Search for \textit{NTRK1} proto-oncogene rearrangements in human thyroid tumours originated after therapeutic radiation

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\textbf{Summary} Rearrangements of \textit{NTRK1} proto-oncogene were detected in ‘spontaneous’ papillary thyroid carcinomas with a frequency varying from 5 to 25% in different studies. These rearrangements result in the formation of chimaeric genes composed of the tyrosine kinase domain of \textit{NTRK1} fused to 5’ sequences of different genes. To investigate if the \textit{NTRK1} gene plays a role in radiation-induced thyroid carcinogenesis, we looked for the presence of \textit{NTRK1}-activating rearrangements in 32 human thyroid tumours (16 follicular adenomas, 14 papillary carcinomas and two lymph-node metastases of papillary thyroid carcinomas) from patients who had received external radiation, using the reverse transcription polymerase chain reaction, Southern blot and direct sequencing techniques. These data were compared with those obtained in a series of 28 ‘spontaneous’ benign and malignant thyroid tumours, collected from patients without a history of radiation exposure and four in vitro culture cell lines derived from ‘spontaneous’ thyroid cancers. Our results concerning the radiation-associated tumours showed that only rearrangements between \textit{NTRK1} and \textit{TPM3} genes (\textit{TRK} oncogene) were detected in 2/14 papillary carcinomas and in one lymph-node metastasis of one of these papillary thyroid carcinomas. All the radiation-associated adenomas were negative. In the ‘spontaneous’ tumours, only one of the 14 papillary carcinomas and one of the four in vitro culture cell lines, derived from a papillary carcinoma, presented a \textit{NTRK1} rearrangement also with the \textit{TPM3} gene. Twenty-five of this series of radiation-associated tumours were previously studied for the \textit{ras} and \textit{RET}/\textit{PTC} oncogenes. In conclusion, our data: (a) show that the overall frequency of \textit{NTRK1} rearrangements is similar between radiation-associated (2/31: 6%) and ‘spontaneous’ epithelial thyroid tumours (2/32: 6%). The frequency, if we consider exclusively the papillary carcinomas, is in both cases 12%; (b) show that the \textit{TRK} oncogene plays a role in the development of a minority of radiation-associated papillary thyroid carcinomas but not in adenomas; and (c) confirm that \textit{RET}/\textit{PTC} rearrangements are the major genetic alteration associated with ionizing radiation-induced thyroid tumorigenesis. © 2000 Cancer Research Campaign

\textbf{Keywords:} thyroid; ionizing radiation; \textit{NTRK1} proto-oncogene; rearrangements; \textit{TRK} oncogene

The first study relating external beam radiation exposure during childhood and thyroid tumorigenesis was described in 1950 (Duffy and Fitzgerald, 1950). Since then, an increased incidence of thyroid cancers has been observed in several populations including atomic bomb survivors (Conrad et al, 1970), patients with a history of external radiation for benign or malignant conditions (Shore et al, 1985), and more recently in children from Belarus and Ukraine after the Chernobyl nuclear power plant explosion (Kazakov et al, 1992). However, little is known concerning the molecular mechanisms originating the radiation-associated thyroid tumours. Radiation is able to induce DNA strand breaks and deletions and stimulates aberrant recombination events, giving rise to chromosomal translocations and intrachromosomal rearrangements (Roth et al, 1995). In ‘spontaneous’ papillary thyroid carcinomas, two proto-oncogenes, \textit{RET} and \textit{NTRK1}, which encode membrane tyrosine kinase receptors, were found activated by rearrangement with a variable frequency (Bongarzone et al, 1996). Recently, we and others have reported a high prevalence of \textit{RET} rearrangements in thyroid tumours from patients who had received therapeutic or accidental radiation (Ito et al, 1994; Fugazzola et al, 1995; Klugbauer et al, 1995; Bounacer et al, 1997; Nikiforov et al, 1997). These data suggested that radiation exposure may be, with a high frequency (more than 60%), a direct inducer of \textit{RET} rearrangements. Studies concerning the research of alterations of other genes in radiation-associated thyroid tumours are also available for \textit{ras}, \textit{gsp} and \textit{p53} (Wright et al, 1991; Challeton et al, 1995; Fogelfeld et al, 1996; Nikiforov et al, 1996). For \textit{NTRK1}, there is only a recent study by Beimfohr et al (1999) concerning exclusively post-Chernobyl tumours.

