Proximity of TPR and NTRK1 Rearranging Loci in Human Thyrocytes

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Abstract
Chromosomal rearrangements are frequently associated with cancer; the mechanisms underlying their cell-type specificity are poorly understood. Papillary thyroid carcinomas are marked by a high frequency of chromosome rearrangements involving the RET and NTRK1 tyrosine kinase receptor genes and producing RET and TRK oncogenes. An explanation for the propensity of thyrocytes to undergo gene rearrangements has been recently proposed by Nikiforova and colleagues, who showed that the recombination between RET and H4 is favored by the loci proximity in interphase nuclei. We investigated whether the spatial proximity is a contributing factor also in the generation of the thyroid-specific TRK oncogenes. The distance between NTRK1 and its oncogenic partner TPR was determined by two-color fluorescence in situ hybridization and two-dimensional microscopy. A three-dimensional reconstruction of the data was also done. We show that the two loci in thyrocytes nuclei display a distance reduced with respect to peripheral blood lymphocytes, thus supporting the notion that spatial proximity of translocation-prone gene loci may favor gene rearrangements. (Cancer Res 2005; 65(7): 2572-6)

Introduction
Cancer cells frequently display disease-specific chromosome rearrangements resulting in the deregulation of gene expression, as well as in the production of chimeric genes with oncogenic activity (1). Chromosomal rearrangements producing fusion genes are a hallmark of human leukemia and sarcoma. Among epithelial tumors, neoplasias arising from thyrocytes are unique insofar as they are associated with frequent chromosomal rearrangements. Follicular carcinomas are associated with the t(2:3)(q13;p25) generating the PAX8/PPARγ fusion oncogene (2). Inversions or translocations of chromosomes 1 and 10 and involving the genes encoding the NTRK1 and RET receptors, respectively, are associated with a consistent portion of papillary tumors. In both cases, the DNA sequences coding for the tyrosine kinase domain of the receptor are fused to a portion of different activating genes. This generates several RET and TRK oncogenes, which display constitutive tyrosine kinase activity (3).

Despite the high frequency of chromosomal rearrangements in human tumors, the molecular bases underlying the predisposition of certain cell types to undergo chromosome rearrangements are not completely understood. The intrinsic capacity of a cell to repair DNA double-strand breaks might contribute to chromosomal rearrangements. On the other hand, several recent reports have proposed that the spatial proximity of translocation-prone gene loci may favor gene rearrangements, thus proposing spatial genome topology as a contributing factor in the formation of specific cancerous chromosomal translocations.

With respect to thyroid-specific rearrangements, sequence analysis of the genomic breakpoints of several RET and NTRK1 oncogenes in papillary tumors revealed the presence of short sequence homology between the rearranging genes (4, 5). Moreover, the rearrangements are conservative, showing only small deletion/insertion with respect to the germ line sequences of the rearranging genes. All these features suggest that the nonhomologous end-joining mechanism, capable to repair DNA double-strand breaks produced by ionizing radiations or carcinogens, may be involved in the generation of RET and TRK oncogenes (6, 7). Interestingly, it has been reported that thyrocytes respond to ionizing radiations by increasing the DNA end-joining activity, unlike other cell types. Therefore, following DNA damage, thyrocytes would be more prone to DNA repair than to apoptosis and this would increase the likelihood of gene rearrangements (8).

The concept that loci proximity may favor chromosomal rearrangements has been shown in different contexts. In normal B cells, the physical proximity of MYC, BCL, and immunoglobulin loci, recurrently translocated in B-cell lymphomas, correlates with their observed clinical incidence of translocation. Moreover, spatial proximity may contribute to tissue specificity of translocations; in fact, the analyzed loci in lymphocytes are further separated from each other in normal fibroblasts where they are not involved in translocations (9).

