Tumor Detection Through the Use of Immunoglobulin Gene Rearrangements Combined With Tissue In Situ Hybridization

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Leukemias and lymphomas can now be classified according to the particular immunoglobulin, T-cell receptor, or growth-affecting genes they are expressing. Recognition of the structural alterations of lymphoid DNA has been used to identify neoplasms of previously uncertain lineage, to aid in diagnosis, and to define the state of differentiation of the neoplasm. We have developed a procedurally simple, rapid turnaround technique for using tumor-specific gene alterations as tumor-specific markers. Probes can be constructed that will recognize only the gene expressed in the tumor and not those in any of the normal cells when used with tissue in situ hybridization. We demonstrate the application of direct sequencing of a specific gene of interest from total RNA from a patient with multiple myeloma. A probe is then generated from this sequence and applied directly to patient material. This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Direct total RNA sequencing. Cell line H929 (Q) was derived from the pleural fluid of a 62-year-old woman diagnosed with IgA kappa multiple myeloma. A. KK125 is a cell line derived from a pleural effusion from a 16-year-old male with a kappa expressing Burkitt's lymphoma. RNA was extracted from the cell lines as described by Chirgwin. The immunoglobulin kappa variable region of each cell line was directly sequenced from 20 µg of total RNA by primer extension using a reverse transcriptase (Promega, Madison, WI) dideoxynucleotide method with a 5' complementary kappa constant region (NT 345-368) 23 base-pair oligonucleotide as a primer.

Oligonucleotide probe synthesis and purification. Forty-five base-pair oligonucleotide probes complementary to the kappa variable regions of cell lines H929 (K,) and KK125 (K,,), the kappa constant region (K,), and the first exon of c-myc were synthesized on an Applied Biosystems DNA synthesizer 380B (Foster City, CA). The kappa hypervariable region probes were complementary to the third complementarity determining regions (CDR) segment. The kappa constant region was complementary to the 5′ end corresponding to NT 400-445. The c-myc oligonucleotide probe was complementary to the 3′ end of the first exon of c-myc corresponding to NT 314-359. All oligonucleotide probes were purified on denaturing gels consisting of 8% polyacrylamide and 8 mol/L urea.

Labeling of oligonucleotide probes. All probes were labeled at the 3′ end, using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis) and [32P] dideoxynucleoside triphosphate (New England Nuclear, >1000 Ci/mmole). The specific activities of the resultant probes were: K, 3.0 x 107 DPM/mole, K, 4.8 x 107 DPM/mole, K, 6.6 x 107 DPM/mole, and c-myc 1.8 x 108 DPM/mole.

Cytospin preparation. Cytospins were made from cells from the pleural fluid of a patient with multiple myeloma. In addition, cytospins of a mixture of cell lines [Burkitt's lymphoma (KK125), T-cell leukemia, and multiple myeloma (H929)] were also prepared.

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1791
Cytospins for a separate mixing experiment were made with known numbers of H929 cells added to $10^7$ normal bone marrow cells. Cytospins were made according to the procedure of Harper and applied to Histostik-coated slides (Accurate Chemical Scientific Corporation, Westbury, NY).9

Frozen sections preparation. Sections from flash-frozen reactive lymph nodes from two separate patients were placed on Histostik-coated slides according to the procedure of Harper.9

In situ hybridization. Hybridization and autoradiography was performed as described.10 Briefly, cytospins were covered with a 1 × 10⁶ disintegrations per minute (DPM) of probe in 45µl of hybridization buffer consisting of 4 × standard saline citrate (SSC) (1 × SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7.2), 50% formamide, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 500 µg/mL yeast tRNA, 10% dextran sulfate, and 10 mm dithiothreitol. Hybridizations were performed at 37°C for 16 hours. Sections were washed in four rinses of 15 minutes each in 50% formamide/2 × SSC at 40°C followed by two one-hour rinses in 1 × SSC at room temperature. After two rapid H₂O dips, the sections were dried and dipped in NTB 2 nuclear emulsion (Kodak) (1:1 with H₂O) and exposed for five days. After autoradiography slides were developed (Kodak D19), they were fixed (Kodak), stained with hematoxylin eosin, and examined using a Zeiss photomicroscope III under lightfield.