The human \textit{NTRK1} proto-oncogene (also called \textit{TRKA}) is located on the q arm of chromosome 1 (Weier et al, 1995) and encodes one of the receptors of the nerve growth factor (NGF) (Kaplan et al, 1991; Klein et al, 1991). \textit{NTRK1} gene transcripts have been detected exclusively in peripheral nervous ganglia, indicating that the gene plays a role in the nervous system development and function (Martin-Zanca et al, 1990). \textit{NTRK1} was originally detected as an oncogene (named \textit{TRK}) in a human colon carcinoma, following transfection of tumoural high molecular weight DNA in NIH 3T3 cells and focus formation (Martin-Zanca et al, 1986). This activated version of the proto-oncogene was generated by a somatic intrachromosomal rearrangement fusing the tyrosine kinase (TK) domain of \textit{NTRK1} with 5’ sequences of the non-muscular tropomyosin gene (\textit{TPM3}). \textit{NTRK1} proto-onco-
gene rearrangements have been described also in human papillary thyroid carcinomas (PTC). In these tumours, the NTRK1 oncogenic rearrangements are the consequence of the fusion of the TK domain of NTRK1 with at least three different genes: the TPM3 gene as found in the original TRK oncogene (Butti et al., 1995); the TPR (translocated promoter region) gene, also located on chromosome 1q and first identified as part of the MET oncogene (TPR-MET) (Park et al., 1986), giving rise at two different oncogenes: TRK-T1 and TRK-T2 (Greco et al., 1992); and the TFG (TRK-fused gene) gene which function is unknown and located on chromosome 3, originating the TRK-T3 oncogene (Greco et al., 1995). All these oncogenic forms of NTRK1 encode cytoplasmic chimaeric proteins which are constitutively phosphorylated on tyrosine. The frequency of NTRK1 activation in ‘spontaneous’ thyroid tumours (exclusively in PTC) varies from 15 to 25% in tumours from Italian patients (Bongarzone et al., 1989; Greco et al., 1992; Butti et al., 1995) to less than 5% in French and Japanese studies (Wajjwalku et al., 1992; Said et al., 1994; Delvincourt et al., 1996).

To determine if the NTRK1 proto-oncogene activating rearrangements play a role in thyroid radiation-induced carcinogenesis, we studied a series of benign and malignant human thyroid tumours which were obtained from patients who had received external radiation therapy. We compared these data with those obtained by us: (1) in a series of ‘spontaneous’ thyroid tumours, collected from patients without any history of radiation exposure and (2) with that previously obtained (Challeton et al., 1995; Bounacer et al., 1997) after the study of 25 of the radiation-associated tumours, looking for the presence of activated ras and Ret genes.

**MATERIALS AND METHODS**

**Patients**

Tumours were collected at the Gustave Roussy Institute (Villejuif, France) and were histologically classified according to the WHO recommendations (Hedinger et al., 1989). A total of 31 tumours obtained from patients with a history of external irradiation for benign or malignant conditions, were examined: 16 follicular adenomas, 14 PTC and two lymph-node metastases of PTC (LNMPPTC) including one from a patient whose primary thyroid tumour was also studied (Table 1). The doses received by the thyroid have been calculated according to Diallo et al. (1996), for 23/31 of our patients treated for their first benign or malignant condition in the Gustave Roussy Institute. As controls, we studied 28 ‘spontaneous’ human thyroid tumours, collected from patients exposed to ionizing radiation.
without any history of radiation: 14 follicular adenomas and 14 PTC (Table 2). Four in vitro cultured human cell lines, three (K2, K5 and K8) derived from ‘spontaneous’ PTC and one (K7) from a follicular less-differentiated carcinoma (Challeton et al., 1997), were also screened.

