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CLINICAL STUDY

Clinical, genetic, and immunohistochemical characterization of 70 Ukrainian adult cases with post-Chornobyl papillary thyroid carcinoma

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Abstract

Background: Increased incidence of papillary thyroid carcinoma (PTC) is observed as a consequence of radiation exposure in connection to the Chornobyl nuclear plant accident in 1986. In this study, we report a cohort of adult Ukrainian patients diagnosed with PTC from 2004 to 2008 following exposure at the age of 18 years or younger.

Methods: In total, 70 patients were identified and clinically characterized. The common *BRAF* 1799T>A mutation was assessed by pyrosequencing, the *RET/PTC1* and *RET/PTC3* (*NCOA4*) rearrangements by RT-PCR, and the expression of Ki-67 (MIB-1 index), BCL2, cyclin A, and cyclin D1 by immunohistochemistry.

Results: In total, 46/70 (66%) cases carried a *BRAF* mutation and/or a *RET/PTC* rearrangement. A *BRAF* mutation was detected in 26 tumors, *RET/PTC1* in 20 cases, and *RET/PTC3* in four cases. In four of these cases, *BRAF* mutation and *RET/PTC* rearrangement were coexisting. The *BRAF* mutation was underrepresented among PTCs with accompanying chronic lymphocytic thyroiditis (CLT) compared with PTCs without this feature (12 vs 44%). MIB-1 proliferation index determined by double staining with leukocyte common antigen was low (mean 0.8%; range 0.05–4.5%). Moreover, increased expression of cyclin A was observed in PTCs with a tumor size >2 cm compared with PTCs ≤ 2 cm (1.2 vs 0.6%). BCL2 and cyclin D1 showed frequent expression but without associations to clinical characteristics or amplification of the *CCND1* locus.

Conclusions: Our results suggest that this cohort has frequent *BRAF* mutation, *RET/PTC1* rearrangement, and low proliferation index. Furthermore, *BRAF* 1799T>A was underrepresented in PTCs with CLT, and cyclin A expression was associated with increased PTC tumor size.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common type of endocrine cancer comprising up to 80% of all malignant thyroid tumors (1). Increased incidence of PTC was observed among Ukrainian children who were exposed to radioactivity after the Chornobyl (Chernobyl) nuclear plant accident in 1986 (2, 3). Specific molecular and genetic features of such childhood PTC have been described (4). Today, it is known that PTC may also develop in adult individuals who were younger than 18 years at the time of the accident and who lived within the contaminated area (5, 6). Molecular changes in such PTC have not been widely studied, and it is presently unclear whether they have similar and/or distinct molecular characteristics compared with PTC in other populations.

PTC commonly exhibits a hotspot *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) mutation or activation of the *RET* or *NTRK* genes through different translocations that lead to abnormal tyrosine kinase activity (4, 7). The common *BRAF* mutation involves a thymine to adenine transversion at position 1799 (1799T>A) in exon 15, which results in an activating missense substitution of valine to glutamic acid at codon 600 (V600E) (4). The frequency of *BRAF* mutation in PTC varies between studies from very low frequencies up to 80% (8, 9, 10), and their presence is reported to have prognostic implications (8). However, a low prevalence of *BRAF* mutation was reported

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for PTCs that developed after the Chornobyl accident (9, 10, 11, 12).

Rearrangements of the *RET* proto-oncogene are also frequently found in PTC and lead to expression of chimerical transcripts termed *RET/PTC* due to fusion of the tyrosine kinase domain of *RET* (TK-*RET*) with various regions of other genes. *RET/PTC1* and *RET/PTC3* are the most common forms of *RET/PTC* constituting up to 90% of all *RET* rearrangements (13). *RET/PTC1* is the result of a translocation between the coiled-coil domain-containing 6 gene (*CCDC6*) and TK-*RET*, while fusion of the *NCOA4* with TK-*RET* leads to the formation of *RET/PTC3*. The frequency of reported *RET/PTC* rearrangements varies largely between studies (7, 12, 13, 14, 15). High frequencies of *RET/PTC3* have been reported in post-Chornobyl childhood PTC, in contrast to adult PTC in which *RET/PTC1* is more common (13, 14, 15).

PTCs are also characterized by expression of certain immunohistochemical markers such as Ki-67, *BCL2*, cyclin A, and cyclin D1 involved in proliferation and apoptosis. *BCL2* is involved in blocking of apoptosis (16) and cell survival (17), and *BCL2* overexpression correlates with PTC aggressiveness (18). Ki-67 is a nuclear protein expressed in proliferating cells, and the MIB-1 MAB against Ki-67 is used for determination of the proliferation index (MIB-1 index). In PTC, increased MIB-1 index has been associated with a worse prognosis in some studies but not in others (19, 20, 21, 22). Cyclin A activates cyclin-dependent kinases to regulate proliferation and cell cycle progression through the S phase to the G2-M checkpoint (23). Cyclin A expression has possible prognostic value in breast cancer (24); however, its role in PTC has been less studied (25, 26). Cyclin D1 is involved in cell cycle control at the G1 checkpoint for progression from G1 to S phase. Expression of cyclin D1 is not observed by immunohistochemistry in normal thyroid cells, while its overexpression has been associated with higher frequency of lymph node metastases (27, 28).

We have identified a cohort of 70 adult patients with PTC who were exposed in their childhood or as teenagers to the Chornobyl radioactive fallout in 1986. Here, we describe the cohort concerning clinical features, expression, and mutation data for some established and some putative prognostic markers: *BRAF*, *RET/PTC1*, *RET/PTC3*, MIB-1 index, *BCL2*, cyclin A, and cyclin D1.

