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Fusion of the Tumor-Suppressor Gene *CHEK2* and the Gene for the Regulatory Subunit B of Protein Phosphatase 2 *PPP2R2A* in Childhood Teratoma[1]

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Abstract

We characterized the molecular genetic consequences of a balanced chromosome translocation t(8;22)(p21;q12), which occurred as the sole cytogenetic aberration in short-term cultured cells from an intrathoracic mature teratoma in a 15-year-old girl. Fluorescence *in situ* hybridization and reverse transcription–polymerase chain reaction disclosed that t(8;22) resulted in the fusion of the genes *PPP2R2A* and *CHEK2*, with an inserted fragment belonging to class I endogenous retrovirus–related sequences at the junction. Sequencing of the two genes did not reveal any additional mutation. None of the three detected *PPP2R2A/CHEK2* fusion transcripts resulted in an in-frame *PPP2R2A/CHEK2* chimerical open reading frame; however, in all of them, the known open reading frame of *CHEK2* was preserved. Thus, promoter swapping leading to deregulated *CHEK2* expression would be the most likely oncogenic mechanism. Whereas inactivating mutations of *CHEK2* previously have been described in a variety of sporadic tumors and in inherited cancer-predisposing syndromes, *PPP2R2A*, encoding a regulatory subunit of the multimeric enzyme phosphatase 2, has not been directly implicated in tumorigenesis. Our findings suggest that deregulation of *CHEK2* and/or *PPP2R2A* is of pathogenetic importance in at least a subset of germ cell tumors.
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Introduction

Mature teratomas are germ cell neoplasms composed of adult-type tissues derived from at least two embryonic layers [1,2]. Most mature teratomas present congenitally in the sacrococcygeal region or within the ovaries or testes of adolescents, but they may occur throughout the body at any age. As the development of teratomas resembles embryogenesis in various aspects, they have been used as a model system to study both embryonic and tumorigenic processes.

Little is known about the genetic mechanisms involved in the pathogenesis of mature teratomas. In contrast to more aggressive subtypes of germ cell tumors, which typically have aneuploid karyotypes with a gain of the short arm of chromosome 12 as the most common feature, mature teratomas often display normal karyotype on cytogenetic analysis, and no consistent pattern of chromosomal gains or losses has been disclosed by comparative genomic hybridization [3,4]. Furthermore, neither in mature teratomas nor in germ cell tumors, in general, has any recurrent, acquired, tumor-specific, balanced chromosome rearrangement been detected. Recently, however, a constitutional t(12;15)(q13;q25), resulting in the fusion of the genes *SENP1* and *MESDC2*, was identified in a patient with infantile teratoma [5].

We have previously reported cytogenetic findings in two teratomas, one of which displayed a balanced translocation t(8;22)(p21;q12) [6]. In the present study, precise mapping of breakpoints was carried out by fluorescence *in situ* hybridization (FISH) on metaphase spreads from this tumor. Based on FISH results implicating a fusion between the *PPP2R2A* and *CHEK2* genes, further molecular investigations were undertaken.

Materials and Methods

Patient

A 15-year-old girl was admitted to a hospital because of viral meningoencephalitis. Due to increasing respiratory difficulty, X-ray and computed tomography scan of the thorax were performed, revealing a large mediastinal tumor. On cytologic analysis of cells obtained from a fine-needle aspiration biopsy of the tumor, no malignant cells were identified, and the tumor was tentatively diagnosed as cystic mature teratoma. After the patient had recovered from meningoencephalitis, the mediastinal tumor was radically excised. Histologic examination of the excised tumor demonstrated a mature teratoma without malignant features. Postoperatively, the patient had a slow

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recovery but remained disease-free for 6 years, after which she was lost to follow-up.

G-banding

Cytogenetic analysis was performed on two occasions [6]. On diagnosis, both a bone marrow sample and tumor cells derived from the fine-needle aspiration biopsy of the mediastinal mass were analyzed. All 25 metaphase cells from the bone marrow sample showed normal female karyotype, whereas short-term cultured cells from the fine-needle aspiration biopsy displayed a balanced translocation t(8;22)(p21;q12) as the sole change in 9 of 14 metaphases; the remaining five mitoses were normal. Cytogenetic investigation of the radically excised tumor showed t(8;22) as the sole change in all 25 metaphase spreads analyzed.

