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Proje Yürütücüsü:

Yard.Doç.Dr. Çetin Saatci

Tıp Fakültesi\ Tıbbi Genetik AD

Prof.Dr.Yusuf Özkul

Tıp Fakültesi\ Tıbbi Genetik AD

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ÖZET

Multiple myeloma hastalarının tedavi öncesi ve sonrası rezidual myeloma hücrelerinin (minimal rezidual hastalık-MRD) tedavisinde görüntüleme tedavinin etkinliği için çok büyük kolaylık gösterecektir.Wilms' tümör genini ekspresyon seviyelerinin real-time k antitatif polimeraz zincir reaksiyonuyla ölçülmesi lösemi ve myelodisplastik sendromda minimal rezidual hastalık indikatörü olarak kullanımın yararı rapor edilmiştir.Bu çalışmanın hedefi WT1 ekspresyon seviyelerini ölçerek,bu genin ekspresyonu ve multiple myeloma arasındaki muhtemel ilişkinin teşhiste bulunmasıdır.Eğer bir ilişki bulunursa,WT1 geni diğer tanı faktörleriyle karşılaştırarak MRD markırı olarak kullanılabilmesidir.Bu çalışmada periferik kandaki WT1 ekspresyonun seviyesini 50 yeni tanı almış multiple myeloma hastasında real-time light cycler kantitatif polimeraz zincir reaksiyonu kullanılarak ölçüldü.Normal WT1 gen kopya sayısı <23µl cDNA bulundu.Multiple myelomalı tüm hastalarda normalWT1-mRNA seviyesi bulundu.Bu çalışmaya göre WT1 ekspresyon analizlerinin multiple myelomalı hastaların teşhisinde rutin klinik uygulamalar için genetik markır olarak kullanımı uygun değildir.

ABSTRACT

Monitoring patients with multiple nyeloma during and after treatment for the presence of residual myeloma cells (minimal residual disease-MRD) has been shown to give a major insight into the effectiveness of treatment. It has been reportad that Wilms tumor gene (WT1) expression levels measured by real time quantitative polymerase chain reaction was useful as an indicator of minimal residual disease in leukomia and myelodysplastic syndrome.The aim of this study was to measure levels of WT1 expression in order to find a possible association between the expression of this gene and multiple myeloma at diagnosis.If an association was found, the WT1 gebe could be evaluated as an MRD marker by comparison with other prognostic factors. We investigated peripheral blood WT1 expression level measured by real time light cycler quantitative polymerase chain reaction in 50 newly diagnosed multiple myeloma patients. The normal WT1 gene copy number was found to be <23µl cDNA and all patients with myeloma were found to have normal WT1-mRNA levels. On this basis WT1 expression analyses is unlikely to be a useful genetic marker for routine clinical use in multiple myeloma patients at diagnosis.

GİRİŞ / AMAÇ VE KAPSAM

Multiple Myelom' lu hastalarda tedavi öncesi ve tedavi sonrası Real-time PCR yöntemini kullanarak, WT1 geninin ekspresyonunun saptanması, saptanan bulguların karşılaştırılıp, hastalığın erken teşhisinde, takibinde veya tedavi başarısının izleminde kullanılabilirliğinin gerekliliğini tespit etmek.Aynı hastalarda 13 nolu kromozomun uzun kolunda bulunan RB1 genine konvansiyonel PCR yöntemi kullanarak 13q delesyona bakmak ve MM' lu hastalarda prognozla arasındaki ilişkinin açığa kavuşturulmasında literatüre değerli bilgilerin kazandırılmasıdır.

