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Matozaki, S
Nakagawa, T
Nakao, Y
Fujita, T

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RAS GENE MUTATIONS IN MULTIPLE MYELOMA AND RELATED MONOCLONAL GAMMOPATHIES

SACHIKO MATOZAKI*, TOSHITARO NAKAGAWA*, YOSHINOBU NAKAO** AND TAKUO FUJITA**

*The Department of Hematology/Oncology
Hyogo Medical Center for Adult
13-70 Kitaoji, Akashi
**The Third Division, Department of Medicine
Kobe University School of Medicine

INDEXING WORDS

RAS oncogene ; point mutation ; multiple myeloma ; monoclonal gammopathy

SYNOPSIS

The presence of RAS gene mutations in precancerous lesions suggests that they participate in the early stages of neoplastic development. Furthermore, neoplastic progression from monoclonal gammopathy of undetermined significance (MGUS) to overt multiple myeloma (MM) have been frequently observed. These observations prompted us to study the pathogenetic role of RAS genes in MM and related monoclonal gammopathies.

DNA from 18 patients with monoclonal gammaglobulinemia including 12 MM were investigated for the presence of N- and K-RAS gene mutations by polymerase chain reaction (PCR) / oligonucleotide hybridization.

Mutations involving codons 12, 13 or 61 of N-RAS gene were identified in 3 of the 12 MM patients, 1 of the solitary plasmacytoma patients and none of the 3 of the MGUS patients. In the case of plasmacytoma, RAS mutations were detected in his bone marrow specimens.

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Authors' names in Japanese : 的崎早智子, 中川俊太郎, 中尾實信, 藤田拓男

These data suggest that RAS genes play a role in the pathogenesis of MM and that detection of RAS mutants would have important clinical implications. However, no apparent correlation was observed between the presence of RAS oncogene mutations and clinical parameters.

INTRODUCTION

The monoclonal gammopathies are commonly seen in medical practice. The clinical diagnosis included monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), primary systemic amyloidosis, macroglobulinemia, lymphoproliferative diseases associated with M-protein, solitary or extramedullary plasmacytoma and others. Although not the most common of these diseases, MM is an important problem. MM is the malignancy of plasma cells and has a progressive course with a median survival of 2 to 3 years[17]. MM may rarely represent in an asymptomatic, stable phase – smoldering multiple myeloma (SMM). It is essential to identify patients who have some features of MM, however, remain clinically stable without therapy for many years. It is often difficult to distinguish a benign from a malignant plasma cell proliferative process. The plasma cell labeling index is helpful in differentiating patients with MGUS or SMM from those with MM[3, 13], however, the most reliable means is the serial measurement of the M-protein level and periodic reevaluation to determine whether features of MM develop. While advances in molecular techniques have clarified the role of cellular oncogenes in some lymphomas and leukemias, no such association has been clarified yet in MM. In some MM cases high levels of c-myc RNA expression[25], the presence of mutated c-myc oncogenes[20], or the presence of rearranged bcl-1[26] have been reported. In addition, the simultaneous presence of a c-myc and a N-RAS oncogene[10], mutated N-RAS genes in MM cell lines[24] or MM patients[22] or elevated H-RAS P21 protein in MM[29] have been reported.

These observations prompted us to investigate the frequency of activating mutations of RAS genes in MM and related monoclonal gammopathies and their correlation between RAS mutations and clinico-pathological features to evaluate the role of RAS genes in disease progression. We studied 32 samples from 18 patients with monoclonal gammaglobulinemia including MM.

MATERILAS AND METHODS

Bone marrow specimens were collected from 18 patients with monoclonal gammopathy including MM during standard diagnostic procedures. Diagnosis was based on analysis of histopathology. Human ovarian teratocarcinoma cells, PA1, human colon carcinoma cells, SW480 and human myelogenous leukemia cells, HL-60 were used for positive controls for point mutations in codon 12 of N-RAS and codon 13 of N-RAS respectively[6, 28]. Mononuclear

cell suspensions were prepared from each specimen by Ficoll/ Hypaque gradient centrifugation. DNA was purified by digestion with proteinase K, extraction with phenol /chloroform, and ethanol precipitation, and dissolved in TE buffer. Samples were stored at 4℃.