Loci proximity has been proposed to play a role also in the H4/RET rearrangement in papillary thyroid tumors. Nikiforova et al. (10) have shown that in thyroid interphase nuclei, H4 and RET loci (both located on chromosome 10) displayed a distance reduced with respect to other cell types and suggested that this spatial contiguity may provide the structural basis for the chromosomal inversion generating the thyroid H4/RET (PTC1) oncogene. We were interested in determining whether the spatial proximity is a contributing factor also in the generation of the thyroid-specific TRK oncogenes, produced by chromosome 1 rearrangements involving the NTRK1 locus. We show that NTRK1 and one of its oncogenic fusion partners, TPR, display a reduced distance in thyrocyte nuclei.

Materials and Methods
Cell culture. Normal thyroid samples (two cases) were obtained from patients undergoing surgery at Istituto Nazionale Tumori (Milan, Italy). Thyroid cell cultures were established according to a previously reported protocol (11). Peripheral blood lymphocytes (PBL) from two healthy donors...
were established according to routine procedures. For the cytogenetic analysis, thyroid cells were serum-starved in medium containing 0.5% FCS.

**Fluorescence in situ hybridization.** Hypotonic treatment was done with 75 mmol/L KCl buffer for PBL, and with 40 mmol/L KCl, 0.5 mmol/L EDTA, and 20 mmol/L HEPES (pH 7.4) for thyrocytes. Cells were fixed in 3:1 methanol/acetic acid and the suspension was dropped on slide and allowed to dry. Fluorescence in situ hybridization (FISH) experiments were done on both metaphase and interphase nuclei. All bacterial artificial chromosomes used in this study are from the RP11 library (P. de Jong, http://www.chori.org/bacpac/): RP11-295K2 for TPR gene and RP11-107D16 for NTRK1 gene. Chromosome preparations were hybridized in situ with TPR and NTRK1 probes labeled by nick translation with Cy3 dUTP (NEB, Boston, MA) and FluorX dCTP (Amersham, Piscataway, NJ), respectively. Labeled probes (500 ng) were combined with 5 μg Cot1 DNA (Roche, Indianapolis, IN) and 3 μg salmon sperm DNA, ethanol-precipitated, air-dried, and dissolved in hybridization buffer (2× SSC, 50% formamide, 10% dextran sulfate). The probes were denatured at 68 °C for 2 minutes and hybridized at 37°C overnight. Afterward, samples were washed thrice at 60°C in 0.1× SSC. Nuclei were counterstained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) and embedded in antifade medium (Vectashield; Vector Laboratories, Burlingame, CA).

Digital images were obtained using a Nikon fluorescence microscope equipped with a digital camera. Cy3 (red) and FluorX (green) fluorescence images, acquired using specific filters, were recorded separately and merged using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA).

The measure of nuclei thickness was obtained using a Bio-Rad Microradiance 2100 CLSM equipped with a 60× oil immersion lens (Bio-Rad, Hercules, CA). HeNe 543 laser and the proper filter set were used. The pin-hole diameter was regulated according the value suggested by the acquisition software to obtain the maximum resolution power. The samples were optically sectioned on Z axis with a 0.3 μm step.

**Distance measure.** The TPR to NTRK1 distances (in micrometers) were measured in interphase nuclei by using a Kontron-Zeiss KS 300 image analyzer (Kontron, Eching, Germany). Nuclei showing two distinct hybridization signals per probe and adequate morphologic shape preservation were selected. For each sample, 170 to 200 nuclei were considered (up to ~1,600 spot couples).