The genetic material used in our study was extracted from frozen tissues of radiation-associated tumours (Table 1) in the case of patients MA5, PA13, PE20 and CA27; for all the other samples the tissues used were paraffin-embedded. Concerning ‘spontaneous’ tumours (Table 2), the genetic material was extracted in all the cases from frozen tissues, with the exception of CO1, CH4, FR7, SZ9, AL11, KR12, MA13, TA14, AM27 and FA28 for which the tissues were paraffin-embedded (Table 2).

RNA extraction

RNA isolation from Duboss or Bouin fixed paraffin-embedded tissue samples, was performed according to a previously described procedure (Bounacer et al., 1997). Total RNA was extracted from frozen tissues, using the RNA-B™ technique (Bioprobe Systems, France) following the manufacturer’s instructions. Total RNA was extracted from in vitro culture cells as described by Michelin et al. (1993). The quality of the RNAs was controlled by reverse transcription polymerase chain reaction (RT-PCR) amplification using β-actin specific primers as described by Viglietto et al. (1995).

RT-PCR method for detecting TRK oncogenes

The reverse transcription reaction was performed as previously described (Bounacer et al., 1997) using half the volume of RNA extracted from paraffin-embedded tissue extracts or 1.5 μg of total RNA from fresh tissue extracts. One fourth of the cDNA was used for PCR amplification with outer primers. For the paraffin-embedded tissue extracts, a second round of PCR was done with nested primers using 1:10 of the first round PCR product. The PCR amplifications were performed as previously described (Bounacer et al., 1997), using an automatic thermocycler (GeneAmp, Perkin-Elmer, France). Ten μl of PCR product were electrophoresed in a 2% agarose gel. PCR primer sequences used in this study are given in Table 3.

### Table 2 TRK rearrangements in human ‘spontaneous’ thyroid tumours (A) and in vitro culture cell lines (B)

| **A** | Patient | Age at tumour diagnosis (years) | Histology | RT-PCR | Southern blot |
|-------|---------|---------------------------------|-----------|--------|--------------|
| CO1‡   | Female  | 37                              | PTC       | –      | ND           |
| SE2‡   | Female  | 15                              | PTC       | –      | –            |
| QU3    | Male    | 39                              | PTC       | –      | –            |
| CH4‡   | Female  | 32                              | PTC       | ND     | –            |
| SE5    | Male    | 75                              | PTC       | –      | –            |
| MA6    | Female  | 46                              | PTC       | –      | –            |
| FR7    | Female  | 27                              | PTC       | ND     | –            |
| BL8‡   | Female  | 44                              | PTC       | –      | –            |
| SZ9    | Female  | 39                              | PTC       | ND     | +            |
| UR10   | Male    | 55                              | PTC       | –      | –            |
| AL11‡  | Male    | 36                              | PTC       | ND     | –            |
| KR12   | Female  | 13                              | PTC       | –      | ND           |
| MA13   | Male    | 57                              | PTC       | ND     | –            |
| TA14   | Female  | 30                              | PTC       | ND     | –            |
| DU15‡  | Female  | 56                              | Macr. Ad. | – | – |
| CO16‡  | Female  | 22                              | Macr. Ad. | – | – |
| RO17   | Female  | 28                              | Mx. Ad.   | –      | –            |
| FA18   | Female  | 43                              | Micr. Ad. | –      | –            |
| CO19   | Female  | 50                              | Macr. Ad. | –      | –            |
| GH20   | Female  | 37                              | Macr. Ad. | –      | –            |
| ME21   | Female  | 42                              | Macr. Ad. | –      | –            |
| DE22   | Female  | 29                              | Micr. Ad. | –      | –            |
| GU23‡  | Female  | 46                              | Mx. Ad.   | –      | –            |
| RE24   | Male    | 69                              | Mx. Ad.   | –      | –            |
| SA25   | Female  | 64                              | Macr. Ad. | –      | –            |
| TH26‡  | Female  | 37                              | Mx. Ad.   | –      | –            |
| AM27   | Female  | 35                              | Macr. Ad. | ND     | –            |
| FA28   | Female  | 43                              | Mx. Ad.   | ND     | –            |

| **B** | Cell lines | Derived from | TRK rearrangement research by RT-PCR |
|-------|------------|--------------|-------------------------------------|
| K2    | PTC        | –            |                                     |
| K5    | PTC        | –            |                                     |
| K7    | FLDC*      | –            |                                     |
| K8    | PTC        | –            |                                     |

