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Investigation of ErbB and Insulin Signaling Pathways on the Pathogenesis of Multiple Myeloma

Multipl Miyelom Patogenezinde ErbB ve İnsülin Sinyal Yolaklarının İncelenmesi

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Abstract

Introduction: Analysis of genes that play roles in Multiple myeloma pathogenesis and their pathways are current research topics. We aim to detect expression of some genes of ErbB and insulin signaling pathways.

Methods: Bone marrows were taken from three healthy volunteers and 17 untreated patients, firstly RNA isolation was made and then cDNA were synthesized. There are eight genes that are related to ErbB and Insulin signaling pathways, after that specific primers for these genes were designed. Gene expression analysis was performed by Real-Time PCR method.

Results: In the patient group, expressions of *MTOR*, *RPTOR*, *PIK3CA*, *AKT1*, *ErbB4*, *PRKAR2A* and *PRKACB* genes were detected to be 3-10 times up-regulated than control group. There were not differences in the expression levels of *RICTOR* and *GYS1* genes between control group and patient group. *GYS1*, *PRKACB* and *PRKAR2A* genes have been analyzed for the first time.

Conclusion: In this study, *PRKAR2A* and *PRKACB* genes expressions were detected to be upregulated and this hasn't been reported in literature before. *MTOR*, *RPTOR*, *PIK3CA*, *AKT1*, *ErbB4* genes expression were detected to be upregulated as had reported in the literature before. All these results will be useful to understand thepathogenesis of the multiple myeloma.

Keywords: Multiple myeloma, qRT- PCR, insulin signaling pathway, ErbB

Öz

Amaç: Multipl miyeloma patogenezinde rol oynayan genlerin ve ilgili yolakların moleküler düzeyde incelenmesi güncel bir araştırma alanıdır. ErbB ve insülin sinyal yolağında bulunan bazı genlerin ekspresyon çalışmasının yapılması amaçlandı.

Yöntemler: Üç sağlıklı gönüllü ve 17 tedavisiz hastadan kemik iliği alındı, ilk olarak RNA izolasyonu yapıldı ve daha sonra cDNA sentezi yapıldı.ErbB ve insülin sinyal yolakları ile ilgili sekiz gen vardır, bu genler için spesifik primerler dizayn edilmiştir. Gen ekspresyon analizi, Real-Time PCR yöntemi ile gerçekleştirilmiştir.

Bulgular: Hasta grubunda *MTOR, RPTOR, PIK3CA, AKT1*, *ErbB4, PRKAR2A* ve *PRKACB* genlerinin ekspresyonu kontrol grubuna göre 3-10 kat arttı. Kontrol grubu ile hasta grubu arasında *RICTOR* ve *GYS1* genlerinin ekspresyon düzeylerinde farklılıklar gözlenmedi. *GYS1*, *PRKACB* ve *PRKAR2A* genleri ilk kez analiz edilmiştir.

Sonuç: Bu çalışmada, *PRKAR2A* ve *PRKACB* gen ekspresyonlarının arttığı saptanmış ve daha önce literatürde bildirilmemiştir. MTOR, *RPTOR*, *PIK3CA*, *AKT1*, *ErbB4* gen ekspresyonunun daha önce literatürde bildirildiği gibi ekspresyonlarının arttığı tespit edildi. Tüm bu sonuçlar Multipl miyelomun patogenezini anlamak için faydalı olacaktır.

Anahtar Sözcükler: Multipl miyelom, qRT-PCR, insulin sinyal yolağı, ErbB

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Introduction

Multiple myeloma (MM) is a clonally B cell malignancy and is described by the accumulation of malignant plasma cells with in the bone marrow, the presence of a monoclonal immunoglobulin in the serum and/or urine, lytic bone lesions, frequent anemia, and renal impairment (1-3). The progression of MM begins as monoclonal gammopathy of undetermined significance, progresses to smoldering myeloma, and becomes eventually (symptomatic) myeloma (4,5). MM accounts for approximately 10% of hematological malignancies (6). MM predominantly affects 71% of cases diagnosed in people aged 65 years and over (7).