FISH

Vital frozen cells stored in liquid nitrogen were thawed and plated in culture flasks with RPMI 1640 medium supplemented with 17% serum and antibiotics. Chromosome preparations for FISH were harvested at passages 2 and 4, as described [7]. To confirm whether the cells still carried t(8;22), they were also subjected to conventional G-banding analysis.

The breakpoint on chromosome 22 was first investigated using commercially available probes for the *EWSR1* (22q12.2) and *BCR* (22q11.23) genes (Vysis, Downers Grove, IL). To further characterize the two chromosomal breakpoints, 32 BAC clones (16 spanning 8p12–8p21 and 16 spanning 22q11.2–22q12.2) were selected based on their location on the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/cgi-bin/Entreez/map-search, 2005) and the UCSC Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway). Clones were propagated, and DNA was extracted by standard methods [8]. BAC DNA was labeled by random hexamer priming (megaprime DNA labeling system; Amersham, Buckinghamshire, UK) with a single fluorochrome or ligand (i.e., biotin-16-dUTP, Cy3-dCTP, digoxigenin-11-dUTP, or FluorX-dCTP). Labeled probes were purified, precipitated, and dissolved in a standard hybridization solution. Slides and probes were denatured simultaneously by incubation on a hot plate at 72°C. After overnight hybridization at 37°C in a humidified chamber, the slides were washed at 74°C in 0.4× SSC for 2 minutes, DAPI-stained, and analyzed.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) and Sequence Analyses

Total RNA was extracted from cultured cells using Trizol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA synthesis was conducted using 5 μg of total RNA in a 20-μl reaction mixture containing 1× first-strand buffer, 10 mM DTT, 1 mM of each deoxynucleoside triphosphate (dNTP), 20 U of RNAse inhibitor (RNA guard; Amersham Biosciences, Piscataway, NJ), 500 pmol of random hexamers, and 200 U of M-MLV reverse transcriptase (Invitrogen). The reaction was carried out at 37°C for 60 minutes, heated at 65°C for 5 minutes, and then kept at 4°C until analysis. PCR amplifications were performed using 1 μl of cDNA as template in a final volume of 50 μl containing 1× PCR buffer, 0.2 mM of each dNTP, 1.25 mM $MgCl_2$, 0.5 μM of each forward primer and reverse primer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen), and run on a PCT-200 DNA Engine (MJ Research, Waltham, MA).

Primers specific for *CHEK2* and *PPP2R2A* were designed to detect possible fusion transcripts (Table 1). Transcripts were amplified using initial denaturation for 5 minutes at 94°C; 30 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C; and final extension for 10 minutes at 72°C. Amplified fragments were run on a 1.6% agarose gel stained with ethidium bromide, purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany), and directly sequenced with the dideoxy procedure using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) on the Applied Biosystems Model 3100-Avant DNA sequencing system. BLAST software (http://www.ncbi.nlm.nih.gov/blast) was used for the analysis of *CHEK2* and *PPP2R2A* sequence data.

Screening for Additional CHEK2 and PPP2R2A Mutations

To search for additional *CHEK2* mutations, PCR amplifications of exons 4 to 9 were performed using 160 ng of genomic DNA as template in a volume of 50 μl containing 1× PCR buffer, 0.8 mM dNTP, 1.25 mM $MgCl_2$, 0.5 μM of each forward primer and reverse primer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen). The annealing temperature for exons 4 to 9 was 58°C. PCR reactions were carried out on a PCT-200 DNA Engine (MJ Research) with initial denaturation for 5 minutes at 94°C; 30 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C; and final extension for 5 minutes at 72°C and for 2 minutes at 26°C. Amplified fragments were run on a 1.6% agarose gel stained with ethidium bromide, purified using the QIAquick gel extraction kit (Qiagen), and sequenced as described above.

