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Original article

Exonic variants in multiple myeloma patients associated with relapsed/refractory and response to bortezomib regimens

Ashraf Kakoo *, Mustafa Al-Attar, Taban Rasheed

Department- College of Science, Salahaddin University, Erbil, Iraq

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ABSTRACT

Novel treatment in multiple myeloma represented by proteasome inhibitors, immunomodulatory drugs and monoclonal antibodies have produced a deep response. However, relapses are possible, and all classes of drugs are refractory to patients. Next-generation sequencing has improved our understanding of the multiple myeloma genome related to drug resistance and has discovered many genomic variants. Therefore, this study was conducted to investigate new variants associated with drug resistance in MM patients who relapsed and refractory to bortezomib regimen and daratumumab treatment using next-generation sequencing for whole-exome sequencing. Peripheral blood samples were collected in EDTA tubes from six patients; four were in relapsed and refractory to bortezomib regimens and daratumumab; two patients responded to bortezomib regimens. Whole-exome sequencing was performed by the MGI-DNBSEQ-G400 instrument. We identified 21 variants in multiple myeloma patients. Seventeen variants were found in relapsed and refractory multiple myeloma in 11 genes (*GNAQ, PMS1, CREB1, NSUNS2, PIK3CG, ROS1, PMS2, FIT4, KDM5A, STK11* and *ZFHX3*). And four variants were identified in two patients with response to bortezomib regimens in 4 genes (*RAF1*, *CREB1, ZFHX3* and *INSR)*. We have observed several genetic variants in many genes that may have been associated with the poor prognosis and poor response to treatment in these patients. These values should be further confirmed in large sample studies using the RNA-seq technique to identify genome expression.

1. Introduction

Multiple Myeloma (MM) is a plasma clonal cell cancer, is distinguished by widespread genomic heterogeneity. As a result, there is a difference in drug response and disease progression (Gupta et al., 2015; Lohr et al., 2014). The novel treatments like proteasome inhibitors, immunomodulatory, and monoclonal antibodies have increased MM patients' survival. However, MM remains irremediable cancer. Most MM patients die of their disease acquired drug resistance limits current therapies' efficacy (Kumar and Rajkumar, 2014).

Bortezomib (Velcade) was the first class of proteasome inhibitors show an effect against MM. It works by blocking activations of the 20S core of the proteasome, which induces apoptosis in myeloma as well as lymphoma cells (Mohan et al., 2017; Ri, 2016). The U.S. FDA approved Daratumumab in 2015 for MM patients, a monoclonal antibody (IgG1) that targets CD38, which is highly expressed in myeloma cells (Nahi et al., 2019).

Next-generation sequencing (NGS) is a method that can sequence millions of fragments of DNA or complementary DNA simultaneously. It has been quickly accepted in the clinical and molecular laboratory due to its capacity to simultaneously analyze many genes or gene regions with a single test compared to traditional methods (Yohe and Thyagarajan, 2017). Development in molecular profiling technology has provided new insight into the

Abbreviations: MM, multiple myeloma; SNP, single nucleotide polymorphism; WES, whole exome sequence; MMR, mismatch repair; VUS, variant unknown significant; NGS, Next-generation sequence; BWA, Burrows-Wheeler Aligner; GATK, Genome Analysis Toolkit; IGV, Integrative Genomic Viewer; NF-κB, nuclear factor kappa B; RANKL, receptor activator of nuclear factors-κB ligand; M−CSF, macrophage colony-stimulating factor; MCL-1, myeloid cell leukaemia-1; BCL-2, B-cell lymphoma 2; RTKs, tyrosine kinases receptors; VEGF-C, vascular endothelial growth factors receptors; MAPK, mitogen-activated protein.

* Corresponding author.
E-mail addresses: ashraf.kako@su.edu.krd, ashrafkako59@gmail.com (A. Kakoo), mustafa.mustafa@su.edu.krd (M. Al-Attar), taban.rasheed@su.edu.krd (T. Rasheed).
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fundamental molecular events that underlie MM's growth and anticancer drug resistance mechanism.