MM is still considered an incurable malignancy (8). MM is a heterogeneous disease with varying clinical evidences, chromosomal aberrations, and molecular characteristics. The disease was to date not disclosed exactly. It's clear that more knowledge of the biological events underlying the development of MM is needed to determine new biomarkers. Interactions of MM cells especially with mesenchymal stromal cells and osteoclast cause to activation of multiple cellular signaling pathways on myeloma cells (PI3K/AKT, JAK/STAT3, RAS/RAF/MAPK/ERK, NFκB) which support their proliferation, survival, migration and even resistance to therapeutic agents (5,9).

We aim to determine the intracellular pathways involved in pathogenesis of the disease with changing expression of the identified genes in insulin signaling pathway and ErbB signaling pathway in MM patients.

Material and methods

Patients

The study group was consisted of between 51-74 years, 11 men and 6 women, including a total of 17 patients. Seventeen patients fulfilling the International Myeloma Working Group diagnostic criteria and Durie-Salmon criteria for MM were studied. The study was approved by the local Ethics Committee of the Istanbul faculty of medicine, İstanbul University (No:2014/927), and all patients provided their informed consent in accordance with the Declaration of Helsinki.

Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from bone marrow using the RNeasy Mini kit (Qiagen Venlo, Netherlands) and RNA samples were quantified using a NanoDrop® ND-2000 spectrophotometer.Total RNA reverse transcribed into total cDNA with the cDNA Synthesis Kit (Thermo Fisher Scientific, Wilmington, Delaware, USA).Gene expression analysis was performed by quantitative reverse transcription (qRT)–PCR (LightCycler 480 II, Roche, Germany).

The PCR reaction started with a denaturation step at 95°C for 10 minutes (1 cycle), followed by 45 cycles at 95°C for 15 seconds, 60°C for 60 seconds and 60°C for 1 second. Subsequently, a melting curve program was applied with continuous fluorescence measurement. A standard curve for genes templates was generated through 4 times dilutions of PCR products and the β-Actin gene was used as reference for normalization of the gene expression levels. Each reaction was performed duplicate. Designed primers are shown in Table 1.

The relative gene expression (fold change) was measured with the comparative threshold cycle (Ct) method using β-Actin as housekeeping gene and the $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

In all statistical analyses were used the SPSS version 13.0. The threshold cycle (Ct) was determined for each sample. ΔCt indicated the difference in expression levels with the Ct value of the related gene and mean of housekeeping gene ($\Delta Ct = Ct_x$ gene $- Ct_{housekeeping}$), and ΔΔCt indicated the difference in the ΔCt value between treatment and control groups ($\Delta\Delta Ct = \Delta Ct_{ES} - \Delta Ct_{control}$). The p values are calculated based on a Student's t-test of the replicate $2^{-\Delta Ct}$ values for each gene in the control and treatment groups. All tests were two-sided, and

Table 1. Primer sequences

MTOR (F)	5'- CTAAGTCTACCACGACAGCCCGG-3'
MTOR (R)	5'- GGCCTTCATGCCACATCTCATGCC-3'
RICTOR (F)	5'-CAACTGGGATGCTGTGAGGCATAG-3'
RICTOR (R)	5'- GTACTAGTAGAGCTGCTGCCAAAC-3'
RAPTOR (F)	5'-GAGAAGCTCTACAGCCTCCTCTCC-3'
RAPTOR (R)	5'- CCGTCCTCTCTGCAGAGTTGC-3'
PIK3CA (F)	5'-ACTTATTGAGGTGGTGCGAAAT -3'
PIK3CA (R)	5'- TGATGTAGTGTGTGGCTGTTGA-3'
AKT1 (F)	5'- GGGTTTCTCCCAGGAGGTTT-3'
AKT1 (R)	5'- GTCCATGGTGTTCCTACCCA -3'
ErbB4 (F)	5'- AGGAGTGAAATTGGACACAGC-3'
ErbB4 (R)	5'- TCCATCTCGGTATACAAACTGGT-3'
PRKAR2A (F)	5'- CCTAGCAGATTTAATAGACG-3'
PRKAR2A (R)	5'- ATCATCTCCTTGGTCAATGA-3'
PRKACB (F)	5'- GTTCTTTCTACCAATCTATATGTCTTTC-3'
PRKACB (R)	5'- ATGGACCAGTGAAATCAATATC-3'
GYS1 (F)	5'- GCCTTTCCAGAGCACTTCAC-3'
GYS1 (R)	5'- CTCCTCGTCCTCATCGTAGC-3'
β-ACTIN (F)	5'- AGAGCTACGAGCTGCCTGAC -3'
β-ACTIN (R)	5'- AGCACTGTGTTGGCGTACAG -3'