For the analysis of exons 10 to 14, the methods described by Sodha et al. [9] were used. Long-range PCR across exons 10 to 14 of *CHEK2* was performed, and the product was used to individually amplify exons 10 to 14.

To search for *PPP2R2A* mutations, exons 1 to 10 were amplified by PCR and sequenced as described above. Primers for the sequencing of *CHEK2* and *PPP2R2A* are available at request.

The BLAST software (http://www.ncbi.nlm.nih.gov/blast) was used for the mutational analysis of *CHEK2* and *PPP2R2A*.

Table 1. Primers for RT-PCR and Direct Sequencing.

Primer	Sequence
PPP2R2A-242F	GTCTCCGCTTCCTGAACTCACCC
PPP2R2A-286F	CCATGTTGCGCTGCAAATGGT
CHEK2-294R	TCCTCAGGTTCTTGGTCCTCAGGA
CHEK2-473R	GGTGCCTCACACCTCTTATCCCAG
CHEK2-686R	ACAAAGGTTCCATTGCCACTGTGAT
CHEK2-982R	AGAGCTGGGTCTGCCTCTCTTGC
CHEK2-906R	CATGTTTTCCTCTCGAAAGCCAGC

Results

FISH Characterization of Breakpoints in 8p21 and 22q12

All metaphase cells harvested from passages 2 and 4 carried t(8;22) (Figure 1*A*). With the use of probes for the *EWSR1* (22q12.2) and *BCR* genes (22q11.23), the chromosomal breakpoint in chromosome 22 was narrowed down to the 6-Mb region between these two loci. Subsequently, 16 BAC clones within this region were selected for further FISH, and the hybridization of one of these clones (i.e., RP11-444G7, covering the *CHEK2* locus) showed split signals (Figure 1*B*). The breakpoint in chromosome 8 was then investigated by the use of 16 BAC clones spanning 8p12–p21. The hybridization of probe RP11-795G8 (containing the *PPP2R2A* locus) resulted in split signals (Figure 1*C*).

Detection and Characterization of PPP2R2A/CHEK2 *Fusion Transcripts*

RT-PCR with various *PPP2R2A* and *CHEK2* primer combinations was carried out to detect fusion transcripts. The use of *PPP2R2A* forward primer PPP2R2A-242F and *CHEK2* reverse primer CHEK2-982R gave rise to three amplified fragments, suggesting the presence of a *PPP2R2A/CHEK2* fusion gene (Figure 2). The three fusion transcripts were then analyzed by direct sequencing with the use of several different *PPP2R2A* forward primers and *CHEK2* reverse primers for further characterization of the fusion breakpoint. Furthermore, a fourth fainter band was seen, presumably representing another splice variant of the fusion product. This band, however, was not sequenced.

In fusion transcripts 1 and 2, exon 2 of *PPP2R2A* (NM_002717) was fused with exon 4 of *CHEK2* (NM_007194.3) (Figures 2 and 3), whereas in transcript 3, exon 2 of *PPP2R2A* was fused with exon 5 of *CHEK2*. In all three transcripts, inserted fragments were found at the junctions (Figure 3). In transcript 1, a 231-bp DNA fragment was inserted between exon 2 of *PPP2R2A* and exon 4 of *CHEK2*. In transcripts 2 and 3, a 98-bp fragment was inserted between the two genes. BLAST analysis revealed that the inserted fragment corresponded to nucleotide positions 22187 to 21953 (231-bp insert) and 22187 to 22086 (98-bp insert) of AL023494 (RP3-366L4), which flanks the telomeric 5′ part of *CHEK2*. Using a repeat masker engine (http://www.repeatmasker.org), the inserts were found to belong to class I endogenous retrovirus (ERV)–related sequences [10].

None of the three amplified transcripts resulted in a chimeric in-frame *PPP2R2A/CHEK2* open reading frame. In all detected fusion transcripts, the first 28 translated amino acids of *PPP2R2A* were followed by amino acids GQ and the terminal codon TGA.

Sequencing of *CHEK2* exons 4 to 14 and of *PPP2R2A* exons 1 to 10 did not reveal any additional mutations.