* Abreviations are the same as in Table 1. ‡ ND: not done. † Samples positive for ras (Said et al., 1994). ‡ Samples positive for RET/PTC (Bounacer et al., 1997). * Follicular less-differentiated carcinoma.
DNA extraction and Southern blot analysis

Genomic DNA was extracted from the frozen tissues as described by Suárez et al. (1990, 1991). Southern blot analysis was performed as previously described (Delvincourt et al., 1996) using as probes, a 1.2 kb BalI-EcoRI fragment of pDM10-1 plasmid and a 2.7 kb KpnI insert of pDM8 plasmid, specific respectively, for the tyrosine kinase domain of the NTRK1 proto-oncogene and the tropomyosin sequences (Martin-Zanca et al., 1986). Both plasmids were kindly provided by Dr Martin-Zanca (Universidad de Salamanca, Spain).

DNA sequence analysis

Direct sequencing of the amplified DNA fragments was carried out by the dideoxy-nucleotide method (Sanger et al., 1977) with 32P ATP, using the double strand DNA cycle sequencing system kit (Gibco-BRL, Life Technologies, France) and the same primers as those used in the amplification, following the manufacturer’s conditions.

RESULTS

Presence of trk rearrangements in radiation-associated and ‘spontaneous’ human thyroid tumours

A total of 31 radiation-associated thyroid tumours (16 follicular adenomas, 14 PTC and two LNMPTC) (Table 1), were screened for the presence of TRK, TRK-T1, TRK-T2 and TRK-T3 chimaeric transcripts, using RT-PCR. As shown in Table 1, the majority of our patients (20/31) were irradiated at a young age (less than 14 years old) and the dose received at the thyroid gland varied from less than 1 to 29 Gy. TRK (TPM3-NTRK1) chimaeric transcripts were only detected in 2/14 PTC. In patient JE, the TRK rearrangement has been detected in both the primary tumour (JE2) and a lymph nodal metastasis (JE3) (Table 1). All the radiation-associated follicular adenomas were negative. Figure 1A illustrates results of positive tumours. The quality of the extracted RNAs is shown in Figure 1B. The TRK bands (254 bp) were observed only after a second round of PCR, probably reflecting the fact that these RNAs were prepared from a small volume of Bouin or Duboss fixed sample. The three positive cases studied by RT-PCR (including the LNMPTC of patient JE2) were confirmed by sequencing the cDNA and one example of these sequences is shown in Figure 2.

As controls, we studied 28 ‘spontaneous’ human thyroid tumours, collected from patients without any history of radiation:

![Figure 1](image)

**Figure 1** Detection by RT-PCR of NTRK1 rearrangements in the RNA of radiation-associated and ‘spontaneous’ human thyroid tumours. (A) Ethidium bromide-stained 2% agarose gel of second PCR round products. Lane 2: NTRK1 expression in a ‘spontaneous’ tumour presenting a C-cell hyperplasia (positive control); lanes 3 and 4: TRK oncogene in the radiation-associated PTC of patient JE and its lymph-node metastasis respectively; lane 5: TRK oncogene in the radiation-associated PTC from patient BO14; lane 6: TRK oncogene in the cell line K5 derived from a human ‘spontaneous’ PTC. The predicted sizes of second PCR round fragments for NTRK1 proto-oncogene and TRK oncogene are 265 bp and 254 bp respectively. Lane 1 shows the PCR amplification of RNA from a positive sample, which was not reverse transcribed prior to PCR amplification (negative control). M: Molecular weight marker 0X174/HaeIII. (B) The same RNAs in (A) were subjected to RT-PCR amplification using β-actin specific primers which generate an 82 bp PCR product. The products of the amplification were run on a 2% agarose gel and ethidium bromide staining of the gel is presented.
14 follicular adenomas and 14 PTC (Table 2). Four in vitro cultured human cell lines, three (K2, K5 and K8) derived from ‘spontaneous’ PTC and one (K7) from a follicular less-differentiated carcinoma, were also screened. The presence of oncogenic TRK, TRK-T1, TRK-T2 or TRK-T3 rearrangements was investigated using the Southern blot and/or RT-PCR techniques (Table 2). Only one papillary carcinoma (patient SZ9) was found positive for TRK oncogene by Southern blot, using as probes a 1.2 kb BalI-EcoRI fragment of pDM10-1 plasmid and (B) with EcoRI and hybridized with a tropomyosin specific probe (2.7 kb KpnI insert of pDM8 plasmid). Co-electrophoresed λHindIII DNA fragments serve as size markers. The arrows indicate the DNA fragments which defined the genetic rearrangement generating the TRK oncogene.