An effective way to understand cancer's molecular basis is to sequence the entire genome or the protein-coding exome by Next-generation sequence (NGS) (Chapman et al., 2011; Zhao et al., 2019). Single nucleotide polymorphisms (SNP) are among the most common forms of genetic variations in the human genome. SNPs in genes that regulate the cell cycle, DNA mismatch repair, metabolism and immunity are connected with genetic susceptibility to cancer (Schirmer et al., 2016). To understand the molecular pathogenesis of different cancers, knowing the mechanisms underlying the effects of SNPs that result in cancer susceptibility is essential. From a clinical viewpoint, SNPs are therapeutic biomarkers and potential diagnostic (Deng et al., 2017).

In this study, we performed whole-exome sequencing (WES) in MM patients who were relapsed and refractory to bortezomib regimen and daratumumab treatment for the discovery of new variants related to drug resistance by using next-generation sequencing,

2. Materials and methods

2.1. Sample collection

The current study was authorized and approved by the Human Ethics Committee of the College of Science, Salahaddin University, Erbil (Approval No: 3/2/2002 Date: 9/6/2019). All patients provided written, informed consent for the publication of data in this study. This study was conducted from August 2019 to June 2020 in Nanakali Hospital for Blood Diseases and Cancers, Erbil City. Peripheral blood samples were collected in an EDTA tube from six patients; four patients were in relapsed and refractory to proteasome inhibitors (bortezomib), immunomodulatory drugs (thalidomide) and monoclonal antibodies (Daratumumab), and two patients had a response to bortezomib regimens.

2.2. DNA extraction and library preparation

QIAamp DNA blood kit (QIAGEN) was used to isolate DNA from peripheral leukocytes, following the manufactures protocol. Twist Human Core Exome Enzymatic Fragmentation (EF) Multiplex Complete kit was used for library construction, and MGIEasy FS DNA Library Prep Kit was performed for formation circular DNA and the library to be ready for sequencing on the MGI system.

2.3. Sequencing and bioinformatics

The library was sequenced on the(MGI-DNBSEQ-G400, China) instrument generating 150 bp paired-end read with 100X mean target coverage. The output of NGS was raw fastq files. This file was quality assessment by FastQC software. Then reads were aligned to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA) software. Variants were identified with Genome Analysis Toolkit (GATK) software. Integrative Genomic Viewer software (IGV) was used for variants visualization.

2.4. Statistical analyses

The statistical analyses were performed with the GraphPad Prism Software (version6.0). D'Agostino-Peaarson omnius test, Shapiro-Wilk normality test and Kolmogorov-Smirnov test were used to determine whether the data were normally distributed or not. Normally distributed data presented as means ± SE (Standard Error), and not normally distributed data presented as median (range)

3. Results

The patient's demographics and baseline characteristics are summarized in Table 1. This study identified 21 variants in MM patients. 17 variants were recorded in 11genes of four patients in relapsed and refractory MM (*GNAQ, PMS1*, *CREB1*, *NSUNS2, PIK3CG, ROS1, PMS2, FIT4, KDM5A, STK11* and *ZFHX3*), Table 2. And four variants were identified in two patients with response to bortezomib regimens in 4 genes (*RAF1*, *CREB1, ZFHX3*, and *INSR),* Table 3.

Out of 17 variants in four relapsed in refractory MM, six variants were detected in the first patient in six genes (*GNAQ, PMS1*, *CREB1*, *NSUNS2, PIK3CG* and *ROS1*); all variants were SNP, except one variant was insertion (c.301-4insT) in the *PMS2* gene. Four variants were found in the second patient in the four genes (*PIK3CG*, GNAQ, *FIT4*, and *PMS2*); the variants were SNP, while one variant was insertion (c.301-4insT) in the *PMS2* gene. Three variants were noted in the third patient in the three genes (*CREB1*, *ROS1*, and *PIK3CG*); the variants were SNP. And four variants were reported in the fourth patient in four genes (*KDM5A*, *STK11*, *ZFHX3* and *PMS1*); the variants were SNP, except one variant was insertion (c.7800–7801 ins AGTGGC) in the *ZFHX3* gene.