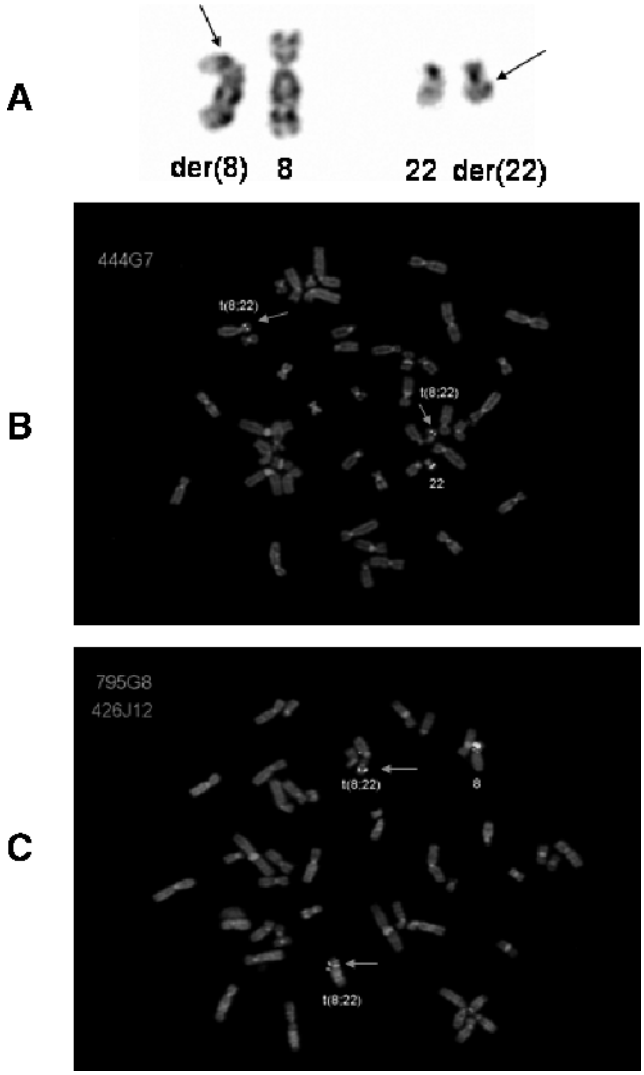

Figure 1. *(A) Partial karyogram showing a reciprocal translocation t(8;22)(p21;q12). Arrows indicate breakpoints. (B) Metaphase cell hybridized with the BAC probe RP11-444G7 (covering the CHEK2 locus; green signals). Arrows indicate split signals on derivative chromosomes 8 and 22. (C) Metaphase cell hybridized with BAC probes RP11-795G8 (covering the PPP2R2A locus; green signals) and RP11-426J12 (mapping to the centromeric side of the breakpoint on chromosome 8; red signal). Arrows indicate split signals for RP11-795G8 on the derivative chromosomes 8 and 22.*

Discussion

In the present study, we used FISH and RT-PCR to characterize a balanced translocation t(8;22) in a mediastinal mature teratoma and showed that this translocation leads to the creation of a novel fusion gene *PPP2R2A/CHEK2*. The fact that t(8;22) was the sole chromosomal change in this tumor makes it reasonable to assume that it was important in pathogenesis. Although the t(8;22) seen in our case has not been described before, it should be emphasized that only a few teratomas, in general, and pediatric extragonadal mature teratomas, in particular, have been cytogenetically analyzed. It should also be noted that various structural rearrangements with breakpoints in 8p or 22q have been reported in teratomas [11].

The mechanism by which t(8;22) may promote tumor development is unclear. All detected *PPP2R2A/CHEK2* fusion transcripts were out of frame, making it unlikely that they would be translated into proper fusion proteins. However, transcripts 1 and 2 preserve the known *CHEK2* open reading

frame, which begins at exon 4, suggesting that one consequence may be aberrant expression of *CHEK2* from the *PPP2R2A* promoter. Such transcriptional deregulation through juxtapositioning with an ectopic promoter (an oncogenic mechanism known as promoter swapping) has been well documented for several other solid tumors, including pleomorphic adenoma of the salivary glands, lipoblastoma, and aneurysmal bone cyst [12–14]. Although this seems to be the most likely mechanism in the present case, it should be noted that, theoretically, the transcripts could be translated into the first 28 amino acids of *PPP2R2A* followed by two amino acids from the inserted ERV sequence, and it cannot be excluded that this chimera could be of pathogenetic importance.