Figure 3

**Combined study of the ras, RET/PTC and TRK oncogenes in radiation-associated tumours**

Twenty-five of the radiation-associated tumours have been also previously screened for the presence of ras mutations and RET/PTC rearrangements (Challeton et al, 1995; Bounacer et al, 1997) (Table 1). Three samples were positive for ras, 15 for RET/PTC and 2 for TRK (Table 1). The overall frequencies of ras, RET and NTRK1 alterations in these radiation associated tumours are 12%, 60% and 8% respectively. Two of the radiation-associated tumours (patients PE1 and PL17) presented simultaneously a Ha-ras mutation and a RET/PTC1 rearrangement (Table 1). In the remaining seven tumours which were not screened for ras (patient JE and patients numbered from 11 to 16), we looked by RT-PCR, for the presence of RET/PTC rearrangements. Three of these tumours [one PTC (FO15) and two LNMPTC (JE3 and PA13)] presented a RET/PTC1 rearrangement (data not shown). Interestingly, one of them (JE2), presented simultaneously a RET/PTC1 and a TRK rearrangement in both the primary tumour and its lymph-nodal metastasis (Table 1).

**DISCUSSION**

In order to determine whether the NTRK1 gene plays a role in radiation-associated thyroid tumorigenesis, we have studied 30 malignant and benign thyroid tumours and two lymph-node metastases, collected at the Gustave Roussy Institute (Villejuif, France) from patients with a history of external radiation (predominantly in childhood) for benign or malignant conditions. The results...
obtained with these samples were compared: (1) with data obtained by screening 32 malignant and benign ‘spontaneous’ thyroid tumours (including four in vitro culture cell lines) and (2) with data previously obtained (Challeton et al, 1995; Bounacer et al, 1997) after the study of 25 of the radiation-associated tumours, looking for the presence of activated ras and RET genes.

In our study, carried out using the RT-PCR and Southern blot techniques, a similar frequency of NTRK1/TPM3 rearrangements was observed in radiation-associated and ‘spontaneous’ samples (6%). These data are similar to that recently published by Beimfohr et al (1999) studying a series of 81 tumours of children from Belarus who had been exposed to radiactive iodine after the Chernobyl reactor accident. The NTRK1-activating rearrangement was also observed in a lymph-node metastasis of one of the radiation-associated PTC (sample JE3; patient JE). In contrast to RET, relatively few studies have been devoted to NTRK1 activation in ‘spontaneous’ thyroid tumours (Bongarzone et al, 1989; Greco et al, 1992, 1995; Wajjwalku et al, 1992; Said et al, 1994; Butti et al, 1995; Delvincourt et al, 1996).