Synthesis of specific RNA. Ta RNA was transcribed from 1.1 kb alpha chain cDNA of the T-cell receptor fragment subcloned into pSP64 vector (Promega) using the SP 6 promoter.

RESULTS

In order to determine the lowest level of specific message that must be present to directly sequence by this method, a mixing experiment was performed. Known quantities of Ta RNA were added to 1 µg of total non-T-cell RNA. As shown in Fig 1, sequencing specific RNA was possible when as little as 1 to 5 µg of specific RNA was added. This suggests that...

Fig 1. Mixing experiment showing the level of specific message that is amenable to sequencing. Quantities ranging from 1 to 100 ng were added to 1 µg of total non-T-cell RNA. Dideoxy sequences reactions were run on 8% denaturing polyacrylamide gel. Message was able to be sequenced when present in the range of 1 to 5 ng. This corresponds to 0.1% of the cellular mRNA.

Fig 2. Immunoglobulin kappa variable region sequenced directly from RNA extracted from cells of a Burkitt's lymphoma. Complementary oligonucleotide to the hypervariable region (CDR3) of the kappa region is noted at the bottom of the figure.
the message must be present at the level of 0.1% of total cellular RNA in order for it to be sequenced. The specific kappa variable regions that would be used as probes were identified by first sequencing the kappa variable regions from 20 μg of total RNA from malignant lymphocytes from a patient with Burkitt's lymphoma (Fig 2) and a patient with multiple myeloma. An oligonucleotide complementary to the 5' of the kappa constant region was used as the primer for the reverse transcriptase-mediated sequencing reaction. The CDR3 portion of the variable regions was identified by sequence comparison.11 Complementary oligonucleotide probes were then generated to the CDR3 segment of the variable region of the Burkitt's (K_k) and the myeloma (K_c). A complementary K region oligonucleotide was generated from the published sequenced kappa constant region.7 The oligonucleotide was complementary to the 5' end of the constant region.

The 32P radiolabeled probes were utilized by in situ hybridization on cytopsins of the cell mixture consisting of lymphocytes from T-cell acute lymphocytic leukemia (ALL), a kappa expressing Burkitt's lymphoma (KK125), and kappa expressing multiple myeloma (H929). Previous work on these cell lines had established the expression of kappa message and kappa protein.4,5 The results of the hybridization of the complementary oligonucleotide probes (K_k, K_c, K_{c'), to the cell mix are shown in Fig 3 (A through C). The K_k probe hybridized to both plasma cells and Burkitt's cells. Plasma cells were labeled with more intensity than the cells from the Burkitt's line (see Fig 3A). Northern blot analysis confirms an approximate tenfold increase in kappa-specific RNA in the plasma cell when compared with the Burkitt's (data not shown). The probe complementary to the kappa hypervariable region of the multiple myeloma hybridized only to the plasma cells in the cell mixture (Fig 3B). The probe complementary to the kappa hypervariable region of the Burkitt's lymphoma hybridized only to the Burkitt's cells, as seen in Fig 3C. The kappa hypervariable region probes complementary to either the Burkitt's lymphoma or multiple myeloma did not hybridize to any cells in the frozen sections from reactive lymph nodes. In contrast, lymphocytes expressing kappa constant message were detected throughout the same reactive lymph nodes (data not shown).