CHEK2, which encodes a checkpoint kinase, is considered a tumor-suppressor gene based on its known role in cellular responses to DNA double-strand breaks, the finding of biallelic inactivating mutations in various sporadic tumors, and epidemiological data showing that certain constitutional mutations confer increased risk for breast cancer, for example [15–17]. In response to double-strand DNA breaks, *CHEK2* becomes activated and phosphorylates various substrates, including CDC25A, CDC25C, BRCA1, E2F1, PML, PLK3, and TP53, thereby promoting a variety of cellular responses, such as cell cycle arrest, apoptosis, and DNA repair. Somatic inactivating mutations of *CHEK2* have been found in small subsets of diverse types of sporadic human malignancies, including osteosarcomas, lymphomas, and carcinomas of the breast, vulva, urinary bladder, and ovary, but they seem to be rare in pediatric cancer [18]. The majority of somatic mutations are missense or truncating mutations clustered in three domains of the CHEK2 protein: the N-terminal SQ/TQ–rich regulatory domain, the protein–protein interaction FHA domain, and the C-terminal catalytic domain [16]. 1100delC mutation, which was first reported in a subset of patients with Li-Fraumeni syndrome, and other constitutional variants are overrepresented in families predisposed to breast cancer and other malignancies [19–21]. It is also well known that *CHEK2* is subject to extensive alternative splicing, with close to 90 splice variants being detected in one study [22]. At present, it is not known whether this complex alternative splicing could influence tumorigenesis. However, the finding of at least three splice variants of the *PPP2R2A/CHEK2* fusion product in the present teratoma is well in line with previously reported data for *CHEK2*.

PPP2R2A, however, has not been directly implicated in tumorigenesis. The PPP2R2A protein is a regulatory subunit of the protein phosphatase 2 (PP2A) enzyme, a highly conserved Ser/Thr phosphatase that regulates cell growth and differentiation through a variety of cellular processes, including signal transduction, DNA replication, apoptosis, and cell cycle progression [23–25]. The PP2A holoenzyme consists of a common dimeric core of a catalytic (C) subunit and a structural (A) subunit, which, in turn, may be associated with a variety of regulatory (B) subunits influencing the subcellular localization and substrate specificity of the phosphatase. Seventy-five different dimeric and trimeric PP2A holoenzymes can thus be generated [23,25]. The *PPP2R2A* gene encodes an α isoform (aka B55α or PR55α) of the regulatory B55 subunit family. *Drosophila melanogaster* mutants carrying an inactivated *B55α/PR55α* gene show an abnormal anaphase distribution of chromosomes; in mammalian cells, the B55α/PR55α subunit seems to be essential for the dephosphorylation of cytoplasmic intermediate filaments [26,27]. Furthermore, PP2A is thought to prevent premature entry into mitosis by negatively regulating CDC2 by keeping CDC25C in a dephosphorylated low-activity state [28]; seemingly, the dephosphorylation/inhibition of CDC25C is exerted by a form of PP2A containing the regulatory subunit B55α [29]. Other support for a suppressor function of PP2A in tumorigenesis stems from the observation that the tumor-promoting agent okadaic acid is a potential inhibitor of PP2A [30]. Additionally, mutations in *PPP2R1A* and *PPP2A1B* genes, encoding the PP2A subunits RP65α and RP65β, respectively, have been detected in melanomas and carcinomas of the lung, breast, and colon [25,31,32]. Of particular interest, it was