Materials and methods

Patients and tissue samples

The 70 cases included in the study were identified from patients surgically treated for a PTC from 2004 to 2008 in Kyiv City Teaching Endocrinological Center, Ukraine. The standard surgical approach used for these patients was total thyroidectomy followed by central lymph node dissection. All patients in the cohort had been exposed to radioactivity from the accident at the Chornobyl nuclear power station in Ukraine in 1986, as determined from the patients' addresses and the geographical pattern of the radioactive fallout. However, data about radiation dosages are not available. At the time of the accident, all patients were 18 years of age or younger and lived near the most heavily contaminated regions Kyiv, Chernihiv, or Zhitomyr (6).

Clinical data were retrieved from medical records, and archival formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were collected for all cases. The tumors were initially classified as primary PTC, classical type, at routine histopathological examination in Kyiv City Teaching Endocrinological Center, whereby presence or absence of coexisting chronic lymphocytic thyroiditis (CLT) was also noted. The diagnosis, presence/absence of CLT, as well as the absence of large lymphocytic infiltrates of the PTC stroma were subsequently confirmed at histopathological revision by one of the authors (A H). In addition, specimens of normal thyroid tissue ($n=4$), goiter ($n=1$), and follicular thyroid adenoma ($n=1$) were collected at the same institution and included as references in the immunohistochemistry and fluorescence *in situ* hybridization (FISH) analysis. Samples were collected, and the study was conducted with ethical permission obtained from the local ethics committees.

Control samples for pyrosequencing constituted 11 PTC samples with *BRAF* T1799A mutation status confirmed by Sanger sequencing as previously reported for ten of the cases by Sofiadis *et al.* (29), as well as three parathyroid adenomas. These samples had been collected as fresh frozen samples at the Karolinska University Hospital, Sweden, with informed consent and ethical approval.

Pyrosequencing of the BRAF *1799T>A mutation*

Genomic DNA (gDNA) was extracted from FFPE sections using a commercially available kit (Qiagen), quantified with a Nano Drop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and used for pyrosequencing. Primers for PCR amplification of *BRAF* exon 15 and subsequent pyrosequencing were designed using the Pyromark Q24 Software 2.0 (Qiagen) and commercially synthesized (biomers.net GmbH, Ulm, Germany). The primer sequences were as follows: forward 5′-GGCCAAAAATTTAATCAGTGGAA-3′, reverse 5′-CTTCATAATGCTTGCTCTGATAGG-3′ (5′-biotinylated) and sequencing 5′-CCACTCCATCGAGATT-3′. PCRs were performed using HotStar Taq DNA polymerase kit (Qiagen) under the following cycling conditions: 95 °C for 15 min, 35 cycles×(94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) and final extension at 72 °C for 10 min. PCR products were

visualized in 2% agarose gel stained with GelRed (Biotium, Hayward, CA, USA). Subsequently, 30 µl biotinylated PCR product was captured to filtered probes using PyroMark Q24 vacuum prep workstation, flushed, and released to Q24 plates with annealing solution according to the protocol recommended by the manufacturer. Plates with annealed samples were processed in a Pyromark Q24 and the results were analyzed using Pyromark Q24 Software 2.0 (Qiagen). Pyrosequencing of additional DNA samples from PTC cases with a *BRAF* 1799T>A mutation or wild-type status previously determined by standard Sanger sequencing was done as positive and negative controls respectively (29). The accuracy of the pyrosequencing was evaluated by analysis of 11 PTCs for which the *BRAF* 1799T>A mutation determined by Sanger sequencing was verified. Furthermore, the sensitivity was demonstrated by detection of the mutation in gDNA diluted one, five, and ten times from one *BRAF* 1799T>A mutation carrying PTC. The specificity of the method was determined by detection of the wild-type *BRAF* sequence only in the three parathyroid adenomas. The cutoff level for *BRAF* 1799T>A was 10%.

Real-time PCR detection of RET/PTC1 and RET/PTC3 fusion transcripts

Total RNA was isolated from all samples using RNA isolation kit for FFPE tissue (Qiagen), according to the protocol recommended by the manufacturer. cDNA was synthesized from 100 ng total RNA using high-capacity cDNA RT kit with random primers (Applied Biosystems) according to the manufacturer's description. Amplification of cDNA was performed by RT-PCR in a StepOnePlus PCR instrument using TaqMan Universal PCR master mix (Applied Biosystems). Primers and probes for *RET/PTC1* and *RET/PTC3* were synthesized according to Rhoden *et al.* (30). The phosphoglycerate kinase 1 gene (*PGK1*) served as endogenous control. Two PTC samples with previously reported expression of *RET/PTC1* or *RET/PTC3*, respectively (14), were included as positive controls, and replacement of cDNA template with water constituted the nontemplate control. RT-PCRs, including negative and positive controls, were performed in duplicate under standard conditions: 50 °C for 2 min followed by 95 °C for 10 min and 45 cycles×(95 °C for 15 s, 60 °C for 1 min). Analysis of RT-PCR results was based on the evaluation of amplification curves for each sample in comparison with positive controls (31).

