RT-PCR Amplicons in the Plasma of Multiple Myeloma Patients

Clinical Relevance and Molecular Pathology

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The occurrence of RNA (RT-PCR amplicons) in the plasma of 70 patients was quantified: 65 patients with multiple myeloma (MM), 3 with Waldenstrom's macroglobulinemia (WM), 2 with monoclonal gammopathy of undetermined significance (MGUS), and 50 from healthy controls. A 713nt amplicon occurred in 16/18 MM patients in relapse, 5/8 untreated patients, 2/3 WM patients and 1/2 MGUS plasmas. None of the initial specimens from 37 patients in remission nor the 50 healthy controls was positive. Homology between 4 sequenced 713nt amplicons was >99.7% and matched (99.6%) a 704nt sequence of the flanking region of the peroxisome proliferator activator receptor gene, located on chromosome 22q11.2. A 255mer within the 713nt amplicon had a 90.2% homology with an AluSc consensus sequence. The occurrence of RNA amplicons seemed to serve as accurate surrogate markers for active disease in MM that provide new insights to the molecular pathogenesis of the disease.

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One of our central interests (1, 2) has been the molecular genetics of the malignant transformation of the B-cell precursors that ultimately become malignant plasma cells. Using a reverse transcriptase-PCR technology (RT-PCR) described previously (3), we found that patients with active multiple myeloma (MM) had RT-PCR amplicons (RNA) in their sera. A 713nt amplicon occurred in the plasma of 62% of MM patients with untreated disease and in 89% with disease in relapse after therapy. Interestingly, the 713nt amplicon was also found in 2/3 patients with Waldenstrom's macroglobulinemia (WM) and in 1/2 patients with monoclonal gammopathy of undetermined significance (MGUS). Remission after therapy was often accompanied by a loss of amplicon detectability. The sequence of the 713nt amplicon was highly homologous (99.6%) to the flanking region of the peroxisome proliferator activator receptor (PPAR) exon sequence located on chromosome 22q11.2, a 'hot spot' for genetic recombination. We discuss possible applications of the findings reported here to the molecular epidemiology and pathogenesis of MM.

MATERIAL AND METHODS

Patients

This study included 70 clinical patients and 50 normal healthy controls. Control samples were obtained from life insurance applicants (Osborne Laboratories, Lenexa, Kansas City, USA). The methods used to diagnose MM and other malignancies, autoimmune diseases, and viral and bacterial infections were those standard for this institution (4). The therapies used to treat patients with MM, WM, and MGUS were described (5). Of the initial specimens taken from patients in the MM cohort, 8 patients had previously untreated disease (PU), 1 had active disease under treatment (ADT), 37 had disease in remission (RM), and 19 had disease in relapse (RL) after therapy. Three patients had WM and 2 MGUS. Four patients had malignancies concurrent with MM: 2 with breast cancer and 2 with uterine and pancreatic cancer, respectively. One MGUS patient had renal adenocarcinoma (atypical low-grade histology). Many of the MM patients had underlying autoimmune diseases: 6 with systemic lupus

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erythematosus; 2 with rheumatoid arthritis; 5 with psoriasis; 3 with amyloid; and 2 with diabetus mellitus. Herpes virus infections (EBV, herpes simplex, and varicella viruses) occurred in from 29 to 38% of PU and RL patients while the frequency was only 2% in RM patients. Active zoster (varicella virus) occurred in 5/65 MM patients. Of these 4/5 occurred in PU or RL patients. Hepatitis virus infections (types A. B, and C considered together) occurred in 12% of MM patients. Upper respiratory tract infections (viral and bacterial considered together) were diagnosed in 12/65 MM patients (18%). Lower respiratory tract infections occurred in 14/65 MM patients (22%). *S. pneumoniae* was the predominant cause of pneumonia.

Plasma specimens

Peripheral blood samples were obtained with informed consent from all patients. Blinded specimens from 50 healthy controls obtained from life insurance applicants (Osborn Laboratories, Lenexa, KS) were matched in so far as possible by gender, age and race. Vacutainer Cell Preparation Tubes (CPT™) (Beckton Dickinson, Franklin Lakes, NJ) were used to collect blood. The blood was centrifuged for 20 min at 1500–1800 rpm. The plasma was separated immediately from the cellular constituents. Specimens were forwarded in cold packs (not frozen) by overnight delivery to the Chronic Illness Research Foundation (Berkeley, CA), and processed for RT-PCR within 48 h.

