Absence of RAS and p53 mutations in thyroid carcinomas of children after Chernobyl in contrast to adult thyroid tumours

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Summary Thyroid carcinomas of an additional series of 34 children exposed to radioactive fall-out after the Chernobyl reactor accident were analysed for mutations in the H-, K- and N-RAS and the p53 gene. Allele-specific oligonucleotide hybridization, single-strand conformation polymorphism (SSCP) and direct sequencing did not disclose mutations in codons 12, 13 and 61 of RAS genes nor mutations in exons 5, 7 and 8 of p53. Considering the recently reported high prevalence of RET rearrangements of the PTC3 type in childhood tumours after Chernobyl (Klugbauer et al, 1995, Oncogene 11: 2459-2467), it follows that RET rearrangements are the most relevant molecular aberration in these radiation-induced tumours. RAS or p53 mutations do not play a role in childhood thyroid carcinogenesis after Chernobyl.

Keywords: RAS, p53, childhood thyroid cancer

The incidence of thyroid carcinomas increased steeply in children exposed to radioactive fall-out after the Chernobyl reactor accident (Baverstock et al, 1992; Kazakov et al, 1992). Papillary thyroid carcinomas (PTC), including solid and follicular variants, predominate (Furmanchuk et al, 1992; Nikiforov and Gnepp, 1994). In adult thyroid carcinomas, genetic changes have been reported, e.g. mutations of RAS, p53 and GSP, as well as c-RET rearrangements and c-MET overexpression (for review see Wynford-Thomas, 1993; Takahashi, 1995; Williams, 1995; Pierotti et al 1996). These genetic alterations observed in adult thyroid carcinomas need to be looked for in thyroid tumours that have developed in children after the Chernobyl accident. Recent studies in our laboratory revealed that RET rearrangement is a highly prevalent molecular alteration in these tumours. It has been found in about two-thirds of the cases analysed (Klugbauer et al, 1995; Rabes and Klugbauer, 1997). This has been confirmed by others (Fuggazola et al, 1995; Nikiforov et al, 1997). However, information about other genetic changes is scarce. In a report on the RAS mutation status of 33 papillary carcinomas of children after Chernobyl that were analysed by single-strand conformation polymorphism (SSCP), RAS gene mutations were not found. In the same tumours, just one p53 missense mutation (codon 160) was observed (Nikiforov et al, 1996). With respect to the G\alpha gene, direct sequencing of exons 8 and 9 comprising the critical codons 201 and 227 did not disclose any mutations (Waldmann and Rabes, 1997).

The possibility cannot be dismissed that RET rearrangement, obviously an important molecular alteration in thyroid carcinomas of radiation-exposed children after Chernobyl, might be effective only in connection with other gene mutations that have been described for thyroid cancer of adults. In the present communication, we report the results of a mutation analysis of RAS and p53 in another large series of thyroid carcinomas that developed in children from Belarus early after the Chernobyl reactor accident to broaden the basis for a molecular evaluation of critical lesions in this most important cohort of human childhood cancers.

MATERIALS AND METHODS

Paraffin-embedded material from thyroid carcinomas and metastases of a total of 34 children was available. The children underwent thyroidectomy at the Department of Surgery, Minsk State Medical Institute and Center of Thyroid Cancer, Minsk, Belarus, after the Chernobyl reactor accident. Paraffin blocks of the tumours were obtained in June 1992. From the paraffin blocks, serial sections were cut for microscopic evaluation and for polymerase chain reaction (PCR) amplification of critical parts of the RAS genes and p53.

In H&E-stained sections (3 \mu m), areas that preferentially contained tumour cells and tumour-free areas of normal tissue were marked. In unstained 8-\mu m paraffin serial sections, congruent areas were scraped off separately, deparaffinized in xylene, washed in ethanol, boiled for 30–40 min and used for PCR. After denaturation for 10 min at 96°C, 40 amplification cycles were performed in a thermal cycler (Perkin-Elmer-Cetus) with Taq DNA polymerase (Boehringer, Germany) and PCR buffer (tenfold: Tris-HCl, 100 mmol l\(^{-1}\), magnesium chloride, 15 mmol l\(^{-1}\), potassium chloride, 500 mmol l\(^{-1}\), gelatin, 1 mg ml\(^{-1}\); pH 8.3, at 20°C), denaturation for 1 min at 95°C, primer annealing for 2 min at 3°C lower than \(T_m\) (melting temperature), and primer extension for 2 min at 72°C. Purity of the PCR products was checked by 10% acrylamide gel electrophoresis.

The primers used for PCR amplification of sequences flanking RAS codon 12/13 and 61 were exactly as reported by Verlaan-de Vries et al (1986) and Tada et al (1990). The p53 primers used for PCR amplification of exon 5, 7 and 8 were chosen according to the sequence data given by Buchman et al (1988).

Allele-specific oligonucleotide hybridization on nylon filters was performed in a dot-blot apparatus as described previously in detail (Rabes et al, 1990; Suchy et al, 1992; Rabes and Suchy, 1995).
Allele-specific oligonucleotides for the dot-blot hybridization were according to Verlaan-de Vries et al (1986) and Tada et al (1990): H-RAS, codon 61, 5'-GTACTCCTCGTGGCGGCC-3' (Gin, wild type), -CCG- (Arg), -CTT- (Lys); K-RAS, codon 61, 5'-CTCTCTTGTGACCTGTG-3' (Gin, wild type), -TGG- (Pro), -TGG- (Arg); N-RAS, codon 61, 5'-GATACTGTCGACAGAAG-3' (Gln, wild type), -GAA- (Glu), -AAA- (Lys), -CTA- (Leu), -CCA- (Pro), -CGA- (Arg), -CAT- (His), -CAC- (His).