Immunohistochemistry

MIB-1 index and expression of BCL2, cyclin A, and cyclin D1 were analyzed on macroarray tissue slides of the 70 PTCs as well as control thyroid samples by immunohistochemistry using a previously described protocol (29). The following primary antibodies were used for antigen detection: monoclonal mouse anti-Ki-67 (clone MIB-1; Dako, Stockholm, Sweden) at dilution 1:300; monoclonal mouse anti-CD45 (leukocyte common antigen, LCA) at 1:50 (clone 2B11+PD7/26; Dako); monoclonal mouse anti-BCL2 (clone 124; Dako) at 1:100; monoclonal rabbit anti-cyclin D1 (clone Sp4; Dako) at 1:250; and monoclonal mouse anti-cyclin A at 1:300 (clone E6E; Novocastra, Leica Biosystems, Newcastle, UK). Macroarrays were prepared by joining and re-embedding of four to nine tissue samples in novel FFPE blocks. For immunohistochemistry, 5 µm paraffin sections were deparaffinized, rehydrated, and treated in preheated citrate buffer pH 6.0 (Dako) at 95–99 °C for 20 min in a microwave oven. After incubation in 0.3% hydrogen peroxide for 30 min and blocking in 1% BSA with 0.01% sodium azide for 1 h at room temperature, endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (SP-2001; Vector Laboratories, Burlingame, CA, USA). Primary antibody diluted in 1% BSA was incubated overnight at 4 °C followed by the biotinylated secondary antibody horse antimouse IgG at 1:700 (BA-1000/BA-2000, Vector Laboratories) for 45 min. Slides were subsequently incubated with the avidin–biotin–peroxidase complex (Vectastain Elite Kit; Vector Laboratories) for 45 min and diaminobenzidine tetrahydrochloride for 6 min and counterstained with hematoxylin for 3 min. Slides analyzed in parallel with omission of the primary antibody served as negative controls and showed expected absence of staining in all cases. Positive controls constituted of tissue sections from anonymous normal tissues of stomach, large and small bowels, as well as lymphoid tissue, which revealed expected staining patterns in accordance with information provided by the antibody manufacturers. Anti-cyclin A, anti-cyclin D, and anti-BCL2 were separately incubated. MIB-1 was incubated separately as well as coincubated with anti-LCA to allow optimal differentiation between proliferating leukocytes and proliferating tumor cells.

Evaluation of immunohistochemistry

Slides were evaluated in a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) equipped with Zeiss Plan-Neofluar objective lenses, and images were captured using a ProgRes C12 Plus camera and the ProgRes Capture Pro 2.5 software program (Jenoptik, Jena, Germany). For each case, the total number of PTC cells was estimated (×16 objective magnification), and the scoring was based on 1500–2000 cells. Non-PTC cells were identified at microscopy and excluded from the scoring of PTC cells. MIB-1 proliferation index and cyclin A expression were determined by counting all positive PTC cells in the areas where the number of immunoreactive nuclei was the highest (hotspot) and by calculating the proportion of positive nuclei. For cyclin D1, only nuclear staining was considered and the proportion of positive PTC cells estimated at

microscopical evaluation. Cytoplasmic staining pattern was observed for BCL2 and the proportion of positive PTC cells was estimated at microscopical evaluation.

Fluorescence in situ *hybridization*

Dual color FISH analysis was performed to evaluate possible regional amplification of the cyclin D1 locus (*CCND1*) on FFPE sections from the 70 PTC cases. A FISH probe kit (Abbott, Scandinavia) containing a Spectrum Orange-labeled *CCND1* probe (11q13) and a Spectrum Green-labeled *CEP11* probe for the D11Z1 alpha centromere satellite repeat was used (11p11.11-q11). FISH was carried out using the Histology FISH Accessory Kit (Dako) according to the recommendations of the manufacturer. Visualization and scoring of FISH signals were performed in a Zeiss Axioplan 2 imaging epifluorescence microscope (Carl Zeiss) using an ×60 objective. For each case, a minimum of 200 interphase nuclei were scored, including only representative PTC cells with nonoverlapping nuclei and two bright green *CEP11* signals. The rationale for this selection was to avoid misscoring of overlapping or sectioned nuclei (32). Sections of an anonymous breast carcinoma with validated *CCND1* amplification were analyzed in parallel as positive controls.

Statistical analyses

Statistical calculations were performed using the data analysis software Statistica version 10.0 (StatSoft Scandinavia AB, Uppsala, Sweden). The Mann–Whitney *U* test was applied to compare the results in sample groups. Spearman rank order correlation test was performed to analyze possible relations between studied parameters. Results with P values <0.05 were regarded as statistically significant.

Results

Clinical description of the post-Chornobyl PTC cohort

The cohort consists of 70 patients who were exposed to radioactivity from the Chornobyl accident in 1986 as children or teenagers ($\leq$18 years) and who were subsequently operated on for a primary PTC from 2004 to 2008. Clinical characterization of patients and tumors was based on medical records and histopathological revision of PTC slides as summarized in Table 1. The mean age of patients was 10.4 years at the time of the Chornobyl accident and 30.4 years at the time of surgery. Female patients were overrepresented 6.8 times compared with male patients (87 vs 13%). For 52 patients, the size of PTC was $\leq$2 cm in maximum diameter, whereas 18 patients had a PTC $>$2 cm. Metastases to local lymph nodes were detected at the time of diagnosis in 19 cases (27%). However, distant metastases were not observed. In 16 of the 70 PTC tumors, coexisting CLT was observed (referred to as PTC/CLT), whereas 54 cases did not show this feature (PTC only). Cases with PTC only and PTC/CLT did not differ significantly concerning gender, age at exposure and surgery, tumor size, or metastasis. Similarly, no statistically significant difference was observed when tumor size was compared with gender, age, metastasis, or presence of CLT.

Table 1 Clinical characteristics for the 70 post-Chornobyl PTC patients.