Out of four variants in two patients responding to bortezomib regimens, two variants were recorded in the first patient in two genes (*RAF1* and *CREB1*); the variants were SNP. And another two variants were recorded in the second patient in two genes (*ZFHX3* and *INSR*); the variant in the *ZFHX3* gene was insertion (c.7800–7801 ins AGTGGC) and variant in the *INSR* gene was SNP.

4. Discussion

The introduction and advancement of new sequencing technologies have opened up new biological scenarios in the last decade, especially in the field of onco-hematologies (Reuter et al., 2015). The MM genome analysis reveals the discovery of novel targets or single pathways controlling the proliferation of myeloma cells will enhance the clinical outcome and survival of patients with refractory MM. NGS has found new insights into the complexity of the intra-clonal heterogeneity of MM. Many new genes have been detected by using NGS. However, some genes have been accepted as markers for diagnosis, prognosis, and treatment (Weaver and Tariman, 2017). In this study, we performed WES for four patients in relapsed and refractory MM. We identified 17

Table 1
Demographic and baseline characteristics of the patients.

Variable	Number of patients (%)
Total number of patients	6
Age, median (range)	64.5 (41 – 81)
Sex	
Male	2 (33.4%)
Female	4 (66.6%)
Myeloma isotype	
IgG	4 (66.6%)
LCMM	2 (33.4%)
Laboratory values	
Creatinine (mg/dl), median (range)	1.5 (2.4–1.2)
Urea (mg/dl), median (range)	73.6 (110–47)
B_2Microglobulin(mg/l), median (range)	8.2 (9.9–4)
ISS	
I	0
II	2 (33.4%)
III	4 (66.6%)
Treatment	
Relapsed to Vel-TD & Daratumub	4 (66.6%)
Response to Vel-TD	2 (33.4%)

LCMM light chain MM, *ISS* International staging system, *Vel-TD* velcade (bortezomib), thalidomide and dexamethasone.

Table 2
Nucleotide variants identified in relapsed and refractory MM patients.

Patients No	Gene names	Variants coordinate	Amino acid change	Zygosity	Variants classification
1	*GNAQ*	NM_002072.5: c.286A > T	p.Th96Ser	Heterozygote	Likely pathogenic
	PMS2	NM_001322009: c.301-4insT	———	Heterozygote	Unknown significant
	CREB1	NM_134442.5: c.179A > T	p.Asn60Ile	Heterozygote	Unknown significant
	NSUN2	NM_017755.6: c.718A > C	p.Asn240His	Heterozygote	Unknown significant
	PIK3CG	NM_002649.3: c.2174G > C	p.Gly725Ala	Heterozygote	Unknown significant
	ROS1	NM_002944.2: c.5100C > A	p.Tyr1700Ter	Heterozygote	Unknown significant
2	*PIK3CG*	NM_002649.3: c.2174G > C	p.Gly725Ala	Heterozygote	Unknown significant
	GNAQ	NM_002072.5: c.286A > T	p.Th96Ser	Heterozygote	Likely pathogenic
	FLT4	NM_001354989.2: c.3919 T > C	p.Ter1307 Argext	Homozygote	Unknown significant
	PMS2	NM_001322009: c.301-4insT	———————	Heterozygote	Unknown significant
3	*CREB1*	NM_134442.5: c.179A > T	p.Asn60Ile	Heterozygote	Unknown significant
	ROS1	NM_002944.2: c.5100 > A	p.Try1700Ter	Heterozygote	Unknown significant
	PIK3CG	NM_002649.3: c.2174G > C	p.Gly725Ala	Heterozygote	Unknown significant
4	*KDM5A*	NM_001042603.3: c.3235G > T	p.Asp1079Asn	Heterozygote	Unknown significant
	STK11	NM-000455.5: c.1150C > T	p.Arg384Trp	Heterozygote	Unknown significant
	ZFHX3	NM_001164766: c.7800–7801 ins AGTGGC	————————	Heterozygote	Unknown significant
	PMS1	NM_000534.5c.1627G > T	P.Glu543Ter	Heterozygote	Likely pathogenic

