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Short Communication

Reverse Transcriptase Polymerase Chain Reaction for the Ki-1 Anaplastic Large Cell Lymphoma-Associated t(2;5) Translocation in Hodgkin's Disease

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Hodgkin's disease (HD) and Ki-1 positive anaplastic large cell lymphoma (Ki-1 ALCL) appear pathologically and immunohistochemically related, and a common histogenesis has been postulated in at least some cases. The breakpoints of the t(2;5) (p23;q35) (p235) translocation, which is reported in about 40% of Ki-1 ALCL, have recently been cloned. They involve a novel tyrosine kinase gene,* ALK*, at 2p23 and the nucleophosmin gene,* NPM*, at 5q35. Reverse transcriptase polymerase chain reaction (RT-PCR) using* NPM *and* ALK *primers consistently detects a fusion product in Ki-1 ALCL cases with the translocation. To determine if this tumor-specific genetic alteration also occurs in HD, we performed* NPM-ALK *RT-PCR on RNA samples extracted from 40 lymph node biopsies of HD (25 nodular sclerosis, 11 mixed cellularity, 2 lymphocyte depleted, 2 lymphocyte predominant). Using control samples, the sensitivity of the* NPM-ALK *RT-PCR assay was shown to be at least 1:10^4. Amplifiable template was confirmed in all samples by RT-PCR using β-actin primers. None of the 40 cases showed the expected 177-bp RT-PCR product indicative of the translocation. We conclude that the most common primary genetic alteration in Ki-1 ALCL, the t(2;5), is absent or very infrequent in typical cases of HD. These results further support the concept that HD and Ki-1 ALCL are pathogenetically distinct entities. (Am J Pathol 1994, 145:1296–1300)

Both Hodgkin's disease (HD) and Ki-1 positive anaplastic large cell lymphoma (ALCL) are characterized by the expression of CD30, the Ki-1 antigen,[1] a cytokine receptor for a ligand related to the tumor necrosis factor family.[2,3] Although a histological distinction is easily made in most cases, there exist cases which constitute a morphological borderline or overlap between the two entities.[4,5] Immunohistochemistry is helpful in this differential diagnosis, but some overlap exists here also.[4,6,7] In addition to the Ki-1 antigen, HD and Ki-1 ALCL are characterized by the expression of some other markers unique among lymphomas, including the KIT receptor, interleukin-9, and the intermediate filament-associated protein restin.[8–10] Thus, the question of whether HD and Ki-1 ALCL represent a continuum of manifestations displayed by a single biological and genetic entity or whether the similarities are merely secondary features of two pathogenetically distinct entities remains current.

Cytogenetic analysis and Southern blotting have not been useful in resolving this question. Cytoge-

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netic analysis, which has been critical in orienting research in other hematopoietic neoplasms including Ki-1 ALCL, has been hampered in HD by the poor yield of abnormal metaphases in chromosome preparations.[11] The use of Southern blotting to analyze genetic alterations in HD has been limited by the generally small proportion of neoplastic cells in lymph nodes involved by HD, usually below the level of sensitivity of this technique.[12]

The recent cloning of the breakpoints of the t(2;5)(p23;q35)(p235) translocation,[13] which is present in at least 40% of non-B-cell ALCLs,[14] has provided a new molecular marker to probe this relationship. The translocation involves a novel tyrosine kinase gene, *ALK*, at 2p23 and the nucleophosmin gene, *NPM*, at 5q35, as shown by one of our groups.[15] Reverse transcriptase polymerase chain reaction (RT-PCR) using *NPM* and *ALK* primers consistently detects a fusion product in Ki-1 ALCL cases with the translocation (unpublished observations). Northern blotting has shown that this fusion RNA is moderately to highly expressed in cell lines with a t(2;5).[15] If this tumor-specific genetic alteration also occurs in HD, it would establish a genetic relationship between subsets of the two entities. To address this question, we performed *NPM-ALK* RT-PCR on RNA samples extracted from 40 lymph node biopsies of typical HD.

Materials and Methods

Representative snap-frozen portions of 42 lymph node biopsies showing HD, diagnosed by conventional morphology and immunohistochemistry, were collected retrospectively. Total RNA was extracted using RNA-STAT (Tel-Test, Friendswood, TX) following the method of Chomczynski and Sacchi.[16] Seven of these cases were included in a previous cytogenetic study of HD (cases 209, 224, 322, 657, 703, 792, and 832 from ref. 17). For control experiments, RNA was similarly obtained from cell lines K562, a blastic chronic myeloid leukemia cell line, and JB6, a Ki-1 positive T-lineage lymphoma cell line with the t(2;5) translocation (gift of M. Kadin), and from a clinical sample of T-cell Ki-1 ALCL (case 230 from ref. 14).