Parameter	Observation
Informative cases (*n*)	70
Gender	
Male	9
Female	61
Ratio (female:male)	7:1
Age at diagnosis (years)	
Mean	30.4
Median (range)	31 (19–39)
Age at Chornobyl (years)	
Mean	10.4
Median (range)	12 (<1–18)
Tumor size (cm)	
Mean	1.9
Median (range)	1.7 (1–6)
Local lymph node metastasis	
No. of cases	19 (27%)
Distant metastasis	
No. of cases	0 (0%)
Chronic lymphocytic thyroiditis (CLT)	
No. of cases with PTC/CLT	16 (23%)
No. of cases with PTC only	54 (77%)

Frequent occurrence of the common BRAF *mutation and/or* RET/PTC *rearrangements*

All 70 cases were screened for the common *BRAF* mutation in exon 15 using pyrosequencing (Fig. 1). In total, 26 (37%) tumors exhibited a base substitution 1799T>A predicted to result in the V600E missense mutation (Table 2). Comparison of *BRAF* mutation status with clinical characteristics did not reveal any significant associations for the parameters gender, sex, age, or lymph node metastasis. However, 24 of the 26 *BRAF* mutated cases had been classified as PTC only while two cases were of PTC/CLT type. Hence, *BRAF* mutations were 3.5 times less frequent in the PTC/CLT group (2/16; 12%) compared with PTC only (24/54; 44%) ($P=0.02$). The cutoff level of 10% was applied to classify cases as positive or negative. Overall, positive cases exhibited proportions of mutant allele, which varied between 12 and 44%. The proportion of mutant alleles was not found to be different between PTC-only cases (mean 28%, range 12–44%) and the two PTC/CLT cases (18 and 34%).

The presence of a *RET/PTC1* or *RET/PTC3* rearrangement was assessed by analysis of amplification curves

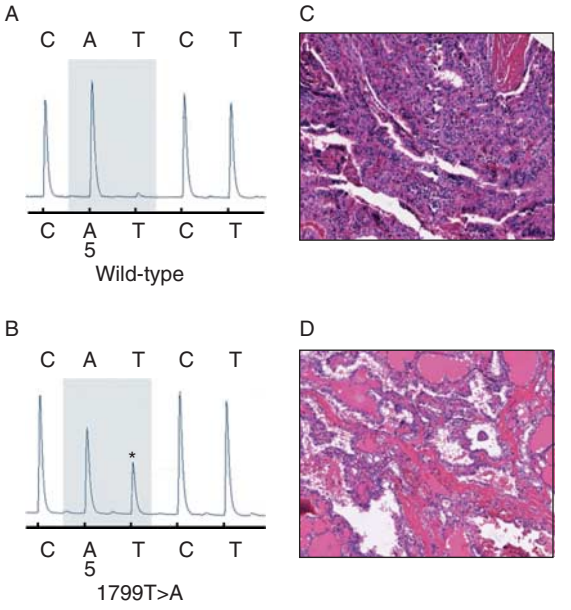

Figure 1 Analysis of the common mutation 1799T>A in exon 15 of *BRAF* by pyrosequencing. (A) Wild-type *BRAF* sequence in a case of PTC/CLT, revealed as a large A peak without a subsequent abnormal T peak in the shaded area of the pyrogram. (B) Detection of a 1799T>A mutation in a case of PTC only, revealed as a decreased peak A combined with an elevated peak T (marked by asterisk). (C and D) Photomicrographs of hematoxylin-stained slides show histopathological findings in (C) a case of PTC/CLT and (D) a PTC only without CLT.

after RT-PCR (Fig. 2). A total of 24 (34%) tumors showed rearrangement of *RET* in which *RET/PTC1* was validated in 20 cases and *RET/PTC3* in four cases (Table 2). Hence, these rearrangements were commonly observed in the cohort and *RET/PTC1* was five times more frequent than *RET/PTC3*. Associations between *RET/PTC1* or *RET/PTC3* and clinical parameters were not observed.

A genetic alteration commonly associated with PTC, i.e. a *BRAF* 1799T>A mutation or a *RET/PTC* rearrangement, was detected in 46 of the 70 PTCs. These included 22 cases with the *BRAF* 1799T>A mutation only, 17 with a *RET/PTC1* rearrangement only, three with *RET/PTC3* only, three with *BRAF* 1799T>A and *RET/PTC1*, and one case with *BRAF* 1799T>A and *RET/PTC3*. In 24 tumors, neither *BRAF* 1799T>A nor a *RET/PTC* rearrangement was revealed.

MIB-1 proliferation index

Proliferation index was determined using MIB-1 immunohistochemistry and counting of cells with positive nuclei (Fig. 3 and Table 2). Lymphocytes used as internal controls showed strong nuclear staining in more than 50% of the cells. Given the frequent occurrence of lymphocytes in the PTC specimens, including 16 cases with PTC/CLT, we performed double staining with LCA to facilitate the distinction between proliferative lymphocytes and tumor cells (Fig. 3). All 70 PTC cases were positive, while normal thyroid tissues included in the FFPE macroarrays of the PTC cohort were completely negative (Table 3). The mean MIB-1 index for the entire cohort determined by combined MIB-1/LCA immunohistochemistry was 0.8% (range 0.05–4.5%). For comparison, MIB-1 index was also determined by regular counting of MIB-1-stained slides, applying visual distinction between proliferating lymphocytes and proliferating tumor cells. This analysis showed that 65/70 PTCs were positive with a mean MIB-1 index of 1.5% (median 1.0%, range 0–7.5%). Comparison of MIB-1 index with clinical characteristics did not reveal statistically significant associations for the MIB-1/LCA- or MIB-1-based analyses.