RT-PCR

To prevent cross-contamination, separate dedicated facilities were used to process specimens and RT-PCR. RNA from 0.25 ml of the plasma specimen was extracted in a laminar flow hood using 0.75 ml TRIZOL LS reagent (Gibco BRL, Gaithersburg, MD). RNA was precipitated with 10 µg of RNAse-free glycogen as a carrier. Both methods were performed as specified by the manufacturer. Precipitated RNA was washed once with 70% ethanol by centrifugation at 4°C, resuspended in 10 µl RNAse-free distilled water, and added to 17 µl of the RT mixture (GeneAmp RNA PCR kit; Perkin-Elmer, Norwalk, CT) containing $MgCl_2$ (5 mM), 1X PCR Buffer II, RNAse Inhibitor (2.5 U), MuLV Reverse Transcriptase (2.5 U), random hexamer primers (2.5 µM), and 1 mM each of dATP, dGTP, dCTP, and dTTP. Poliovirus, Sabin type 1, RNA (National Institute for Biological Standards and Control, Hertfordshire, UK) was used as a positive control. RT was omitted from the reaction mixture as a negative control. The RT mixture was incubated for 10 min at 22°C, 30 min at 42°C, and 5 min at 95°C using a Perkin Elmer Thermocycler. The RT mixture then was added to the top of a Hot Start PCR reaction using a melted Ampliwax bead (Perkin Elmer, Norwalk, CT) as the barrier. The 70 µl top PCR mixture contained 1X PCR Buffer II and Amplitaq (2.5 U). The 30 µl bottom PCR mixture contained 1X PCR Buffer II, 2 mM $MgCl_2$, and the appropriate primer pairs (15 µM). Primers from the enteroviral (ENT) non-translated region (PG01: 5′-AAGCACTTCTGTTTCC-3′, PG02: 5′-CATTCAGGGGCCGGAGGA-3′) and the poliovirus viral protein region (P2/P3) junction of poliovirus types 1 and 2 (PG03: 5′-GAAATGTGTAAGAACTGTCA-3′, PG04: 5′-GTAACAATGTTTCTTTTAGCC-3′) were used as primer pairs or in a multiplex combination. After 35 cycles of amplification (1 min at 94°C, 2 min at 48°C, and 1 min at 72°C), 8 ul of the PCR mixture was electrophoresed using a precast 6% polyacrylamide gel in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA) (NOVEX, San Diego, CA) for 45 min and 60 min, respectively, at 200 volts. Gels were stained for 20 min in a 0.5 µg/ml ethidium bromide solution and photographed under UV light.

Cloning and sequencing

The RT-PCR amplicons from 4 representative MM patients were run on and excised from a 2% NuSieve GTG low melting point agarose gel (FMC BioProducts, Rockland, ME). Bands were blunt-end cloned using the Prime PCR Cloner Kit (5 PRIME–3 PRIME, Inc., Boulder, CO) according to the manufacturer's specifications. Sequence analysis was performed using the automated sequencer from ABI PRISM (Operon Technologies, Inc., Alameda, CA).

Blast analysis

Genetic analysis was applied to the 713nt sequence (GenBank # AF018254) using the Advance Blast program (http://www.ncbi.nlm.nih.gov/BLAST/). The following parameters were set: the 'none' database and nr (all non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF) were selected, the expected threshold was set at 1, and the MM amplicon was queried.

Nucleotide sequence accession numbers

The sequence of the 713nt amplicon is listed as GenBank # AF018254.

Statistical analysis

A 2× 2 contingency analysis (Table 1) was carried out using Graphpad InStat (Graphpad Program Software, San Diego, CA).

RESULTS

Characteristics of patients

In the MM cohort the youngest patient was 30 years old; the oldest was 76. Five patients were less than 40 (8%), 15 were 40 to 49 (23%), 17 were 50 to 59 (26%) and 28 were 60 years or older (43%). Thus, the mean age (52.7 years) was less than that typically reported in the literature. The ratio of males to females was essentially 2 : 1.

We are unable to account for the young age distribution of the MM patient population, i.e., 57% were less than 60 years old. It is not known whether environmental pollutants may have been a factor in the occurrence of MM in the described cohort, and whether such pollutants had an effect on the occurrence of amplicons in plasma specimens from MM patients.