In this type of tumour, it has been shown that NTRK1 rearrangements seem to be present exclusively in PTC with a frequency varying from 25% in Italian studies (Bongarzone et al, 1989; Greco et al, 1992; Butti et al, 1995) to less than 5% in French and Japanese studies (Wajjwalku et al, 1992; Said et al, 1994; Delvincourt et al, 1996). This difference may be the consequence of geographical factors, as suggested by Delvincourt et al (1996) studying a homogeneous population from the Champagne-Ardennes region of France. However, the possibility cannot be excluded that using the technique of transfection of high molecular weight tumour DNA in 3T3 cells, as was the case in the Italian studies, Delvincourt et al (1996) and Said et al (1994) would have also found a higher frequency of activation and perhaps new NTRK1 chimaeric genes.

Up to date, there is no report concerning NTRK1 activation in radiation-associated thyroid tumours. In our present study, the overall frequency of NTRK1 rearrangements considering only the radiation-associated and ‘spontaneous’ PTC, is 14% (2/14) and 12% (2/17) respectively. In contrast with results previously reported by us concerning the RET/PTC oncogene (Bounacer et al, 1997), all the radiation-associated follicular adenomas were negative for the presence of NTRK1 activating rearrangements, in agreement with previous and present data concerning ‘spontaneous’ thyroid follicular adenomas (Wajjwalku et al, 1992; Said et al, 1994; Delvincourt et al, 1996).

The type of activating rearrangement of the NTRK1 proto-oncogene observed by us in our radiation-associated and ‘spontaneous’ thyroid tumours, involved exclusively the TPM3 gene. Little is known about the mechanism by which the NTRK1 proto-oncogene is damaged by genotoxic agents, to generate a chimaeric gene. Until present, the only study carried out to characterize the sequence of the genomic regions involved in the NTRK1 activating rearrangements, has been done by Butti et al (1995) in three ‘spontaneous’ PTC and concerns exclusively the TRK oncogene (TPM3-NTRK1). These authors showed that the different breakpoints occurred in intrinsic regions of both genes. They identified in these regions the presence of some recombinogenic elements including palindromes, direct and inverted repeats and Alu sequences. However, the significance of these results in the process of rearrangement after irradiation is still unknown, because there are no similar studies concerning thyroid radiation-associated tumours. Whether or not the mechanism of rearrangement is the same in ‘spontaneous’ and radiation-associated tumours, will probably be elucidated studying the breakpoints in both types of tumours.

Twenty-five of our radiation-associated tumours were previously screened for the presence of ras mutations and RET/PTC rearrangements (Challeton et al, 1995; Bounacer et al, 1997) (Table 1). The overall frequencies of ras, RET and NTRK1 alterations in these radiation associated tumours are 12%, 60% and 8% respectively. This result confirms, as previously reported by us for tumours originated after therapeutic radiation (Bounacer et al, 1997) and by others in tumours appearing after the Chernobyl fallout (Ito et al, 1994; Fugazzola et al, 1995; Klugbauer et al, 1995; Nikiforov et al, 1997), that RET oncogenic activation by rearrangement represents the major genetic lesion associated with radiation-induced thyroid tumorigenesis. Three of the radiation-associated tumours presented simultaneously two different genetic alterations: in two cases a RET/PTC1 rearrangement and a Ha-ras mutation (patients PE1 and PL17) and in one case a RET/PTC1 and a TRK rearrangement (patient JE2) (Table 1). It is tempting to speculate about an eventual mechanism of cooperation between the simultaneously altered ras and RET genes in the initiation or progression of our human radiation-associated tumours, as previously described by Santoro et al (1993) studying an in vitro culture rat thyroid epithelial cell line. The fact that only a low number of our positive radiation-associated tumours present two simultaneous genetic alterations (3/25), pleads in favour of an alternative role of the ras, RET and NTRK1 genes in the induction of the tumorigenic process. However, an eventual cooperation between these genes and/or with other unknown genes must not be neglected.

In conclusion, taken together our data: (a) show that the NTRK1 proto-oncogene is activated by rearrangement with a similar frequency in ‘spontaneous’ and radiation-associated thyroid tumours; (b) show that the NTRK1 proto-oncogene activating rearrangements play a role in the development of a minority of radiation-associated PTC but not in adenomas and (c) confirm that RET oncogenic activation by rearrangement is the major genetic event associated with ionizing radiation-induced thyroid tumorigenesis.

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