Expression of cyclin A in relation to size of PTC

Cyclin A expression was determined by scoring of immunohistochemical nuclear expression (Fig. 4A and B). In normal thyroid tissue, no staining was observed (Table 3). In the PTC cohort, the mean level of

Table 2 Summary of genetic and immunohistochemical findings in the 70 cases of post-Chornobyl PTCs studied. Cutoff level for positive cases was 0% for all antibodies.

Parameter studied	Observation in 70 PTCs
BRAF 1799T>A mutation	
No. with 1799T>A	26 (37%)
No. with wild-type	44 (63%)
RET/PTC1 rearrangement	
No. with *RET/PTC1*	20 (29%)
No. without rearrangement	50 (71%)
RET/PTC3 rearrangement	
No. with *RET/PTC3*	4 (6%)
No. without rearrangement	66 (94%)
BRAF and *RET/PTC*	
No. with *1799T>A* and *RET/PTC1*	3 (4%)
No. with *1799T>A* and *RET/PTC3*	1 (1%)
No. with *BRAF* wild-type and no *RET/PTC*	22 (31%)
MIB-1 proliferation index (MIB-1 only)	
Mean proportion positive nuclei	1.5%
Median proportion positive nuclei	1.0%
MIB-1 proliferation index (MIB-1+anti-LCA)	
Mean proportion positive nuclei	0.8%
Median proportion positive nuclei	0.7%
No. of positive cases	70 (100%)
Cyclin A immunohistochemistry	
Mean proportion positive cells	0.7%
Median proportion positive cells	0.4%
No. of positive cases	64 (92%)
Cyclin D1 immunohistochemistry	
Mean proportion positive nuclei	27%
Median proportion positive nuclei	20%
No. of positive cases	68 (97%)
BCL2 immunohistochemistry	
Mean proportion positive cells	48%
Median proportion positive cells	50%
No. of positive cases	53 (76%)

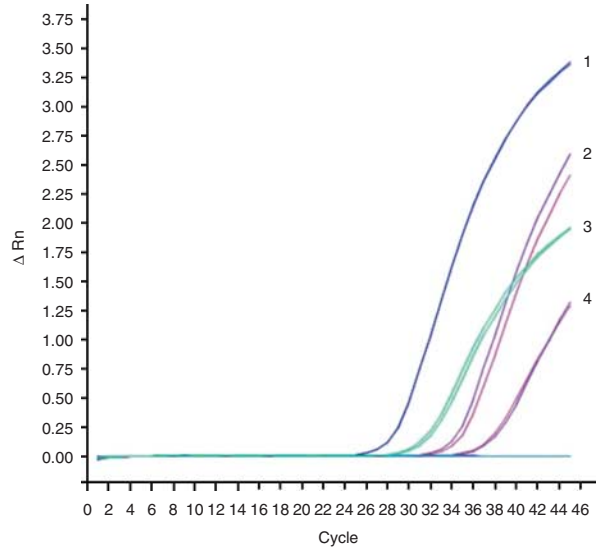

Figure 2 Detection of a *RET/PTC1* fusion by reverse-transcribed PCR. The amplification curves 1 and 3 reveal the presence of *RET/PTC1* in a PTC case (curve 1), and the positive control with previously validated *RET/PTC1* (curve 3). Curves 2 and 4 correspond to amplification of the endogenous control *PGK1*.

expression was 0.7% positive cells ranging from 0 to 3.9%. Six PTC cases were negative with lack of immunoreactive PTC cells. Among the 64 tumors with cyclin A expression, 48 cases showed $<1\%$ positive cells and 16 exhibited 1–4% positive cells according to the previously published recommendations of classification (33). When compared with the clinical characteristics, we found that the expression of cyclin A differed significantly according to the size of the PTC. Specifically, expression of cyclin A was higher in PTCs >2 cm than in PTCs ≤ 2 cm ($P=0.004$; mean 1.2 vs 0.6% respectively). No other association between cyclin A expression and clinical parameters was noted.

Frequent expression of cyclin D1 without associated CCND1 *amplification*

Cyclin D1 was evaluated concerning both protein expression and regional amplification of the *CCND1* locus. Cyclin D1 immunohistochemistry was negative in normal thyroid tissue (Table 3). In the PTC cohort, we detected nuclear immunoexpression of cyclin D1 (Fig. 4C and D), and in addition, cytoplasmic staining was also noted in some cases, which was not included in the scorings. The mean proportion of positive PTC cells was 27% ranging from 0 to 90%. Sixty-eight cases showed expression with $<10\%$ positive cells in 25 cases, 10–49% positive cells in 25 cases, and $\geq 50\%$ positive cells in the remaining 18 cases according to the previously applied cutoff levels for subgroups (34). No association was detected between the expression level and clinical characteristics. FISH analysis in normal thyroid tissue showed two signals for the *CCND1* probe. Moreover, FISH analyses revealed two bright green and two bright orange signals in all representative PTC cells for all cases. This observation suggests that the observed cyclin D1 expression was not a consequence of *CCND1* regional amplification.

Expression of BCL2

Immunohistochemical expression of *BCL2* was identified in the majority of PTC cases and observed in normal thyroid tissue and goiter (Table 3 and Fig. 4E and F). Among the 70 PTC cases, the mean level of expression was 48% ranging from 0 to 100% positively stained cells. Altogether, 53 cases exhibited *BCL2* expression, in 1–25% of the cells for 13 cases, in 26–50% of cells for eight cases, and in $>50\%$ of cells in 32 cases. The remaining 17 cases were negative without immunoreactive PTC cells. Normal thyroid tissue and goiter were strongly positive with $>75\%$ positively stained cells in 4/5 samples (Table 3). No association with clinical parameters was identified.