In the MM population approximately 28% suffered from underlying autoimmune disorders, systemic lupus erythematosus and psoriasis being the most common. The occurrence of intercurrent bacterial, viral, and mycotic infections was typical of MM patients. However, the frequency (12%) of viral hepatitis markers (types A, B, and C considered together) appeared to be high in this patient group. The occurrence of breast cancer in two MM patients, and uterine and pancreatic cancer in each of two other MM patients, also seemed to be elevated compared to that in normal controls.

Studies of larger population groups are needed to lend statistical validity to these observations. The described secondary diseases in MM patients undoubtedly played a role in the pathogenesis of the disease possibly through the agency of one or more immunologic deficiencies.

RNA in plasma specimens

We reported (3) that RNA amplicons (414nt and 759nt in size) that were derived from the sera of subjects with the Persian Gulf War Syndrome served as molecular signatures for that disorder. Here we report similar findings for MM. Figure 1 shows the bands (amplicons) derived from plasma specimens obtained from 4 representative MM patients with active disease. No attempt was made to resolve amplicons >713nt in size. The 350nt and 713nt amplicons typically found in MM patients with active disease were detected by RT-PCR but not by direct PCR. In Table 1 we show that the 713nt amplicon was detected in plasma specimens from 89% of RL patients, and in 62% of PU patients, respectively. The 350nt amplicon occurred in 33% and 62% of RL and PU patients, respectively. The 350nt and 713nt amplicons appeared to be applicable surrogate markers for MM patients with active disease (0.0001–0.0008 for PU and RL patients). The 713nt amplicon, but not the 350nt amplicon, was found in 2 out of 3 plasma specimens obtained from WM patients. One out of 2 plasma specimens taken from MGUS patients was positive for the 713nt amplicon only. None of the initial specimens obtained from patients in remission contained the 713nt amplicon. Similarly, no 713nt amplicons were derived from plasma specimens obtained from the 50 healthy controls.

Occurrence of amplicons in serial specimens

Since the data in Table 1 were for initial specimens only, it was of interest to determine the pattern of results for serial specimens taken from the same patient during the course of disease. Studies (Table 2) were carried out on 12 patients: 2 serial specimens were taken from 10 patients; 3 from 1 patient; and 4 from 1 patient. Four patients were in RM when initial and subsequent specimens were taken. Of the 13 specimens obtained from these patients, 1 was

Table 1

Amplicons derived from initial plasma specimens

Patients (% positive)						
Amplicons (bp)	PU/MM[1]	RL/MM[1]	RM/MM[1]	WM[1]	MGUS[1]	Controls
ENT 297[2]	6/9 (67)	5/18 (28)	2/36 (6)	0/3	1/2	21/50 (42)
p-value[3]	0.28	0.40	0.0001	NA	NA	
P2/P3	2/9 (11)	1/18 (6)	0/37	0/3	0/2	11/50 (22)
p-value	1.00	0.16	0.002			
200	5/8 (62)	10/18 (56)	0/37	2/3 (67)	1/2 (50)	15/50 (50)
p-value	0.011	0.86	<0.0001			
350	5/8 (62)	6/18 (33)	0/37	0/3	0/2	0/50
p-value	<0.0001	0.0002	NA			
700	1/8 (12)	5/18 (28)	0/37	1/3 (33)	0/2	0/50
p-value	0.14	0.0008	NA			
713	5/8 (62)	16/18 (89)	0/37	2/3 (67)	1/2 (50)	0/50
p-value	<0.0001	<0.0001	NA			

[1] Abbreviations: MM = multiple myeloma; WM = Waldenstrom's macroglobulinemia; MGUS = monoclonal gammopathies of undetermined significance; PU = patients previously untreated; RL = patients in relapse; RM = in remission.

[2] ENT 297 refers to the untranslated sequences of enteroviruses. P2/P3 refers to the PG03–PG04 primers used to detect a 565 bp band of the P2/P3 junctures of Sabin types 1 and 2 of oral poliovirus vaccine strains.

[3] Compared with normal controls.