Oligonucleotide synthesis

Primers 5'-dCTGGTGTGAAATGACTGAGT-3' and 5'-dCATCTACAAAGTGGTTCTGG-3' are 20-mers for the amplification of a 101 base pair (bp) region in the first exon of the N-RAS gene including critical codons 12 and 13. Primers 5'-dGTTATAGATGG-TGAAACCTG-3' and 5'-ACACAGAGGAAGCCTTCGCC-3' are 20-mers for the amplification of a 110 bp region of the N-RAS gene including codon 61. These oligonucleotides were synthesized on an Applied Biosystems (Foster city, CA) model 380A synthesizer for use in the polymerase chain reaction (PCR). Oligonucleotide probes for codon 12 of N-RAS were purchased from NEN Research Products (Boston, MA). Seven different oligonucleotide probes for codon 61 of N-RAS were purchased from Oncogene Science, INC.(Manhasset, NY).

Polymerase chain reaction (PCR)

DNAs from samples were incubated with 100ng of primer complimentary to sequences upstream and downstream of the RAS codons to be screened in the presence of Taq polymerase (Cetus). PCR was performed by a modification of the method described by Saiki et al.[23]. Thirty cycles of denaturation at 94℃ for one minute, annealing at 60℃ for two minutes were done on an automated heat-block (DNA thermal cycler ; Cetus).

Oligonucleotide hybridization

1 μl aliquots of the PCR product were transferred to a nylon filter (Gene screen plus, New England Nuclear) with a dot-blot manifold (BRL) and hybridized to a panel of 19-mer synthetic oligonucleotide probes which are representative of the normal codons 12, 13, and 61 as well as of all possible activating mutations affecting these codons. Oligonucleotide probes were labeled with [γ-^{32}P] ATP (Amersham International plc, UK, ; specific activity, 5000Ci/mmol) by means of T4 polynucleotide kinase (BRL) and purified through a Qick Spin Column G-25 (BMY). Pre-hybridization and hybridization were performed in a 3 M tetramethylammoniumchloride salt solution at 56℃ as described previously[30]. The filters were washed twice in 2 × SSPE, 0.1% SDS for 10 min, at room temperature, followed by a 30 min wash at 60℃.

Subsequently, the filters were washed for 30 min in hybridization buffer without

Denhardt's solution and salmon sperm DNA at various temperatures (63℃ for N-RAS codon 12 and K-RAS codon 12, 59℃ for N-RAS codon 61). The washing conditions were critical, because probes differing by a single base change have various melting temperatures. Finally the filters were exposed to Kodak XAR films at −70℃ over night using an intensifying screen.

RESULTS

Analysis of RAS oncogene mutations

DNAs extracted from 18 cases of MM, solitary plasmacytoma and MGUS specimens were amplified by PCR with the thermostable Taq polymerase enzyme and analyzed by hybridization to a panel of oligonucleotide probes of mutated codons 12, 13, or 61 of N-RAS and codon 12 of K-RAS as described in the methods. Because in pathologic biopsies the percentage of neoplastic cells may vary greatly depending on the presence of contaminating normal cells, we first assessed the sensitivity of our method. DNA from a cell line with a known RAS mutation was diluted with normal human placental DNA, then each dilution was amplified by PCR and hybridized with appropriate oligonucleotide probes. Our experimental conditions allow detection of mutations present in 1% of a given cell population (Data not shown).

Representative patient samples as well as positive and negative controls are shown in Fig. 1. Since there are some residual spots of at least 5% intensity compared to the wild type in some placental DNA, we considered them to be negative. As shown in Table 1 RAS gene mutations were detected 3 of 12 cases of MM (25%) and 1 of 3 cases of solitary plasmacytoma (33%), whereas no mutations were detected in 3 cases of MGUS. All mutations were codon 12 and/or 13 and/or 61 of N-RAS, no mutations were found in codon 12 of K-RAS. While, there was not found any particular codon affected. Some patients were analized several times in their clinical course and the same results were obtained. As shown in Table 2, all 4 RAS mutants showed multiple point mutations.