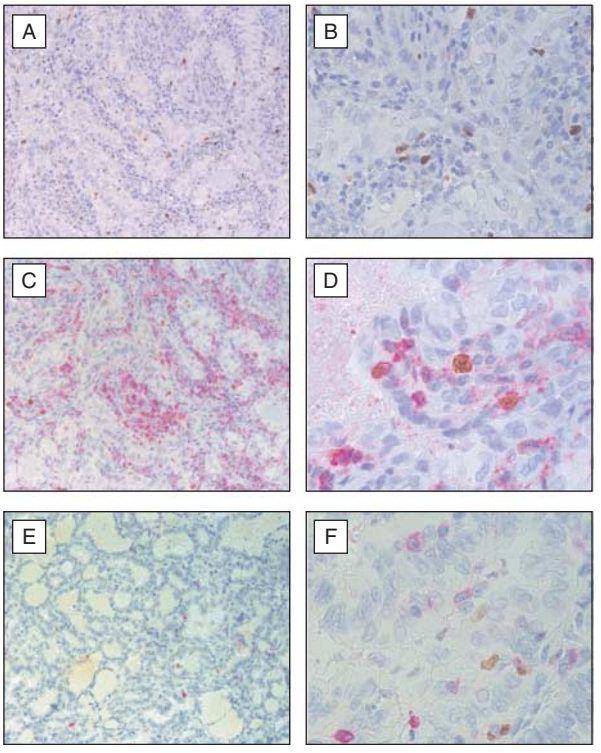

Figure 3 Analysis of MIB-1 proliferation index by immunohistochemistry using MIB-1 only or double staining with MIB-1 and anti-LCA. (A and B) A case of PTC/CLT shown after regular MIB-1 staining with immunoreactivity in PTC cells and proliferating lymphocytes. (C and D) The same case after double staining with MIB-1 and anti-LCA showing proliferative PTC cells (brown), proliferative lymphocytes (red and brown), and nonproliferative lymphocytes (red). (E and F) A case of PTC only without CLT after MIB-1 and anti-LCA double staining. Slides are shown in different magnifications using (A, C and E) objective $\times 16$; (B) $\times 40$; or (D and F) $\times 63$.

Table 3 Expression of MIB-1, BCL2, cyclin A, and cyclin D1 in nonmalignant tissues. Cutoff level for positive cases was 0% for all antibodies.

Parameter	MIB-1	BCL2	Cyclin A	Cyclin D1
Normal thyroid ($n=4$)				
Negative	4	0	4	4
Positive	0	4	0	0
Follicular thyroid adenoma ($n=1$)				
Negative	0	0	0	0
Positive	1	1	1	1
Goiter ($n=1$)				
Negative	1	0	1	1
Positive	0	1	0	0

Comparison between genetic and immunohistochemical phenotypes

Possible relationships between the genetic findings and immunohistochemical parameters assessed in the study were determined by Spearman's rank order correlation test. Several statistically significant observations were made. MIB-1 index showed a positive correlation with expression levels of both cyclin A ($r=0.38$, $P<0.05$) and cyclin D1 ($r=0.34$, $P<0.05$). Cyclin D1 expression levels showed a positive correlation with cyclin A expression ($r=0.39$, $P<0.05$) and a negative correlation with the presence of *RET/PTC1* ($r=-0.26$, $P<0.05$). Finally, a positive correlation was found between *BRAF* mutation and *BCL2* expression ($r=0.24$, $P<0.05$). However, for all identified correlations, Cohen's effect size was <0.5, suggesting that the detected correlations are relatively weak.

Discussion

In this study, we present a comparably large cohort of patients operated on for PTC, who were exposed to radioactive fallout in their childhood or as teenagers after the Chornobyl nuclear plant accident in 1986. The clinical features do not appear to be significantly different compared with other cohorts of PTC patients who were not exposed to radioactivity. Whether this cohort has a significantly different clinical course awaits follow-up; however, the time allowing for prognostic evaluation is presently too short. One weakness of the study is the lack of a control group in the experiments, in order to shed light over the specificity of the findings. A control group, however, would require recruitment from a totally different age group, or from another, noncontaminated, geographic area with a different demographic profile. Therefore, we decided not to include a control group in the experiments but to compare all the findings with existing data on similar cohorts found in the literature.

To further characterize the cohort, we have applied some established markers often used in the work-up of PTC patients. Some of these markers are summarized in Table 4, containing details of observations from published studies of postradiation PTCs and nonradiation-associated PTCs. The frequency of *BRAF* mutation in the entire cohort was 37%, which is similar to many series of nonradiation PTC (35, 36). By contrast, *BRAF* mutation has been less frequently observed in postradiation PTC, i.e. 4–24% (Table 4). It is worth noticing that *BRAF* mutation was significantly underrepresented among the patients with PTC/CLT compared with PTC only, which is in accordance with the previous studies (37, 38). The finding may also reflect the facts that *BRAF* mutation has been associated with more aggressive PTC (39, 40) while the presence of CLT in PTC seems to lead to a better prognosis (41, 42). Thus, the fewer occurrences of *BRAF* mutation in PTC/CLT patients may also be connected to good prognosis.