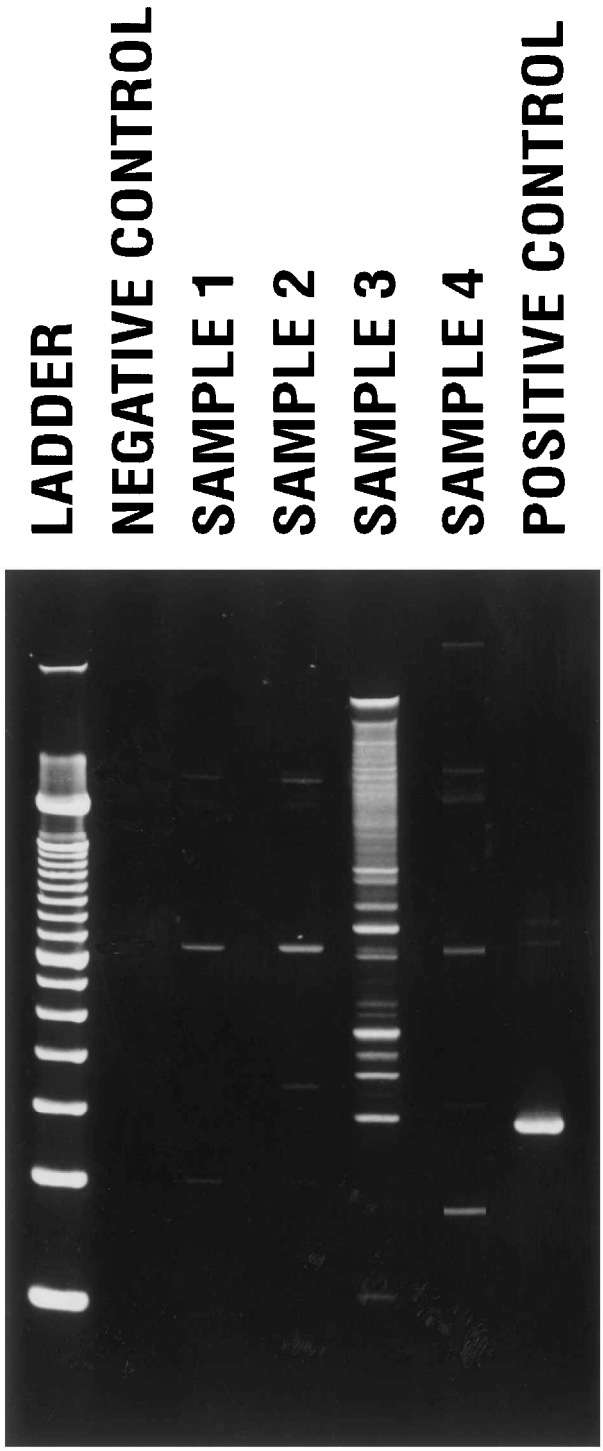

Fig. 1. Polyacrylamide gel electropheresis patterns of RT-PCR amplicons derived from 4 representative multiple myeloma (MM) patients with active disease. Lane 1, 100-bp ladder; Lane 2, poliovirus without RT as a negative control; Lanes 3–6, 4 representative MM patients with active disease; Lane 7, poliovirus-ENT positive control.

positive for the 350nt amplicon. All of the remaining specimens were negative. These results show a high degree of consistency between RM and amplicon undetectability, thus confirming the results presented in Table 1. In this MM cohort 6/12 patients had active disease when initial specimens were taken. Five in this group were tested positive for either the 350nt or 713nt amplicon. When therapy converted disease to remission, there was often a loss of one or more amplicons. However, results for 2 patients were not consistent. Further studies of serial specimens, in conjunction with therapeutic modalities, are needed to confirm and extend these preliminary findings. Possibly the 350nt and 713nt amplicons act as independent variables.

Sequence analysis of the 713nt amplicon

The 713nt amplicons derived from 4 representative MM patients were cloned and sequenced. Sequence homology (Table 3) among the 4 amplicons was $> 99.7\%$. Table 3 also shows that when differences occurred, they involved single base substitutions at different locations. A 709nt sequence within the 713nt amplicon was homologous (99.6%) with the flanking region of the PPAR gene located on chromosome 22q11.2. A 255mer at position 106 to 360 of the 713nt amplicon had 90.2% homology with an AluSc subfamily (6) consensus sequence (GenBank # U14571).

DISCUSSION

The occurrence of RNA amplicons (350nt to 713nt in size) in the majority ($23/27 = 85\%$) of initial plasma specimens obtained from MM patients with active disease, along with their absence in patients with disease in remission (0/37) or in 50 healthy controls, appears to have application to the clinical evaluation and diagnosis of this disease. These findings provide a new platform for analyzing the pathogenesis of MM at the molecular level. The occurrence of such amplicons in plasma specimens from patients with WM (2/3) and MGUS (1/2) is of added interest. They indicate that the described amplicons may be basic indicators of pathologic B-cell ontogeny and regulation. The conversion of active MM to remission by therapy, accompanied by a loss of amplicons, is consistent with these considerations. However, the number of patients studied is too few to permit formal conclusions to be drawn.