Table 3
Nucleotide variants identified in patients response to bortezomib.

Patients No	Gene names	Variants coordinate	Amino acid change	Zygosity	Variants classification
1	*RAF1*	NM_002880.3: c.1516A > G	p.Thr506Ala	Heterozygote	Unknown significant
	CREB1	NM_134442.5: c.179A > T	p.Asn60Ile	Heterozygote	Unknown significant
2	*ZFHX3*	NM_001164766: c.7800–7801 ins AGTGGC	_____	Heterozygote	Unknown significant
	INSR	NM_001079817: c.653-5ins TC	_____	Heterozygote	Unknown significant

variants in 11 genes (*GNAQ, PMS1, CREB1, NSUNS2, PIK3CG, ROS1, PMS2, FIT4, KDM5A, STK11,* and *ZFHX3*), Table 2. These variants lead to change in the amino acid, may affect the expression of these genes and affect protein function and stability. Many of these genes have been related to drug resistance pathways and cell survival in MM and other cancers.

In Table 2, our results revealed variant likely pathogenic in the *GNAQ* gene in two patients; this gene is a guanine nucleotide-binding protein regulating B-cell development and survival (Jonsson et al., 2017). This variant is previously reported in a study on natural killer / T cell lymphoma patients (Li et al., 2019). A study suggests that the variation of *GNAQ* stimulate the nuclear factor kappa B (NF-κB) pathway in cancer, which increases receptor activator of nuclear factors-κB ligand (RANKL) and macrophage colony-stimulating factor (M−CSF) expression (Choi et al., 2020). It has been suggested that activating the NF-κB pathway is essential in MM's pathogenesis and resistance to treatments (Ikeda et al., 2015). A variant likely pathogenic in the *PMS1* gene was observed in one patient, which is a splice nonsense variant. *PMS1* is a tumour suppressor gene. Such a null variant most probably cause a loss of function mutation. Moreover, variant unknown significant (VUS) in the *PMS2* gene was detected in two patients; this is a splice site variant. Human Splicing Finder predicts this variant as likely pathogenic. *PMS2* gene is a tumour suppressor such a splice site variants most probably cause a loss of function mutation. *PMS1* and *PMS2* genes are mismatch repair system components. DNA mismatch repair (MMR) enzymes take action as proofreading complexes that preserve genomic integrity and MMR-deficient cells disply an increase mutation rate (Fukuhara et al., 2015). MMR is one of the main biological pathways implicated in cancer development and drug resistance (Torgovnick and Schumacher, 2015). The change of DNA repair pathways can enhance tumorigenesis and can induce drug resistance. In particular, several lines of evidence confirm the strong connection between DNA damage repair mechanismes and response to treatment in MM and patients' survival (Walker et al., 2015a; Kassambara et al., 2014). Mice that have the *PMS2* gene deleted develop lymphomas (Chen et al., 2005).