The results of hybridization of the same three complementary oligonucleotide probes to the patient's pleural fluid at the time of relapse are depicted in Fig 3 (D through F). Complementary oligonucleotide probes to the kappa constant and kappa hypervariable regions of H929 hybridized to the majority of cells in the pleural fluid. Only plasma cells within the pleural fluid appeared to be positively labeled and appeared to express more message than the plasma cells from the cell line. Other cell types did not hybridize with the probes (Fig 3 D and E). The probe complementary to the hypervariable region of the Burkitt's lymphoma did not hybridize to the cells of the patient's pleural fluid (3F).

As an extension of this work, we used a complementary oncogene probe. Previous work has demonstrated unique alterations in the c-myc oncogene for specific tumors.6 We constructed an oligonucleotide probe complementary to the 3' end of the first exon of c-myc from the multiple myeloma cell line (H929). The hybridization of the oligonucleotide probe complementary to the first exon of c-myc is shown in

Fig. 3. (A) Tissue in situ hybridization using a complementary probe to the kappa constant region. Cell mix contains cells from kappa expressing Burkitt's lymphoma (B), kappa expressing multiple myeloma, and T-cell ALL (T). Complementary oligonucleotide probe to the kappa constant region hybridizes to the plasma cells and Burkitt's cells of the cell mixture, as demonstrated by silver grains in the autoradiographic emulsion layer above the cells. Original magnification (hematoxylin and eosin [H & E]) x 126. (B) Tissue in situ hybridization using a complementary probe to the kappa constant region. Cell mix contains cells from kappa expressing Burkitt's lymphoma (B), kappa expressing multiple myeloma, and T-cell ALL (T). Complementary oligonucleotide probe to the kappa constant region hybridizes to the plasma cells and Burkitt's cells of the cell mixture, as demonstrated by silver grains in the autoradiographic emulsion layer above the cells. Original magnification (hematoxylin and eosin [H & E]) x 126. (C) Cell mix hybridization with the complementary kappa variable region oligonucleotide probe specific for the kappa variable region of the multiple myeloma. Note hybridization only to the plasma cells. Original magnification (H&E) x 126. (D) Hybridization of the cell mix with the complementary oligonucleotide probe specific for the kappa variable region of the multiple myeloma. Note hybridization only to the plasma cells. Original magnification (H&E) x 126. (E) Cell mix hybridization with the complementary kappa variable region oligonucleotide probe specific for the kappa variable region of the multiple myeloma. Note hybridization only to the plasma cells. Original magnification (H&E) x 126. (F) Hybridization of the cell mix with the complementary kappa variable region oligonucleotide probe specific for the kappa variable region of the multiple myeloma. Note hybridization only to the plasma cells. Original magnification (H&E) x 126.
The results of the hybridizations using the c-myc oligonucleotide probe cannot be compared directly to those using the kappa region probes, since the exposure time differed. The exposure time for the c-myc probe was at least twice as long as for the kappa region probes. More grains would have been detected with a longer exposure time using the c-myc probe. However, there is much less c-myc message present in the plasma cells than kappa message.

Hybridization of reactive lymph nodes, with the kappa hypervariable oligonucleotide probes for the Burkitt's lymphoma and multiple myeloma, failed to reveal message complementary to these CDR3 regions. However, numerous cells were identified in the same lymph node sections using a complementary oligonucleotide probe to the kappa constant region.

In a separate mixing experiment, we added known numbers of H929 myeloma cells to samples of $10^4$ normal bone marrow cells and hybridized them to the oligonucleotide unique for the myeloma CDR3 region. No hybridization was detected in the control sample containing only normal bone marrow. Rough estimates of predicted v observed hybridization, given the number of myeloma cells added, indicated that essentially every myeloma cell added was detected (data not shown).

DISCUSSION

We report here how it is possible to use specific gene rearrangements detected within tumors as markers for the tumor cells. This permits us to distinguish the tumor cell in its transformed and clonally proliferative state. The immunoglobulin kappa rearrangement studied here most likely had nothing to do with the malignant event; however, it does provide a specific marker for a malignant clone. Since only the cells expressing the variable region of interest hybridize with the probe, those cells expressing the unique hypervariable region are detected among the negative cells present within the field.