Because we were concerned that the occurrence of amplicons in plasma specimens may have been caused by secondary diseases in MM patients, careful clinical studies were conducted to evaluate such a possibility. None of the intercurrent infections described for the MM cohort (see METHODS) had an observable effect on amplicon occurrence. Similarly, concurrent malignancies in MM patients (2 with breast adenocarcinoma, and two with pancreatic and uterine cancer, respectively) and underlying autoimmune disorders in MM patients (28%) likewise had no observable effect. The common occurrence of autoimmune disorders in MM patients may reflect basic predisposing

immunologic abnormalities involving undefined genetic and environmental factors.

Primers for the enterovirus non-translated PGO1 and PGO2 sequences (ENT), and poliovirus protein regions PGO3 and PGO4 (P2/P3), were used for the RT-PCR assays. As shown in Fig. 1, these primers generated bands (amplicons) of various sizes in polyacrylamide gels. ENT and P2/P3 bands occurred (Table 1) with lower frequency in specimens obtained from MM patients. The 200nt amplicon occurred with high frequency in specimens from MM, WM, and MGUS patients and normal controls. Therefore it was not a discriminatory parameter. The 350nt band occurred in 5/8 (62%) of PU/MM and 6/18 (33%) of RL/MM patients. None occurred in RM/MM patients or in healthy control specimens. By contrast, the 713nt band occurred in 5/8 (62%) of PU/MM and 16/18 (89%) of RL/MM specimens. Interestingly, it also occurred in 2/3 WM and 1/2 MGUS specimens. The occurrence of the 700nt amplicon appeared to be anomalous, occurring in specimens from 1/8 PU/MM, 5/18 RL/MM, 1/3 WM, and 0/2 MGUS patients, but absent from healthy controls. Three of the MM patients were unusual. Two were positive for all bands; both were in relapse. One serial specimen obtained ftom one patient in RM was positive for a 350nt band. These results suggest that the occurrence of the 713nt band may be a valuable surrogate marker for active clinical disease in MM and WM. Compared with healthy controls, the p-values were ≤ 0.0002 for the 350nt and 713nt bands. Since the 713nt amplicon occurred with the highest frequency, it appears to be the more applicable surrogate marker. How well the 350nt and 713nt amplicons compared with other surrogate markers (7) for MM remains to be determined. Similarly, the disappearance of the 713nt band in association with therapy may be a useful

Table 2

*Occurrence of amplicons in serial specimens from the same patients**

Patient	Months after initial specimen	Therapy at time of specimen	Clinical status	Occurrence of amplicons (nt)		
				350	700	750
Same clinical condition when sampled						
1	0	Off Rx	RM	−	−	−
	3	Off Rx	RM	−	−	−
2	0	VAD	RL	+	−	+
	7	Cytoxan	RL	−	−	+
5	0	Decadron/Biaxin	RM	−	−	−
	17		RM	+	−	−
6	0	Prior Transplant	RM	−	−	−
			RM	−	−	−
8	0	No Rx	RM	−	−	−
	4	Melphalan	RM	−	−	−
9	0	No Rx	RM	−	−	−
	2		RM	−	−	−
Different clinical conditions when sampled						
3	0		PU	+	+	+
	3	Decadron and biaxin	RM	−	−	−
			RL	+	+	−
4	0	Melphalan, Prednisone, Radiation Rx				
	7		RM	−	−	−
7	0	Alpha interferon	ADT	−	−	−
	3		RL	+	+	+
	6		RL	−	−	−
	12		RM	−	−	−
10	0	None	PU	+	−	+
	4	Melphalan	RL	−	+	−
	9	Prednisone	RL	−	−	−
11	0	None	RM	−	−	−
	9		RL	+	−	+
12	0	None	PU	+	−	+
	3	Melphalan	RL	−	+	+

* See Table 1 for abbreviations; ADT = active disease under treatment; Rx = therapy. See ref. (5) for therapy modalities.