As previously shown, two pairs of heterologous signals are located in two separated areas of the interphasic nucleus with distances several times shorter than any other possible distance between heterologous signals. Thus, it was assumed that the two shorter distances were between loci on the same chromosome. The measured distance is the bidimensional projection of the real three-dimensional distance on the upper surface of the specimen. This measure can be considered representative of the real distance, independently from the status of DNA, because all the dispositions of DNA in the nucleus have statistically the same probability. We applied the Pythagoras theorem to maximize the underestimation of the threedimensional distance. If d represents the distance between spot A and B measured on the two-dimensional images and h is the slice thickness, \( x = \sqrt{h^2 + d^2} \) is the maximum possible distance between the two loci (it corresponds to the real three-dimensional distance only when the two points lie on the opposite sides of the slice). Thus, if we consider x in place of d, the maximum relative error is:

\[
y = \frac{x - d}{x} = 1 - \frac{d}{h} \cdot \frac{\sqrt{h^2 + d^2}}{h^2 + d^2}
\]

The mean error is then

\[
\bar{y} = \frac{y}{h} = 1 - \frac{d}{h} \cdot \frac{\sqrt{h^2 + d^2}}{(h^2 + d^2)}
\]

where \( \bar{y} \) is the probability for the spot to lie in any position between h and 0.

**Statistical analysis.** All data were subjected to statistical analysis, using SPSS 12.0 software (SPSS, Inc., Chicago, IL). Variance analysis with fully factorial model was adopted. The analysis was done both on the distances obtained from the projected images and the maximized ones.

**Results and Discussion**

Thyroid-specific TRK oncogenes are generated by rearrangements of the NTRK1 gene with three different activating genes, namely TPR, TPM3, and TFG (3). We were interested in assessing whether the spatial contiguity model, recently proposed...
for H4/RET rearrangement (10), could be applied to the TRK oncogenes. We chose the TPR and NTRK1 combination, involved in the generation of TRK-T1 and TRK-T2. These two oncogenes display different structure mostly with respect to the portion contributed by TPR (5). Both TPR and NTRK1 genes are located on chromosome 1 (q25 and q21-22, respectively) and are separated by 30 Mb. The type of chromosomal rearrangement generating TRK-T1 and TRK-T2 oncogenes is unknown because no cytogenetic studies are available for tumors carrying TPR/NTRK1 rearrangements. However, the presence in the tumor DNA of the reciprocal products of the rearrangement and the evidence that TPR and NTRK1 have opposite transcriptional orientation indicate that chromosome inversion is the only mechanism capable to produce TRK-T1 and TRK-T2 oncogenes (12). H4/RET and TPR/NTRK1 oncogenes are produced by similar chromosome rearrangements. However, the distance between the rearranging loci is different in

### Table 1. Mean values of two-dimensional distances between TPR and NTRK1 loci in the two cases (1 and 2) of both thyrocytes and PBLs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>THY 1</td>
<td>2.5406 (1.49559)</td>
<td>322</td>
</tr>
<tr>
<td>THY 2</td>
<td>2.4440 (1.23385)</td>
<td>508</td>
</tr>
<tr>
<td>Total</td>
<td>2.4815 (1.34141)</td>
<td>830</td>
</tr>
<tr>
<td>PBL 1</td>
<td>4.1790 (2.28545)</td>
<td>304</td>
</tr>
<tr>
<td>PBL 2</td>
<td>3.3982 (1.81209)</td>
<td>408</td>
</tr>
<tr>
<td>Total</td>
<td>3.7316 (2.06276)</td>
<td>712</td>
</tr>
</tbody>
</table>

NOTE: Mean values are expressed in micrometers.
Abbreviations: THY, thyrocyte; n, number of measures.
the two cases, being 18 Mb for H4/RET and 30 Mb for TPR/NTRK1. At variance with Nikiforova et al. (10), who compared the two-dimensional distance between H4 and RET to a theoretical Rayleigh distribution, we used a method by which the two-dimensional distances between TPR and NTRK1 were maximized in function of slice thickness.