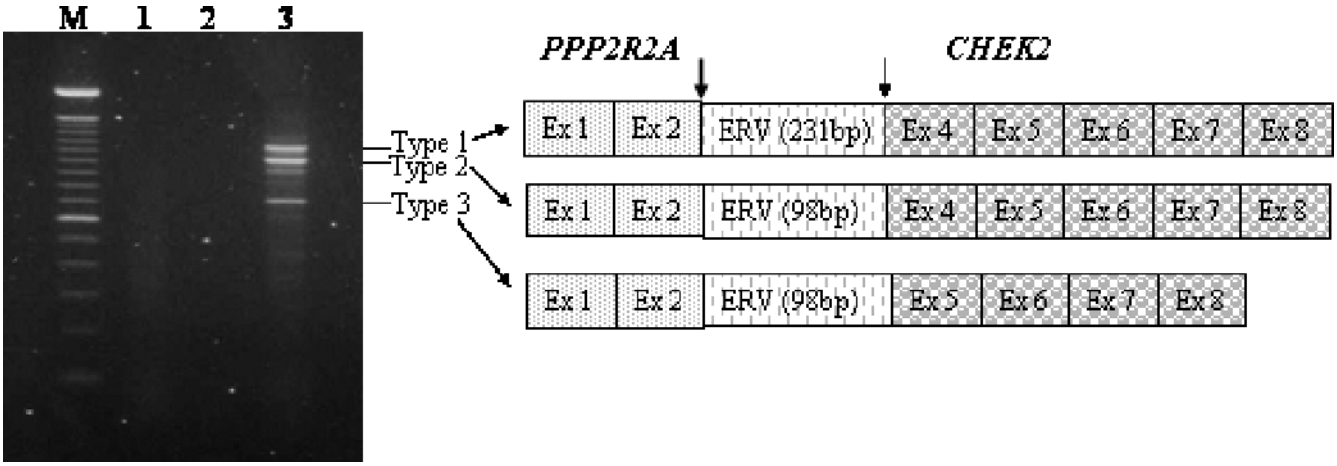

Figure 2. *Analysis of the PPP2R2A/CHEK2 fusion transcripts. M: 100-bp DNA ladder. Lane 1: No fusion transcript was found while using the forward primer CHEK2-82F and the reverse primer PPP2R2A-956R. Lane 2: Blank. Lane 3: Three fusion transcripts were detected with the forward primer PPP2R2A-242F and the reverse primer CHEK2-982R.*