Table 3

Sequence of the 713-nt amplicon (GenBank # AF018254)

Sequence #							
1	AAGCACTTCT	GTTTCCTGAA	TCTAAAGAAA	GACAACATGC	TGCTTTTTAA	TCATAGGATG	GAGAATTTTA
71	AAGAACTGTT	TGGGCCAGGC	ACAGTCGCTC	ATACTTGTAA	TCCCAGCACT	TTGGGAGGCC	GAGGCGGGTG
141	GATCACAAGG	TCAGCAGATC	GAGACCATCC	TGG[2]CCAACAT	GGTGAAACCC	TGTCTCTACT	AAAAATACAA
211	AAATTAGCCG	GGTGTGGTGG	CACATGCCTG	TAATCCCAGC	TACTCGGGAA	GCTGAGGCAG	GAGAATTGCT
281	TGAACCAGGG	AGTTGGAGGT	TGCAGTGAGC	TAAGACTGCA[3]	CCACTGCACT	CCAGCCTGGT	GACAGAACGA
351	GACTCTGTCT	TAAAAACAAA	CAAACAAAAA	AAAAATCTGT	TAGATAG[4]GCT	ATCAAAATGC	AGCTGTTGTT
421	TTGTTTTTGG	CTCACTGTTT	TCGTGGT[5]TGT	AACTAATATG	TGGAAAGGCC	CATTTCCAGG	TTTGCGTAGA
491	AGAGCCCAGA	AAACAGAGTC	TCAAGACCCC	CGCTCTGGAC	TGTCATAAGC	TAGCACCCGT	GGTAAGCGGG
561	ACGAGACAAG	CTCCCGAAGC	CCGCCAGCTT	CCTGCTCCAC	TCAGCTCCGT	CCAGTCAACC	TGAACCCACC
631	CAGTCCAGCT	GTCTGTGGGA	ATGGTGGTGT	TCTTAGGGAC	AGACTGACAC	CTTACTTGTC	AGTGTTCCTC
701	CGGCCCCTGA	ATG					

[1] Amplicons were sequenced from four different patients. The sequence for patient 1 is listed as GenBank # AF018254. Underlined and italicized letters indicate differences in the flanking region of the PPAR gene (GenBank # Z79997).
* Enclosed box indicates an Alu sequence. The start and end of the sequence are marked by an asterisk.
[2] C for patient 3.
[3] G for patient 4.
[4] A for patients 2, 3 and 4.
[5] C for patient 2.

parameter of therapeutic efficacy. Since 20–30% of MGUS patients progress to MM (8), the occurrence of the 713nt amplicon in the plasma of a MGUS patient suggests that it may possibly be of prognostic value in this disorder.

The occurrence of RNA in the plasma of MM patients raised the question of their origin and how they resisted degradation by RNAses. Since there are enormous numbers (10^{10} to 10^{13}) of malignant plasmatoid cells in MM patients with active disease, it is reasonable to speculate that plasma RNA was derived from such cells. Lysis of such cells may not be required for release of RNA. Kolodny et al. reported (9, 10) that cells in culture secrete RNA that is transferable between contiguous cells and that such RNA may have been informational.

There are multiple lines of evidence indicating that RNA may be protected in vivo from RNAse degradation by complexing with cellular or serum constituents. Wieczorek et al. found (11) that RNAs in the sera of patients with a variety of malignancies persisted as RNAse-resistant RNA-proteolipid complexes. Sarrowa et al. showed (12) that small cytoplasmic RNA transcripts combined with the SRP/14 protein subunit of the 7SL signal the recognition particle. Chang & Maraia documented (13) that ALU RNA transcripts bind to cellular protein. Hohjoh et al. (14) found that LINE-1 retroposon RNA transcripts associated specifically with a cytoplasmic protein called P40. Heller et al. reported (15) that some of the RNA found in the sera of some MM patients was associated with alpha globulin. These results indicate that RNA can be protected in vivo by complexing with one or more cellular or extracellular proteins or structures.

RNA in the plasma of patients with MM may be of basic importance to the pathogenesis of this disease. In studies of plasmacytoma in mice, Heller et al. (15) defined a number of its pleiotropic effects of plasma RNA. When RNA contained in the plasma of plasmacytoma-bearing mice was injected into normal recipients, the distribution of peripheral blood polyclonal lymphocytes was shifted to a population consisting mainly of lymphocytes bearing surface immunoglobulins of the same idiotype as their plasmacytomas. When lymphocytes were incubated in vitro with plasma RNA (16), their surface immunoglobulins were 'converted' (synthesized *de novo*) to those of the transplanted plasmacytoma, i.e., 'surface immunoglobulin conversion (SIg)'. When plasma RNA was injected into mice (17) before immunization with sheep erythrocytes, the primary immune response was suppressed markedly.