GENEL BİLGİLER

Multple myeloma (MM), terminal differansiye B hücrelerinin malignansisidir.Yıllık insidansı yaklaşık milyonda 40' dır yani ülkemizde yılda 2800 yeni vaka ortaya çıkmaktadır.İnsidansı yaşla artar (ortalama 65 yaş), 40 yaşın altında nadirdir, Afro karayiplilerde iki kat daha sıktır.Sadece radyasyon mazuriyet kesin epidemiyolojik risk faktörü olarak tanımlanmıştır.Hastalık birkaç aydan 10 yıla kadar değişebilen süre ile oldukça heterojenöz sonuç göstermektedir.Konvansiyonel olarak kullanılan prognostik faktörler [serum b-2 mikroglobulin (b-2 m), albumin ce C reaktif protein (CRP) düzeyi, Durie- Salmon evreleme sistemi] hem kantitatiftir hem de her yerde kolaylıkla elde edilebileceğinden MM' li hastalarla ilgili klinik çalışmalarda oldukça kullanışlıdır.Bununla birlikte bu hastalardaki temel genetik anormallikleri gösteremezler.

Rpse et al. (1990) WT1 geninin 11p13 bölgesinde yerleştiğini saptamış ve Gessler et al.(1992) WT1 geninin genomik organizasyonunu tanımlamış, 10 exondan oluştuğunu bulmuştur.İki bölgedeki alternatif splicing in paterni, epidermal growth factor reseptör (EGFR), insulin-like growth factor-ıı (IGF-II), IGF-I reseptör (IGFR-I) ve androgen receptor gibi büyüme faktörleri ve onların reseptörlerini transkripsiyon faktöründe, ayrıntılı olarak karakterize edilmiştir.wtWT1 proteininde, trans-aktivasyon domaini WT1'nin N-terminal kısmındadır ve onun transkripsiyonal aktivasyonu, bu transkripsiyon faktörlerinin hücre büyümesinin süprese etme yeteneği için kritiktir.Eğer bu trans-aktivasyonu damainini kodlayan dizide trWT1 transkripti yokken DNA bağlayan domaini mevcutsa, dominant negatif gibi etki gösterebilir ve direkt onkogenezise dahil olabilir.Wilm's tümöründe, WT1 geninde birçok farklı delesyonlar ve nokta mutasyonlar bildirilmiştir ki bununda mutasyona uğramış WT1 geninin ürünlerinin karsinogeneziste rol oynayabileceğini düşündürmektesir.Yüksek ekspresyon düzeyleri akut lenfositik lösemiler, akut myeloid lösemiler ve kronik myelositik lösemilerde saptanmıştır.Ama ekspresyonu multple myelomada araştırılmamıştır.

WT1 transkripsiyon faktörünün gerçekten lökomogenezise katkı mı yaptığı veya sadece immatur fetotipi gösterdiği immatür CD34+ progenitor hücrelerde normal olarak ekprese edildiği ve farklılaşmanın WT1 downregülasyonu ile ilişkili olduğunun bulunmasına kadar açık değildi.Tüm lösemili hastalarda lösemi hücrelerindeki WT1 ekpresyonunun yüksek olması kötü prognozla ilişkilidir.Üstelik WT1 ekpresyonunun yüksek olması kötü prognozla ilişkilidir.Üstelik WT1 ekpresyonu ve çoklu ilaç rezistansı bazı hematolojik malignensilerle ilişkilidir ki bu yüzden hematolojik malignensilerde kemorezistansta potansiyel diagnostik bir marker olabilir.WT1 ekpresyon düzeyinin lösemili vakaların sonucu ile direkt olarak korele olduğu düşünülmektedir.Bu yüzden transkripsiyon miktarının ve onun ana varyantlarının tam olarak değerlendirilmesi kritiktir ve hastalığın prognozu ve tanının doğruluğunu sağlar.

