

Detection of Mutations in the Fas Antigen of Lymphoma Tumors by RT-PCR and Denaturing Gradient Gel Electrophoresis

Terry Landowski, Ibrahim Buyuksal, and William Dalton, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, 12902 Magnolia Drive, Tampa FL 33612

Introduction

Identifying of mutations within specific genes has become an important strategy in determining the diagnosis and prognosis for many diseases, including cancer. Several techniques have been developed to examine heterogeneous tissue samples for specific mutations, including restriction fragment length polymorphism (RFLP), single-stranded conformation polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE), among others. These techniques are useful for rapid screening of heterogeneous tissues, and each has its individual strength (Eng and Vijg 1997). Using a reverse transcription polymerase chain reaction (RT-PCR) and DGGE, we have examined the cytoplasmic region of the Fas antigen known as the death domain in tumor specimens from patients with lymphoma of various histiocytic origins. These mutations may contribute to the failure of the tumor cells to undergo apoptosis, and thus contribute to the pathogenesis and progression of the disease (Landowski et al. 1997).

Methods

Tumor biopsies were obtained during postoperative resection, and peripheral blood lymphocytes for controls were obtained from normal volunteers. Total RNA was isolated from cryopreserved specimens, and 50-100 ng of RNA was reverse transcribed by oligo(dT) priming. PCR primers for the Fas III region were as previously described (Landowski et al. 1997) with the addition of a 42 bp GC clamp on the forward primer (Sheffield et al. 1989). Following 35 cycles of amplification in a 9600 PerkinElmer thermocycler, the PCR products were denatured for 5 min at 95°C and reannealed for 1 hr at 55°C to allow heteroduplex formation.

Melting temperature of the 430 bp PCR product was predicted using MacMelt™ software and confirmed by analysis on a 6% acrylamide gel (37.5:1) with a perpendicular gradient of 12.5–37.5% denaturant. Electrophoresis in the DCode™ universal mutation detection system was at a constant temperature of 60°C in 1x TAE buffer. Gels were run at 100 V for 2.5 hr. Optimum strand separation occurred at 23–24% denaturant, which correlated to the predicted melting temperature of 65–70°C. All further analyses were done in a parallel gradient of 15–30% denaturant at 60°C, with running time extended to 4.5 hr. PCR products were detected by ethidium bromide staining.

Results and Discussion

Hematological tumors typically contain a heterogeneous population of cells that includes both neoplastic and normal cells. The identification of tumor-specific mutations requires a method that can separate mutant sequences from normal sequences for isolation and identification. Sensitivity of the techniques is important in diseases such as lymphoma, which typically contain large numbers of normal stromal cells and infiltrating lymphocytes.

To determine the sensitivity of the DGGE analysis, we took advantage of a previously identified polymorphism in the Fas antigen, in which base pair 836 is changed from C to T (Fiucci and Ruberti 1995). Direct sequencing of PCR products has shown that the T-cell leukemia cell line CEM is heterozygous for the alternative codon, ACT, while the myeloma cell line 8226 expresses only the higher frequency codon, ACC. RNA was isolated from CEM and 8226 cell lines, and titrated at 1%, 5%, 10%, 30%, and 50% to determine the minimal amount of polymorphic cDNA detectable (Figure 1). Using ethidium bromide staining, we were able to easily identify 5% polymorphic RNA, and even as little as 1% was detectable.