Table 1 Frequency of N-RAS mutations in MM and related monoclonal gammopathies

Diagnosis	No. of samples analyzed	No. of patients analized	No. of patients with mutations	Frequency (%)
MM	25	12	3/12	25
Solitary plasmacytome	4	3	1/3	33
MGUS	3	3	0/3	0

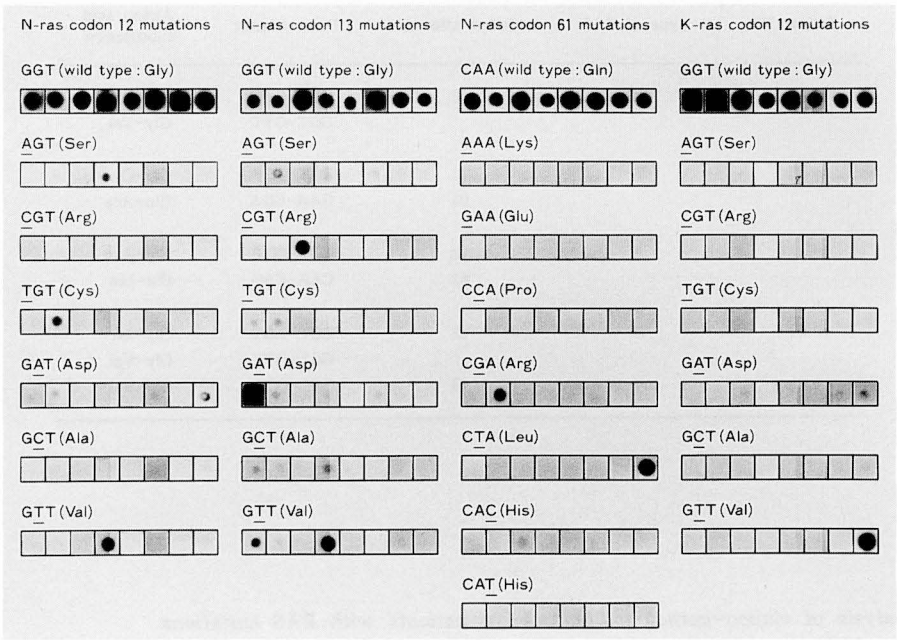

Figure 1.

Dot blot hybridization analysis of mutations at N-RAS codons 12, 13, and 61 and K-RAS codon 12 from representative patients. The regions across the codons 12, 13, and 61 of N-RAS, or 12 of K-RAS in the DNAs of 18 patients were amplified, spotted onto nylon filters, and hybridized with wild-type or specific mutated probes. Conditions for PCR and hybridization are described in the text. DNAs from cell lines PA1, SW480, and HL-60 were used as positive controls for the mutated codon 12 of N-RAS, codon 12 of K-RAS, and codon 61 of N-RAS respectively. Human placental DNAs were used as controls for the wild type RAS gene.

Each grid number and corresponding amplified DNA preparation are : 1, case 1 ; 2, case 2 ; 3, case 7 ; 4, case 15 ; 5, case K.Y.(MM) ; 6, case T.N.(MM) ; 7, Human placental DNAs ; 8, positive controls ; PA1 for codons 12 and 13 of N-RAS ; HL-60 for codon 61 of N-RAS, and SW-480 for codon 12 of K-RAS.

Table 2 Summary of RAS gene mutations in MM and solitary plasmacytoma

Patient No.	Type of RAS	Codon affected	Base chlange	Amino acid substitution
1	N	13	GGT-GAT	Gly-Asp
			GGT-GTT	Gly-Val
2	N	12	GGT-TGT	Gly-Cys
		61	GAA-CGA	Gln-Arg
7	N	13	GGT-CGT	Gly-Arg
		61	CAA-CAC	Gln-His
15	N	12	GGT-AGT	Gly-Ser
			GGT-GTT	Gly-Val
		13	GGT-GTT	Gly-Val