The 5′NPM and 3′ALK primers and the NPM-ALK junction oligonucleotide have been previously described,[15] and their sequences were, respectively, 5′-TCCCTTGGGGGCTTTGAAATAACACC-3, 5′-CGAGGTGCGGAGCTTGCTCAGC-3′, and 5′-AGCACTTAGTAGTGTACCGCCGGA-3′. RT-PCR was performed on total RNA, using the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT) on an automated thermal cycler (Omnigene; Hybaid, UK). Reverse transcription was performed for 30 minutes at 42 C on 1 μg of total RNA, using random hexamers. The reverse transcriptase was inactivated at 99 C for 5 minutes. The PCR reagents were then added. The final $MgCl_2$ concentration was 1.5 mmol/L, and the amount of each primer was 30 pmol/reaction. The cycling parameters were: 40 cycles of 94 C for 1 minute, 60 C for 1 minute, 72 C for 30 seconds, followed by a final extension at 72 C for 5 minutes. The RT-PCR products were electrophoresed on 3% NuSieve agarose gels (FMC, Rockland, ME) and visualized with ethidium bromide staining. The PCR products were blotted by capillary transfer onto nylon membranes, which were then hybridized with end-labeled NPM-ALK junction oligonucleotide, washed at high stringency, and applied to X-ray film for 2 to 24 hours. The presence of amplifiable RNA was confirmed by performing RT-PCR for β-actin transcripts using the following primers, 5′-AGGCCAACCGCGAGAAGATGACC-3′ and 5′-GAAGTCCAGGGCGACGTAGCAC-3′, spanning nucleotides 1387 to 2160 of the published genomic sequence,[18] which yield a 343-bp RT-PCR product.

Results

Using control cell lines, the sensitivity of the *NPM-ALK* RT-PCR assay was first assessed. A serial dilution was performed using total RNAs extracted from the K562 myeloid leukemia cell line and the t(2;5)-bearing JB6 Ki-1 ALCL cell line. The expected 177-bp *NPM-ALK* product was faintly visible on agarose gels at the highest dilution, 1 in 10^4 which consisted of 0.1 ng of JB6 RNA in 1 μg of K562 RNA (Figure 1). The same dilution was strongly positive following transfer and hybridization with the *NPM-ALK* junction oligonucleotide (Figure 2). The sensitivity of the RT-PCR assay including hybridization was therefore determined to be at least 1 in 10^4 (greater dilutions not tested).

RNA samples were extracted from 42 lymph node biopsies of HD. The adequacy of the RNA samples was assessed in all cases by RT-PCR using β-actin primers which amplify a 343-bp product (Figure 3). Two samples yielded no β-actin product and were excluded from further analysis. The remaining 40 samples, including 25 nodular sclerosis, 11 mixed cellularity, 2 lymphocyte-depleted, and 2 lymphocyte predominant (1 diffuse, 1 nodular), were subjected to the *NPM-ALK* RT-PCR assay. None of the 40 cases showed the expected 177-bp product indicative of the translocation, nor did they show products of different size; the negative results in five of these cases are shown in Figure 4. All HD cases remained completely negative following transfer and hybridization with the *NPM-ALK* junction oligonucleotide (Figure 2).

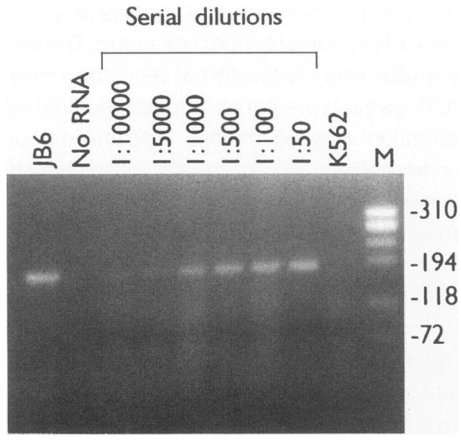

Figure 1. *Sensitivity of RT-PCR analysis for chimeric NPM-ALK RNA. Serial dilution of total RNA from the t(2;5)-bearing JB6 Ki-1 ALCL cell line in total RNA from the K562 acute myeloid leukemia cell line. The expected 177-bp product is faintly visible on the agarose gel at the greatest dilution, 1 in 10^4, which contains 0.1 ng of JB6 RNA in 1 μg of K562 RNA. Product from the same dilution was clearly seen following hybridization with the* NPM-ALK *junction oligonucleotide (Figure 2). As expected, the K562 and "No RNA" lanes show no product. The marker lane (M) contains* Hae *III-digested PhiX174 DNA (sizes of selected bands indicated in bp).*