Anöploidi (flow cytometry ve fluorescence in situ hybridization (FISH) analizi ile değerlendirilebilir) esas olarak MM'lu tüm hastalarda olmaktadır.Akut myeloblastik l ve lenfoblastik lösemili hastalarda

sitogenetik çalışmaların prognostik önemi uzun süre önce tanımlanmıştır.MM da benzer çalışmaların sistematik değerlendirilmesi, temel olarak hastalığın düşük proliferatif doğası nedeniyle geri kalmıştır.Bu yüzden hastaların büyük çoğunluğu (yaklaşık 2/3 ü) non-myeloma hücrelerinin (normal hematopoietik) metafazlarını yansıtan non-informatif karyotipik bulgulara sahiptir.Konvansiyonel sitogenetik analizler diğer araştırmacılar tarafından HDT veya standart tedavilerle tedavi edilmiş MM' lı hastalarda kötü prognostik faktör olarak bildirilmiştir.Kötü prognozda del 13 arasında kesin bir ilişki henüz tespit edilmemiştir.Ama biz düşünmekteyizki del 13 olduğu zaman 13q14 bölgesindeki RB1 tümör süpresör geninin haplo yetersizliği ortaya çıkmaktadır.Gen ekpresyon profil teknolojisi,FISH ile del 13 saptanan hastalarda iki eksizyon tamir geninin önemli derecede down-regulasyon olduğunu tespit etmiştir.Böyle genlerin kaybı, genomik instabiliteye yol açabileceklerini ve böyle hastalarda kötü bir sonuç olacağını düşündürtmektedir.

WT1 geninin akut myeloid lösemi (AML), akut lenfoblastik lösemi ve kronik myeloid lösemili hastalarda yüksek oranda ekprese olduğu bulunmuştur ve lösemik hücrelerin viabilitesinde anahtar bir rol oynadığı düşünülmektedir.WT1 geninin real time kantitatif zincir reaksiyonu ile kantitatif değerlendirilmesi son yıllarda değişik tip lösemilerde allojenik kemik iliği transplantasyonundan sonra minimal rezidüel hastalığın tespit edilmesinde marker olarak kullanılmıştır.Halbuki multiple astalarda WT1 geninin tedavi öncesi tedavi sonrası ekpresyonunun karşılaştırılıp hastalığın erken teşhisinde saptamak temel amacımızdır.Ayrıca 13. Kromozomun uzun kolunda bulunan RB1 genine konvansiyonel pcr yöntemiyle bakarak 13q delesyonu ile MM arasındaki ilişkinin açığa kavuşturulmasında literatüre değerli bilgilerin kazadırılması da ikinci amacımızdır.Ek yararlar olarak WT1 ekpresyonunun çoklu ilaç direnci ile ilişkisi hakkında şimdiye kadar bazı hematolojik malignensilerde bildirilmiş bilgilere ek veri sağlanabilecek ve ayrıca bu çalışmanın bir uzantısı olarak Insulin-like growth factor I-reseptörünün, myelomada otokrin büyüme sinyali döngüsünde etken olabileceği bulunmuştur ki (WT1 'in IGF1-reseptör geninin ekpresyonunun kontrolünde rol aldığı düşünülürse) bu bağlamda IGF1 reseptör geni ile ilgili çalışmalar planlanabilecektir.

GEREÇ VE YÖNTEM

Eylül 2005 ve Mart 2007 tarihleri arasında yeni multiple myelom tanısı alan 50 yeni tanılı hasta (75 yaşından küçük, hiç terapi almamış) alındı.Multiple myeloma çalışmasına serum ve idrar protein elektroforezi ve immünoelektroforez, immünglobülinlerin serum seviyeleri kantitasyon, biyopside kemik iliği plazma hücre seviyesi, serum B2M, albümin, LDH ve CRP seviyeleri.Hastalar VAD (vincristine 1 mg, gunde 1; deksametazon 40mg/m2, günde 1-4; deksorubusin 50mg/m2 günde 1) veya MP (melphalan ve prednisolone) kemoterapi protokolüne göre tedavi edildi.