Direct sequencing of PCR products was performed after purification using spin columns (Promega) by the dideoxy method (Sanger et al, 1977) with [32P] dATP (Amersham-Buchler, Braunschweig, Germany) using the T7-Sequencing Kit (Pharmacia, Freiburg, Germany) as described (Klugbauer et al, 1995). One of the two flanking primers that had been selected for PCR was used for direct sequencing.

SSCP analyses (Orita et al, 1989) were performed using 32P-labelled PCR products after 95% formamide denaturation and electrophoresis either in 10% acrylamide or with Hydrolink-MDE gel (Soto and Sukumar, 1992) at 4°C or 10°C. Some gels were run in 3% glycerol. Each sample was studied, at least, to two different temperatures or with glycerol. Samples that showed mobility shifts were subjected to direct sequencing.

Primers and analytical oligonucleotides were synthesized in an Applied Biosystems Synthesizer (381A) or were purchased from Pharmacia, MWG or Stratagene. The exact sequence of all primers used for this study are available on request.

RESULTS

In 44 tumour and metastasis samples evaluated either by allele-specific oligonucleotide hybridization or direct sequencing, mutations were not observed at the critical codons 12, 13 or 61 of either H-, K- or N-RAS. Examples of allele-specific oligonucleotide hybridization are given in Figure 1. At other codons, aberrations from the wild type were found in two tumour samples. A solid variant of a papillary carcinoma (no. 24426) showed a silent base exchange (valine to valine) in H-RAS, codon 14 at the third position [CAC (GTG) to CAT (GTA)], with the wild-type allele CAC (GTG) still present (Figure 2). The normal sequence CAC (GTG) is found in the surrounding normal tissue.

The second aberration was found in tumour No 21445, a lymph node metastasis of a papillary thyroid carcinoma with a missense mutation at the first position of H-RAS codon 15, CCG to TCG (GGC to AGC), which leads to a glycine to serine exchange. The wild-type allele is still present in the sequencing gel (Figure 2). In none of the other 58 direct-sequencing runs of exon 1 of H-, K- or
N-RAS nor in the 81 allele-specific oligonucleotide hybridizations for codon 61 were any further aberrations from normal found in the tumour samples.

p53 mutation hot spots of exons 5, 7 and 8, for which mutations have been described in anaplastic thyroid carcinomas of adults, were evaluated by SSCP in a total of 22 samples (exon 8, 19 tumour samples). Analyses in different test systems gave no indication of the presence of mutations in the tumours. Examples are given in Figure 3. Samples for which a mobility shift in the SSCP could not be ruled out completely were directly sequenced (Figure 4). p53 mutations at the investigated exons did not occur in these childhood thyroid carcinomas.

DISCUSSION

The complete lack of RAS mutations at the critical codons 12, 13, or 61 and the absence of p53 mutations in thyroid tumours of the Chernobyl-affected children contrasts with molecular alterations found in sporadic thyroid carcinomas of adults, which show RAS mutations in a considerable number of cases (Lemoine et al, 1988, 1989; Wright et al, 1989, 1991; Namba et al, 1990, Suarez et al, 1990; Karga et al, 1991; Shi et al, 1991; Ham et al, 1994; Manenti et al, 1994; Challet et al, 1995). The single case of a missense mutation in our series with a glycine to leucine exchange concerns codon 15 of H-RAS. A mutation at this site does not interfere with GTPase activity or protein binding capacity of p21ras. This and the silent mutation (val-val) found at codon 14 of H-RAS seem to reflect the clonality of these tumours. p53 mutations have only been found in adult thyroid carcinomas of the anaplastic type (Ito et al, 1992; Nakamura et al, 1992; Fagin et al, 1993; Ho et al, 1996). Childhood thyroid carcinomas after Chernobyl are predominantly of the papillary type (Furmanchuk et al, 1992; Nikiforov and Gnepp, 1994). Thus, the absence of p53 mutations is not unexpected.

Recently, we reported a high prevalence of RET rearrangements in thyroid carcinomas of children after the Chernobyl reactor accident. ELE/RET (PTC3) is the predominant type of rearrangement (Fugazzola et al, 1995; Klugbauer et al, 1995, 1996; Nikiforov et al, 1997; Rabes and Klugbauer, 1997). Missense mutations of RAS or p53 may certainly occur in cells of radiation-exposed thyroid glands but will probably not show up in a tumour because the clonogenic potential of thyrocytes bearing RAS or p53 mutations is apparently less penetrant than that of cells with RET rearrangement after radiation-induced DNA double-strand break and recombination repair.

This second communication after the earlier report by Nikiforov et al (1996) appears justified as the collection of more data is important for evaluation, on a broad basis, of the critical molecular events in this cohort of radiation-induced tumours. In principle, the data presented here, which were obtained from another set of tumours, confirm the results of Nikiforov et al (1996). However, the possibility cannot be excluded that thyroid carcinomas that develop in exposed areas after longer latency periods may exhibit a wider spectrum of genetic changes, including RAS or p53 mutations. It is tempting to predict that these later appearing tumours will less frequently show RET rearrangements because the probability of DNA double-strand breaks decreases with lowering dose of irradiation. Instead, other less penetrant molecular alterations, e.g. in RAS or p53 genes, or a combination of various genetic changes may take the lead in the development of thyroid carcinomas occurring late after Chernobyl. At present, RET rearrangements of the PTC3 type.
are obviously a highly significant genetic aberration in thyroid carcinomas of young children exposed to radioactive fall-out. All other genetic changes known from adult thyroid carcinomas, RAS and p53 in particular, appear to be irrelevant at this stage.

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