Discussion

HD is one of very few common neoplasms of which our understanding at the genetic level remains grossly inadequate. One approach to this problem has been based on the hypothesis that Ki-1 ALCL is closely related to HD and that the study of Ki-1 ALCL, which is more amenable to genetic analysis, may therefore yield insights into the nature of HD. Indeed, HD and Ki-1 ALCL share many features, including expression of the CD30 antigen and other relatively restricted antigens and cytokines.[8–10] Occasional cases sit on the morphological borderline between the two entities, eg, "Hodgkin's related" Ki-1 ALCL and cellular or lymphocyte depleted variants of HD.[5,19] Composite and sequential cases have also been described.[6,20] At the genetic level, although studies of HD are fraught with difficulties of interpretation, both lesions appear to display an unusual "dissociation" of immunophenotype and immunogenotype.[21]

Ki-1 ALCL is characterized by the translocation t(2;5)(p23;q35)(p235).[13] We have previously found that approximately 40% of non-B-cell Ki-1 ALCL show this translocation or a variant thereof.[14] As a whole, non-B-cell Ki-1 ALCL account for about 85% of Ki-1 ALCL.[1,21–23] In contrast, it has only been reported once in an apparent B-cell Ki-1 ALCL.[24] Interestingly, in the limited cytogenetic literature on HD, two cases with a 5q35 breakpoint have been described,[17,25] as del(5)(q14q35) in one case, and add(5)(q35) in another case, case 7 in the present series (case 832 from ref. 17).

Clearly, if subsets of HD and Ki-1 ALCL were genetically related, the identification of the genes involved in the pathogenesis of Ki-1 ALCL would greatly facilitate the study of HD by PCR-based methods. This approach was recently made possible by the cloning of the translocation breakpoints of the t(2;5) by one of our groups.[15] The translocation fuses a novel tyrosine kinase gene, *ALK*, at 2p23 with the nucleophosmin gene, *NPM*, at 5q35.[15] The *NPM* gene encodes a nucleolar phosphoprotein; the t(2;5) results in a putative chimeric product consisting of the amino-terminal portion of this protein fused to the catalytic domain of ALK.[15] The chimeric RNA transcript is at least moderately abundant in cell lines bearing the translocation.[15] How this predicted hybrid protein is involved in the pathogenesis of Ki-1 ALCL and whether it has any relationship to the overexpression of CD30 remains to be determined. Nonetheless, the consistency of the rearrangement at the

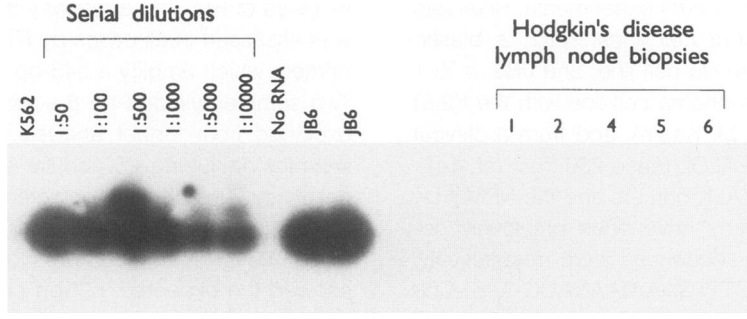

Figure 2. *Hybridization analysis of NPM-ALK RT-PCR products using a NPM-ALK junction oligonucleotide.* Left: *serial dilution of total RNA from the t(2;5)-bearing JB6 Ki-1 ALCL cell line in total RNA from the K562 acute myeloid leukemia cell line, corresponding to samples in Figure 1. The expected 177-bp product is clearly demonstrated down to the greatest dilution, 1 in 10^4 which contains 0.1 ng of JB6 RNA in 1 μg of K562 RNA. As expected, the K562 and "No RNA" lanes are negative, although partly obscured by the signals in adjacent lanes.* Right: *RT-PCR analysis for chimeric* NPM-ALK *RNA in five HD cases from Figure 4. The samples are completely negative even after hybridization analysis. RT-PCR products from a clinical sample of Ki-1 ALCL (no. 230) and the JB6 Ki-1 ALCL cell line are strongly positive.*