GENETİK ANALİZ:
Periferik kan örnekleri (5ml, Etilendiamin tetra asetik asit) tanı anında her bir hastadan alındı.Toplamda 50 örnek analiz adildi.Altı (6) sağlıklı kişinin periferik kan örnekleri kontrol olarak kullanıldı.
Real –Time light cycler kantitatif polimeraz zincir reaksiyonu (QRT-PCR)
RNA eldesi Qiagen RNA Blood mini kite (Qiagen , Hilden, Germany) göre yapıldı.cDNA sentezi RT-PCR kitine göre yapıldı.Real-Time PCR Light Cycler Fast Start DNA SYBR Green kiti ile çalışıldı.Tüm primerler Search-LC (Heidelberg, Germany) ‘ den alındı.Hausekeeping gen olarak ABL geni kullanıldı.Her çalışma için pozitif ve negatif kontrol kullanıldı.Her bir örnekteki hedef molekül sayısı farklı hedeflerden elde edilen standart curve analizine göre yapıldı.Tüm örnekler ikişer kez çalışıldı.

BULGULAR

WT1 ekpresyon seviyeleri negatif kontrol ve hasta grubu arasında anlamlı bir fark bulunamamıştır. (WT1 gen kopyaları < 23µl cDNA) MRD pozitif örneklerdeki WT1 seviyeleri bizim hastalarımıza göre oldukça yüksek bulundu. 50 örnekte de WT1 ekpresyonu tespit edilememiştir. İkincil hedefimiz WT1 geninin MRD takibinde marker olarak kullanılmasıdır.

TARTIŞMA VE SONUÇ

WT1 geninin akut myeloid lösmi (AML), akute lenfoblastik lösemi ve kronik myeloid lösemili hastalarda yüksek oranda ekprese olduğu bulunmuştur ve lösemik hücrelerin viabilitesinde anahtar bir rol oynadığı düşünülmektedir.WT1 geninin real time kantitatif zincir reaksiyonu ile kantitatif değerlendirilmesi son yıllarda değişik tip lösemilerde allojenik kemik iliği transplantasyonundan sonra minimal rezidüel hastalığın tespit edilmesinde marker olarak kullanılmıştır. Fakat multiple myelomalı hastalarda WT1 gen ekpresyonu hakkında bir bilgi yoktur.Bu bağlamda MM'lı hastalarda WT1 geninin tedavi öncesi tedavi sonrası ekpresyonunun karşılaştırılıp hastalığın erken teşhisinde veya takibinde veya tedavi başarısının izleminde kullanılabilirliğinin tespitini real time PCR yöntemiyle saptamak temel amacımızdır.

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EKLER

1-Makale ekte sunulmuştur.

Expression of WT1 gene in multiple myeloma patients at diagnosis: is WT1 gene expression a useful marker in multiple myeloma?

Cetin Saatci1, A. Okay Caglayan1, Ismail Kocyigit2, Hilal Akalin1, Leyla G. Kaynar2,Fevzi Altuntas2, Bulent Eser2, Muzaffer Demir3, Mustafa Cetin2 and Yusuf Ozkul1

1Department of Medical Genetics,
2Department of Hematology, Medical Faculty, Erciyes University,
Kayseri and
3Department of Hematology, Medical Faculty, Trakya University, Edirne, Turkey

Monitoring patients with multiple myeloma during and after treatment for the presence of residualmyeloma cells (minimal residual disease – MRD) has been shown to give a major insight into theeffectiveness of treatment. It has been reported that Wilms' tumor gene (WT1) expression levels measured by real-time quantitative polymerase chain reaction was useful as an indicator of minimal residual disease in leukemia and myelodysplastic syndrome. The aim of this study was to measure levels of WT1 expression, in order to find a possible association between the expressionof this gene and multiple myeloma at diagnosis. If an association was found, the WT1 gene could be evaluated as an MRD marker by comparison with other prognostic factors. We investigated peripheral blood WT1 expression level measured by real-time light cycler quantitative polymerase chain reaction in 50 newly diagnosed multiple myeloma patients. The normal WT1 gene copy number was found to be ,23/ml cDNA and all patients with myeloma were found to have normal WT1-mRNA levels. On this basis WT1 expression analyses is unlikely to be a useful genetic marker for routine clinical use in multiple myeloma patients at diagnosis.

