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Resequencing Analysis of the Candidate Tyrosine Kinase and *RAS* Pathway Gene Families in Multiple Myeloma

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Abstract

Multiple myeloma (MM) is an incurable, B-cell malignancy, characterized by the clonal proliferation and accumulation of malignant plasma cells in bone marrow. Despite recent advances in understanding of genomic aberrations, a comprehensive catalogue of clinically actionable mutations in MM is just beginning to emerge. The tyrosine kinase (TK) and *RAS* oncogenes, which encode important regulators of various signaling pathways, are among the most frequently altered gene families in cancer. To clarify the role of TK and *RAS* genes in pathogenesis of MM, we performed a systematic, targeted screening of mutations on prioritized *RAS* and TK genes, in CD138 sorted bone marrow specimens from 42 untreated patients. We identified a total of 24 mutations in *KRAS*, *PIK3CA*, *INSR*, *LTK* and *MERTK* genes. In particular, seven novel mutations in addition to known *KRAS* mutations were observed. Prediction analysis tools, PolyPhen and SIFT were used to assess the functional significance of these novel mutations. Our analysis predicted that these mutations may have a deleterious effect, resulting in functional alteration of proteins involved in the pathogenesis of myeloma. While further investigation is needed to determine the functional consequences of these proteins, mutational testing of the *RAS* and TK genes in larger myeloma cohorts might be also useful to establish the recurrent nature of these mutations.

Keywords

Multiple Myeloma; Tyrosine Kinase; *RAS*; Resequencing; Mutation analysis; Cancer

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Authorship Contribution

M.H.T, J.F.D, R.V and S.K Conceived the project, R.V, M.H.T and C.M Identified and collected samples, V.H, R.M, R.N Analyzed the data, V.H, R.M and S.K wrote the paper.

Disclosure of Conflict of Interest

None

Introduction

Multiple myeloma (MM) is the second most common hematopoietic malignancy, characterized by uncontrolled accumulation of plasma cells in the bone marrow [1]. Despite advances in systemic and supportive therapies, MM remains a fatal disease. Mutations in receptor tyrosine kinase (TK) and *RAS* oncogene gene families resulting in constitutive activation of the gene product have been reported in myeloma [2-6]. However, a recent mutational screening of 31, receptor TK gene domains revealed few disruptive mutations in MM [2]. Furthermore, mutational studies in *RAS* pathway genes, have described conflicting results in terms of their existence and frequency in MM [3, 5-9].

Emerging technologies such as genome-wide screening of mutations have been used in several cancers including myeloma [2, 10] and numerous somatic mutations in many genes have been identified and catalogued. It is worth noting that the distributions of mutations have differed in each of these cancer types and in individual samples [11]. Very recently, whole genome sequence and exome analyses in myeloma patients reported numerous putative mutations in a large number of genes [12]. Although this powerful technology shows great promise, its application to routine clinical or research practice is not viable considering the time and cost involved. Using a targeted resequencing approach, we performed a systematic and focused profiling of the more commonly involved candidate tyrosine kinase and *RAS* oncogenes in a large panel of myeloma patients. Our objective was to identify mutations that could reveal a genetic signature for MM and further our understanding of molecular events and potentially identify novel therapeutic targets.

Methods

DNA for resequencing analysis was extracted from CD138 selected bone marrow cells using AutoMacs (Miltenyi Biotec, CA). The study cohort consisting of 42 newly diagnosed, untreated, MM patients was approved by the Human Research Protection Office at Washington University School of Medicine. Informed consent from the patients was obtained in accordance to the declaration of Helsinki.

Expression data from GEO accession GSE755 [13] was used to generate a heat map (s.Figure 1a-d) as a way of prioritizing the RTK and *RAS* pathways genes. The GEO data set, consisting of 173 MM samples with and without skeletal lesions as diagnosed by MRI, was analyzed by two different methods: (1) Function Express, an annotation-based microarray analysis [<http://cbmi.wustl.edu/html/FEClient.html>] and (2) Affymetrix GeneChip Operating Software (Affymetrix Inc, Santa Clara, CA). The Affymetrix data was generated using a baseline correction of 1500 to generate absence/presence calls.