the p values less than 0.05 were considered statistically significant.

Results

The average and standard deviation values of the clinical parameters of the patients are shown below (Table 2). The expression levels of genes in the ErbB and Insulin signaling pathways are shown in Figure 1, Figure 2 and Table 3.

Discussion

Currently, development of novel targeted therapies for multiple myelom is a very active area of research. Advances in molecular biologic thechniques and understanding the interactions between genes in pathways related to disease pathogenesis and prognosis are expected to allow the use of new targeted therapies in the near future. Signal transduction is now considered to be the one of the key mechanisms impaired in many types of cancer. Therefore, identifying the main pathways, the genes interacting with each other in these pathways and novel prognostic markers will help not only early diagnosis of MM, but also effective treatment of this disease.

In 2002 Sukru Ozturk et al. made a project named "Comparative Gene Expression Profilling of Multiple Myeloma, Smoldering Myeloma and Monoclonal Gammopathy Undetermine Significance Caces" and found 405 fusion sequences. This project was support by Istanbul University (Project No: 7348). These fusions were analyzed using the UCSC website (https://genome.ucsc.edu/). After that with Venny program we found the genes that can be related with disease. These genes were

Table 2. The average and standard deviation values of the clinical parameters of the patients

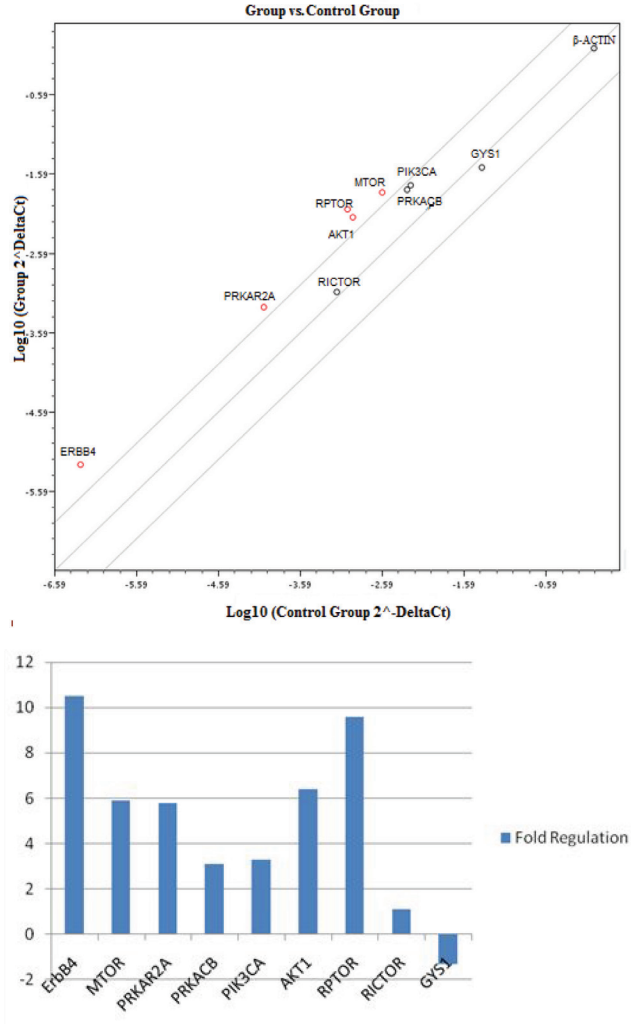
	Average	Standard deviation	Normal
Albumin(g/dL)	3.13	0.824	3.75-5.01
Creatin (mg/dL)	1.41	1.324	0.52-1.08
Hb (g/dL)	10.331	2.606	12.1-15.9
Hct (%)	30.77	6.85	-
White blood cell ($x10^3$/µl)	7.09	2.5	3.2-10.6
Red blood cell ($x10^6$µl)	3.63	0.809	3.88-5.46
Platelet ($x10^3$/µl)	210.8	88.26	150000-440000
M spike (g/dL)	2.39	2.62	2.39
Kappa light chain (mg/L)	9876.7	11341.44	3.3-19.4
Lambda light chain (mg/L)	3006.55	4267.3	5.71-26.3
Age	62.76	7.51	-
Beta microglobulin (mg/L)	0.06	0.147	-