Keywords: Expression, multiple myeloma, WT1

Introduction

Multiple myeloma (MM) is a tumor of bone marrow plasma cells. It accounts for 10% of hematologic malignancies and 1% of all cancer deaths.1 Incidence increases with age (median 65 years), it is rare under 40, and twice as common in Afro-Caribbeans. The only clearly defined epidemiological risk factor is radiation exposure.2 Several adverse prognostic factors including paraprotein levels, Hb count, serum calcium level, lytic bone lesions, and renal function have been identified. These factors are combined in the widely used Durie-Salmon staging system.3 Renal impairment is a risk factor only because of its association with high tumour burden.4 Additionally, the international prognostic index (IPI) consisting of beta-2 microglobulin and serum albumin level is also used as a prognostic factor. However, the parameters used do not address the fundamental genetic abnormalities of the disease. Deletion of 13q is an important adverse prognostic factor5 and the presence of hypodiploidy is strongly associated with poor prognosis in MM.6 In contrast to the acute myeloblastic and lymphoblastic leukemias7,8 where the prognostic importance of cytogenetic studies has long been recognized, the systematic evaluation of similar studies in MM has lagged behind, mainly because of the low proliferative nature of the disease. Therefore, the majority of patients (approximately two-thirds at presentation) have non-informative karyotypic studies which reflects the analysis of only the metaphases of (normal hematopoietic) non-myeloma cells.

Correspondence to: A. Okay Caglayan, MD Erciyes University, Medical
Faculty, Department of Medical Genetics, Talas Caddesi, 38039, Kayseri,
Turkey
E-mail: aocaglayan@erciyes.edu.tr
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In recent years, the use of Wilms' tumor gene as a 'panleukemic' marker has been investigated in various studies.9–11 WT1 encodes a transcription factor involved in the pathogenesis of Wilms' tumor. Although the role of WT1 in tumorogenesis has not been well defined, further analyses of the gene have shown that the level of expression is high in most patients with acute leukemia and CML.12,13 Longterm monitoring of WT1 levels has been used to detect early relapse and predict the prognosis after chemotherapy or allogeneic stem cell transplantation. 14–16 An association between WT1 expression and relapse has been found a number of studies.17–19 However, there is very little data concerning the monitoring of WT1 levels in myeloma patients and its effect on prognosis. Our primary goal was to evaluate peripheral blood (PB) WT1 level as genetic marker at diagnosis and the secondary goal was to determine the association with the WT1 gene as an MRD marker by comparing the other prognostic factors.

Methods

Patients Between September 2005 and March 2007, 50 newly diagnosed patients with symptomatic MM (75 years or younger, no prior therapy) were enrolled in this prospective study (Table 1). Written informed consent had been obtained from all participants in keeping with institutional and National Cancer Institute guidelines. Multiple myeloma work-up included analysis of serum and urine protein electrophoresis and immunoelectrophoresis, quantitation of serum levels of immunoglobulins, bone marrow plasma cell level on biopsy and aspirate samples, serum B2M, albumin, LDH and CRP levels. Baseline MRI examinations were performed of the axial skeleton (head, shoulders, sternum, spine and pelvis). 20 Patients were treated with VAD (vincristine 1 mg, day 1; dexamethasone 40 mg/m2, days 1–4; doxorubicin 50 mg/m2, day 1) or MP (melphalan and prednisolone) chemotherapy protocol.21

Genetic analysis

Peripheral blood samples (5 ml, ethylene daimine tetra acetate) were drawn from each patient at diagnosis. A total of 50 samples were analyzed. PB samples from six healthy volunteers were used as controls. Real-time light cycler quantitative polymerase chain reaction (QRT-PCR) RNA preparation and reverse transcription: The total RNA was isolated from 1 ml of buffy coat, obtained through centrifugation (400 g for 10 min). The buffy coat was transferred to 6 ml of red cell lysis buffer included in the Qiagen RNA Blood Mini-kit (Qiagen, Hilden,

Germany). RNA extraction was then performed according to the manufacturer's instructions. The cDNA was prepared using first strand cDNA synthesis kit for RT-PCR according to the manufacturer's instructions. Real-time PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit. All primers were obtained from Search-LC (Heidelberg, Germany). The calculated number of specific transcripts was normalized to the housekeeping gene ABL and presented as adjusted transcripts/ml cDNA. We used negative and positive controls of each run. The number of target molecules in each unknown sample was calculated automatically using the standard curves from each different target. All samples were run in duplicate and a negative control was included in each run.