In this study, we determined the proportion of *BRAF* mutant alleles to be below 50% (mean 28%), which could be related to contamination of nontumor cells in the samples studied as well as intratumoral heterogeneity of the *BRAF* mutation. The latter situation was recently shown by Guerra *et al.* (43). In our study, histopathological examination of all samples indicated a high PTC representativity with minor proportions of non-PTC cells. This was also true for the PTC/CLT cases in which large areas of lymphocytic infiltrations were not observed. Taken together with the sensitivity of the

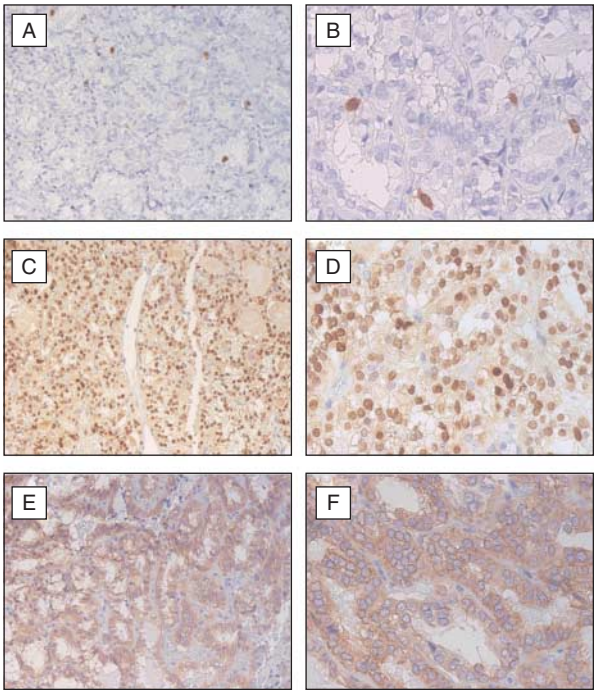

Figure 4 Immunohistochemical analysis of cyclin A, cyclin D1, and *BCL2* expression shown in small (objective ×16, left) or large magnification (objective ×40, right). (A and B) Cyclin A expression of 2.8% in a large-sized PTC >2 cm; (C and D) cyclin D1 expression of 70% in a sample of PTC only; and (E and F) *BCL2* expression of 90% in a PTC-only sample.

Table 4 Comparison between published series of PTC.

PTC case no.	Area of exposure	Age at exposure mean years (range)	Age at surgery mean years (range)	*BRAF* mutation no. (%)	*RET/PTC1* or three rearrangement			MIB-1 index mean, % nuclei (range)	Reference
					RET/PTC1 no. (%)	*RET/PTC3* no. (%)	Total no. (%)		
Postradiation PTC cases									
70	**Ukraine**	**10 (<1–18)**	**30 (19–39)**	**26 (37)**	**20 (29)**	**4 (6)**	**24 (34)**	**0.8 (0.05–4.5)**	–[a]
12	France	13 (6–24)	38 (20–61)	1 (8)	1 (8)	2 (17)	3 (25)	–	(15)
30	USA	3 (0–16)	29 (10–59)	1 (4)	–	–	26 (87)	–	(11)
27	Ukraine	<16	14 (8–16)	1 (4)	–	–	12 (45)	–	(12)
55	Belarus, Ukraine	<17	– (12–31)	2 (4)	6 (11)	26 (47)	32 (58)	–	(10)
34	Ukraine	6 (1–17)	19 (13–30)	4 (12)	5 (15)	9 (26)	14 (41)	–	(9)
33	Ukraine	<17	24 (>15)	8 (24)	–	–	12 (36)	–	(45)
15	Ukraine	<17	14 (<15)	0	–	–	5 (33)	–	(45)
PTC cases without previous radiation									
28	–	–	–	4 (14)	–	–	–	–	(38)
107	–	–	45 (14–77)	31 (29)	24 (22)	5 (5)	29 (27)	–	(37)
55	–	–	–	16 (29)	10 (18)	6 (11)	16 (29)	–	(7)
60	–	–	39 (20–77)	24 (40)	4 (6.5)	5 (8)	9 (15)	–	(36)
61	–	–	54	–	1 (1.6)	2 (3)	3 (5)	–	(14)
10	–	–	43 (25–97)	5 (50)	–	–	–	–	(29)
54	–	–	– (≤45–>45)	42 (78)	1 (1.8)	4 (7)	5 (9)	–	(39)
169	–	–	– (≤45–>45)	–	40 (23.7)	5 (3)	45 (27)[b]	–	(44)
18	–	–	49 (36–63)	–	–	–	–	1.7 (0.1–3.8)	(28)
30	–	–	62 (27–80)	–	–	–	–	1.9 (0.3–11.8)	(19)
185	–	–	49 (12–94)	–	–	–	–	2.9 (0–40)	(20)
108	–	–	– (<35–>55)	–	–	–	–	(>1–<10)	(18)
371	–	–	49 (17–83)					(<1–>5)	(22)

'–', not analyzed, not available or not applicable.
[a]Current study.
[b]In this study, three cases showed both RET/PTC1 and three rearrangements.

pyrosequencing (by which *BRAF* 1799T>A was observed in gDNA of a PTC after dilution), our observations would support intratumoral heterogeneity for *BRAF* 1799T>A.

RET/PTC rearrangements in the form of *RET/PTC1* and *RET/PTC3* were demonstrated in 29 and 6% respectively. While previous studies on *RET/PTC* have reported highly varying frequencies from 5 to 87% (Table 4), postradiation cases have generally shown the highest frequencies. In comparison with these reports, our finding of 34% *RET/PTC* positivity falls within the lower range of postradiation PTC and is comparable to the highest frequencies among nonradiation PTCs (15, 37, 44). However, with regard to the specific fusion type involved, we found *RET/PTC1* to be five times more common than *RET/PTC3*, which is in contrast to other reported postradiation PTCs but in agreement with nonradiation PTCs (45). Overall, the presence of *RET/PTC* is usually considered to be a sign of poor prognosis in PTC. Moreover, *RET/PTC* accompanied by a *BRAF* 1799T>A mutation is associated with a high risk of disease recurrence and metastases. In the current study, co-occurrence of *RET/PTC* and *BRAF* mutation was detected in four PTCs. Although the clinical features at surgery were not indicative of poor prognosis, these cases should be considered for close follow-up for early recognition of signs for PTC recurrence as reported in the literature (13, 39).