Bacterial artificial chromosome probes containing the loci of interest were selected from RP11 library (P. de Jong): 295K2 for TPR gene, and 107D16 for NTRK1 gene. They were directly labeled with Cy3 (red spots) and FluorX (green spots), respectively. Hybridization on PBL metaphase nuclei showed a specific reaction of probes 295K2 and 107D16 with the chromosome 1 regions containing the TPR and NTRK1 loci, respectively (data not shown). We did dual-color FISH on both metaphase and interphase nuclei from human primary thyrocytes obtained from two donors and, as control, PBL, obtained from two unrelated individuals. Samples were analyzed by fluorescence microscopy. Analysis of metaphase spreads showed separated red (TPR) and green (NTRK1) spots in both thyrocytes and PBL (data not shown), thus excluding the presence of TPR/NTRK1 rearrangement in our samples.

The two-dimensional distance between red (TPR) and green (NTRK1) signals was determined on recorded images (Fig. 1), as described in Materials and Methods. The frequency histograms of two-dimensional distances and the mean distance detected in each sample are reported in Fig. 2A and Table 1, respectively. The two thyrocyte samples showed very similar distance distribution and mean values (2.5406 and 2.4440 μm). The two PBL samples showed a slightly different distribution. However, the difference between the mean distance values (4.1790 and 3.3982 μm) was not statistically significant. By comparing the distribution of distances between TPR and NTRK1 loci detected in thyrocytes and PBL, marked differences were observed. In both thyrocyte samples, 45% of nuclei showed a distance in the range 0 to 2 μm, whereas in the two PBL samples only 15% and 25% of nuclei were detected in this range. The difference between the two cell types is more evident if we consider the nuclei showing TPR/NTRK1 distances in the range 0 to 1 μm: 15% in thyrocytes versus 2% and 5% in PBL. Statistical analysis revealed a significant difference between the mean distance of each cell type (2.4815 μm for thyrocytes, 3.7316 μm for PBL; Fisher’s F = 216.8, P < 0.001), suggesting that TPR and NTRK1 loci are closer in thyrocytes with respect to PBL.

To exclude possible artifacts of bidimensional analysis, we did a three-dimensional reconstruction of the data by applying a mathematical correction considering the maximized error derived from the nuclei thickness (see Materials and Methods). In Table 2, the maximized mean TPR/NTRK1 distances in thyrocytes and PBL are reported; in Fig. 2B, frequency histograms of the maximized distances in the two cell types are shown. Also, in this case, the two cell populations showed different distributions. Statistical analysis revealed a significant difference between the maximized mean distance of each cell type (4.8217 μm for thyrocytes and 5.7468 μm for PBL; Fisher’s F = 290.3, P < 0.001).

On the whole, our data show that NTRK1 is usually nearer its translocation partner TPR in thyroid cells than in other nonrelated tissues, such as PBL.

The present work supports the model proposed by Nikiforova et al. (10) by using a gene pair separated by a wider distance and a different data analysis method. Altogether, the data show that in thyrocytes, RET and NTRK1 loci display a reduced distance from their rearrangement partners (H4 and TPR, respectively) with respect to other cell types. This proximity may favor thyroid-specific oncogenic rearrangements, which may represent the consequence of DNA double-strand breaks repair. Such type of DNA damage is produced by several agents, such as ionizing radiation, oxygen free radicals, DNA replication, and topoisomerase failure. The role of ionizing radiation is unequivocally shown for RET rearrangements because they are prevalent in papillary thyroid tumors from children exposed to radiation after the Chernobyl accident (13). On the contrary, the frequency of NTRK1 rearrangements in post-Chernobyl papillary thyroid tumors is equivalent to that of sporadic tumors (14). Nevertheless, the association of thyroid malignancies with therapeutic or accidental radiation is epidemiologically documented (15). In addition, the features of genomic breakpoints in both RET and NTRK1 rearrangements suggest the occurrence of similar DNA damage (6, 7).

In conclusion, our data support the notion that cancer-specific chromosomal rearrangements are probably enhanced by relative loci proximity (16–18), which depends on nuclear organization, and it is related to specific pattern of gene expression or replication in cellular differentiation.

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References

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