Tumor specimens from patients with lymphoma were examined for mutations in the signal-transducing domain of the Fas antigen by RT-PCR and DGGE analysis. Figure 2 shows a representative ethidium-stained DGGE gel including

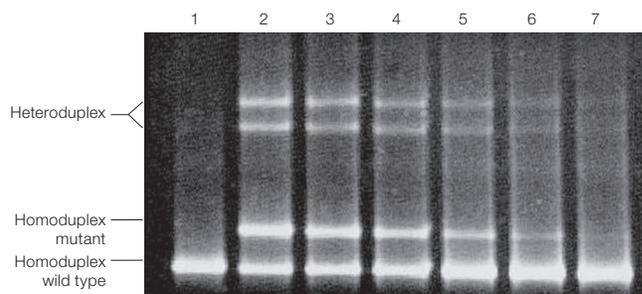


Fig. 1. Titration of total RNA isolated from the human myeloma cell line 8226, with total RNA isolated from the T-cell leukemia cell line CEM. Total RNA was mixed at the indicated concentrations, reverse transcribed, and the Fas III domain amplified with 35 cycles of PCR. Products were analyzed on a 6% acrylamide DGGE as described. Lane 1, 100% 8226 RNA (wild type); lane 2, 100% CEM RNA (polymorphic); lane 3, 50% CEM RNA/50% 8226 RNA; lane 4, 30% CEM RNA/70% 8226 RNA; lane 5, 10% CEM RNA/90% 8226 RNA; lane 6, 5% CEM RNA/95% 8226 RNA; lane 7, 1% CEM RNA/99% 8226 RNA.

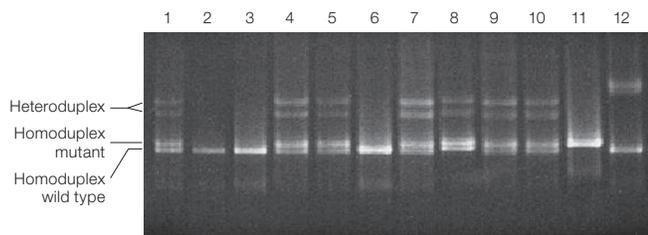


Fig. 2. DGGE analysis of the Fas III domain in lymphoma patients. Total RNA was isolated from lymphoma tumor tissues, reverse transcribed, and the Fas III domain amplified with 35 cycles of PCR. Products were analyzed on a 6% acrylamide DGGE gel as described. Lane 1, CEM (polymorphic); lane 2, 8226 (wild type); lanes 3–12, lymphoma patient specimens.

10 patient samples. The CEM T-cell line serves as a positive control and demonstrates the heteroduplex bands formed by polymorphic and wild-type cDNA. The myeloma cell line 8226, which serves as a negative control, has been fully sequenced and shown to be wild-type sequence. Of the patient specimens demonstrated in Figure 2, two display a single band characteristic of wild-type sequence. Patient specimens 4, 5, 7, 9, and 10 demonstrate heteroduplex band formation, indicating an alteration in the cDNA sequence. These bands were extracted from the gel and reamplified by PCR for direct sequencing. In addition, the altered mobility of the single band seen in patient 8 is suggestive of a homozygous mutation. This band was also extracted for sequence analysis.

With the high frequency of polymorphisms reported at base pair 836 in the Fas antigen, we sought to develop a screening method to distinguish the known polymorphism from other unknown mutations that could have functional significance. Expression of the alternative codon, ACC, introduces a unique restriction site for the enzyme *DraI*, which is not present in the wild-type cDNA sequence. Cleavage of the Fas III cDNAs with *DraI* results in a 368 bp fragment when the polymorphic codon is expressed, while this enzyme does not cut the wild-type cDNA. Patient samples that demonstrated heteroduplex formation on DGGE analysis were incubated overnight at 37°C with 0.5 U *DraI*, denatured for 5 min at 95°C, and allowed to reanneal at 55°C for 1 hr. Digested products were loaded on the DGGE acrylamide gel and electrophoresed 3.5 hr under conditions described above (Figure 3). This treatment resulted in the disappearance of one of the heteroduplex bands in specimens with the polymorphism and the appearance of a truncated homoduplex band (see lanes 1, 4, 5, 8, and 9 of Figure 3). Patient specimens that are homozygous for the polymorphism display only the truncated homoduplex (lane 10), while patient specimens with mutations in addition to the polymorphism retained two heteroduplex bands (lanes 7 and 11).