Moreover, different frequencies of *RET/PTC1* and *RET/PTC3* were reported in childhood post-Chornobyl PTC. However, these studies showed significant variation of these genetic aberrations depending on the histopathological type of PTC. Thus, *RET/PTC1* was associated with the classical and diffuse sclerosing variants of PTC, whereas *RET/PTC3* was associated with the solid follicular type (46, 47). On the other hand, the solid follicular type of PTC is more common in pediatric patients, while the classical PTC is more common in adults (47). The patient's age is also an important factor for the *BRAF 1799T>A* mutation, which is commonly found in adult patients, but is rare in childhood PTC, which is consistent with our finding (9, 10).

MIB-1 index is increasingly used in the immunohistochemical work-up of several cancer types. The MIB-1 MAB is directed toward the nuclear antigen Ki-67 and is used for identification of proliferative cells and areas of tumors with a high degree of proliferation. This index has been suggested to predict the prognosis in many cancer varieties, including PTC (19, 20, 21). PTC exhibits varying proportions of infiltrating lymphocytes, which was pronounced in the PTC/CLT entity and was less abundant in several PTC-only cases. As MIB-1 immunostaining targets proliferating cells, both proliferating lymphocytes and tumor cells will be stained, with associated risks of misclassification and false-positive or negative scoring as a consequence. To achieve optimal scoring conditions, we used double staining with MIB-1 and LCA in addition to regular MIB-1 staining of all cases. Typical examples of the result are illustrated in Fig. 3. Overall, lower MIB-1 proliferation index was revealed using the MIB-1/LCA-based analysis compared with MIB-1 only (mean 0.8 vs 1.5%; Table 2). If substantiated in follow-up studies, the observations suggest that double staining of MIB-1 and LCA should be considered for use in clinical routine work-up of PTC instead of regular MIB-1-only analysis. Associations between MIB-1 proliferative index and clinical features were not observed. In the five cases with MIB-1 index above the $\geq 1.85\%$ border applied in our previous studies (19, 20), signs of aggressive clinical features were not observed concerning histopathological and clinical features present at the time of surgery. However, in some previous publications, increased MIB-1 index in PTC has been associated with adverse outcome during follow-up (19, 20, 22). Possible prognostic implications of MIB-1 index in this cohort cannot be presently assessed given the lack of follow-up.

Expression of the antiapoptotic protein BCL2 was observed in the majority of PTCs. All normal thyroid tissue samples and 53/70 PTCs expressed BCL2, suggesting that BCL2 could have a protective role to prevent apoptosis in normal thyroid, which is partly lost in malignancy. In contrast to a previous study, no correlation was observed between BCL2 expression and MIB-1 index in PTC/CLT cases (48). However, a positive correlation was observed between *BCL2* expression and *BRAF* mutation. Although this correlation was not strong, it is consistent with the study by Preto *et al.* (49), showing inhibition of BCL2 in PTC cell lines treated with the BRAF and kinase inhibitor sorafenib. Moreover, the lack of association between clinical features and BCL2 expression in our cohort is consistent with the observations by Siironen *et al.* (18). Given that phosphorylation is needed for the antiapoptotic effect of BCL2 (50), further determination of phosphorylated *BCL2* expression levels would add more information about the antiapoptotic status of PTC.

We observed significantly elevated expression of cyclin A in PTCs larger than 2 cm. Previous studies of this protein have reported overexpression in poorly differentiated and undifferentiated thyroid cancers, indicating a role in thyroid carcinoma de-differentiation (25). Our finding of an association between cyclin A expression and tumor size implies that cyclin A could have prognostic value in irradiation-associated PTC; however, much longer follow-up is required to prove or disprove this. Although the possible utility of cyclin A for routine clinical practice is presently unclear, the observed association warrants further investigation of cyclin A in relation to follow-up.

Cyclin D1 expression was detected in the majority of PTCs. This was not accompanied by regional amplification of the *CCND1* locus, suggesting that cyclin D1 is deregulated at the transcriptional, translational, or posttranslational level. In our scorings,

we have included nuclear expression of cyclin D1 as suggested elsewhere (34). However, we have also observed cytoplasmic staining, which could be explained by cytoplasmic sequestration of cyclin D1 due to inhibition of its transportation to the nucleus (51, 52). Elevated expression of cyclin D1 was found to be correlated with elevated MIB-1 index, an association that was also demonstrated by Alama *et al.* (53) in meningioma. No other associations to clinical or pathological features were observed, which is in agreement with the previous studies (34, 54). However, others have reported that cyclin D1 overexpression may be a prognostic marker for PTC (55, 56).

In summary, we report a cohort of adult PTC patients exposed to the radioactive fallout from the Chornobyl accident during their childhood or as teenagers. Our results from genetic and molecular characterization suggest that this cohort is characterized by frequent *BRAF* 1799T>A mutation and *RET/PTC1* rearrangement as well as low proliferation, which are partly overlapping and partly distinguishing from other reported cohorts of postradiation- and nonradiation-related PTC. Moreover, *BRAF* mutation was significantly underrepresented in the PTC/CLT group, and cyclin A expression was associated with tumor size in this entity. Long-term follow-up in this cohort will eventually identify possible effects on patient outcome in this patient group.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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