Based on the top hit expression profiles and the availability of previously established primer and sequencing conditions, we prioritized these candidate genes for analyses using the standard resequencing pipeline implemented at the Genome Institute (GI) at Washington University Medical School [<http://genome.wustl.edu/>] [14, 15]. Gene sequences from GenBank [www.ncbi.nlm.nih.gov] and Ensembl [www.ensembl.org] were used as reference for assembly and primer construction. The primers were either designed by ABI VariantSEQR [www.ncbi.nlm.nih.gov/projects/genome/probe/doc/ProjVariantSEQR.shtml] or with Primer 3 [<http://primer3.sourceforge.net>] based algorithms. Exonuclease/SAP purified amplicons were directly sequenced using BigDye Terminator chemistry on an ABI3730XL automated sequencer (Applied Biosystems, Foster City, CA). Mutations were detected using two separate automated detection methodologies: (1) Mutation profiling pipeline [<http://cbmi.wustl.edu/html/index.html>] and the PolyScan informatics suite (<http://genome.wustl.edu/software/polyscan>) [16]; Mutations from the resequencing pipeline

were viewed in a graphical user interface using Mutation Viewer [http://cbmi.wustl.edu/html/Mutation_Viwer.html]. This allowed us to validate mutations called independently by the two automated calling methodologies.

PolyPhen (<http://genetics.bwh.harvard.edu/pph2/index.shtml>) and SIFT (<http://sift.bii.a-star.edu.sg/index.html>) prediction tools were further used to assess the functional impact of the observed mutations. In addition, we performed a sensitivity test of mutation detection technology by serially diluting and mixing the mutant to wild type allele in a varying range of 0 – 100 %. The G12D mutation of NRAS gene from H929 line was differentially mixed with wild type allele in U266 cell line and sequenced with ABI 3730XL automated sequencer using standard procedures. Student's t-Test was employed to find the significance of the genotype frequencies.

Results

We identified 24 mutations, of which 7 were novel in our cohort (Table 1 and Figure 1). To establish the sensitivity of detection, we performed serial dilution and mixing experiments using a known mutant allele (*NRAS*-G12D) from the H929 cell line to the normal allele of U266 cell line. Spectral difference of mutant allele was observed when at least 10-20% of cells harbored the mutant allele (s.figure 2). Lack of germline DNA availability limited us from characterizing the somatic events of the observed mutations.

Mutations p.G12D, p.G12C and p.Q61H in *KRAS* gene were observed (Figure 1a). In addition, a 9bp deletion (p.E62_Y64) was also identified. This in frame deletion and mutations at the G12 position might be involved in disrupting the binding partners in the activating *RAS* signaling pathway (Figure 1b). Prediction analysis, both sorting of intolerant from tolerant and PolyPhen analysis predicted that these alterations are most likely damaging. In the *PIK3CA* gene, apart from the p.I391M alteration we also discovered a novel p.R310C mutation (Figure 1c) but the classic hotspot mutations reported in most cancers were not observed. The p.R310C mutation has been recorded as a somatic event in the catalogue of somatic mutations in cancer (COSMIC) database and is highly conserved. In silico analysis predicted that this mutation is most probably damaging, suggesting that this alteration might be pathogenic (Figure 1d). Novel mutations p.A119V and p.S748L in the Insulin receptor gene were discovered (Figure 1e). These conserved sequences of the *INSR* mutation, signify a potentially critical role for these mutations in myeloma. Novel mutations p.R243Q and p.D535N in *LTK* gene and p.T690I in *MERTK* gene were also observed. Most of these mutations, viz. A119V, V1012M, D535N, P227L, E823Q and R865W were also predicted to probably damage protein functions. Notably, a significant number of missense variants, predominantly in the *MERTK* gene were identified. Since our patients were mostly Caucasians, we compared the genotype frequency of these polymorphic mutations to the dbSNP database and found most but not all were significantly different from the normal population (Table 1).