Results

The WT1 expression expression levels were not significantly different between negative control and the patient group (WT1 gene copies ,23/ml cDNA). In addition, WT1 levels in MRD positive samples were significantly higher than in our patients (P,0.05). Because WT1 expression was not detected in 50 samples, the secondary goal to determine the association with the WT1 gene as an MRD marker by comparing the other prognostic factors was not performed.

Table 1 Patients clinical and laboratory characteristics

Number of patients	50
Median age (range), years	57.5 (30–81)
Males females, n	32 male, 18 female
M component isotype, n	50
IgG	31
IgA	12
Non-secretuar	7
Light chain, n	50
Kappa	20
Lambda	14
Non k, non l	9
Stage (Durie-Salmon)	50
I	9
II	22
III	19

Median B2MG level (range), mg/l	11.85 (2.6–24.3)
Median CRP level (range), mg/l	18.95 (2.3–152)
Median LDH level (range), U/l	145 (100–190)
Median Hb level (range), mg/dl	9.8 (6 . 8–13.7)
Median BMPC (range),	% 55% (20–95%)

B2MG: b2-microglobulin; CRP: C-reactive protein; BMPC: bone marrow plasma cells; LDH: lactat dehydrogenase.

Discussion

The clinical course of patients with multiple myeloma is highly variable. A large part of newly diagnosed cases of MM have a normal karyotype and abnormalities are more often seen in advanced disease that is associated with an increased proliferative activity of malignant cells. FISH testing for specific chromosomal abnormalities in patients with normal chromosome studies has identified an abnormal clone in 45% of cases.22 Deletions of 13q14, 17p13, and 11q23 are all associated with poor prognostic factor.23–25 14q32 translocations involving chromosomes 4 and 16 have also been identified.8 Additional common cytogenetic changes can be seen as hyper- or hypodiploidy and abnormalities involving the chromosome 1q. Also further genetic instability is initiated through alteration of proliferation or apoptosis pathways. Pathways with identified mutations include the nuclear factor-kB pathway, ras and p53. Sensitive MRD assays are essential for detection of a threatening relapse and the early start of immunotherapeutic interventions. However, current MRD assays are heterogeneous and disease specific approaches are sometimes needed. Therefore, the idea of using WT1 as a MRD marker was an obvious area of study in myeloma patients. Hatta et al. examined the association of clinical parameters and WT1 expression in bone marrow for 17 newly diagnosed multiple myeloma patients and found WT1 transcripts increased when clinical factors deteriorate, including the stage, amount of M protein, Hb, platelet count, blood urea nitrogen, creatinine, serum alkaline phosphatase, calcium, beta2-microglobulin, thymidine kinase activity, and C-reactive protein.26 In contrast, an association between WT1 expression and multiple myeloma was not shown in present study. This difference may be partly explained by differences in sensitivities, the use of qualitative versus quantitative analyses and sample type. The concrete role of WT1 in hematopoiesis and leukemogenesis still remains unclear. It is reported that WT1 expression levels in PB samples by quantitative RTPCR were

significantly lower when compared to bone marrow samples in healthy individuals and the WT1 expression level was quite low and sometimes undetectable by quantitative RT-PCR.27 On the other hand, Inoue et al. reported that WT1 expression inBM was one log of magnitude higher than in PB.14 In present study, a comparison of the range of WT1 levels in patients and MRD positive samples showed that all of the patients are within the same range as the MRDnegative ones. Therefore, WT1 analysis in BM may be more sensitive than PB in myeloma patients. In conclusion, there is a constitutive low expression of WT1 in myeloma patients in peripheral blood and WT1 expression analysis may not be a practical genetic marker for routine clinical use in myeloma patients at diagnosis.

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