Analysis of clinico-pathologic features of patients with RAS mutations

Clinical data from MM cases are summarized in Table 3. No apparent correlation was observed between the presence of RAS mutations and the parameters listed in Table 3, however, there was a tendency that we found RAS gene mutations in patients in advanced stages or in those with more severe bone changes. In Case 1, we detected N-RAS point mutation at codon 13. We found massive osteolytic lesion in his sacrum and lt. femur. After 12 courses of melphalan and prednisolon therapy, he remains in complete remission (CR) state. In Case 2, we found N-RAS mutations at codon 12 and 61, her X-rays revealed multiple punched-out lesions and many pathological fractures. Her disease has become refractory to chemotherapy. In Case 7, we detected N-RAS mutationsat codon 13 and 61. He was admitted to hospital because of renal failure. His disease became refractory to chemotherapy and he died of intracranial bleeding. In Case 15, he was admitted to hospital because of solitary plasmacytoma. We found N-RAS mutations at codon 12 and 13 in his BM specimens, although we couldn't study his tumor specimen. After radiotherapy to his tumor and systemic chemotherapy, he remains in a CR state.

Table 3 Clinical parameters of MM patients analyzed

clinical parameters	No. of positive/No. of analyzed
Stage (12pt.)	
I A	0/2
II A	0/4
III A	2/5
III B	1/1
Percent BM plasma cells (10pt.)	
> 10 < 30	1/4
> 30 < 50	0/2
> 50	0/4
Protein typez (12pt.)	
IgG	0/7
IgA	3/4
IgM	0/0
Light chain	0/1
Hb (g/dℓ) (12pt.)	
> 10	2/5
> 8.5 < 10	0/2
< 8.5	1/5
Bone status index (12pt.)	
0	0/2
1	0/3
2	1/4
3	2/3

DISCUSSION

Point mutations of RAS genes have been described in various hematological disorders. In lymphoid malignancies, the cumulative data indicate that RAS gene mutations occur in about 15 to 20% of ALL and are absent in CLL and non Hodgkin's lymphoma[1, 21].

Recently, phenotypic data has suggested that MM is a disease in which the majority of tumor cells are aneuploid with a differentiated B-cell phenotype but with subpopulations that also express early B, possibly T, and even myelomonocytic features[9,14]. In addition, the introduction of activated H- or N-RAS oncogenes was shown to cause both the tumorigenic conversion and the plasmacytoid differentiation of human lymphoblastoid cells immortalized by EBV[27]. These observations suggest that a possible role of RAS mutation in MM pathogenesis by leading to both malignant transformation and the plasmacytoid differentiation of the clonal pre-B cell populations or even the early stem cells that are

thought to represent the precursors of MM cells.

Our data showed a preferential involevement of the N-RAS gene in MM similar to the previous observation in various hematopoietic malignancies[6, 7]. While we didn't find any particular codon affected or any amino acid substitution, this confirms the suggestion by Neri et al[22]. that RAS gene mutations are generated through multiple and/or relatively nonspecific mechanisms.

In Case 15 of solitary plasmacytoma, N-RAS mutations were found in the bone marrow in which the plasma cell ratio was only 2.4 %, although we couldn't study his tumor specimen. Recently, Kumar et al[16] reported that activated RAS genes can precede the onset of neoplasia and can exist in apparently normal cells for long periods of time without inducing malignant transformation. These activated RAS genes would remain silent until an additional trigger of neoplastic development, for instance, an additional genetic alteration or exposure to a carcinogen. In this case, after he received both radiotherapy and chemotherapy, he remains in a CR state. However, uneless he got systemic chemotherapy, he may have developed MM in the near future. Detection of RAS gene mutations may be useful to distinguish benign from a malignant plasma cell proliferative processes.

The occurrence of RAS gene mutations in plasma cell disorders would be variant with recent observations in colon adenoma[4, 5] and MDS[15, 18], where the mutations are detected in premalignant stages of tumor development. Indeed, the frequently observed neoplastic progression from MGUS to an aggressive disease (overt MM or plasma cell leukemia) probably reflects a multistep process of tumorigenesis that leads to progressively deregulated cell growth. While we couldn't detect RAS gene mutations in MGUS, it could be because of the small numbers of case studies or because of their clinical staging of MGUS. It is necessary to study longitudinally, large numbers of cases at different stages of tumor development.

Our data suggest that the tendency that RAS mutants are found at relatively advanced stages and in patients who have severe bone change. Neri et al.[22] suggested that preliminary correlations between the presence of RAS mutations and poor therapeutic response.

These observations suggest that further investigations into the correlations between RAS mutations and clinical features, and detection of RAS mutants will have important clinical implications.

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