Additionally, we found VUS in the *CREB1* gene in two patients. There is no data found about this variant and its functional effect. Cyclic AMP Response Element Binding (CREB) protein is a transcription factor having a major role in the nuclear responses to various external signals that lead to differentiation, proliferation, survival and apoptosis (Dauria and Di, 2013). Knockdown of *CREB1* significantly inhibits cell proliferation, colony formation, migration and invasion in addition to induced cell arrest at G1/G0 phase in vitro. Many genome-wide association studies have shown that the number of putative target genes for CREB is around 5000 (Sakamoto and Frank, 2009; Mayr and Montminy, 2001). Previous studies have shown that *CREB* is a proto-oncogene which overexpression promotes cellular proliferation in haematopoietic cells (Sassone-Corsi, 1995; Montminy, 1997). In *vitro* and in *vivo*, abnormal proliferation and survival of myeloid cells appear due to the upregulation of CREB target genes such as *CyclinA1*(Cho et al., 2011). Several factors associated with the growth and survival of MM cells related to CREB family members have been identified (Zhang and Fenton, 2002), like the myeloid cell leukaemia-1 (MCL-1) protein and an anti-apoptotic member of the B-cell lymphoma 2 (BCL-2) family. These have been considered critical regulators of MM cell survival and suggested an attractive therapeutic target (Zhang and Fenton, 2002).

Furthermore, VUS in the *NSUN2* gene was recorded in one patient. This gene's effect on cancer mechanism is epigenetic; no medical record exists about this variant. NOP2/Sun tRNA methyltransferase family member 2 (NSUN2) encoded by this gene. This enzyme catalyzes the methylation of cytosine to 5-methylcytosine of intron-containing tRNA precursor. This is an essential step in pairing anticodon-codon, and therefore crucial for proper messenger RNA translation (Blanco and Frye, 2014). In addition, this modulation plays a vital role in tissue homeostasis, cell division. Increase the production of protein by various mechanisms such as promoting mRNA stability and enhancing protein synthesis and translation (Chellamuthu and Gray, 2020). The alterations to *NSUN2* are common in breast cancer (Manning et al., 2020), colon cancer (Okamoto et al., 2012), and lung cancer

(Saijo et al., 2001). Generally, there is limited information about variants in the *NSUN2* gene and multiple myeloma.

We identified two unknown variants in two genes of tyrosine kinases receptors (RTKs), the *FLT4* gene in one patient and the *ROS1* gene in two patients. In various stages of neoplastic growth and progression, RTKs are involved. Their signalling affects cell growth, adhesion, differentiation, motility and death (Berenstein, 2015; Robinson et al., 2000). When subverted, these processes may give origin to cancer (Blume-Jensen and Hunter, 2001). Fms-related tyrosine kinase 4 (*FLT4*) is a member of class III receptor tyrosine kinases, including vascular endothelial growth factors receptors (VEGF-C). Silent mutations, nonsense mutations, missense mutations, and frameshift deletions in the *FLT4* gene are detected in cancers like stomach, intestinal, and skin cancer (Mendes Oliveira et al., 2018; Melikhan-Revzin et al., 2015). *ROS1* (ROS proto-oncogene1, receptor tyrosine kinase) is a gene that encodes the proto-oncogene tyrosine-protein kinase ROS protein. The rearrangement of this gene is detected in lung adenocarcinoma, breast invasive ductal carcinoma, cholangiocarcinoma, gastric, ovaries, and colorectal cancer (Lin and Shaw, 2017). as well as ROS1 fusions have been recorded in MM (Morgan et al., 2018). This missense variant in these genes and altered amino acid may affect protein function or stability, and there is no data about these variants.

Whole exome sequencing revealed a missense variant in the *PIK3CG* gene (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma) in three patients. Missense variant may cause loss or gain of function. *PIK3CG* is a tumour suppressor gene (Samuels and Ericson, 2006; Li et al., 2015) and lipid kinases family that integrates cues from cytokines, growth factors and other environmental signals, then translating them to intracellular signals that regulate various signalling pathways (Thorpe et al., 2014). These pathways regulate many cellular processes and physiological functions, including cell proliferation, metabolism, growth, survival and motility (Vanhaesebroeck et al., 2010). This pathway has been shown to be constitutively activated in MM cells and to have a pleiotropic effect influencing drug resistance, angiogenesis, proliferation, and cell adhesion (Sahin et al., 2014).