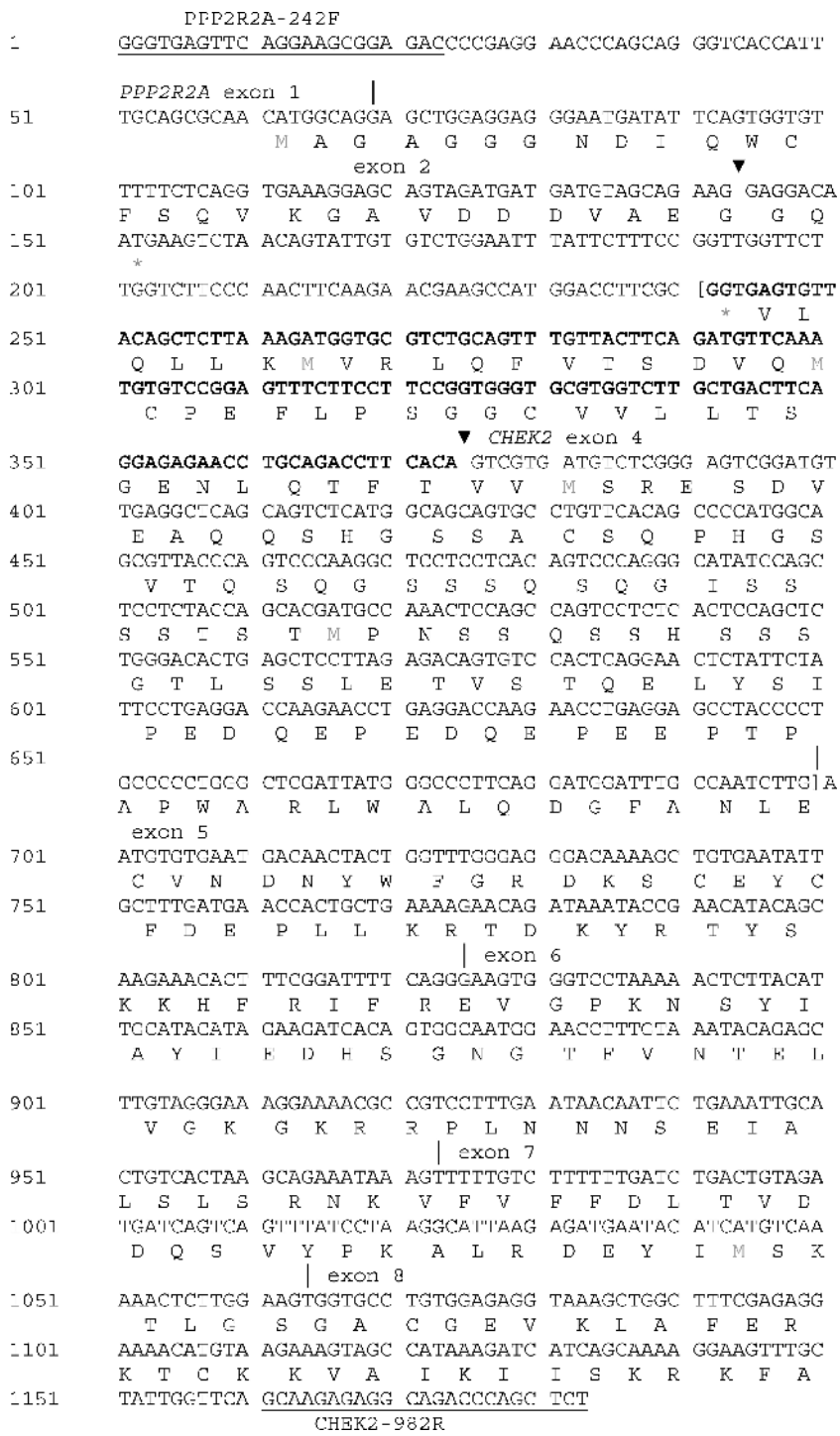

Figure 3. *Complete nucleotide sequence of PPP2R2A/CHEK2 chimeric transcript 1 detected by RT-PCR. The primers PPP2R2A-242F and CHEK2-982R are underlined. Arrowheads indicate breakpoints, and vertical lines indicate exon boundaries. The inserted DNA between exon 2 of PPP2R2A and exon 4 of CHEK2 is a repetitive sequence from chromosome 22, which belongs to type 1 of ERV-related sequences. Sequence in bold (part of the inserted DNA) is absent in type 2 chimeric transcript. Sequence in brackets is not present in type 3 of the PPP2R2A/CHEK2 cDNA fragment (part of inserted sequence and exon 4 of CHEK2).*

recently shown that PP2A can dephosphorylate and inactivate *CHEK2* and, *vice versa*, that *CHEK2* can phosphorylate the B subunit of PP2A, thereby increasing its activity [33].

The present study showed that the outcome of t(8;22), found as the sole chromosome aberration in a mature teratoma, is the fusion of *PPP2R2A* and *CHEK2*—the first acquired fusion gene to be detected in germ cell tumors. In the majority of cases, previous identification of gene fusions in human malignancies has been guided by the finding of balanced chromosome rearrangements in short-term cultured tumor cells, and it has been hypothesized that the present relative lack of known fusion genes in certain tumor types, such as epithelial malignancies and germ cell tumors, might be partly explained by the small number of such tumors

that have been properly analyzed by chromosome banding techniques [34]. In line with this reasoning, it was recently shown that a large fraction of prostate carcinomas carries subtle chromosome rearrangements, leading to highly recurrent fusion genes [35]. Whether the presently detected *PPP2R2A/CHEK2* fusion is a common and tumor-specific event needs to be analyzed in a larger series of teratomas. In addition, if the essential molecular outcome in the present case were deregulation of *CHEK2* and/or *PPP2R2A* rather than creation of a specific fusion transcript, it might be worthwhile to study the expression patterns of these genes in germ cell neoplasms.

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