Discussion

We identified mutations in *KRAS*, including previously known mutations and a 9bp deletion. *NRAS* sequencing did not uncover any mutation in our cohort. *RAS* pathway genes are known to play an important role in most cancers and are among the well studied genes involving activating mutations [17]. Studies in MM have shown that *NRAS*, codon-61 mutations are more prevalent compared to *KRAS* mutations [6], though some other reports contradict this [3]. Results from many of these mutational studies run the gamut from 100% *RAS* mutations to none [3, 5, 6, 8, 12]. These conflicting results may be accounted by potential factors involving sample selection and the mutational techniques employed [9]. To

overcome these issues, we selected MM patients at diagnosis, without including MGUS or smoldering MM and used DNA from CD138 positive cells. In order to assess the sensitivity of our mutation detection tool, we also performed a mixing experiment of wild type with mutant allele of *NRAS*G12D at varying proportions and sequenced the mixture. The mutant allele was readily detected when present at concentrations as low as 10%. This range of sensitivity found in our study is consistent with Sanger sequencing and other methodologies used to detect *RAS* mutations in MM [6, 9].

Using the whole genome sequencing approach, Chapman *et al* [12] identified a higher frequency of *NRAS* and *KRAS* mutations in myeloma compared to our report. While we cannot rule out variability due to technique, the most likely explanation for this discrepancy in mutation frequency may be related to differences in sample characteristics between the two studies. An important difference between the Chapman study and this study needs to be stated. The Chapman study included both treated and untreated patients. When only untreated patients are compared, the frequency of *KRAS* mutation is comparable: 6 vs. 4 found in our study. That said, there is no clear explanation for the discrepancy of *NRAS* mutations which is yet to be clearly established in MM patients. By and large, resequencing of MM patient samples identified low frequency of *KRAS* mutations and no *NRAS* mutations, and these findings are in keeping with earlier studies [7, 8].

PIK3CA plays an important role in regulating cell growth, transformation, adhesion, apoptosis, survival and motility, and mutations in this gene have been reported in many cancers [18]. Our ressequencing analysis of the *PIK3CA* gene confirmed recent findings in MM patients who did not show three *PIK3CA* hot spot mutations [19]. Interestingly, the initial whole genome sequencing of MM too failed to identify any mutations in *PIK3CA* [12]. Taken together, the low incidence of *PIK3CA* mutations and the absence of the hotspot mutations in MM studies suggest that *PIK3CA* gene might not be critical in myeloma's pathogenesis. Considering *PIK3CA*'s critical function in many other cancers, additional studies in larger cohorts are needed to further our understating of the role played by *PIK3CA* in myeloma.

Mutations in the TK genes have also been extensively studied and it has been shown that high frequency of somatic mutations exists in many types of cancers [20]. In addition, this family of genes has also shown great promise in identifying druggable targets in cancer. Claudio *et al* [2] used high throughput screening in receptor TK pathway gene domains and identified mutations in some members of the TK gene family in MM. We hoped to identify novel mutations by screening the entire gene in this family as opposed to gene domain screening and discovered novel mutations in the insulin receptor gene, *LTK* and *MERTK* genes. The *INSR* protein product is expressed in MM cells and is shown to increase throughout the normal plasma cell differentiation [21]. These mutations were highly conserved and some of them were predicted to be intolerant using prediction analysis. Surprisingly, none of these novel kinase mutations observed in our study were reported in the initial MM whole genome sequencing report [12]. Although we observed these mutations at a low frequency in our study, conserved kinase mutations are known to affect protein expression, and could play a pathogenic role in MM. The role of kinase genes in targeted therapeutics in cancer is well established and modulating or inhibiting these mutated genes or their downstream pathway might also have a potential role in MM pathogenesis. In addition, significant differences in these genotype frequencies might serve as a useful biomarker for detecting myeloma specific targets especially if validated in a larger study groups.