The specificity of the probes is shown in the cell-mixing experiment, since the complementary kappa hypervariable region probe for the multiple myeloma hybridizes to the plasma cells and not to the Burkitt's cells. The complementary kappa hypervariable probe for the Burkitt's lymphoma hybridizes only to the Burkitt's cells and not the plasma cells. Neither of the hypervariable region probes hybridize to the T cells. Fortuitous expression of an identical CDR3 region by normal cells could potentially confuse the analysis. Although theoretically possible, this common hypervariable segment expression is expected (on the basis of the combinational possibilities of VJ or VDJ recombination) to be of very low frequency. Indeed, in reactive lymph nodes, kappa hypervariable message for the Burkitt's lymphoma and multiple myeloma was not detected, although kappa messages in general were easily detected. Results of the mixing experiment using known numbers of H929 myeloma cells with normal bone marrow demonstrate that essentially every myeloma cell added was being detected. Thus, this technique is applicable to detection of minimal residual disease, tumor progression, and metastases, as well as ultimately staging the patient on a molecular level, and CDR3 segments make for quite specific probes.

The aim of this study was to develop a rapid turnaround technique that could be used on patient material to search for specific tumor cells in the tissue of interest. This can be accomplished with this technique within two weeks of making the RNA with slight variability in exposure time related to the abundance of message and the specific activity of the probe. As demonstrated in the cell mixture, the amount of mRNA in the Burkitt's cells was sufficient to detect those cells among the negative cells present in the same field.

This method is not limited to the use of immunoglobulin or T-cell receptor gene rearrangements; it can be used to detect genes that potentially contribute to malignancy. Alterations of oncogenes in the form of amplifications, translocations, and somatic mutations, such as insertions and point mutations have been described. Many of these alterations occur within the transcriptional unit, resulting in expression...
of the specific alterations within the gene. In our study, using probes specific for altered first exons of c-myc genes from different tumors, we were able to show specificity of cell identification by in situ hybridization. Theoretically, it should be possible to detect single-point mutations through complementary oligonucleotide probes, although we have not tried this. The sensitivity of this technique may not be refined enough to detect this minor difference among homologous genes.

Another factor that affects this procedure is the level of mRNA expressed by the cell. The RNA sequencing protocol we use can accommodate up to 100 μg of total RNA. Assuming mRNA to be 1% to 3% of total, it is then possible to sequence the specific mRNA of interest when the gene is expressed at the level of 0.1% of the mRNA population (see Fig 1). At this level of sensitivity, sequencing the variable region from a variety of B- and T-cell tumors as well as oncogenes from hematopoietic and solid tumors is possible. Amplification of minute quantities of DNA or cDNA is now possible with the use of polymerase chain reaction (PCR). Applied within this setting, PCR could enable the sequencing of transcripts expressed at a much lower level than 0.1%.

Once the sequence has been obtained through the use of complementary oligonucleotide probes, the sequence can be detected in the cells via in situ hybridization. The level of sensitivity using RNA–RNA in situ hybridization is estimated to be around 30 copies of mRNA per cell, or 0.01% of the mRNA (data not shown). This is consistent with previous reports by others. We have begun this study using relatively large oligonucleotide probes consisting of 45 nucleotides. The next stage in our study is the use of smaller oligonucleotide probes. By using smaller probes, the specificity for minor variations in a particular gene may be increased.

The two constraints of this procedure appear to be only that the specific tumor DNA alteration be part of the transcription unit of the particular gene being studied (that is, that the alteration be transcribed into mRNA), and that the level of transcript be high enough to be amenable to rapid, direct RNA sequencing and subsequent detection. Given the rapid progress in the refinement and increasing sensitivity of these techniques, this second constraint would not appear to be a serious limitation to their general applicability.

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Tumor detection through the use of immunoglobulin gene rearrangements combined with tissue in situ hybridization

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