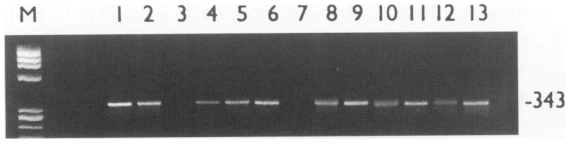

Figure 3. *RT-PCR analysis of β-actin RNA in HD. Agarose gel showing the presence of the expected 343-bp product in 11 of 13 HD cases from the current series, confirming the adequacy of the RNA samples. Cases 1 to 6 correspond to cases 1 to 6 in Figure 4. Case 3 yielded no β-actin product and was not included in the final study group. A repeat RNA extraction in case 7 yielded amplifiable β-actin RNA (not illustrated). Lane M contains the DNA size marker,* HaeIII*-digested PhiX174.*

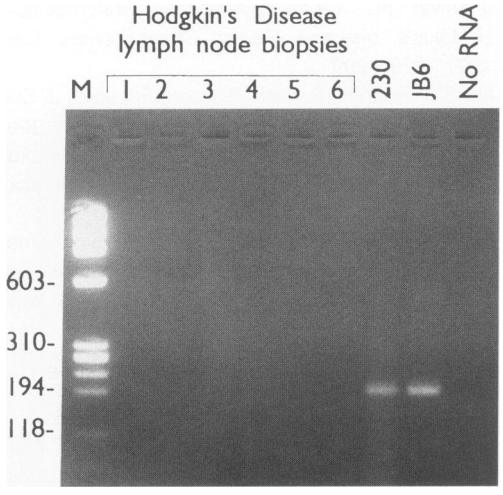

Figure 4. *RT-PCR analysis of chimeric NPM-ALK RNA in HD. Agarose gel showing negative results in five HD cases (HD case 3 excluded; see Figure 3). RNAs extracted from a clinical sample of Ki-1 ALCL (no. 230) and the JB6 Ki-1 ALCL cell line provide the positive controls. Lane M contains the* HaeIII*-digested PhiX174 size marker (sizes of selected bands indicated in bp).*

RNA level allows convenient detection of the gene fusion by RT-PCR.[15]

In the present study, we performed *NPM-ALK* RT-PCR on 40 lymph node biopsies of HD. We have found the primers used for the RT-PCR to detect the chimeric RNA in all cytogenetically proven t(2;5)containing lymphomas (unpublished observations). Moreover, the level of sensitivity of the RT-PCR assay, 1 in 10^4, exceeded the estimated proportion of neoplastic cells in lymph node tissue involved by HD, 1 in 10^2 to 1 in 10^3. Nevertheless, none of the 40 cases showed a positive RT-PCR result, including case 7, previously reported to contain a 5q35 breakpoint [(add(5)(q35); see case 832 in ref. 17]. Our results thus indicate that a molecularly identical translocation is absent or very infrequent in HD. Thus, there appears to be no genetic relationship between HD and the subset of T-cell Ki-1 ALCL characterized by this translocation. The present results do not, however, allow us to exclude the possibility that HD and the subset of Ki-1 ALCL lacking the t(2;5) are related or that a structurally different translocation involving *NPM*, *ALK*, or both is present in some HD. Furthermore, our study focused on typical cases of HD; the infrequent "borderline," composite, and sequential cases of HD and Ki-1 ALCL will need to be similarly examined.

Recently, the detection of rearrangement of the *NPM* gene by Southern blotting has been reported in 2 of 9 cases of HD.[26] The same samples were not studied by RT-PCR for the chimeric *NPM-ALK* transcript. It is possible that these two rearranged HD samples may harbor an *NPM* rearrangement other than the t(2;5). The further characterization of the apparent alteration of the *NPM* gene in these two cases of HD will be of great interest.

Beyond the pathologically evident differences between typical forms of HD and Ki-1 ALCL, some biological differences include more frequent extranodal involvement in Ki-1 ALCL, a strong association with EBV in HD but less so in Ki-1 ALCL,[5] and contrasting responses to the CD30 ligand.[27] Indeed, this last observation may point to a fundamental difference between the two entities: CD30 ligand was found to enhance the proliferation of "T-cell-like" HD cell lines, but resulted in cell death of CD30 Ki-1 ALCL cell lines.[3,27] One possibility is that HD and Ki-1 ALCL may result from different genetic lesions arising in the same or closely related precursor cells. Although our results further support the view that their molecular pathogenesis is unrelated, a postulated common precursor cell could account for their occasional morphological similarities.

Acknowledgments

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