Hct: Hematocrit, Hb: Hemoglobin concentration

Table 3. The expression levels of genes, confidence interval and p value

Genes	Fold regulation	95% CI	p value
ErbB4	10.5968	6.93-14.26	0.004517
MTOR	5.9518	3.00-8.91	0.013228
PRKAR2A	5.8204	1.39-10.25	0.006539
PRKACB	3.1383	1.57-4.71	0.029055
PIK3CA	3.3122	1.80-4.82	0.023204
AKT1	6.498	0.92-12.08	0.054141
RPTOR	9.6911	5.88-13.50	0.006912
RICTOR	1.1783	0.83-1.52	0.342337
GYS1	-1.3493	0.42-1.06	0.205086
B-ACTIN	1	1.00-1.00	0

CI: Confidence interval



analysed by WebGestalt database and ten pathways were detected, because of financial means we choosed two of them. Insulin and ERBB signaling pathway which takes part especially in cell poliferation and protein synthesis were determined by bioinformatics analysis.

ErbB4 gene is tyrosine kinase transmembrane receptors that regulate cell proliferation and differentiation (10). *ErbB4* gene doesn't arise from hematopoietic origin, but is known to be associated with poor prognosis in endometrial cancer (11). *ErbB4* gene expression levels decreases in pancreas and kidney tumors. In many studies its shown that *ErbB4* is overexpressed in series of breast cancer, colorectal cancer and osteosarcoma (12). In study of the Matlouk and et al (2008) reported expression of *ErbB4* gene in 9 of the 17 human myeloma cell lines. Also primary myeloma cells were expressed in 14 of 21 patients, and they showed that the gene wasn't expressed in normal plasmablastic cell and bone marrow plasma cells (13). In this study, *ErbB4* gene was found 9 times upregulate in bone marrow of MM patients.

MTOR is a molecular sensor that regulates cell proliferation, protein synthesis, transcription also this molecular sensor promotes cell cycle progression from G_1 to S phase (14). The study of Maison and et al. (2011) showed that MM1S, U266 and U266LR7 cell lines have low *RPTOR* gene expression, RAPTOR and *RICTOR* genes was expressed in OPM1, OPM2, MM1R, H929 and RPM18226 cell lines (15). In our study, we didn't find a significant expression of genes *RICTOR*, we found that RAPTOR 9 times and *MTOR* 5 times upregulated.

The regulation of *PI3K* / AKT /*MTOR* pathway degenerate in human cancers and this effect cell survival, proliferation and metastasis. Phosphotidylinositol, messenger molecular are lipid kinases that are a subclass of the PI3Ks. PI3Ks is activated by cell membrane receptors. When PI3Ks is phosphorylated, are actsas an activating second messengerin downstream pathways including AKT (14). *PI3K* / AKT signaling pathway is important for MM cell proliferation, survival and anti-apoptosis. Reduced activation of *PI3K* gene leads to MM cell death but increased activation of *PI3K* gene lead to proliferation (16). Study of Azabu and et al. (17) in 2013, they detected increased expression of genes in *PI3KCA*. Our results are consistent with the literature; we showed that *PI3KCA* gene expression was upregulated 3 times.