Many of the events described in the mouse plasmacytoma model (15) appear to occur in human MM. Chen et al. (18) showed that plasma RNA obtained from patients with MM caused SIg conversion of peripheral lymphocytes to the idiotype of the patients' myeloma. Chen et al.'s

results (18) suggest that in humans the shift from a normal distribution of polyclonal lymphocytes to one in which the idiotype of the myeloma is predominant is mediated by tumor-cell-derived plasma RNA. The various immune suppressive mechanisms described by Katzmann et al. (19) in the mouse plasmacytoma model appear to be operative in human MM (20). In their studies of patients with MM, Paglieroni & MacKenzie (21) found that the number of lymphocytes that bound pneumococcal polysaccharide, tetanus toxoid, and diphtheria toxoid was normal. From this the authors suggest that one of the immunologic defects in MM patients lies beyond the antigen recognition step and occurs at the level of antigen-induced blast transformation. The implication is that the defect probably involves the cytokines that regulate lymphocyte mitogenesis and B-cell ontogeny. The finding by Potter (22) that the exposure of gnotobiotic mice to environmental antigens is required for the genesis of plasmacytomas is consistent with this hypothesis.

Heller et. al. reported (15) that when mice received a prior injection of plasmacytoma-derived plasma RNA, it changed a sublethal dose of *L. monocytogenes* to one that was uniformly fatal. This correlates with the high susceptibility of MM patients to infection (23). Pneumonia caused by *S. pneumoniae* is an important cause of terminal illness in MM patients (24). The primary immune response to pneumococcal polysaccharide is B cell mediated. Heller et al. showed (15) that the primary immune response to sheep erthryocytes (also B cell mediated) was suppressed markedly by plasmacytoma derived RNA. A similar suppression of B-cell response by tumor-cell RNA in MM patients could explain the susceptibility to pneumococcal pneumonia and the failure of *S. pneumoniae* vaccine to protect such patients.

Since the 713nt amplicon was the most consistent molecular marker for active MM, 4 representative amplicons were cloned and sequenced to determine their homology and their similarities to sequences recorded in the GenBank. Homologies among the cloned amplicons were 99.7%. Differences (Table 3) were limited to single nucleotides. There were only two differences between the 713nt amplicon and the flanking region of the fourth exon of the PPAR gene (GenBank # Z7997), e.g., at position numbers 1–3 and 704–713. Several other interesting sequence homologies were found in the 713nt amplicon. The ALU sequences at nucleotides 106 to 360 were the best matched (90.2%) to the ALU sequence of the Sc subfamily (GenBank U14571).

There is reasonable evidence (25–28) that an association exists between MM and the exposure to industrial chemicals, pesticides, or other environmental insults. Our observation of a high frequency of MM in young adults is consistent with such an association. In this regard, the occurrence of the 350nt/713nt amplicons as molecular markers for MM provides a basis for carrying out molecular epidemiologic studies. The first requirement, however, is to study a larger number of MM patients and controls to quantify the sensitivity and specificity of their occurrence. An insight to this approach is afforded by our finding that the 709nt sequence of the 713nt amplicon had a $>99.7\%$ homology to the flanking sequence of the PPAR gene on chromosome 22q11.2. Peroxidase proliferators regulate gene expression and are known (29) to participate in the induction of liver tumors in rodents. Thus, it can be hypothesized that environmental toxins may predispose to MM because they alter PPAR gene regulation, i.e., alteration of gene regulation may lead to B-cell malignancy in MM.

The high degree of homology between the ALU sequences in the 713nt amplicon and chromosome 22q11.2 also raises the question of their possible involvement in the molecular pathogenesis of MM. It has become clear (30) that ALUs are not simply inert nucleotide sequences in human DNA, but that they have important regulatory functions in gene expression. ALU expression can be induced by exposure (31, 32) of cells to heat shock proteins or/and cytotoxic chemicals. Since environmental toxins (33) and infectious agents (34) may have an effect on ALU-mediated gene expression, and on ALU transpositions in germline and somatic cells, there is reason to consider whether environmental toxins and infectious agents could affect ALU-mediated gene expression during the steps in B-cell ontogeny that lead to MM.

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