In summary, we identified 3 known somatic mutations in *KRAS* gene and 7 novel mutations in cytokine signaling genes in MM. A large number of these polymorphic mutations were

significantly predominant in our patients suggesting a possible role in these alleles specific to MM pathogenesis. Although the role of *RAS* mutations is not yet clear in defining the pathogenesis of MM, the novel TK mutations identified in this study have to be functionally validated and its relevance needs to be measured in the milieu of other genetic aberrations in myeloma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Reference

1. Fonseca R, Bergsagel PL, Drach J, et al. International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. *Leukemia*. 2009; 23:2210–21. [PubMed: 19798094]
2. Claudio JO, Zhan F, Zhuang L, et al. Expression and mutation status of candidate kinases in multiple myeloma. *Leukemia*. 2007; 21:1124–7. [PubMed: 17344920]
3. Bezieau S, Devilder MC, Avet-Loiseau H, et al. High incidence of N and K-Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. *Hum Mutat*. 2001; 18:212–24. [PubMed: 11524732]
4. Onwuazor ON, Wen XY, Wang DY, et al. Mutation, SNP, and isoform analysis of fibroblast growth factor receptor 3 (FGFR3) in 150 newly diagnosed multiple myeloma patients. *Blood*. 2003; 102:772–3. [PubMed: 12835230]
5. Liu P, Leong T, Quam L, et al. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. *Blood*. 1996; 88:2699–706. [PubMed: 8839865]
6. Kalakonda N, Rothwell DG, Scarffe JH, et al. Detection of N-Ras codon 61 mutations in subpopulations of tumor cells in multiple myeloma at presentation. *Blood*. 2001; 98:1555–60. [PubMed: 11520807]
7. Intini D, Agnelli L, Ciceri G, et al. Relevance of Ras gene mutations in the context of the molecular heterogeneity of multiple myeloma. *Hematol Oncol*. 2007; 25:6–10. [PubMed: 17036375]
8. Martin P, Santon A, Garcia-Cosio M, et al. RAS mutations are uncommon in multiple myeloma and other monoclonal gammopathies. *Int J Oncol*. 2005; 27:1023–8. [PubMed: 16142319]
9. Chng WJ, Gonzalez-Paz N, Price-Troska T, et al. Clinical and biological significance of RAS mutations in multiple myeloma. *Leukemia*. 2008; 22:2280–4. [PubMed: 18528420]
10. Stephens P, Edkins S, Davies H, et al. A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. *Nat Genet*. 2005; 37:590–2. [PubMed: 15908952]
11. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. *Nature*. 2007; 446:153–8. [PubMed: 17344846]
12. Chapman MA, Lawrence MS, Keats JJ, et al. Initial genome sequencing and analysis of multiple myeloma. *Nature*. 2011; 471:467–72. [PubMed: 21430775]
13. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med*. 2003; 349:2483–94. [PubMed: 14695408]

14. Ley TJ, Minx PJ, Walter MJ, et al. A pilot study of high-throughput, sequence-based mutational profiling of primary human acute myeloid leukemia cell genomes. *Proc Natl Acad Sci U S A*. 2003; 100:14275–80. [PubMed: 14614138]
15. Tomasson MH, Xiang Z, Walgren R, et al. Somatic mutations and germline sequence variants in the expressed tyrosine kinase genes of patients with de novo acute myeloid leukemia. *Blood*. 2008; 111:4797–808. [PubMed: 18270328]
16. Chen K, McLellan MD, Ding L, et al. PolyScan: an automatic indel and SNP detection approach to the analysis of human resequencing data. *Genome Res*. 2007; 17:659–66. [PubMed: 17416743]
17. Quinlan MP, Settleman J. Isoform-specific ras functions in development and cancer. *Future Oncol*. 2009; 5:105–16. [PubMed: 19243303]
18. Karakas B, Bachman KE, Park BH. Mutation of the PIK3CA oncogene in human cancers. *Br J Cancer*. 2006; 94:455–9. [PubMed: 16449998]
19. Ismail SI, Mahmoud IS, Msallam MM, Sughayer MA. Hotspot mutations of PIK3CA and AKT1 genes are absent in multiple myeloma. *Leuk Res*. 2010; 34:824–6. [PubMed: 20022634]
20. Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. *Nat Rev Cancer*. 2004; 4:177–83. [PubMed: 14993899]
21. Sprynski AC, Hose D, Kassambara A, et al. Insulin is a potent myeloma cell growth factor through insulin/IGF-1 hybrid receptor activation. *Leukemia*. 2010; 24:1940–50. [PubMed: 20844560]