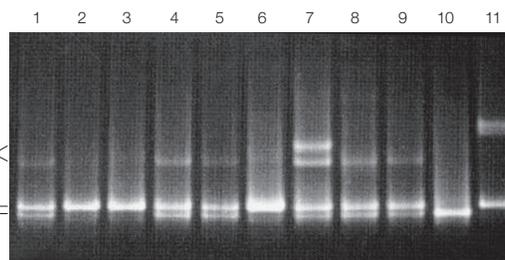


Fig. 3. DGGE analysis of restriction enzyme digested RT-PCR products. RT-PCR products were cleaved by *DraI* and separated on a DGGE gel as described. Lane 1, 50% CEM RNA/50% 8226 RNA; lane 2, 8226 (wild type); lane 3–11, lymphoma patient specimens.

Conclusions

Crosslinking of the Fas antigen by antibody or specific ligand induces apoptosis in susceptible cells. Mutations within the signal-transducing domain may render the cells resistant to programmed cell death, and contribute to the progression of neoplastic disease. We have utilized the methods of RT-PCR and DGGE to detect small changes in the coding sequence of the Fas antigen in hematological tumors.

In contrast to many other rapid screening techniques, DGGE is based on known physical parameters of a particular gene. By calculating the predicted melting temperature of a segment of DNA, analytical conditions can be optimized for maximum accuracy and sensitivity (Myers et al. 1987). We have demonstrated that DGGE can be used to detect as little as 1% of total RNA containing a single-base change with nonradioisotopic techniques. In addition, with a combination of restriction digest and DGGE analysis, we have developed a technique that can be used to distinguish between previously characterized noncoding changes in the Fas antigen, and unknown mutations. Identification of the known polymorphism reduces the number of bands which must be fully sequenced, and contributes to the overall efficiency of the genetic screening.

References

- Eng C and Vijg J, Genetic testing: the problems and the promise, *Nat Biotechnol* 15, 422–426 (1997)
- Fiucci G and Ruberti G, Detection of polymorphisms within the Fas cDNA gene sequence by GC-clamp denaturing gradient gel electrophoresis, *Immunogenetics* 39, 437–439 (1994)
- Landowski TH et al., Mutations in the Fas antigen in patients with multiple myeloma, *Blood* 90, 4266–4270 (1997)
- Myers RM et al., Detection and localization of single base changes by denaturing gradient gel electrophoresis, *Methods Enzymol* 155, 501–527 (1987)
- Sheffield VC et al., Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes, *Proc Natl Acad Sci USA* 86, 232–236 (1989)

Practice of the polymerase chain reaction (PCR) may require a license.

Information in this tech note was current as of the date of writing (1998) and not necessarily the date this version (rev B, 2007) was published.

BIO-RAD

**Bio-Rad
Laboratories, Inc.**

Life Science
Group

Web site www.bio-rad.com USA 800 4BIORAD Australia 61 02 9914 2800 Austria 01 877 89 01 Belgium 09 385 55 11 Brazil 55 21 3237 9400
Canada 905 364 3435 China 86 21 6426 0808 Czech Republic 420 241 430 532 Denmark 44 52 10 00 Finland 09 804 22 00 France 01 47 95 69 65
Germany 089 318 84 0 Greece 30 210 777 4396 Hong Kong 852 2789 3300 Hungary 36 1 455 8800 India 91 124 4029300 Israel 03 963 6050
Italy 39 02 216091 Japan 03 6361 7000 Korea 82 2 3473 4460 Mexico 52 555 488 7670 The Netherlands 0318 540666 New Zealand 0508 805 500
Norway 23 38 41 30 Poland 48 22 331 99 99 Portugal 351 21 472 7700 Russia 7 495 721 14 04 Singapore 65 6415 3188 South Africa 27 861 246 723
Spain 34 91 590 5200 Sweden 08 555 12700 Switzerland 061 717 95 55 Taiwan 886 2 2578 7189 United Kingdom 020 8328 2000