence databases, however, it was not possible to identify the entire RFG8 sequence and to achieve information on the function of the corresponding protein. Therefore, we performed bioinformatic analyses to characterize the rfg8 amino acid sequence shown in Fig. 3 to postulate the role of the protein of the PTC8 rearrangement.

The partial rfg8 protein contains 145 negatively charged versus 112 positively charged amino acids, with leucine being the most abundant (13.6%). The hydrophilicity profile of the protein was plotted by the method of Kyte and Doolittle (23), but no hydrophobic region that could code for a transmembrane domain (data by the EXPASY PROTPARAM tool) was found. These predictions were confirmed by the PSORT II analysis (24, 25) according to which the rfg8 amino acid sequence codes for a protein that is in all probability cytoplasmic. In detail, the predictions are based on two different methods: (a) the NNCN score that discriminates the tendency of the protein to be either at the nucleus or in the cytoplasm is calculated based on the amino acid composition (24). NNCN predicted with a probability of 70.6% that rfg8 is a cytoplasmic protein; (b) the algorithm k-Nearest Neighbors Classifier (k-NN) was used for assessing the probability of a protein localizing at various candidate sites (25). The prediction is performed using the k-nearest data points, where k is a predefined integer parameter. According to the k data points, rfg8 has a probability of 65.2% of being a cytoplasmic protein and of 13% of being localized to the nucleus. The nuclear component of the sequence might be attributable to a putative nuclear localization signal that is shown in Fig. 3 (26). The correct localization of the rfg8 protein may be important for the function of the rfg8/ret fusion protein, because the nuclear localization signal is found upstream of the breakpoint and, therefore, would be transcribed. It could then be possible for the ret TK part of the fusion protein to interact directly with nuclear substrates.

We performed a ProfileScan to compare the partial rfg8 amino acid sequence with current PROSITE (27, 28) and Pfam (29) profile libraries. Both databases are collections of protein motifs and families with Pfam generally focusing on classical domains with a high proportion of extracellular modules. In contrast, the PROSITE profile collection emphasizes domains in intracellular proteins and proteins involved in signal transduction, DNA repair, cell cycle regulation, and apoptosis.

The ProfileScan predicted the existence of a leucine zipper in the NH2-terminal part of the rfg8 amino acid sequence (Fig. 3). To obtain more precise results, we analyzed additionally the rfg8 amino acid sequence using the Paircoil program (30). This program is designed to predict the location of coiled-coil regions. By this method, the rfg8 protein seemed to contain two NH2-terminal coiled-coil domains (Fig. 3) with the second domain appearing as a typical leucine zipper. Further analysis using the MultiCoil program (31) revealed with a probability of 75% that this second domain forms two-stranded coiled-coils. Coiled-coil domains, which confer the ability to dimerize, have been shown to play a crucial role in the ligand-independent activation of ret TK oncoproteins (32). All of the described RET rearranged proteins including rfg8 contain coiled-coils that are located upstream of the breakpoints to become part of the rfg8/ret products (5, 6, 11).

Additionally, we found three putative heat repeat motifs in the COOH-terminal part of the rfg8 amino acid sequence (Fig. 3). This motif is very common for helical repeat proteins where 3 to 36 repeat units form a rod-like helical structure that appears to function as a protein–protein interaction surface. Many of the heat repeat-containing proteins seem to be involved in intracellular transport processes (for review, Ref. 33). The heat repeat motifs are located downstream of the breakpoint in the RFG8 sequence; therefore, the putative interaction function might be important for the ret/rfg8 fusion protein.

Recently, we published the PTC6 and PTC7 types of RET rearrangements (6) and identified two transcriptional coactivators for nuclear receptors as being involved in the rearrangements. These are HTIF1α (34) and γ19, which is the same as RFG7 (6). Furthermore, ELE1, the partner of RET in the PTC3 rearrangement, has been identified as a transcriptional coactivator of the androgen receptor (35). Therefore, we compared the rfg8 amino acid sequence with those of transcriptional coactivators. We detected a LxxLL motif that is known to be responsible for the interaction of transcriptional coactivators with nuclear receptors (36). This motif is located downstream of the breakpoint in RFG8 (Fig. 3) and, therefore, is part of the rfg8/ret fusion product. However, we could not find more sequence similarities that would allow us to conclude that rfg8 is a transcriptional coactivator.

In conclusion, we identified the novel PTC8 type of RET rearrangement in two childhood PTCs from Belarus. Like all of the other RET
rearrangements described thus far, it is caused by a balanced chromosomal translocation, and reciprocal transcripts are produced. The gene involved was designated RFG8 and, like the previously detected RFGs, is expressed ubiquitously. The analysis of this novel RET rearrangement is reported in this study for the first time. The importance of each new type of RET rearrangement is not reflected in a high rearrangement frequency, but rather in the identification of similarities among the involved fusion proteins. This permits us to gain greater insight into their mode of action as rearrangement partners. As bioinformatic tools gradually improve, it becomes easier to obtain characterizations of amino acid sequences. rfg8 is most likely a cytoplasmic protein that might be involved in intracellular transport processes. As bioinformatic tools gradually improve, it becomes easier to obtain characterizations of amino acid sequences. rfg8 is most likely a cytoplasmic protein that might be involved in intracellular transport processes.

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Note Added in Proofs

After submission of this manuscript, an additional gene fusion involving RET has been reported for which the designation PTCS was chosen [Salassidis, K., Bruch, J., Zitzelsberger, H., Lengfelder, E., Kellerer, A. M., and Bauch, M.].

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