A missense variant in the Lysine(k)-specific demethylase 5A (KDM5A) gene was detected in one patient. This gene encodes a protein that acts as gene regulation by eliminating di-and trimethyl marks from lysine four on histone H3, making them potential players in tumour suppressors' downregulation. Still, it can also report that their action represses oncogenes. Depending on the methylation site, their effect on transcription may either suppress or activate (Plch et al., 2019). Histone methylation is essential in regulating gene expression, and its dysregulation has been detected in many cancers (Højfeldt et al., 2013). It has been suggested that the KDM5 family of demethylases have a role in drug tolerance (Plch et al., 2019). Upregulation of this gene is associated with chemotherapy resistance detected in gastric cancer (Li et al., 2014), lung cancer (Teng et al., 2013) and breast cancer (Hou et al., 2012). Moreover, Whole-exome sequencing revealed a missense variant in the serine/threonine kinase11 (STK11) gene in one patient, VUS. This gene encodes a protein that belongs to the serine/threonine kinase family. The protein functions in the regulation of cell polarity, apoptosis, DNA damage repair (Li et al., 2020b). Recently STK11 gene has been identified as a tumour suppressor gene, which often is silenced in the wide spectrum of truncating mutation (Li et al., 2020b).

Finally**,** VUS in the zinc-finger homeobox 3 (ZFHX3) was found in one patient. This gene is a large transcription factor containing four homeodomains, 23 zinc-finger domains, and many other motifs (Hu et al., 2019). This is a splice site variant. Human Splicing Finder predicts this variant as likely pathogenic. This gene is detected as a tumour suppressor in several cancers (Walker et al., 2015b). Such splice site variants most probably cause loss of function mutation.

As part of this study in Table 3, we performed WES for two patients in response to bortezomib regimens, and we identified four variants in 4 genes (*RAF1*, *CREB1*, *ZFHX3*, and *INSR).* Two variants in two genes (*CREB1* and *ZFHX3)* were recorded in relapsed and refractory MM patients. VUS in the *RAF1* gene was detected. Such a missense variant may cause loss or gain of function. RAF-1 is a proto-oncogene, serine/threonine kinase that encoded a protein is a mitogen activated protein kinase (MAPK) that functions downstream of small GTPase (RAS) and activates mitogen activated protein kinase kinase 1/2 (MEK1) and (MEK2) (Li et al., 2020a). In several cellular processes, the MAPK pathway is critically involved**.** The dysregulation of this pathway leads to uncontrolled cellular proliferation, survival, and dedifferentiation. Consequently, the MAPK pathway is changed or inappropriately activated in most cancers (Yaeger and Corcoran, 2019). A splice site variant in the Insulin Receptor gene (INSR) was recorded; this variant was VUS. The insulin receptor, a tyrosine kinase protein, is encoded by the *INSR* gene. This splice site variant may cause loss or gain of function. The insulin receptor is overexpressed in some malignancies, affecting abnormal response to proinsulin, insulin, and insulin-like growth factors, with predominant mitogenic rather than metabolic effects. The biological function of the overexpressed insulin receptor in cancer is not yet well understood (Vella et al., 2018).

In this study, the genes that recorded genetic change in relapsed and refractory MM patients may have a critical role in tumour progression and drug resistance like *GNAQ, PMS1, PMS2*, *PIK3CG* and *KDM5A*. Variation in these genes may affect the expression of genes and affect coding protein.

In conclusion, we detected several genetic alterations in many genes (*GNAQ, PMS1, CREB1, NSUNS2, PIK3CG, ROS1, PMS2, FIT4, KDM5A, STK11* and *ZFHX3*). These variants lead to change in the amino acid, may affect the expression of these genes and affect protein function and stability. That may have been associated with the poor prognosis and poor response to treatment in these patients. These values should be further confirmed in large sample studies using RNA-seq technique to identify genome expression.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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