The most important down-stream effector of the *PI3K* pathway is a protein kinase B, also known as AKT. AKT is involved in cellular processes such as cell proliferation survival and migration, glucose metabolism and transcription of genes (18,19). In study of Lopez-Corral and et al. (20) (2014) showedthat *AKT1* gene was more expressedin MM than MGUS. In our study, we found that the expression of *AKT1* gene increased 6 times.

There was no statistically significant difference in the level of *GYS1* gene expression. It depends on two reasons; first *GYS1* gene is a catalyst in rate limited step in biosynthesis. This catalyst role is arranged with phosphorylation by kinases. Especially the expression levels of the gene change according to stage of disease. Second in cardiovascular disease *GYS1* expression levels increase because *GYS1* gene is found mostly in skeletal and heart muscle.

PKA is acAMP-depent protein kinase and take part in AKT signal transduction pathway. *PKA* play role in antilipolisis mechanism, cell growth, apoptosis and gene transcription. Recently, it was shown that the disruption in regulation of *PKA* causes deterioration of D-type cyclins including cyclin D1. Schroders and his colleagues (21) observed that the expression of *PRKACB* gene decreases in the half of mantle cell lymphoma. The levels of cAMP are so important because of effects on cell cycle proliferation, apoptosis and cyclin D1. In Many types of cancer it was shown that *PKA* genes subtypes had different expression levels. It is upregulated in stomach cancer and breast cancer (22,23). In our study, *PRKACB* gene was 3 times upregulated also, there is no another study that analyze the connection between *PRKACB* gene level and MM.

AKT gene gets induced by PI3K, which is in insulin signal pathway and gets phosphorylated by PDK1/2. AKT leads to protein synthesis by phosphorylating *MTOR* and *RAPTOR* genes and glycogenesis by dephosphorylation of *GYS* and *PHK* genes. The *RAS* gene, which exists in the same pathway leads to differentiation and proliferation by phosphorylating *RAF*, *MEK* and *ERK1/2* genes. All these events make think that all the genes are used by different mechanism and the genes make changes mRNA levels. All these effect are in the same direction and connected to each other. The results of our study shown that especially these gene expression levels are same-directed and increase approximately same rate with each other in MM patients. This result is important because of showing our study consistency.

PKA, which takes part in insulin signal pathway is active after phosphorylating. *PRKAR2A* and *PRKACB* are the most remarkable subtypes among the proteins' subtypes because the genes have both catalytically and regulatory function. There are so many studies that show PRKAR2A gene is upregulated. For example; Bidkhori and et al. (24) show that PRKAR2A is overexpressed in lung adenocarcinoma in their study in 2013. In 2004, Neben and at al. found that PRKAR2A is related to centromere structure and functions their study with 29 patients. According to the cell growth results, PRKAR2A is 2 times upregulated in AML patients (25). Our results

are matching with Neben and et al. results. Expression increase in breast, colorectal and various human non-endocrine cancers (26) and especially in cervical cancer this increased expression is related with poor prognosis. In our study, we determined that PRKAR2A gene is 5 times expressed; also there aren't any studies, which reveal this gene's relation with MM.

Conclusion

As conclusion, Insulin signaling pathway is involved in protein synthesis, gluconeogenesis and proliferation. ERBB signaling pathway also is involved in protein synthesis and cell cycle. In this study some gene expressions, which exist in insulin and ErbB signal pathways (*MTOR*, *RPTOR*, PIK3CA, *AKT1*, *ErbB4*, PRKAR2A, and *PRKACB*) were examined. There is no significant expression difference about *GYS1* and *RICTOR* genes expression levels in MM patients. Especially *ErbB4* and *MTOR*, *RPTOR*, PIK3CA, *AKT1*, PRKAR2A, *PRKACB* genes expression levels were found 3-10 times upregulated than control group. The genes that were involved in this study and our results can be useful for explanation of etiopathogenesis in MM.

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