Table 1

DNA alterations in candidate *Ras* and *TK* genes in multiple myeloma

Gene	Gene ID ^a	Nucleotide change ^b	Amino acid change	Zygosity ^c	dbSNP Id	Minor allele Frequency (%)
<i>KRAS2</i>	3845	c.35 G>A	p.G12D	Hetero		
<i>KRAS2</i>	3845	c.34 G>T, c.33 T>C	p.G12C, A11A	Hetero		
<i>KRAS2</i>	3845	c.183 A>C	p.Q61H	Hetero	rs17851045	
<i>KRAS2</i>	3845	c.186_194 del	p.E62_Y64	Homo		
<i>PIK3CA</i>	5290	c.928 C>T	p.R310C	Hetero		
<i>INSR</i>	3643	c.356 C>T	p.A119V	Hetero		
<i>INSR</i>	3643	c.2243 C>T	p.S748L	Hetero		
<i>LTK</i>	4058	c.728 G>A	p.R243Q	Hetero		
<i>LTK</i>	4058	c.1603 G>A	p.D535N	Hetero		
<i>MERTK</i>	10461	c.2069 C>T	p.T690I	Hetero		

					Normal	Patient	
<i>PIK3CA</i>	5290	c.1173 A>G	p.I391M	Hetero	rs3729680	6	7.14
<i>INSR</i>	3643	c.3034 G>A	p.V1012M	Hetero	rs1799816	-	2.4
<i>LTK</i>	4058	c.125 G>A	p.R42Q	Hetero	rs2305030	6.7	24 [*]
<i>LTK</i>	4058	c.680 C>T	p.P227L	Hetero	rs55739813	-	7.14 [*]
<i>MERTK</i>	10461	c.60 A>T	p.R20S	Hetero	rs35898499	5.6	7.14
<i>MERTK</i>	10461	c.1552 A>G	p.I518V	Hetero, Homo	rs2230515	68.3	100 [*]
<i>MERTK</i>	10461	c.1397 G>A	p.R466K	Hetero, Homo	rs7604639	68	91.3 [*]
<i>MERTK</i>	10461	c.353 G>A	p.S118N	Hetero, Homo	rs13027171	37.5	42.5
<i>MERTK</i>	10461	c.2608 G>A	p.V870I	Hetero	rs2230517	3.4	18.8 [*]
<i>MERTK</i>	10461	c.844 G>A	p.A282T	Hetero	rs7588635	0	7.5 [*]
<i>MERTK</i>	10461	c.1493 A>G, c.1494 C>T	p.N498S	Hetero	rs35858762	0	2.5
<i>MERTK</i>	10461	c.878 G>A	p.R293H	Hetero	rs34072093	1.7	2.5
<i>MERTK</i>	10461	c.2593 C>T	p.R865W	Hetero	rs2230516	1.7	2.5

				Normal	Patient
MERTK	10461	c.2467 G>C	p.E823Q	Hetero	rs55924349
				-	1.25

^a Gene ID at National center for biotechnology information (<http://www.ncbi.nlm.nih.gov/sites/entrez>)

^b del., deletion

^c Homo, homozygous; hetero, heterozygous

^{*} p<0.05